Demonstrated Benefits of Low-Flow Analytical Methods for Biopharmaceutical Applications

Elevating low-flow LCMS beyond bottom-up proteomics can bring analytical advantages to the biopharma space.

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INTRODUCTION

The growing prevalence of next generation biopharmaceuticals means that drug product characterization is no longer confined to simple small molecule analysis. This necessitates incorporation of analytical methods capable of performing in-depth macromolecule characterization while maintaining requisite levels of sensitivity, precision, and throughput, such as that obtained using liquid chromatography-mass spectrometry (LCMS) methods. While many may associate the low-flow range of ultra-high performance liquid chromatography (UHPLC) with bottom-up proteomics, the true versatility of UHPLC brings many appealing advantages to bioanalysis in conjunction with high-resolution mass analysis. Low-flow systems are effective for intact protein and peptide determination with robust results, and even carry improvements in sensitivity relative to conventional LC separations. Systems such as the Thermo Scientific™ Vanquish™ Neo UHPLC system are well suited for applications as diverse as monoclonal antibody assessment, host cell protein analysis for initial process screening, and characterization of next generation biotherapeutics, such as Adeno-associated virus (AAV) proteins. Examples of these applications will be discussed herein along with key facets of UHPLC instrumentation.

ADVANTAGES OF LOW-FLOW VANQUISH NEO UHPLC PLATFORM

Optimizing the sensitivity of analytical methods is crucial to ensure sufficient analytical depth for discovery, quality control, or product safety assessments of biopharmaceutical samples. Unlike conventional LC systems, low-flow separations are specifically tailored for maximizing sensitivity in LCMS workflows when needed. In electrospray ionization, lower flow rates generate smaller sized droplets, which have an increased surface-to-volume ratio that enables more efficient ionization, which is especially beneficial for biomolecules of limited abundance. In systems like the Thermo Scientific Vanquish Neo UHPLC platform, a broad range of flow rates

are accessible including nano-, capillary-, and micro-flow, which allow analysts to balance sample throughput. The Vanquish Neo pump affords a wide operating range across six orders of magnitude, as low as 1 nL/min up to 100 µL/min for columns with inner diameters (IDs) from 20 µm up to 1 mm. All fittings in the flow path of the Vanquish Neo UHPLC are Thermo Scientific™ nanoViper™ Fingertight Fittings, which are compatible with operating pressures up to 1500 bar for rapidly achieving column equilibrium. Moreover, the flow rate and ID flexibility allow the Vanquish full compatibility with a range of different electrospray configurations, including the typical nanospray, microspray, and the analytical flow Thermo Scientific™ OptaMax™ ion source.

Beyond offering a flexible LC platform capable of high sensitivity and throughput, the Vanquish Neo brings a suite of smart capabilities that streamline method development and workflow execution. The entire platform is controlled by a single system driver coupled with a system-wide interlink to create direct communication between each component and intelligent coordination of operations. This complete system integration acts as a framework for automating common procedures that can be performed throughout the system. Additionally, the software suite includes a large library of common LCMS mobile phases for easy gradient programming, along with integrated guides for compiling new methods or the addition of other solvents. To ensure system operation within appropriate bounds, the software interface provides active notifications if the operator sets a parameter outside of the recommended range, such as when an analyst sets too small of a volume to appropriately equilibrate the column.

ANALYSIS OF REPRESENTATIVE INTACT PROTEIN MIXTURE USING LOW-FLOW LCMS

While low-flow chromatography presently has the perception of being largely used for bottom-up proteomics, wherein the smaller components of peptide digests can be easily separated with reverse phase (RP) columns, UHPLC can also separate larger intact proteins for a variety of biopharma applications.

