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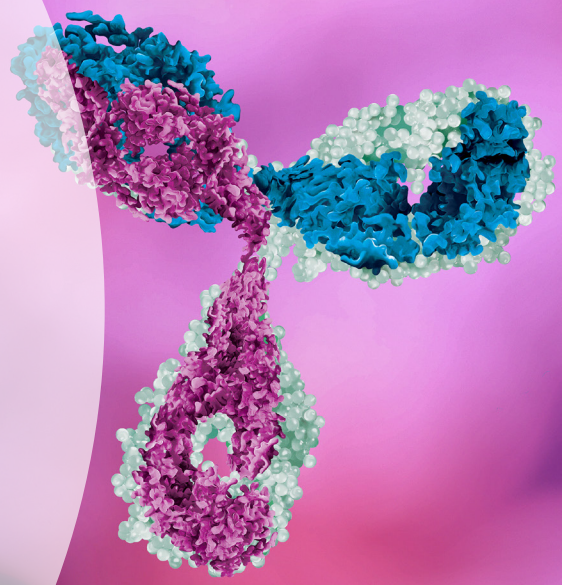
Know Your Protein, Know Your Impurities



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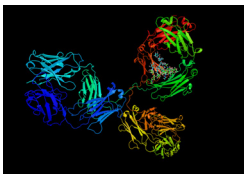
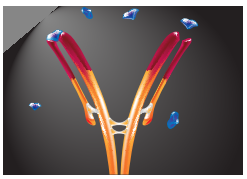


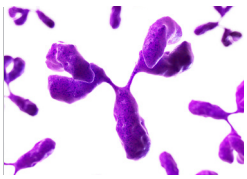
Rapid Low-Level Identification and Quantitation of Host Cell Proteins

Steve Madden and Linfeng Wu



A Fast and Sensitive Workflow for In-Depth Peptide Mapping and PTM Analysis

Linfeng Wu



Application Note: Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies

Linfeng Wu

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INTRODUCTION

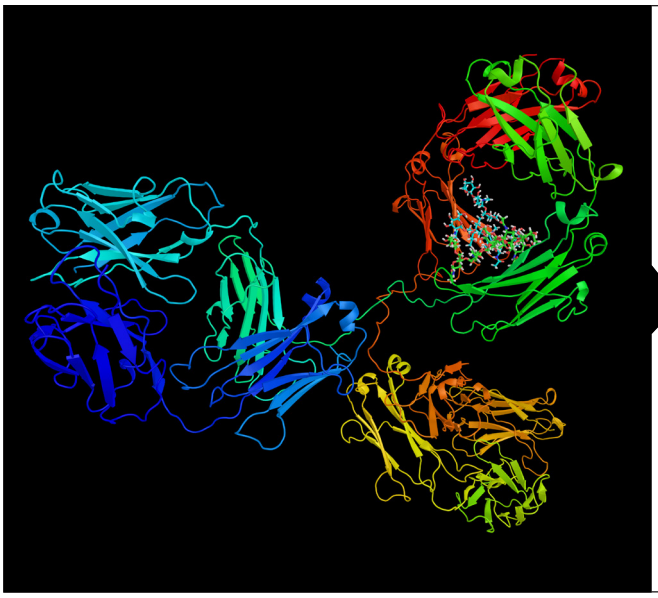
Analytical laboratories working in the biopharmaceutical segment face several challenges when characterizing monoclonal antibodies. How might improvements in technology for peptide mapping, post-translational modification (PTM) analysis, and host cell protein identification and quantitation help streamline workflows and give developers everything they need to know about their molecules?

Know Your Protein, Know Your Impurities takes an in-depth look at several workflows intended to give laboratories more information about the proteins they are working with in less time.

First, explore two automated, streamlined workflows for host cell protein (HCP) analysis using LC–MS systems: a discovery workflow that identifies proteins down to 2 ppm using an advanced LC/Q-TOF system with sample fractionation by AssayMAP Bravo and data acquisition by Iterative MS/MS, and a triple-quadrupole LC–MS workflow for targeted protein quantification at the sub-ppm level.

In a separate piece, learn how a peptide mapping workflow—including sample preparation, LC separation, MS detection, and data processing and reporting—addresses various challenges and offers a streamlined, user-friendly, and reproducible solution for laboratories.

Rounding out this ebook is an application note that demonstrates simultaneous identification and quantification of chemical-induced deamidation and oxidation on recombinant mAbs with a peptide-mapping method using an integrated workflow.



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Rapid Low-Level Identification and Quantitation of Host Cell Proteins

Steve Madden and Linfeng Wu

Evaluating automated, streamlined workflows for the identification and characterization of HCP impurities in biopharmaceuticals.

Overview

Safety, purity, and potency are basic requirements of biopharmaceutical products for human use. Inevitably, the biological systems used to generate therapeutic molecules will themselves contribute impurities to the product. Host cell proteins (HCPs) are one class of process-related impurity that may be present, even after extensive purification. Since they fit the definition of Critical Quality Attributes (1, 2), HCPs must be monitored and controlled (3). Enzyme-linked immunosorbent assays (ELISA) are widely used but have limitations. We report here on the evaluation of two HCP analysis workflows using liquid chromatography–mass spectrometry (LC–MS) systems that are designed for HCP discovery and routine analysis, respectively.

HCP Issues and Options

ELISA is a widely accepted method for quantifying HCPs in protein therapeutics, but it lacks the specificity and coverage needed to identify individual HCPs and misses those that do not react with the immune reagent. Consequently, LC–MS approaches are increasingly being used as a complement to HCP analysis. One of their advantages is that specific antibodies are not required. LC–MS systems allow the identification of individual proteins and, with its high analytical sensitivity, delivers both qualitative and quantitative information. This should enable improvements to the early purification process development for HCPs. However, the use of LC–MS is not without its challenges, primarily because of the tendency of low-abundance HCPs to co-elute with highly abundant peptides from the drug product. If the analysis is to be successful, there must be excellent separation of the peptides and the use of LC–

Figure 1: Experiment to evaluate HCP analysis.

- Spike-in UPS2 standards in purified CHO-cultured mAb before digestion
  - UPS2 mix of 48 proteins spanning 6 orders of magnitude
  - standard protein levels from 0.0004 to 313 ppm
  - mAb without UPS2 was used as a negative control
- Automated sample preparation using AssayMAP Bravo
  - Denaturation, reduction, alkylation, digestion, desalting
- 60min LC method on an AdvanceBio Peptide Plus column (2.1x150 mm)
- Data-dependent acquisition by 6545XT AdvanceBio LC/Q-TOF
  - Iterative MS/MS vs. Auto MS/MS

MS systems that have a broad dynamic range.

Evaluation: HCP Analysis Discovery Workflow

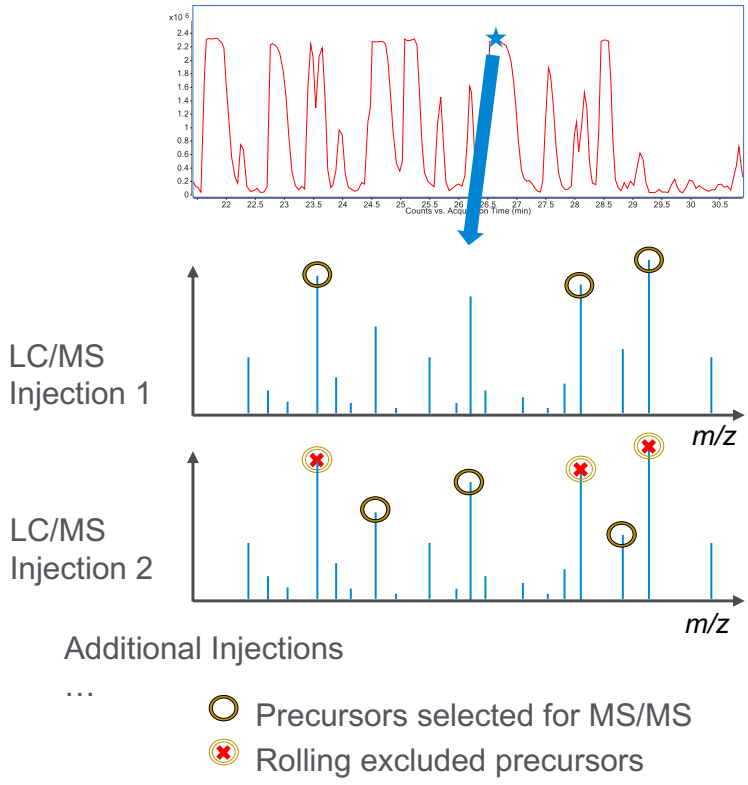
A new HCP analysis workflow comprising automated sample preparation (Agilent AssayMAP Bravo), an advanced quadrupole time-of-flight LC–MS system (Agilent 6545XT AdvanceBio LC/Q-TOF), and vendor-neutral data processing software (Protein Metrics) was evaluated for HCP discovery. The setup and samples used are summarized in **Figure 1.** Samples were generated by spiking a purified Chinese hamster ovary (CHO)-cultured monoclonal antibody (mAb), before digestion, with commercially available Proteomics Dynamic Range Standard UPS2 (a mixture of 48 proteins spanning six orders of magnitude). This produced the standard protein at concentrations from 0.0004 ppm to 313 ppm, a range that mimics the amount of HCPs present in therapeutic proteins. Unspiked mAb was used as the negative control, which is important in avoiding potential false positives arising from low-quality spectra or false assignment of endogenous peptides from proteins homologous to the spiking proteins.

The overall experimental approach for the evaluation was designed to:

- Compare iterative MS/MS with traditional auto MS/MS
- Assess the chromatography reproducibility and dynamic range of the LC/Q-TOF platform
- Examine further ways of improving identification of HCPs.

Introducing Iterative MS/MS. A key element in this workflow is the quadrupole time-of-flight LC–MS system,

Figure 2: Iterative MS/MS acquisition, An Easy way to Dig Deeper



which is already widely used for intact protein analysis, peptide mapping, and glycan method called Iterative MS/MS (see **Figure 2**), which improves the identification of low-abundance precursors. Taking the initial MS1 spectrum, the precursor ions that will gather the data are selected and are used to acquire the MS/MS spectra. Those precursor ions with customizable mass and retention time tolerances are then automatically excluded such that the next analysis goes lower and digs deeper with the next set of precursor ions. With subsequent injections, this proceeds further down the sample to find the HCPs. As a result, more precursors are automatically interrogated by LC–MS/MS.

Outcomes. The comparison of Iterative MS/MS versus auto MS/MS analysis of UPS2-spiked monoclonal antibodies and unspiked controls is shown in **Figure 3.**

Overall, Iterative MS/MS identified more unique peptide sequences per protein across a wider dynamic range than did auto MS/MS, especially for low-abundance proteins. All the spiked-in proteins above 8 ppm were identified with high confidence. At the low level, Iterative MS/MS identified two additional proteins. For other proteins, it identified one or two additional peptide sequences which is important in

Figure 3: Comparison of unique peptide sequences between Iterative MS/MS and Auto MS/MS.

