IMPROVING PERFORMANCE OF MULTI-ATTRUBUTE METHOD ASSAYS USING AN LC-MS WITH A NOVEL INERT FLUDIC PATHWAY

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INTRODUCTION

- The Peptide Multi-Attribute Method (MAM) is an LC-MS assay for direct measurement of product and critical quality attribute (pQA and CQA) levels in a digest of a biotherapeutic protein.
- One challenge faced in MAM assay optimization is assay sensitivity and reproducibility limitations due to class-specific analyte loss.
- Interactions between "acidic" peptides and metal surfaces inside chromatographic hardware can impact the MAM assay quality through analyte peak tailing, low peptide recovery, and irreproducible chromatography of metal sensitive peptides.
- In this study, we demonstrate that MAM assay performance can be optimized using instrumentation and columns containing a novel inert LC fluidic pathway.

METHODS

Samples

Waters NISTmAb tryptic digestion standard was prepared in 0.1% formic acid . 0.1 - 2.0 µg of mAb digest was loaded per analysis.

Instrumentation

The BioAccord System with ACQUITY Premier was used for this study. The ACQUITY Premier UPLC features novel MaxPeak High Performance Surfaces (HPS) that improves the overall chromatographic performance of analytes.

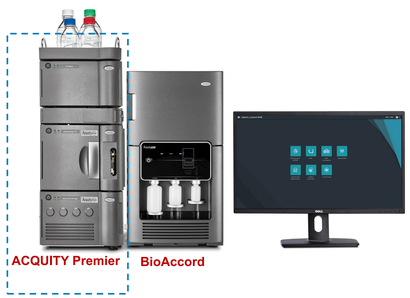

Figure 1. BioAccord System with ACQUITY Premier. The system is controlled by waters_connect informatics for compliant ready acquisition, data processing, and reporting.

LC method: Peptides were separated on an ACQUITY UPLC Peptide CSH C18 column or ACQUITY Premier CSH C18 columns, 130Å, 1.7 µm, 2.1 mm, 150 mm using a 78 min linear 1 – 35% acetonitrile gradient in 0.1% Formic acid.

MS method: The data acquired in ESI+ mode from *m/z* 50-2000 in data independent acquisition mode (MS^E) + Fragmentation.

Informatics: Data were acquired and processed using the waters_connect UNIFI, MS-Toolkit and Peptide MAM Apps.

RESULTS

Increased Recovery

Improved Recovery of Acidic Peptide Modifications

Peptides containing multiple acidic residues or modifications are susceptible to adsorption on the metal flow path surfaces of LCs and columns. The mAb PENNYK peptide, susceptible to multiple deamidations is an ideal example. These deamidations are an attribute routinely monitored for most humanized mAb products.

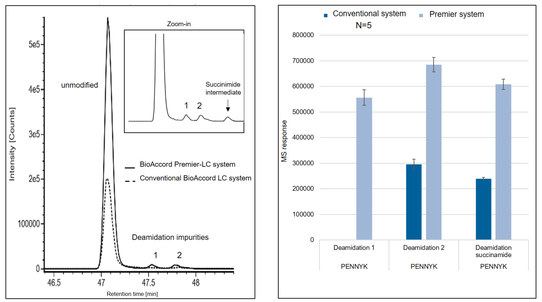

Figure 2. PENNYK Peptide Recovery Comparison

Left: *Extracted Ion Chromatograms (XIC) for the HC:T37 peptide (sequence: GFYPSDIAVEWESNGQ**PENNYK**) and associated deamidation/succinimide variants. Peptides monitored from a tryptic NISTmAb reference material digest separated on a conventional BioAccord System (dashed line) or a BioAccord System with ACQUITY Premier featuring MaxPeak HPS technology (solid line).*

Right: *Average normalized peak area for HC:T37 variants (5 Injections) for both systems*

Improved MS fragmentation data quality

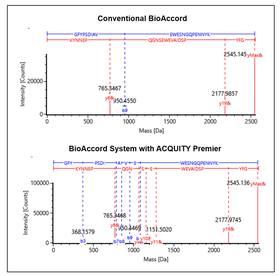

Figure 3. The improved MS signal obtained using Premier HPS Technology also improves the quality of fragmentation data for the metal sensitive PENNYK peptide deamidation-2 variant.