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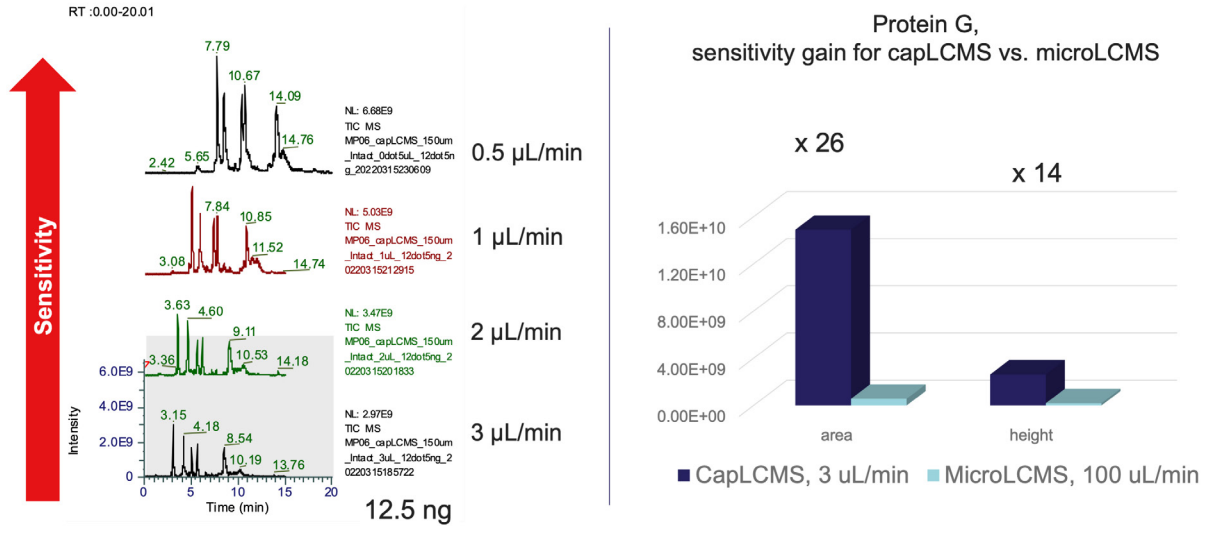
To demonstrate the utility of UHPLC for intact protein analysis, the standard Thermo Scientific Pierce™ Intact Protein Standard Mix was analyzed using a Vanquish Neo UHPLC system with the Thermo Scientific™ Obitrap Exploris™ 240 mass spectrometer. Both micro-flow and capillary-flow separations were completed on Thermo Scientific™ MAbPac™ RP columns with IDs 1.0 mm and 150 µm, respectively. When analyzed with micro-LC at 30, 50, 70, and 100 µL/min on a 1.0 mm column, the resultant peak areas maintained a linear relationship with flow rate, indicating flexibility for adjusting methods across different flow rates. Similarly, varying injection volume from 10 nL to 1 µL—which fall along the range of sample mass equivalents from 2.5 ng to 250 ng—produced linear correlation between mass injected and the resulting peak heights, as well as peak areas. Transitioning to even lower flow rates and a narrower ID 150 µm column, the same Pierce protein mixture yielded excellent separation and sensitivity.

As shown in **FIGURE 1**, the separations performed at 0.5, 1, 2, and 3 µL/min demonstrate that analytical fidelity can be maintained for intact proteins even at sub-microliter per minute flow rates and nanogram-level sample sizes. Examination of the signal for a representative analyte, Protein G, showed high sensitivity gains obtained for the low flow separations. When the flow rate was decreased by >30x from 100 µL/min down to 3 µL/min, the corresponding signals were enhanced by a factor of 26x for peak area and 14x for peak height.

FIGURE 1: Cap LCMS Analysis of Intact Proteins



EXAMPLE USES OF UHPLC IN BIOPHARMA APPLICATIONS

Beyond the analysis of neat, unmodified protein standards, the Vanquish UHPLC system is equipped to meet the challenge of characterizing samples of greater complexity, like those taken from biological matrices. This allows platforms such as the Vanquish Neo to determine product quality attributes (PQAs) of low abundant species, sparse post-translational modifications (PTMs), or from limited sample quantities. Moreover, in-depth analyses can be performed on a diverse assortment of sample classes, such as monoclonal antibody (mAb) digests, proteins from mammalian cell culture, and AAV-derived peptides.

For a representative demonstration of UHPLC characterization of mAb samples, a NIST mAb standard was subjected to an in-solution digest procedure involving reduction of disulfide bridges followed by alkylation, a cleanup stage, and trypsin digestion for 30 minutes at 37 °C. The resultant solution was analyzed via nano-flow LCMS at 250 nL/min on a 75 µm x 50 cm Thermo Scientific™ EasySpray™ HPLC column using a 2% to 35% acetonitrile gradient over 60 minutes, with the aim of detecting key PQAs for mAbs like low abundant PTMs.

FIGURE 2 displays the sequence coverage for both the NIST mAb heavy chain and the NIST mAb light chain proteins, which were obtained using triplicate 10 ng injections.