Protein Accession	Protein Spiking Level (ppm)	3 Injections per Method	
		Iterative MS/MS	Auto MS/MS
mAb_HC	NA	419	382
mAb_LC	NA	201	186
ALBU_HUMAN_spike	313.0	46	43
CAH2_HUMAN_spike	137.3	19	15
CAH1_HUMAN_spike	135.6	9	9
LEP__HUMAN_spike	76.2	4	1
HBB_HUMAN_spike	74.8	12	10
HBA_HUMAN_spike	71.3	7	6
UBIQ_HUMAN_spike	50.0	6	6
CO5_HUMAN_spike	40.3	4	4
CATA_HUMAN_spike	28.1	2	2
SUMO1_HUMAN_spike	18.3	3	1
NQO1_HUMAN_spike	14.5	2	0
PRDX1_HUMAN_spike	10.4	3	0
PPIA_HUMAN_spike	9.5	4	4
MYG_HUMAN_spike	8.0	2	1

- Iterative MS/MS acquisition method identified more unique peptide sequences

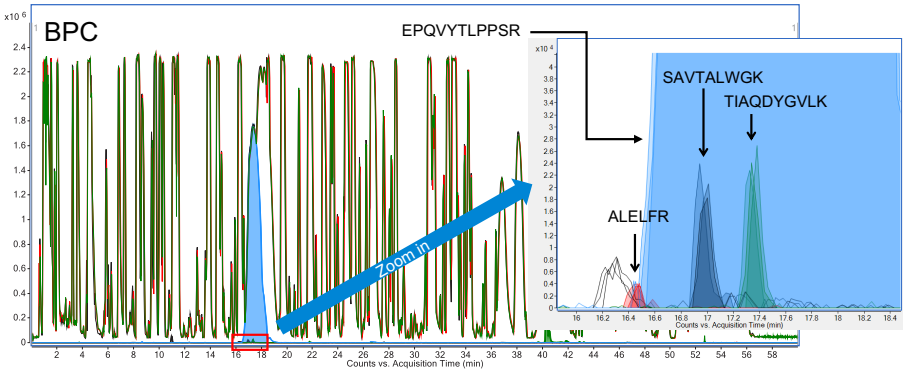
giving confidence for such low-level protein identification.

In addition to the system's ability to identify specific proteins, chromatographic reproducibility and the dynamic range of the platform are critically important. **Figure 4** shows the overlaid chromatograms of the base peak chromatogram (BPC) and extracted ion chromatogram (XEIC) of four co-eluting precursor ions from three Iterative LC–MS/MS runs.

Each injection loads 32 µg of sample. Peak intensity and retention times are highly reproducible, peak shape is symmetric even for very abundant peptides, thus demonstrating that column loading capacity is high. The zoomed-in area shows four peptides across a large dynamic range. The intensity ranges from 6.76E+0.3 to 1.38E+0.8, giving a spectral dynamic range of more than four orders of magnitude. Reproducibility is good, even for the lowest peptide (from myoglobin), which was spiked at 8 ppm. This all suggests that MS1 intensity extracted from Iterative MS/MS has the potential for use in label-free quantification.

To assess the label-free quantitative capabilities of this workflow, the EIC for each identified UPS2 protein was normalized to the highest protein in the sample, and the values were exported from the data analysis software. The normalized EIC showed correlation with the real spiking levels, illustrating that these values can be used for semi-quantification of the HCP (see **Figure 5**). As expected, no UPS2 spiking proteins were identified in the control sample, demonstrating the speci-

Figure 4: Overlaid chromatograms of three LC–MS/MS runs.



In-spectrum Dynamic Range
~ 4.3 orders of magnitude

Peptide	Mass Error (ppm)	Intensity	Intensity %RSD	Protein spiking Level (ppm)	Protein Name
ALELFR	-1.1	6.76E+03	10.3%	8	Myoglobin
TIAQDYGVLK	-1.8	1.51E+05	6.2%	10.4	Peroxiredoxin 1
SAVTALWGK	4.8	1.36E+05	6.0%	74.8	Hemoglobin subunit beta
EPQVYTLPPSR	1.0	1.38E+08	1.2%	NA	mAb

ficity of this approach. Having also shown that the data acquired by one-dimensional LC–MS/MS can identify all the spiking proteins above 8 ppm, it then becomes possible to perform label-free quantification for both cross-sample comparison as well as semi-quantification of the protein level in the same sample.

Further improving identification. Further improvements to identification can be achieved by adding peptide fractionation at the sample preparation stage (Agilent AssayMAP Bravo with peptide fractionation cartridge). Using the default setting of high-pH reversed-phase fractionation (HPRP) on a reverse phase cartridge, the same samples were run again, and all fractions were analyzed by Iterative MS/MS, with two injections per fraction. The table in **Figure 6** compares the number of peptide sequences identified by one-dimensional LC–MS/MS with those identified by adding HPRP into the process. Overall, more unique peptides were identified with the addition of HPRP. New proteins were seen, including several at the single-digit ppm level, and it was possible to identify all proteins above 2 ppm, with clear demonstration of good spectral quality at that low level. The experimental work also showed the identification of 138 endogenous CHO proteins using HPRP fractionation, compared with 38 for one-dimensional LC with three Iterative MS/MS injections (no fractionation), a three-fold increase.

In conclusion, the workflow described for HCP discovery demonstrated:

- Excellent chromatography reproducibility with a broad dynamic range

Figure 5: Label-free quantification.

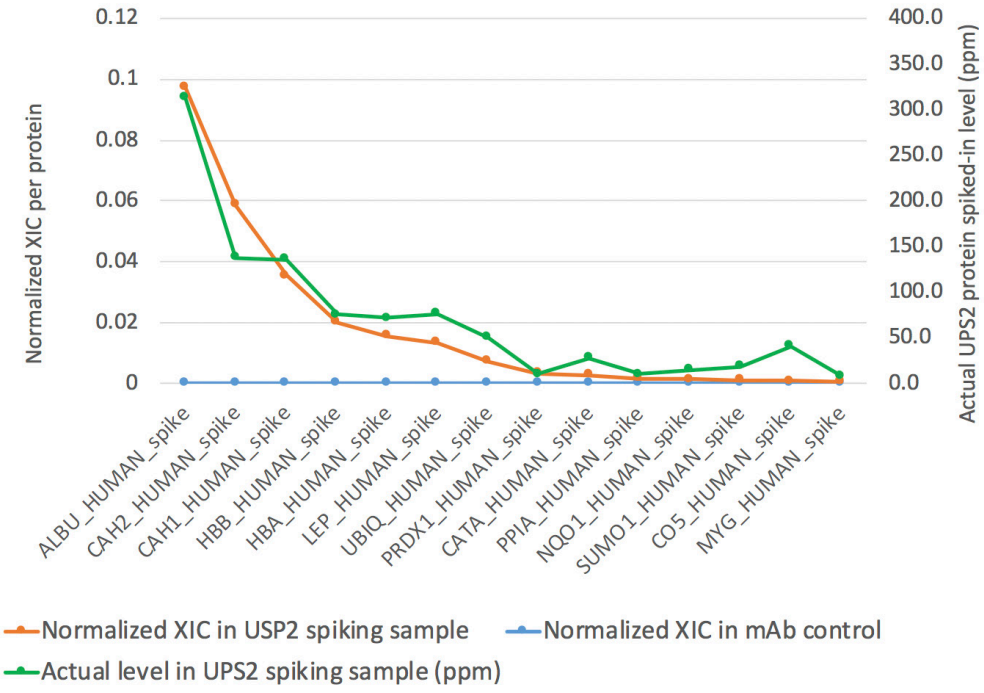


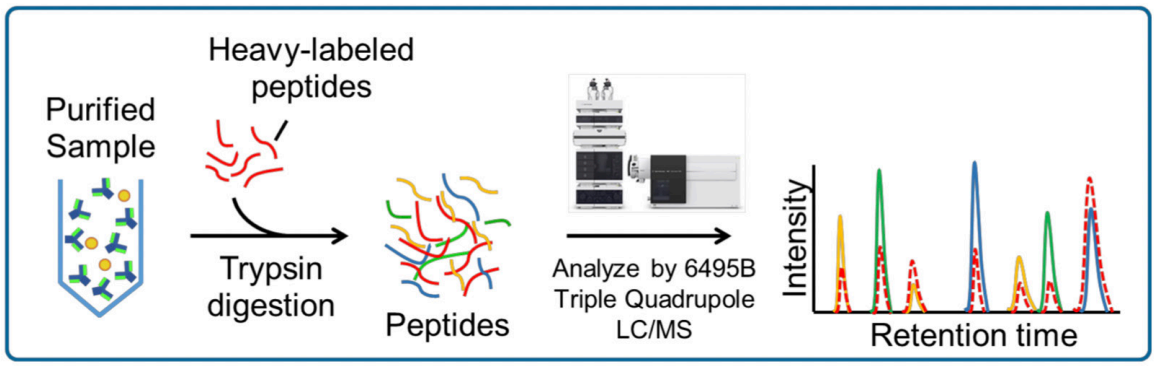
Figure 6: Improve ientification by fractionation using AssayMAP Bravo.

Protein Accession	Protein Spiking Level (ppm)	# Unique Peptide Sequences	
		1D LC-MS/MS	HPRP + LC-MS/MS
ALBU_HUMAN_spike	313.0	46	**79**
CAH2_HUMAN_spike	137.3	19	**32**
CAH1_HUMAN_spike	135.6	9	**15**
LEP_HUMAN_spike	76.2	4	**6**
HBB_HUMAN_spike	74.8	12	**22**
HBA_HUMAN_spike	71.3	7	**14**
UBIQ_HUMAN_spike	50.0	6	**9**
CO5_HUMAN_spike	40.3	4	**6**
CATA_HUMAN_spike	28.1	2	**14**
SUMO1_HUMAN_spike	18.3	3	**11**
NQO1_HUMAN_spike	14.5	2	**8**
PRDX1_HUMAN_spike	10.4	3	**9**
PPIA_HUMAN_spike	9.5	4	**11**
MYG_HUMAN_spike	8.0	2	**2**
CYB5_HUMAN_spike	7.6	0	**2**
EGR_HUMAN_spike	3.0	0	**1**
SYHC_HUMAN_spike	2.7	0	**5**
KCRM_HUMAN_spike	2.0	0	**3**

← **2 ppm**

Identification of all the spiking proteins above 2 ppm with Iterative MS/MS acquisition

Figure 7: Targeted protein quantification by isotope dilution strategy and MRM.



- The use of data-dependent acquisition methods for easy data analysis
- The ability to perform simultaneous protein identification and semi-quantitation
- An automated Iterative MS/MS approach for deeper protein identification
- Identification of all spiked proteins down to 8 ppm with one dimensional LC-MS/MS
- Detection down to 2 ppm with the addition of HPRP fractionation using AssayMAP Bravo.

From Discovery to Target Protein Quantification

Once HCP discovery analysis is complete, the logical next step is routine analysis to monitor the concentrations of those proteins. Multiple reaction monitoring (MRM)-based methods are commonly used for this type of routine analysis because of their high sensitivity and robustness. In a typical workflow for targeted protein quantification by isotope dilution strategy and MRM (see **Figure 7**), several peptides are chosen for protein quantification. These heavy isotope-labeled peptides are added into the sample and the digested sample is analyzed by a triple-quadrupole LC–MS instrument.

Optimization of the LC/MRM method is critical to success. One convenient optimization tool for LC–MS (Agilent Automation Tool) is integrated within the freely available Skyline software (4), developed at the University of Washington. The software was originally founded as part of the National Cancer Institute's Clinical Proteomics Tumor Analysis Consortium (CPTAC), a multi-lab effort aimed at assessing targeted proteomics as a clinical technique. The software was developed to provide a common platform for integrating and evaluating results from different laboratories working with triple-quadrupole systems from a range of vendors and has become widely used in the proteomics community. The automation tool allows users to select all the parameters for the steps typically performed during optimiza-

tion and leads to the creation of a method with optimized collision energy, retention time windows for targeted peptide transitions, and optionally can run the samples. The method can then be used directly on the user's Agilent triple- quadrupole LC/MS system.

Evaluation: HCP Analysis Workflow for Targeted Protein Quantification at the Sub-ppm Level

Experimental work was carried out to evaluate a MRM workflow for targeted HCP quantification. The workflow uses automated sample preparation as before, detection with a triple- quadrupole LC/MS (Agilent 6495B), and automated data processing (Agilent MassHunter). Samples were similar to those used in Evaluation 1. The same UPS2 proteomics standard was spiked into the mAb, followed by reduction, alkylation and trypsin digestion. Then, after the addition of stable isotope-labeled (SIL) peptide, samples were analyzed by LC–MS equipment.