(Top) *Conventional BioAccord System and Column*

(Bottom) *BioAccord System with ACQUITY Premier and Premier Peptide Column.*

Consistent Performance

Accurately measuring peptide attributes: column loading

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%
Glycopeptide		
EEQYNSTYR	unmodified	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. Multiple NIST mAb critical quality attributes of higher and lower abundance were monitored across varying mass loads (0.1 µg – 2.0 µg) of the digest on a BioAccord System with ACQUITY Premier to identify optimal loading levels.

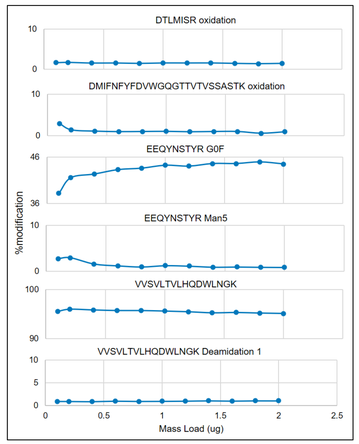

Figure 4. %modification levels for NISTmAb attributes based on normalized MS response vs. increasing mass load. Values for even the lowest level attributes monitored stabilize at ~0.2 µg of digest loaded on-column.

Reproducibility

Results spanning three-orders of useful dynamic range

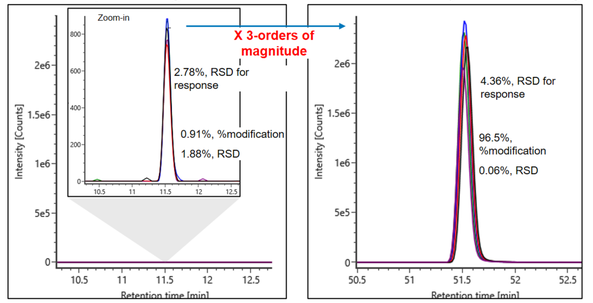

*Figure 5. Extracted Ion Chromatograms of the T26: VVSVLTVLHQDWLNGK base peak **(RIGHT)** at 1.0 ug optimum loading levels. Data for the T25:EEQYNSTYR Man5 Glycosylation peak **(LEFT)** is seen with a 0.08% relative MS response to the T26 peak, but generates comparable %RSD for response and % Modification determined from five replicate analyses.*

Peptide sequence	Modification	%Mod Conventional System	%Mod ACQUITY Premier	%RSD Conventional System	%RSD ACQUITY Premier
VVSVLTVLHQDWLNGK	(base peak)	95.88	96.55	0.04	0.06
DIQMTQSPSTLSASVGDR	oxidation	0.86	0.86	7.00	4.00
DMIFNFYFDVWGQGTTVTVSSASTK	oxidation	1.69	1.06	7.30	1.18
DTLMISR	oxidation	1.33	1.66	3.05	3.45
GFYPSDAVEWESNGQPENNYK	Deamidation 1	2.10	1.71	7.40	2.81
GFYPSDIAVEWESNGQPENNYK	Deamidation 2	-	2.10	-	1.33
GFYPSDIAVEWESNGQPENNYK	Deamidation succinamide	1.99	1.87	2.68	0.89
VTNMDPADTATYYCAR	oxidation	0.46	0.67	7.45	2.40
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	1.00	3.06	1.88

Table 2. NISTmAb quality attributes (1 µg mass load) with the BioAccord System and BioAccord System with ACQUITY Premier. Comparable % modification levels determined for the abundant attributes, with the Premier Technology enabling additional low level attribute quantification with generally superior precision across 5-injections.

CONCLUSIONS

The BioAccord System with ACQUITY Premier was developed to produce superior results by removing bias in chromatographic separations of metal sensitive analytes. When applied for peptide level attribute monitoring experiments, these benefits are realized in the resulting data quality improvements:

- Improved acidic peptide recovery , particularly for lower-level variants such as deamidation, generating better quantification and higher quality confirmatory fragmentation data
- Ability to measure comparable %modification levels across wider range of on-column loading levels, improving assay flexibility and robustness.
- Enabling measurements over 3-orders of magnitude useful dynamic range for analysis of both lower and higher abundance peptide attributes in a single experiment.

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