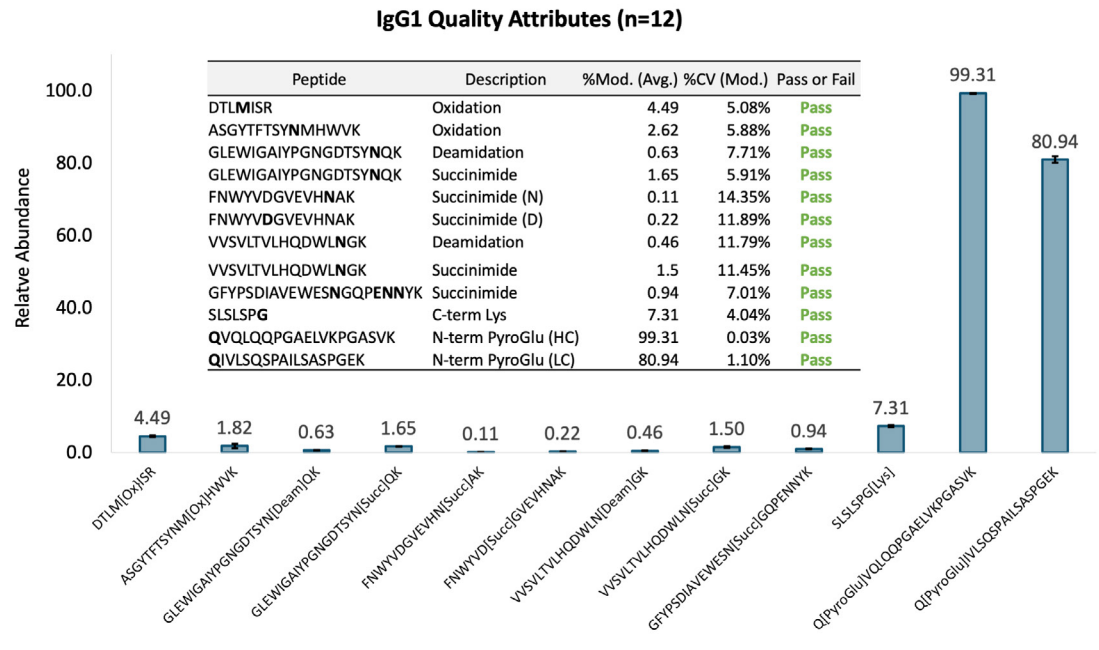
FIGURE 2: Sequence Coverage

NIST mAb injection	Sequence Coverage [%]		Number of MS peaks	
	Light Chain	Heavy Chain	Light Chain	Heavy Chain
1000 ng	96.24	98.44	229	813
750 ng	100.00	98.44	210	805
500 ng	100.00	98.44	200	776
250 ng	100.00	98.00	198	714
100 ng	100.00	97.10	167	584
50 ng	100.00	97.10	132	489
10 ng	98.59	96.21	73	274

The NIST mAb samples were analyzed using injection masses covering a full two orders of magnitude, from 10 ng to 1000 ng, while maintaining sequence coverage of >96% for both the light and heavy chains, even for 10 ng samples. A set of 10 specific peptides were then selected for evaluating system performance, which was accomplished with 100 ng injections performed in triplicate. All ten peptides yielded highly consistent values across the measured metrics, with the %CV of ≤10% for peak area, ≤2% for retention time, and ≤10% for the peak FWHM. Further assessment of a single representative peptide, SLSLSPG, found excellent linearity (R^2 = 0.9979) across 10-250 ng with limits of detection (LOD) and quantification (LOQ) of 6.63 ng and 20.10 ng, respectively. For detection of pertinent post-translational modifications as PQAs, the Vanquish Neo platform proved to be capable of detecting PTMs even present at less than 2% relative abundance, such as deamidation and oxidation, measured from 6 replicates of 100 ng NIST mAb. Higher abundance PTMs, such as a *C*-terminal Lys or *N*-glycosylation, were also identified, with %CV of <15% for each sample, indicating appreciable precision for all PTMs.