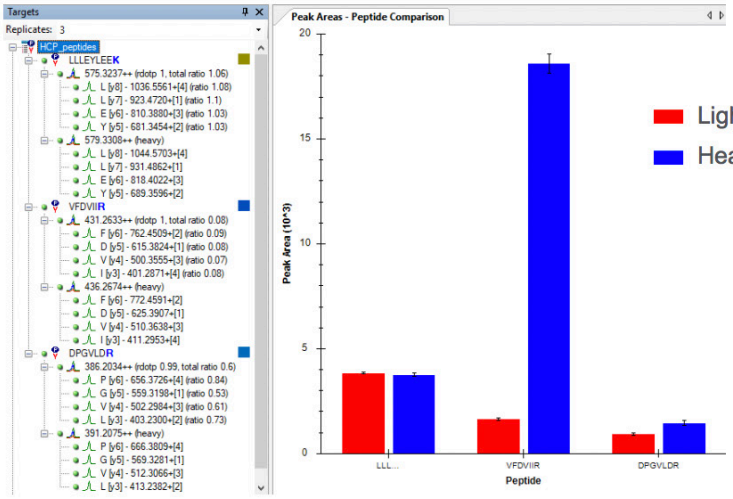
The three peptides chosen to evaluate this targeted HCP quantitation workflow match to two of the UPS2 proteins, SUMO1 and SYHC, together with the endogenous CHO protein S100-A11. Standard curves for these three peptides in the range 6.25 attomole per µg to 125 femtomole per µg showed good linearity and reproducibility. Detection and quantification levels were as follows, where LOD is the lower limit of detection and LLOQ lower limit of quantification:

Absolute quantification. For HCP analysis, absolute quantification of the targeted proteins is essential. The gold standard for absolute quantification by mass spectrometry is the addition of a SIL version of the targeted protein to the sample at an early stage of sample processing. However, equivalent SIL proteins are often not commercially available and the cost of custom synthesis may be prohibitive. Consequently, heavy peptides containing the same amino acid sequence of the trypsin peptides have also been used for absolute protein quantification.

Protein	LOD (ppm)	LLOQ (ppm)
SUMO1	Data not available	0.24
SYHC	0.35	0.70
Protein S100-A11	0.065	0.13

In the SIL approach to evaluation reported on here, peptides were generally added to the sample following protein digestion, making it impossible to correct for protein losses during sample preparation. Similarly, no account is taken of the yield of the protein digestion step. Thus, the calculated protein absolute concentration using heavy peptides often underestimates the real protein concentration. **Figure 8** shows the light and heavy peptide ratios in the samples with calculations of protein abundance based on the heavy peptide spiking levels and these ratios. As expected, there is approximately 50% underestimation of the standard proteins, consistent with several published reports. This indicates that when using the heavy isotope-labeled peptide approach for absolute protein quantification, recovery

Figure 8: Absolute quantification of targeted proteins.



Targeted Protein	SUMO1_HUMAN	SYHC_HUMAN	Protein S100-A11 (G3HUU6)
Protein MW	38,815 Da	58,233 Da	11,241Da
Peptide sequence	LLLEYLEEK	VFDVIIR	DPGVLDR
Spiked protein level	18.3 ppm	2.7 ppm	NA
Measured protein level	10.1 ppm	1.2 ppm	1.6 ppm

of the targeted peptide from the sample preparation must be carefully evaluated. Reproducible sample preparation in the laboratory will also be essential for accurate cross-sample and cross-batch comparison.

Conclusion

The evaluation of two workflows for HCP analysis using LC–MS highlights their role in automating and streamlining the identification and characterization of HCPs. The first, a discovery workflow, was shown to identify all proteins down to 2 ppm using an advanced LC/Q-TOF system with sample fractionation by AssayMAP Bravo and data acquisition by Iterative MS/MS. Vendor-neutral data processing software enables simultaneous protein identification and semi-quantification. The second workflow uses a triple-quadrupole LC–MS for targeted protein quantification at the sub ppm level. The importance of optimizing LC/MRM method development and the availability of an automation tool within the freely available Skyline software were also discussed.

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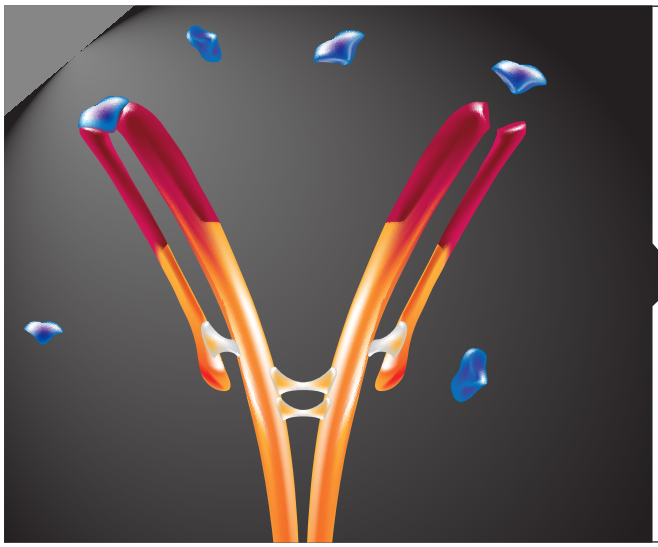

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A Fast and Sensitive Workflow for In-Depth Peptide Mapping and PTM Analysis

Linfeng Wu

The characterization of monoclonal antibodies (mAbs) typically includes analyzing intact protein mass, protein aggregation, C-terminal lysine processing, and protein disulfide bond shuffling. Scientists also characterize post-translational modifications (PTMs) such as deamidation/oxidation and glycosylation using various chromatography separation methods. For example, reversed-phase chromatography may be used for peptide mapping, hydrophilic interaction liquid chromatography for glycan analysis, ion-exchange chromatography for charged variant analysis, size-exclusion chromatography for aggregation analysis, and mass spectrometry (MS) for detection.

Liquid chromatography–mass spectrometry (LC-MS) is the major platform for monitoring many key critical quality attributes. For example, it is used to determine intact protein mass, peptide sequence confirmation, and glycan identification. It is the most popular method today for peptide mapping. For peptide mapping, sensitivity, reproducible identification and quantification with a quick turnaround time is often preferred.

To address these challenges, Agilent Technologies created the Agilent Peptide Mapping Workflow solution, which includes sample preparation, LC separation, MS detection, data processing and reporting.

Workflow Step 1: Sample Preparation Using AssayMAP Bravo

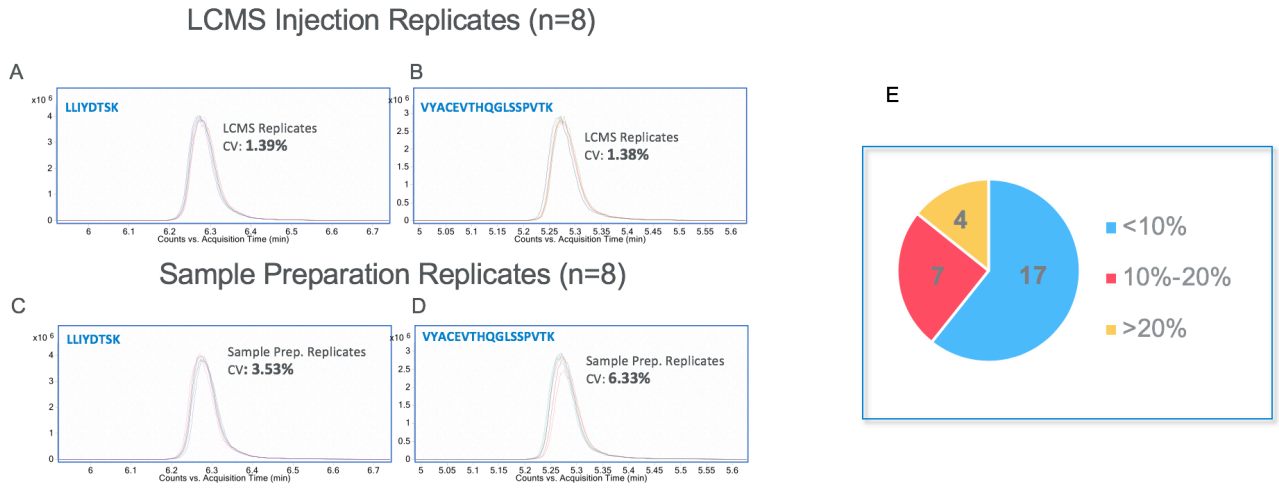
For peptide mapping, the value of sample preparation automation is especially important because it often necessitates analysts to use various enzymes for digestion, perform replicate analysis, and compare multiple sample results. To obtain reliable data, ensuring reproducible sample preparation is critical.

Within the Agilent Peptide Mapping Workflow, the Agilent AssayMAP Bravo platform is designed mainly for improved protein sample preparation, and is composed of four parts:

- The Bravo automated liquid handler.
- Microchromatography cartridges, which are disposable packed bed columns with a 5-µL bed held in place by a membrane. These cartridges are packed with a variety of solid medium for all common protein sample preparation workflows including protein purification, peptide desalting, strong cation exchange and reversed-phase fractionation, and phosphopeptide enrichment.
- 96 positive displacement syringes engage the AssayMAP cartridges, creating a closed system that enables high-precision flow rate control across the resin bed. Flow rate control, coupled with the packed bed columns, enables sample preparation with chromatography quality.
- Intuitive software and a simple user interface are designed for analytical scientists, not automation experts. No programming experience is needed. Scientists simply select the appropriate protocol from a pulldown menu targeting a few key parameters such as number of samples and sample volume, and then press "go."

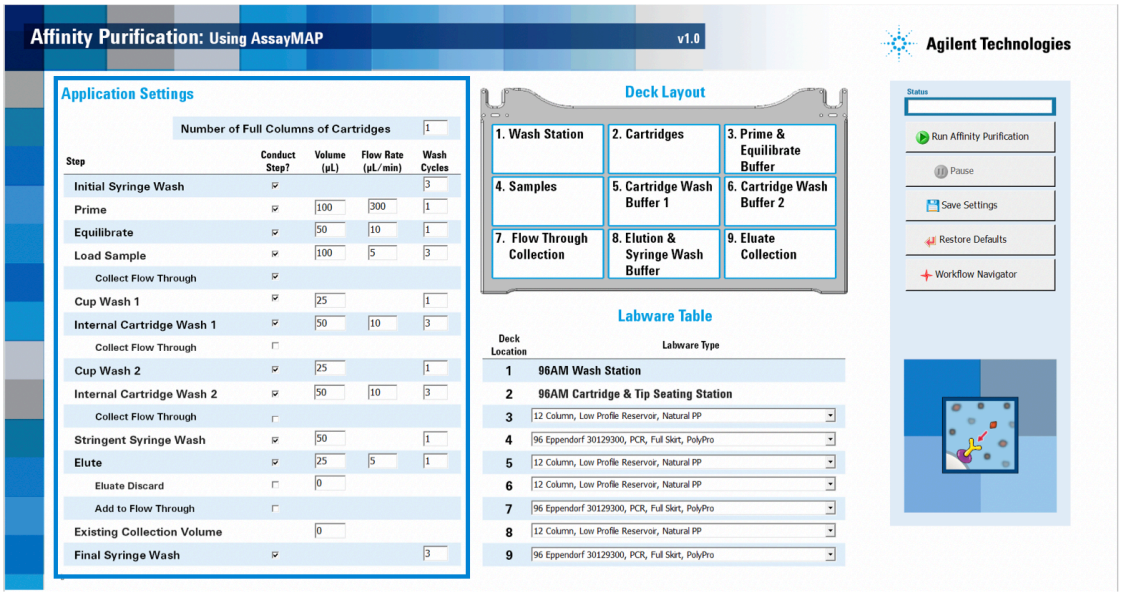
AssayMAP sample prep reproducibility. The reproducibility of sample preparation with the AssayMAP Bravo platform using 5 µg of NIST mAb is shown in **Figure 1.** Panels A and B show overlaid extracted ion chromatograms of two representative peptides from replicate injections of the same sample. Both peptides show the coefficient of variation (CV) less than 1.4% by replicate injections. Panels C and D show the overlaid EIC of these two peptides from replicate sample preparation by AssayMAP Bravo platform. The CV only

Figure 1: Peptides quantitation reproducibility using AssayMAP.



Extracted Ion Chromatography (EIC) of Representative Peptides Digested from 5 µg NIST mAb. Panel A & B: LCMS injection replicates of the same sample (n=8) and Panel C & D: Sample preparation replicates (n=8). Panel E: Pie chart of the number of peptides with %CV less than 10%, between 10% and 20% and over 20%.

Figure 2: AssayMAP Bravo user interface.



Features
- Designed for bench scientists
- Easy-to-use with minimal inputs required
- Harmonized interfaces across applications

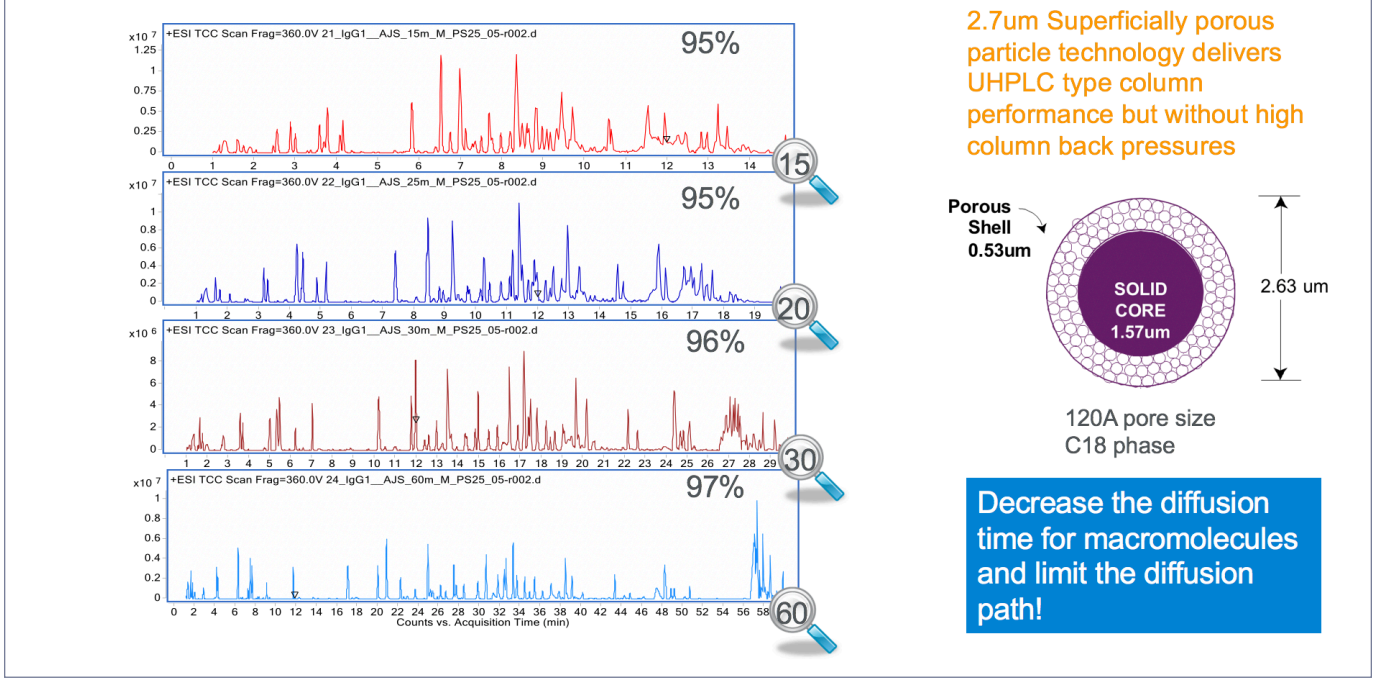
Benefits
- Minimal training required
- Rapid implementation
- Simple protocol transfer between sites

slightly increased to 3.53% and 6.33%, respectively. Panel E shows that of the 28 quantified peptides, 17 peptides had a CV less than 10%, seven peptides had a CV of 10–20%, and four peptides had a CV higher than 20%.

AssayMAP Bravo user interface. In addition to peptide digestion, AssayMAP Bravo also provides a series of preconfigured protocols including antibody purification, immunoaffinity purification, phosphopeptide enrichment, N-glycan sample preparation, peptide cleanup, and the peptide fractionation.

The user interface (**Figure 2**) for affinity purification is designed to be easily used by bench scientists. Users only need to input a few key parameters required for their specific experiment. Since the interface is very similar across different applications, after becoming familiar with one application, moving to another one is very intuitive. Using the software requires minimal training so it can be readily adopted in the laboratory. Protocol transfer between users and sites is simple and dramatically decreases the risk that key information gets lost or miscommunicated. The ability to rapidly setup and run experiments with automation allows scientists to do more important work while the automation handles the tedious tasks.

Figure 3: Reducing peptide mapping time without losing resolution.



Workflow Step 2: Peptide Mapping Workflow Separation

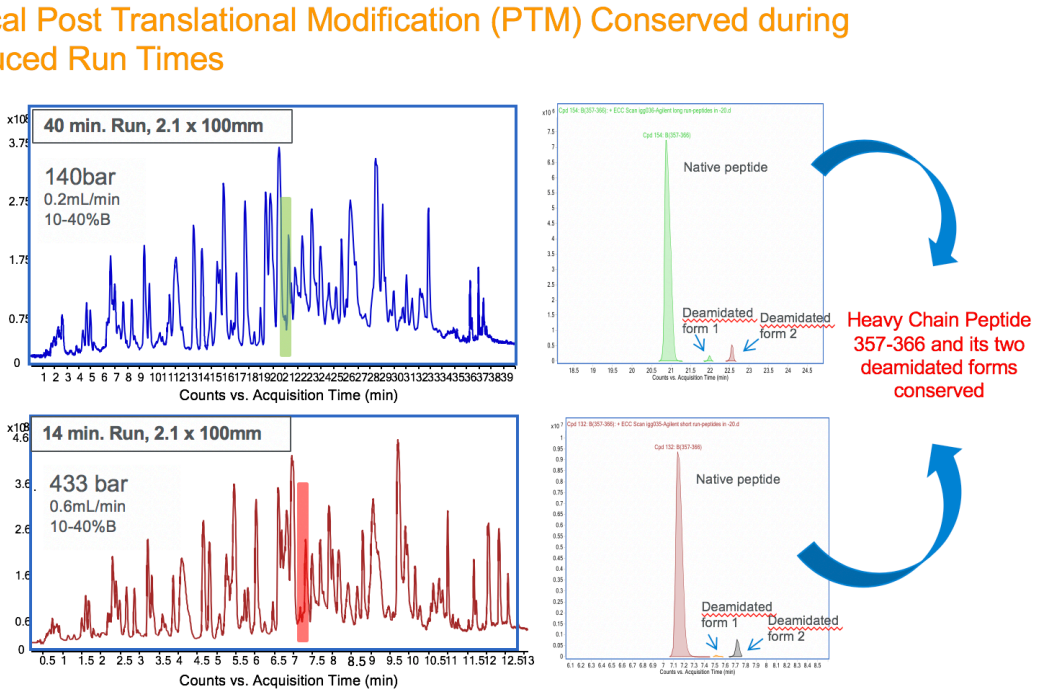
The next part of the peptide mapping workflow is separation. The system used is Agilent 1290 Infinity II LC System, which increases efficiency on three fronts:

- The new design of 1290 Infinity binary pump and new detector provides higher resolution separation and retention time precision.
- Thousands of samples placed in the autosampler can be run in a sequence. The dual-needle design also provides a faster injection cycle.
- Method transfer from legacy equipment to 1290 Infinity II is optimized. As a result, there is no need to revalidate existing methods on the new instrument.

Instrument runtime versus resolution. Another critical component in peptide mapping is the instrument runtime. The Agilent AdvanceBio Column fills a critical gap in biotherapeutics' characterization. It allows users to generate both rapid and highly efficient peptide maps at a traditional LC system pressure.

The AdvanceBio Peptide Mapping Column particle is 2.7 µm with a solid core and is superficially porous. With this technology, this column achieves substantial improvements in peptide mapping during very fast runtimes and low system pressures (**Figure 3**). At the same time, it can still maintain high peak performance efficiency. During reversed-phase LC–MS analysis, long gradients are typically used to enable higher resolution and to ensure

Figure 4: Rapid LC-MS IgG tryptic digest peptide mapping.

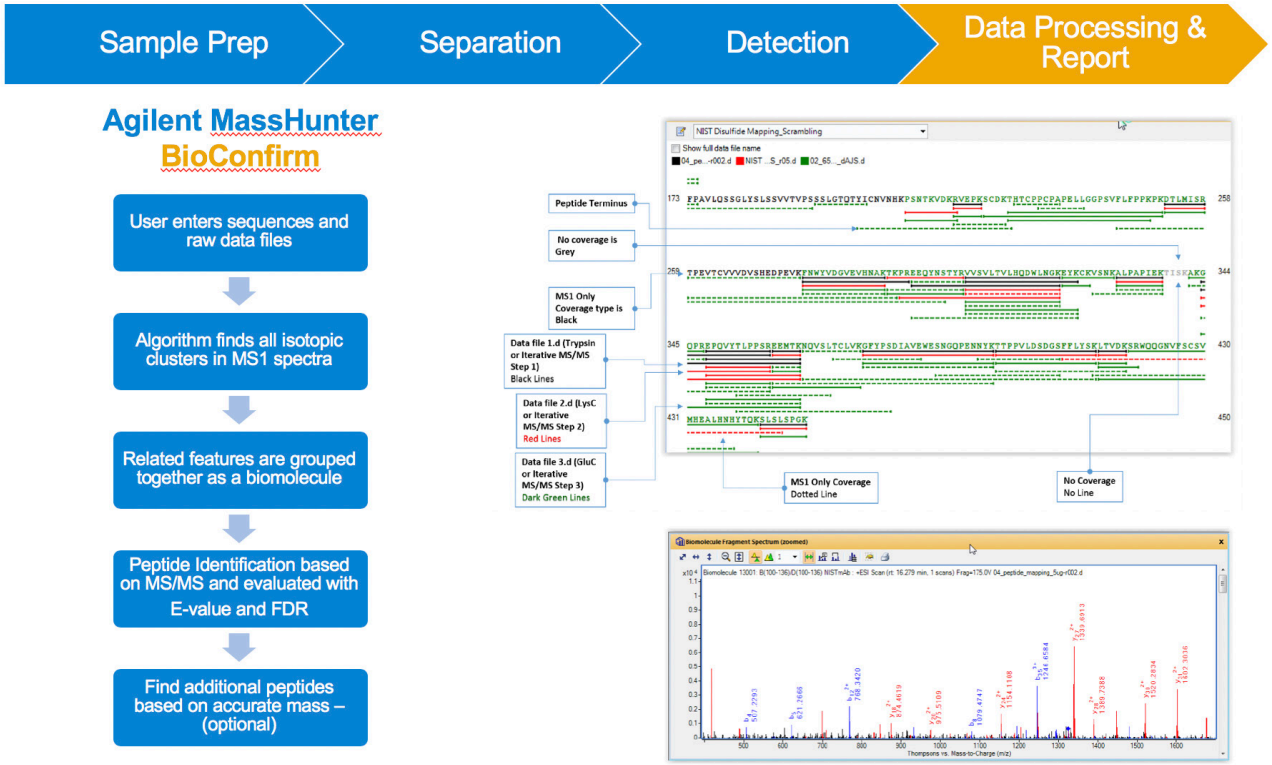


modified peptides are detectable from their native form, as well as other closely eluting the peptides. To reduce runtime, steeper gradients are often employed. However, this is typically at the cost of lost resolution and overall mapping quality. It is, therefore, important not to sacrifice separation quality or compromise the mass spectral information when runtimes are reduced.

The left side of Figure 3 shows the results from an experiment performed to compare sequence coverage using different gradient times. When the gradient time was reduced from 60 minutes to 30 minutes, 20 minutes, and 15 minutes, the sequence coverage was maintained at a high level (95–97%).