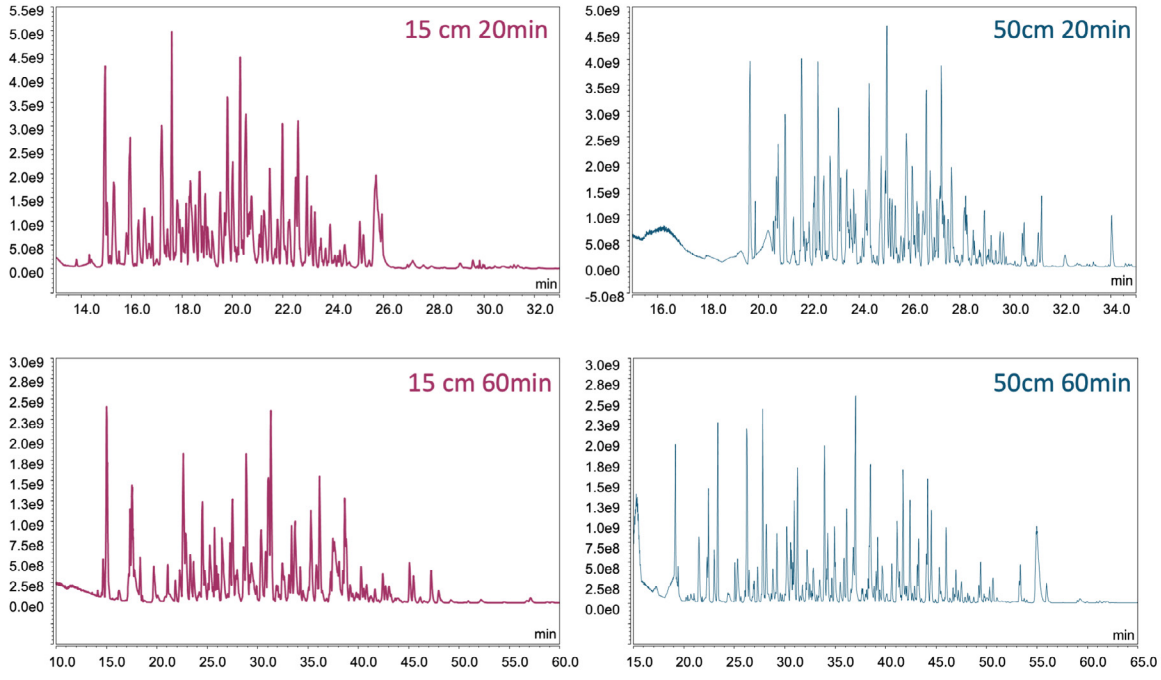
The often-limited supply of cell culture products can render granular analysis challenging, but UHPLC permits thorough product characterization without requiring large quantities of analyte. To assess how low-flow LCMS fits into the cell culture characterization process, IgG1 was spiked at concentrations according to previously reported cellular productivity levels, with Protein A purified by antibody-coated magnetic beads followed by SP3 sample preparation. The four samples created were intended to represent high- and low-IgG1-producing cells after either three days or five days of production, thereby yielding between 6 µg and 92 µg of expressed IgG1. Much like the high reproducibility in retention times observed for the mAb digest, the IgG1 peptides had excellent retention time stability, with %CVs of ≤0.5%. When processing the resultant data, a total of 138 distinct components were identified and correspond to 29 separate PQAs, including 12 different PTMs characterized within the sample (**FIGURE 3**).

FIGURE 3: PQA Analysis



Peptide	Description	%Mod. (Avg.)	%CV (Mod.)	Pass or Fail
DTL**M**ISR	Oxidation	4.49	5.08%	Pass
ASGYTFTSY**N**MHWVK	Oxidation	2.62	5.88%	Pass
GLEWIGAIYPGNGDTSY**N**QK	Deamidation	0.63	7.71%	Pass
GLEWIGAIYPGNGDTSY**N**QK	Succinimide	1.65	5.91%	Pass
FNWYVDGVEVH**N**AK	Succinimide (N)	0.11	14.35%	Pass
FNWYV**D**GVEVHNAK	Succinimide (D)	0.22	11.89%	Pass
VVSVLTVLHQDWL**N**GK	Deamidation	0.46	11.79%	Pass
VVSVLTVLHQDWL**N**GK	Succinimide	1.5	11.45%	Pass
GFYPSDIAVEWES**N**GQP**E**NN**Y**K	Succinimide	0.94	7.01%	Pass
SLSLSP**G**	C-term Lys	7.31	4.04%	Pass
QVQLQQPGAELVKPGASVK	N-term PyroGlu (HC)	99.31	0.03%	Pass
QIVLSQSPAILSASPGEK	N-term PyroGlu (LC)	80.94	1.10%	Pass