Rapid LC–MS IgG tryptic digest peptide mapping. In another experiment, peptide mapping total ion chromatograms from AdvanceBio peptide mapping column during a 40-minute run and a 14-minute run were compared. In this comparison, the column dimensions remained constant and the gradient slope was adjusted to keep the ratio of gradient change in each column volume equal, thereby ensuring that the chromatography selectivity remained similar. The short gradient was run with a higher flow rate. The TIC comparison (**Figure 4**) showed that the resolution selectivity and separation performance had not been compromised during the reduced runtime of 14 minutes. The pressure at the higher flow rate, which is a 0.6 mL per minute, is around 400 bar and very manageable by this LC system. In comparison with the 40-minute run, the

Figure 5: Agilent MassHunter BioConfirm.



native and the deamidated species in the 14-minute run was still well separated and readily identified by the Q-TOF analysis.

Workflow Step 3: Detection Using the Agilent 6545XT AdvanceBio LC/QTOF System

The third part in the workflow is MS detection. The Agilent 6545XT AdvanceBio LC/Q-TOF system is designed to handle multiple workflows in biopharmaceutical characterization. Examining intact proteins, mapping peptide sequences, identifying PTMs, and analyzing glycans can all be accomplished on the same instrument. The Agilent SWARM autotune allows users to quickly optimize the instrument performance for different applications.

Additional features on the Agilent 6545XT AdvanceBio LC/Q-TOF system provide improved peptide mapping. The capillary removal valve allows capillary cleaning without venting the instrument, making basic instrument maintenance fast. The advanced decision engine, which selects precursor ions for tandem MS fragmentation, is now performed by the logic in the software, which improves Auto MS/MS acquisition performance. A new acquisition mode, Iterative MS/

MS, allows identification of low abundant peptides with tandem MS/MS and in this mode, protein digest sample is subjected to a multiple LC–MS/MS analysis as needed.

Workflow Step 4: Data Processing and Reporting Using MassHunter BioConfirm

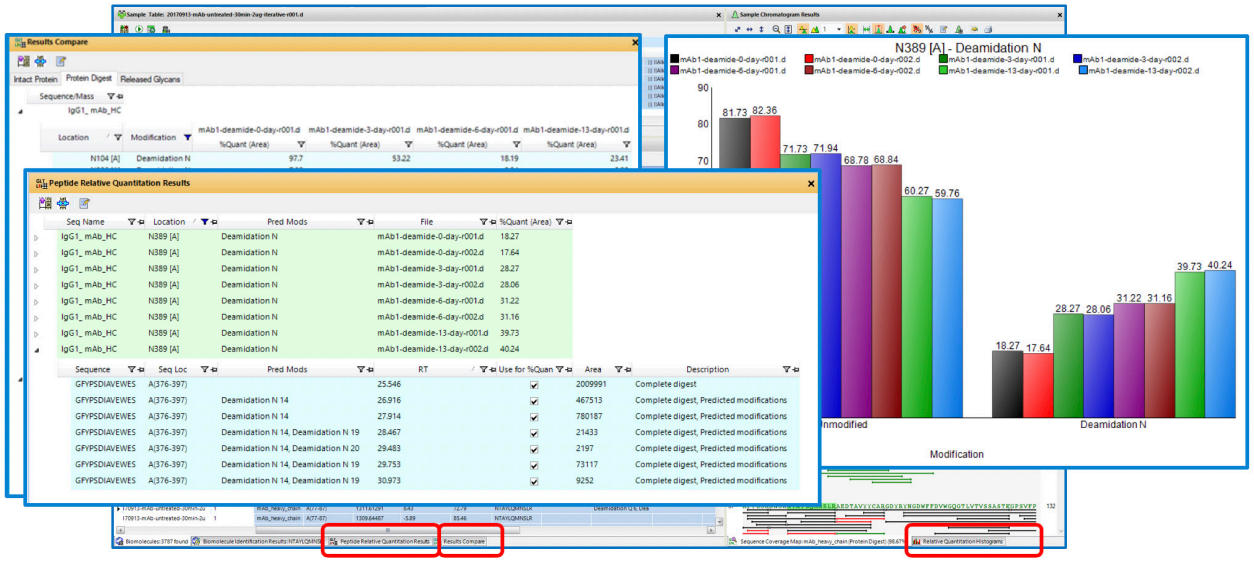
For data processing, the Agilent MassHunter BioConfirm dedicated software can handle the entire protein analysis, peptide mapping, and glycan analysis. For peptide mapping, the protein digest workflow is first selected and will automatically mine the data and match identified peptides to the sequence. Peptide identification based on MS2 are evaluated by e-value and false discovery rate (FDR). On the sequence coverage map (**Figure 5**), peptides identified by MS2 patterns are solid lines and those identified by MS1 mass accuracy are dotted lines. The modifications for consideration, such as oxidation and deamidation, are defined in the profile. In addition, users can select the enzyme that was used for protein digest. In the next step, users select which found features to display. They can choose to display peptide identified only based on MS2 features or to display peptides found by MS2 features and by MS1 only. In the following steps, users select MS1 and MS2 mass tolerance, FDR cutoff, and peptide matching rules. Displayed are missed cleavage, maximum number of matches per molecule, peptide length, truncation specificity, and maximum modification.

BioConfirm software allows both sample centric and peptide centric data navigation. A panel displays the list of uploaded data files and users can select multiple data files from the list for displaying data. Other panels displays the biomolecule result table MS spectra, fragment spectra, which allows users to navigate from mapping result to mass spectra. Linked MS1 and MS2 spectra and extracted compound chromatograms show synchronously when clicking the biomolecule in the result table. A sequence coverage map appears as well.

BioConfirm software also offers faster relative quantification of peptide post-translational modification (**Figure 6**). On the bottom of the display screen are several tabs. One of the tabs is the result compare table, which offers an overview of the PTM quantitation results for all the modified residues across all the selected sample files. It calculates the percentage peak area of the modified peptide relative to the sum of native and modified forms and summarize the comparison result in the table. In addition to the result compare table, BioConfirm also visualizes the quantitation result with a histogram, which is in the same panel as the sequence coverage map.

For a peptide mapping workflow, the therapeutic protein is first digested to produce peptide fragments. Due to the complexity of the sample, scientists sometimes inspect peptide forms used for PTM quantification. BioConfirm software generates a peptide relative quan-

Figure 6: BioConfirm relative quantitation of peptide PTMs.



titation results table, which lists all the modified residues and their corresponding peptides from each data file.

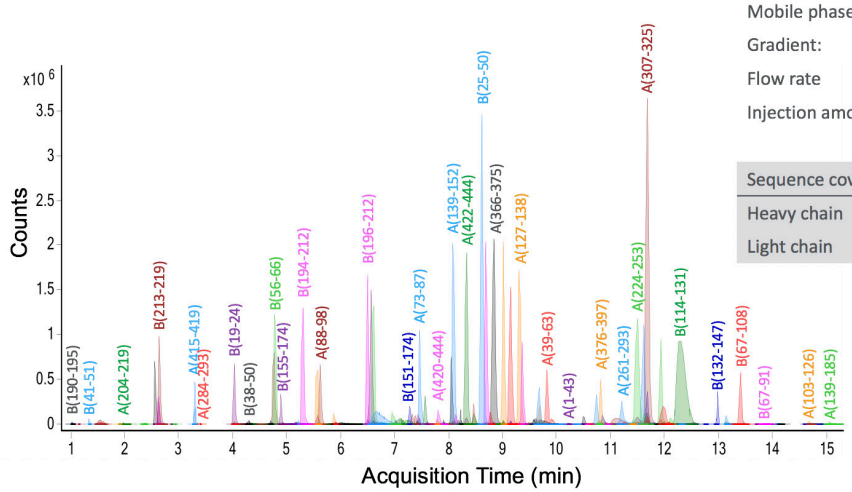
BioConfirm applies a series of rules related to protein digestion and PTM analysis to automatically determine the peptide forms used for PTM quant. The software also allows users to adjust the peptide selection. The percolated percentage of quant, the result compare table, and the histogram will be synchronized and will update the result immediately.

Peptide Mapping Data

The peptide mapping data using this workflow shows the extracted compound chromatogram of a cultured purified mAb digested with trypsin (see **Figure 7**). Identified peptides were labeled with corresponding sequence numbers in antibody light and heavy chain.

With a 15-minute gradient, peptide identification with 0.1% false discovery rate, 96.24% of the heavy chain and 98.63% light chain sequences were covered. The only missed peptide in light chain is the three-amino acid peptide. Relaxing false discovery rate to 1%, a missed cleavage peptide overlapping with the short peptide was identified, improving the sequence coverage of light chain to 100%. For the heavy chain, the difficulty is in two regions. One region contains four amino acids. The other region is a 13-amino acid peptide containing N-glycosylation site. The glycan of a glycopeptide tends to fragment first leading to poor peptide backbone fragmentation, so the resulting identification score generally is much

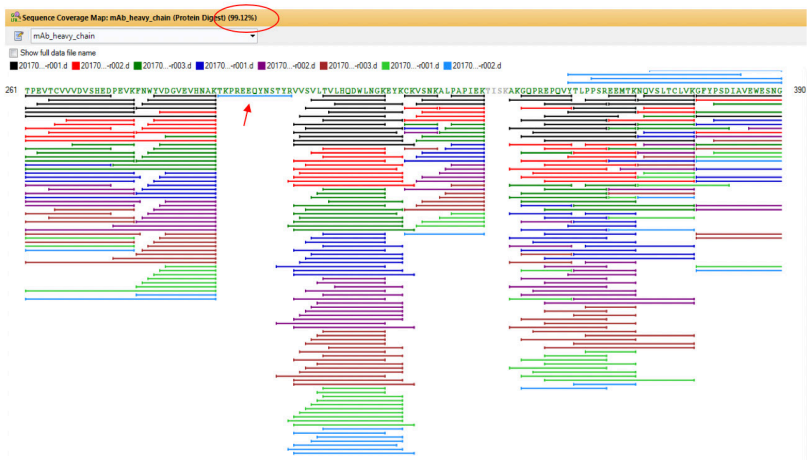
Figure 7: Peptide mapping of mAb digest.



Column:	AdvanceBio Peptide Mapping, 2.1 x 150 mm
Mobile phase A:	Water + 0.1% FA
Mobile phase B:	Water/acetonitrile (10:90 v:v) + 0.1% FA
Gradient:	3% - 40% B, 15min
Flow rate	0.4 mL/min
Injection amount:	0.6 µg

Sequence coverage	0.1% FDR	1% FDR
Heavy chain	96.24%	96.24%
Light chain	98.63%	100%

Figure 8: Iterative MS/MS acquisition for improved sequence coverage.



Overlaid sequence coverage of eight LC/MS/MS analyses, including

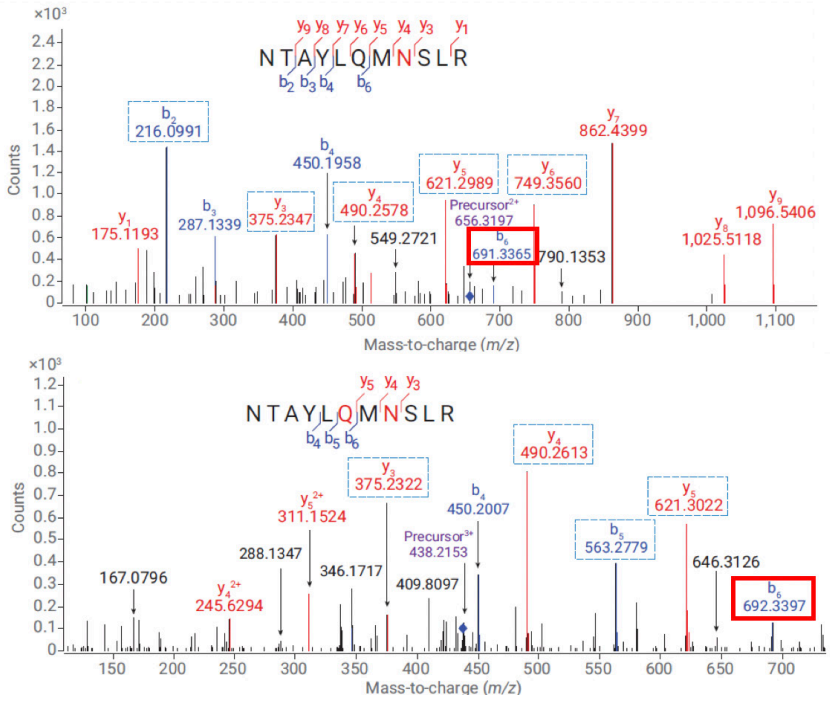
- 3 injections, 15 min, Auto MS/MS
- 3 injections, 30 min, Auto MS/MS
- 2 injections, 15 min, Iterative MS/MS

lower than for an unmodified form. In the unmodified form, it generally exists at a very low level in the sample.