FIGURE 4: AAV Peptide Base Peak Chromatograms



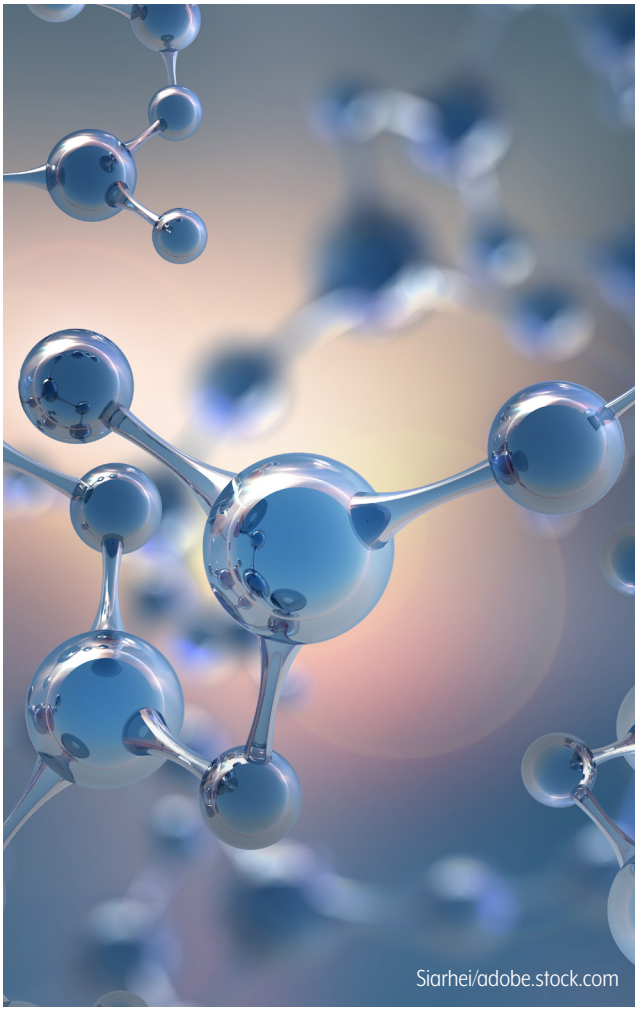
As shown in both the bar chart and table in **FIGURE 3**, two *N*-terminal pyroglutamates were the PTMs found in greatest abundance. Less abundant species were also located, such as a *C*-terminal lysine residue, oxidation, deamidation, and addition of a succinimide, of which 3 PTMs were at <0.5% relative abundance in the sample. Similarly, a set of 12 distinct *N*-terminal glycosylation PTMs were successfully identified within the peptide mixture. This demonstration of high sensitivity for low abundant species is also beneficial for host cell protein (HCP) detection, where HCPs expressed at low levels can evade detection but still cause harm to the product or to patients receiving the therapeutic products. To that end, the IgG1 samples were analyzed and a total of 141 HCPs were detected based upon a minimum of 2 unique peptides each. Of the 141 detectable HCPs, 65 were quantifiable and included both common HCPs—such as Cathepsin, which can impact product stability—as well as heat shock proteins, which may be immunogenic and therefore are critical for monitoring to maintain product safety. Following detection and quantification of HCPs, principal component analysis (PCA) can be used to differentiate between cells that tend to yield a higher level of HCPs and those with a lower HCP yield. This provides valuable process development information without requiring high volume cell cultures.

Finally, gene therapies are a growing segment of the biopharmaceutical industry wherein AAVs are employed frequently to deliver genetic material through a trimetric protein capsid containing the therapeutic cargo genome. Correspondingly, manufacturers must be able to characterize these AAV components, even though the expensive cost of AAVs can result in limited AAV sample for analysis. For an example of the AAV peptide mapping workflow performed on the Vanquish Neo, three technical replicates of 200 ng samples of AAV protein capsids containing the VP1, VP2, and VP3 proteins in a 1:1:10 ratio were separated on 15 cm and 50 cm EasySpray Neo columns using 20 minute and 60 minute gradients (**FIGURE 4**).

Each combination of gradient time and column size produced good separation and exceptional overall sequence coverage, with sequence coverages of ≥98% obtained for the three proteins in all four methods. A high degree of PTMs were found within all four sample sets, with 55 PTMs identified with the 60 minute separation on the 50 cm column as the fewest number of PTMs located, while the greatest number of PTMs located, 84, was obtained with the 15 cm column over a 60 minute gradient. Regardless of the method selected, high coverage of potential PQAs was observed using a low-flow LCMS approach.

CONCLUSION

All stages of biopharmaceutical development and manufacturing require careful product assessment, but obtaining results with adequate analytical depth can be difficult due to limited sample quantities or low target analyte concentrations. Conventional LCMS provides robust results in bioanalysis, but lacks the sensitivity necessary to detect elusive components. Low-flow UHPLC systems coupled with high-resolution mass spectrometry can meet the sensitivity needs across all segments of the biopharma industry, while also bringing the excellent precision and high throughput expected of modern LCMS instrumentation.



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