Improved Sequence Coverage

An overlaid sequence coverage map (**Figure 8**) on the typical region on a mAb heavy chain shows peptide identification from eight LC–MS/MS analysis including

Figure 9: Deamidation N8 versus Q6N8.



two sets of triplicate Auto MS/MS analysis using 15- minute and 30-minute gradient, respectively. It also included two-injection Iterative MS/MS with a 15-minute gradient. Peptides identified from different data files were coded with different colors. The nonglycosylated peptide sequence location 294 to 306 was not identified in any of the replicate Auto MS/MS analysis or the first injection of Iterative MS/MS, and it only identified in the second injection of iterative MS/MS, increasing the sequence coverage of the heavy chain to 99.12%. This result demonstrates the advantage of Iterative MS/MS acquisition for in-depth peptide mapping and provides an alternative approach to identify low-abundant peptides.

Characterization of Peptide PTM

In addition to sequence coverage, characterization of peptide PTM is critical during drug development and manufacture. Protein oxidation and deamidation are two of the prominent PTMs to have biological significance. Methionine oxidation results in +15.9949 Dalton mass shift. As for deamidation, asparagine or glutamine is susceptible to a spontaneous, not enzymatic, reaction. The reaction results in a +0.984 Dalton mass shift. A similar reaction can occur in aspartate light chain, yielding a partial conversion to an isoaspartate. *In vivo,* this unusual form of aspartate is repaired by an enzyme. The balance between the

isomerization and repairing affects the organism's physiology.

As an example, a sample of a peptide located at heavy chain 77 to 87 amino acids containing one methionine, two asparagine, and one glutamine was subjected to oxidation and deamidation generating a variety of peptide species. The experiment was performed to compare replicate injections with Auto MS/MS acquisition with the result acquired by Iterative MS/MS. Using a 30-minute gradient with two microgram loading amount, both Auto MS/MS and the first injection of Iterative MS/MS can identify a native form and M7 oxidated forms, as well as N1 and N8 deamidated forms.

The N8 deamidated form was identified at two different retention times, which shows the presence of aspartate isomerization. At these conditions, a double-deamidated form, Q6N8 deamidated peptide, was identified in the second injection of Iterative MS/MS, but not in the replicate Auto MS/MS analysis. The relative abundance of this double-deamidated peptide was only 0.16%. When examining the extracted compound chromatograms, the Q6N8 deamidated peptide convoluted with one of the N8 deamidated peptide forms.

PTM Analysis

To confirm the accuracy of deamidation identification, the MS/MS spectra of this co-eluted peptide forms were inspected (**Figure 9**). The Q6N8 double deamidated form was identified on a precursor with +3 charge state and the N8 deamidated form was identified on a precursor with +2 charge state.

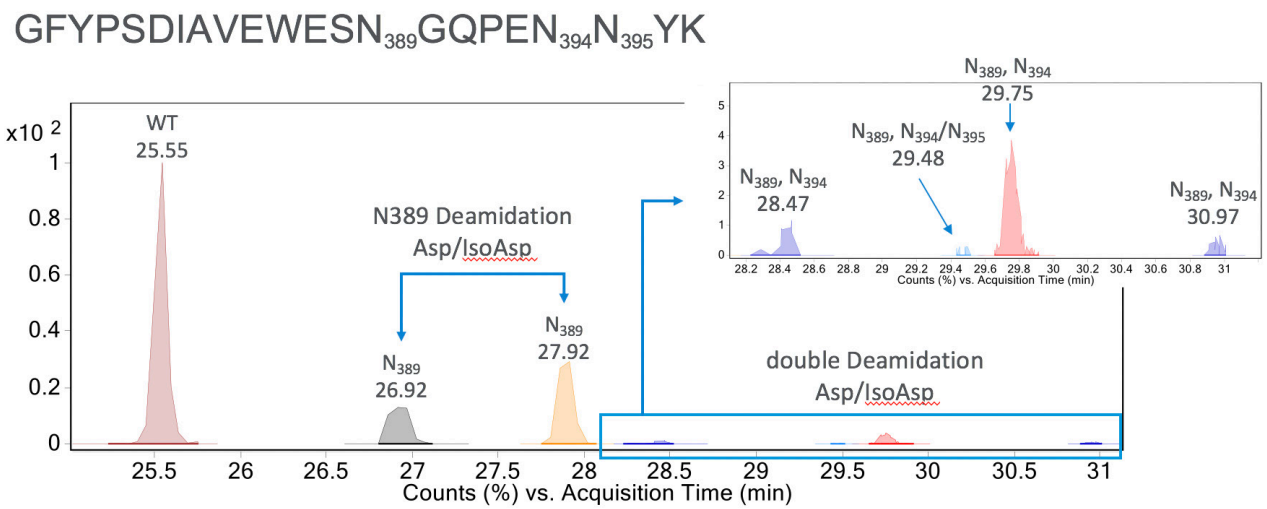
The b6 proton ion, which is highlighted with a red box, shows a one Dalton mass shift on the bottom panel. It comes from the double deamidated form, which confirms one of the deamidations was located at the Q6 location. This inspection confirms the deamidation localization; for these two co-eluting peptides, it also demonstrates the identification quality of low-abundance peptides by Iterative MS/MS.

To evaluate the relative quantitation of PTM analysis, two forced degradation studies were carried out. One is a forced deamidation experiment in high pH condition in which samples were collected at four time points including zero day, three days, six days, and 13 days. The other experiment is forced oxidation with hydrogen peroxide. For this experiment, mAbs were incubated in a buffer containing different concentrations of hydrogen peroxide and then incubated overnight. All of the samples were reduced, alkylated, digested, and desalted in parallel by AssayMAP Bravo system automatically, then analyzed by the Agilent 6545XT AdvanceBio LC/Q-TOF system.

Separation of Deamidated Peptides Forms

Asparagine deamidation is one of the most frequently observed modifications in proteins and peptides. It is a major source for instability in formulation and storage conditions. The PENNY peptide

Figure 10: Excellent separation of deamidated peptides forms.



on mAb heavy chain, has been reported as being solvent accessible and sensitive to chemical degradation. It contains the three asparagine residues and each one with a different susceptibility to deamidation when exposure to high pH condition. Therefore, this peptide is used to demonstrate the simultaneous identification and quantification of asparagine deamidation and aspartate isomerization using the Agilent workflow.

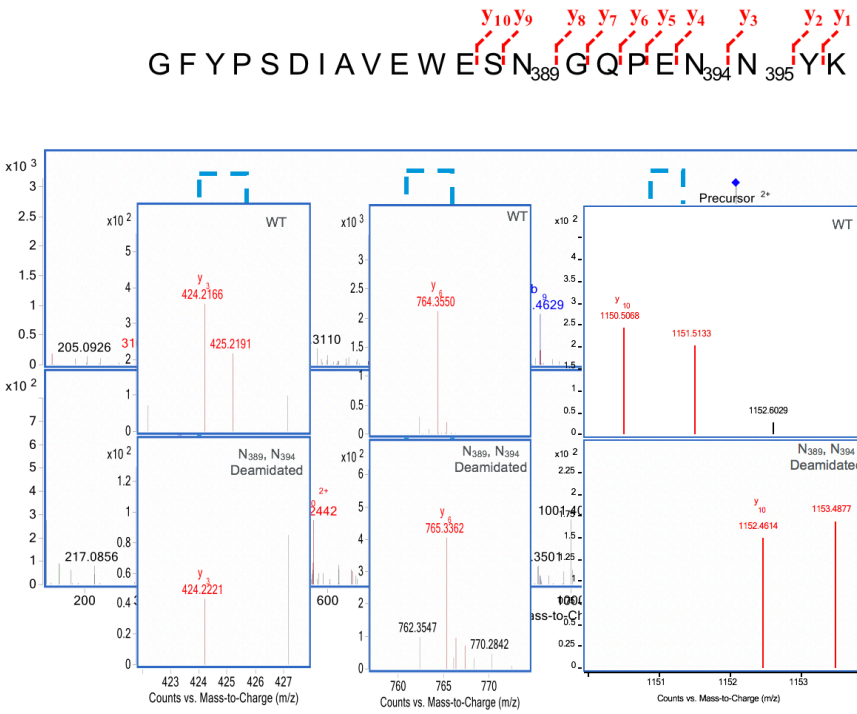
Shown below (**Figure 10**) is the extracted compound chromatograms for the various deamidated forms of the PENNY peptide using an Agilent surface charged reversed-phased column with a 30-minute gradient. Seven different peptide forms of the PENNY peptide are shown, including the wild-type peptide, the N389 asparagine deamidation and isoaspartate forms, as well as four double-deamidated and aspartate isomerization forms. It shows that all the forms were dispersed within a six-minute time window and the modified forms were separated from the wild-type.

Confirmation of Deamination Localization

BioConfirm software provides a user-friendly interface for PTM analysis. Users can select peptides of interest for side-by-side comparison of the MS2 spectrum. **Figure 11** is an example comparing wild-type form and a double-deamidated form of the PENNY peptide. The spectra can be zoomed in synchronously for detail fragment ion comparison.

In this example, the y3 proton ion displayed the same m/z on both wild-type and deamidated forms, thus confirming there is no deamidation on N395. The y6 proton ion has a one Dalton mass shift.

Figure 11: Confirmation of deamination localization.



The y10 proton ion has a two Dalton mass shift on the deamidated peptides showing that double deamidation is located at N389 and N394.

Forced Deamidation Example

The relative quantitation histogram of these three asparagine deamidation can be directly reported by BioConfirm software (**Figure 12**). It was observed that N389 has the highest deamidation level of all the three asparagine residues during the stress study, and that the deamidation level increased during the time course. Two technical replicates were performed for each time point and indicates the two technical replicates performed at each time point is very reproducible. In addition to deamidation, the relative quantitation of methionine oxidation was assessed using BioConfirm software.

Forced Oxidation Example

Figure 13 shows an example of the relative quantitation histogram of light chain methionine oxidation for mAb 1 and NISTmAb in response to accelerated hydrogen peroxide exposure. It shows that both mAbs exhibited an increasing level of oxidation at M4 and have very different oxidation rates.

Figure 12: Forced deamidation.

GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK

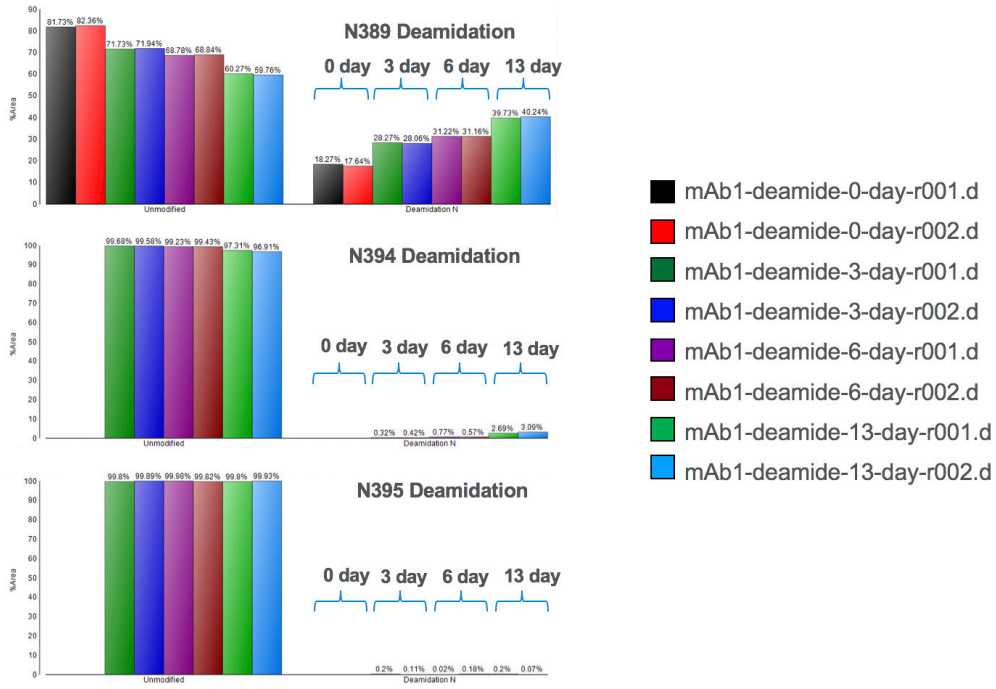
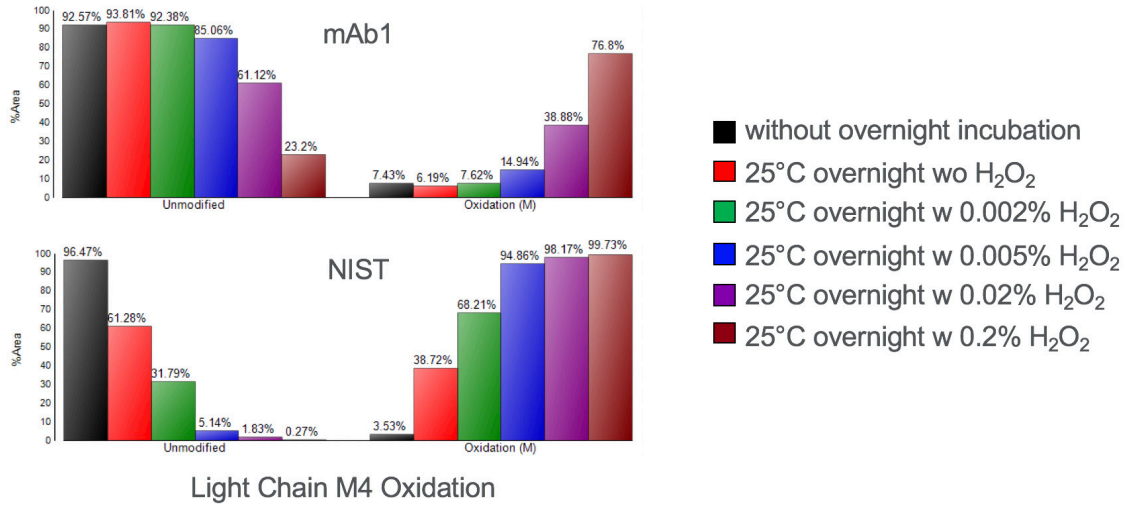
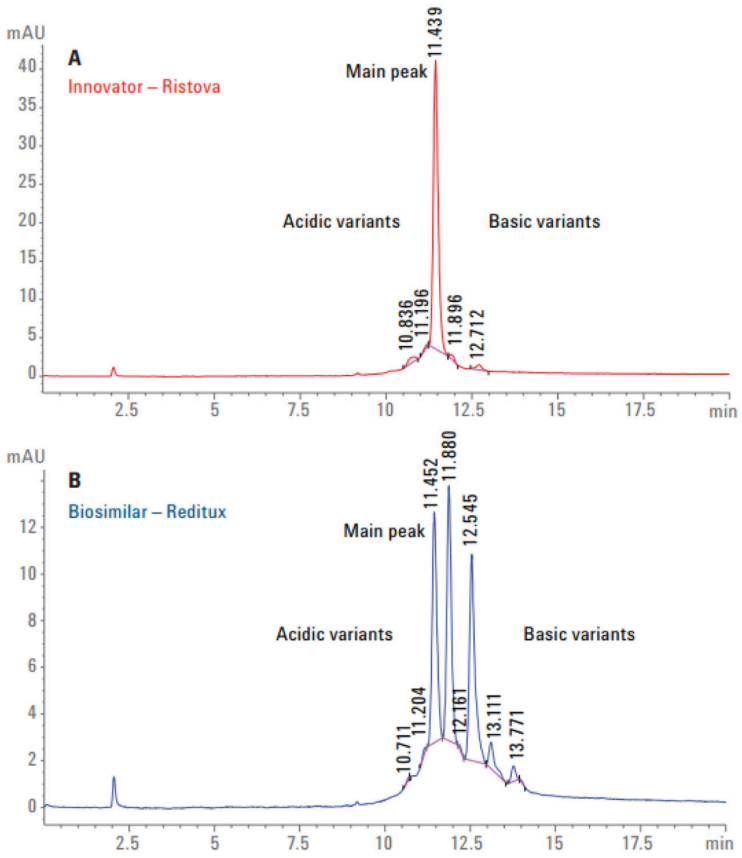


Figure 13: Forced oxidation.



Light Chain M4 Oxidation

Figure 14: Innovator versus biosimilar.



Innovator and Biosimilar Example

Another study to compare a pair of innovator and biosimilar drugs (**Figure 14**) shows the charged variant analysis profile using ion exchange chromatography. The innovator, which is seen in the top panel, shows one major peak and acidic and basic variants at very low abundance. The biosimilars (bottom panel) show three major peaks. The first peak is the same as the main peak in the innovator, and there are two basic forms that are very significant.

Carboxypeptidase (CBP) Treatment

When CBP was used to treat both molecules, it cleaves the C-terminal lysine (**Figure 15**). On the left is the overlay of the innovator and biosimilar. On the right is the overlay after CBP treatment. Now, the biosimilars showed only one major peak, which comigrated with the main peak of the innovator. The two basic peaks disappeared after CBP treatment, which suggests the basic forms are C-terminal lysine variants.

Peptide mapping using trypsin to digest the two proteins was performed for fur-

Figure 15: CBP treatment.

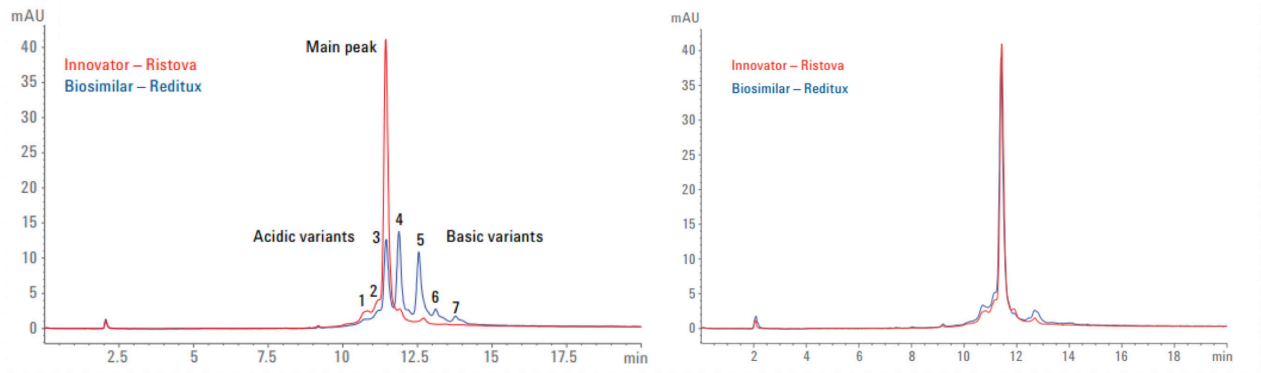
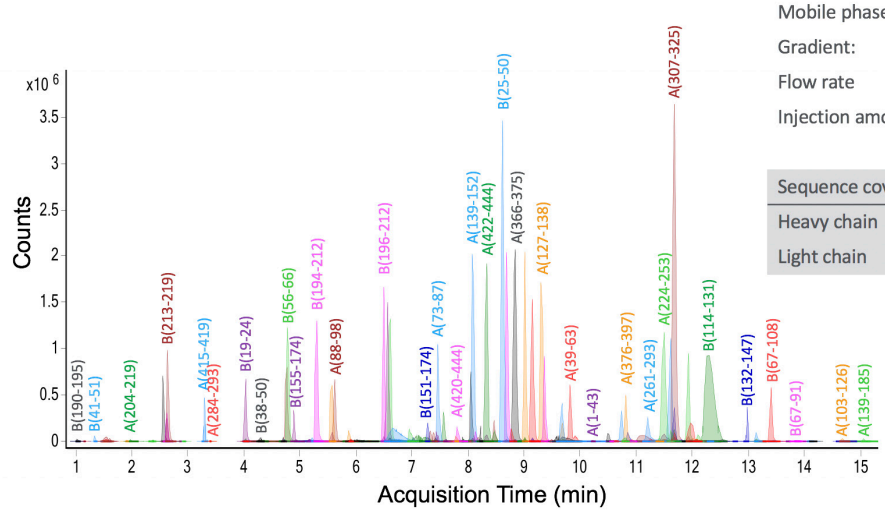


Figure 16: Peptide mapping.



Column:	AdvanceBio Peptide Mapping, 2.1 x 150 mm
Mobile phase A:	Water + 0.1% FA
Mobile phase B:	Water/acetonitrile (10:90 v:v) + 0.1% FA
Gradient:	3% - 40% B, 15min
Flow rate	0.4 mL/min
Injection amount:	0.6 µg

Sequence coverage	0.1% FDR	1% FDR
Heavy chain	96.24%	96.24%
Light chain	98.63%	100%

ther validation (**Figure 16**). Although most of the peaks are identical, a closer look to compare the two digested samples indicates differences in two regions. They are corresponding with C-terminal peptides and show the C-terminal peptide of the innovator having no lysine in the end while the biosimilar contains lysine. Also, when looking at PTM differences in the two samples, the biosimilar sample had a slightly higher methionine oxidation at the DLTMISR peptide than the innovator. The deamidation of asparagine is similar for both samples.

Conclusion

In this study, peptide mapping was a critical technique to characterize protein sequence and compare similarity for biosimilars. The Agilent Peptide Mapping Workflow Solution contains the Agilent AssayMAP Bravo system, which enables automated sample digestion with excellent reproducibility. The Agilent 1290 Infinity II LC system and AdvanceBio columns enables rapid separation for improved laboratory efficiency. The highly accurate Agilent 6545XT AdvanceBio LC/Q-TOF system with the new Iterative MS/MS acquisition mode enhances the identification of low abundant peptides in consecutive LC–MS/MS analysis while eliminating extra effort in method optimization. The Agilent MassHunter BioConfirm software automates and streamlines the data processing for this complex process. Agilent has streamlined peptide mapping from sample to answer providing a more user-friendly and reproducible solution.

Linfeng Wu, PhD is an application scientist at Agilent Technologies.

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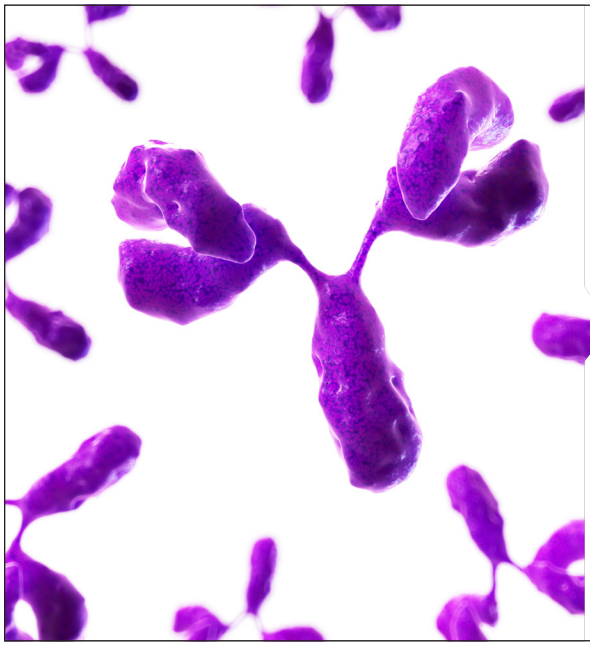

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Application Note

Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies

Linfeng Wu

Introduction

Modifications such as asparagine (Asn) deamidation, aspartate (Asp) isomerization, and methionine (Met) oxidation are typical degradation products for recombinant antibodies. Previous studies have shown that degradation of Asn, Asp, and Met residues in mAbs can affect protein activity[1-4]. Therefore, those modifications in a protein drug candidate, for example, a mAb, are critical quality attributes (CQAs), and are closely monitored under storage and formulation conditions. They are often the focus of stress and forced degradation studies conducted during drug development. To assess these CQAs, simultaneous identification and quantification are needed. This Application Note demonstrates simultaneous identification and quantification of chemical-induced deamidation and oxidation on recombinant mAbs with a peptide-mapping method using an integrated workflow including an Agilent AssayMAP Bravo platform, an Agilent 1290 Infinity II LC, an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software (**Figure 1**).

Experimental

Materials

The mAb1 sample was a recombinant CHO-cultured IgG1 mAb, which was produced and purified by a third-party partner. NISTmAb was purchased from the National Institute of Standards and Technology (NIST).

Instrumentation

- Agilent AssayMAP Bravo system (G5571AA)
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler

Figure 1: Integrated workflow for peptide mapping.

(G7167B) with sample cooler (option 100)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549A)
- Dual Agilent Jet Stream ESI source (G1958-65268)

Chemical induction and sample preparation

To induce significant levels of Asn deamidation, mAb1 samples were exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for 0, 3, 6, and 13 days, respectively. To induce Met oxidation, mAb1 and NISTmAb samples were incubated in Tris-HCl buffers containing different concentrations of oxidizing agent H_2O_2 (ranging from 0 to 0.2 % v/v) overnight at room temperature. All samples were lyophilized and stored in –80 °C before sample digestion.

All samples were reduced, alkylated, trypsin-digested, and desalted using the AssayMAP Bravo platform[5]. Digested samples were subjected to LC/MS analysis.

LC/MS analysis

LC separation was performed on an Agilent reversed-phase C18 column with a charged surface (2.1 × 150 mm, 2.7 µm) using a 30-minute gradient (**Table 1**). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (**Table 2**).

Table 1: Liquid chromatography parameters.

LC parameters	
Analytical column	Agilent reversed-phase C18 column with a charged surface
Mobile phase A	H_2O, 0.1 % formic acid
Mobile phase B	90 % acetonitrile and 0.1 % formic acid in H_2O
Column temperature	60 °C
Flow rate	0.4 mL/min
Gradient	0.0 minutes → 3 %B 30.0 minutes → 22 %B 32.0 minutes → 90 %B 35.0 minutes → 90 %B 37.0 minutes → 3 %B
Stop time	40 minutes

Data processing

Data acquired from LC/MS/MS analysis were processed using MassHunter BioConfirm 10.0 software. For the chemical-induced deamidation study, searching parameters were set up as trypsin digest allowing semitryptic peptides and maximum two missed cleavages, fixed modifications containing cysteine (C) alkylation and N-terminal pyroGlu (E/Q), variable modifications containing asparagine (N) or glutamine (Q) deamidation, and methionine (M) oxidation. Mass tolerance allows 10 ppm for MS1 and 20 ppm for MS2. Peptide length was limited to 5 to 60 amino acids (AAs). Peptide-spectrum matches required MS/MS features, and were filtered by a 0.1 % false discovery rate (FDR). For the chemical-induced oxidation study, the searching parameters were the same as the induced deamidation study. An exception was that tryptophan (W) oxidation (+4 Da, +16 Da, or +32 Da) was added[6].

Results and discussion

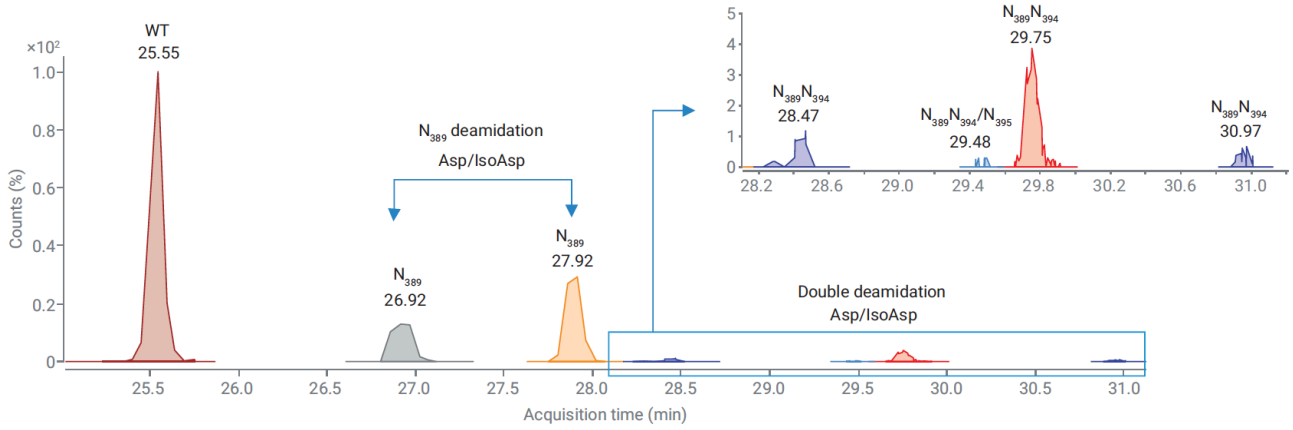
Separation and identification of deamidated peptides

Asn deamidation is one of the most frequently observed modifications in proteins and peptides. It is a major source of instability in formulation and storage conditions during biopharmaceutics production. The Asn residue can be deamidated either

Table 2: MS parameters.

Parameter	Value
Instrument	6545XT AdvanceBio LC/Q-TOF
Gas temperature	325 °C
Drying gas flow	13 L/min
Nebulizer	35 psig
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
VCap	4,000 V
Nozzle voltage	0 V
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	*m/z* 300 to 1,700
Acquisition rate	8 spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ *m/z* 1.3)
Precursors/cycle	Top 10
Collision energy	3.1*(*m/z*)/100+1 for charge 2; 3.6*(*m/z*)/100−4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 counts and 0.001 %
Dynamic exclusion on	1 repeat, then exclude for 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000 counts/spectrum
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By charge state then abundance; +2, +3, >+3

Figure 2: ECC of Asn deamidation, Asp isomerization, and wild type forms of the GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK peptide on a reversed-phase C18 column with charged surface using a 30-minute LC gradient .



through hydrolysis to form Asp or through a cyclic succinimide intermediate to form both aspartate or iso-aspartate. Since there is only a 0.984 Da shift for deamidation, it could be difficult to accurately identify and quantify deamidation and aspartate isomerization when the different forms coelute.

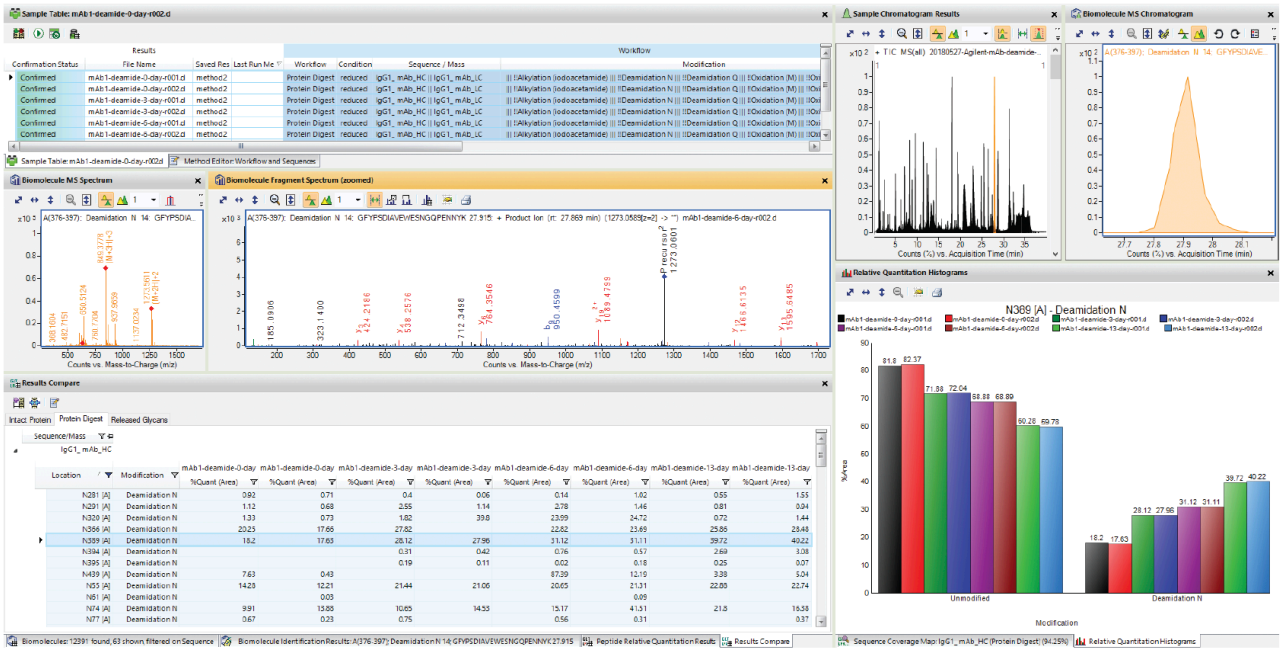
The deamidation of Asn in the conserved CH_3 region has been identified as being solvent-accessible and sensitive to chemical degradation in previous reports[3,4]. The PENNY peptide matched to this region (heavy chain sequence location 376–397, GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK) contains three Asn residues, each with a different susceptibility to deamidation under exposure to high pH conditions. We used this peptide to demonstrate the simultaneous identification and quantification of Asn deamidation and Asp isomerization using the Agilent peptide-mapping workflow.

Figure 2 shows the extracted compound chromatograms (ECCs) for the various PTM forms of the PENNY peptide using a 30-minute LC gradient. Seven different forms of the PENNY peptide are shown, including the unmodified wildtype (WT) peptide, the N$_{389}$ Asn deamidation/Asp isomerization, and the double deamidation/Asp isomerization forms. It shows that all the forms were dispersed within a six-minute time window, and the modified forms were well separated from the unmodified form.

MassHunter BioConfirm software provides a user-friendly interface for PTM analysis (**Figure 3**). One of the software features allows an analyst to select the peptides of interest in the biomolecules table for side-by-side comparison of their MS/MS spectra. **Figure 4** shows the MS/MS spectrum comparison of the wild type (WT, RT = 25.55 minutes) and the double deamidated form (N$_{389}$, N$_{394}$, RT = 30.97

Quantitation of Chemical-Induced Deamidation

Figure 3: Screenshot of MassHunter BioConfirm 10.0 software for PTM identification and quantitation.



minutes) of the PENNY peptide using BioConfirm software. The spectra can be zoomed synchronously for detailed fragment ion comparison. The y_3 product ions show the same *m/z* on both WT and deamidated forms, confirming that there is no deamidation on N_{395}. The y_6 product ion has +1 Da mass shift, and the y_{10} product ions has +2 Da mass shift on deamidated peptides, showing that the double-deamidated sites are at N_{389} and N_{394}.

All the MS/MS spectra for the peptide forms shown in Figure 2 have been inspected to verify the assignment of deamidation localization. Almost all the deamidated forms were unambiguously identified with their deamidation localization. An exception was that the double deamidated forms (N_{389}, N_{394}/N_{395}) with a retention time of 29.48 minutes contain an ambiguous Asn deamidation assignment on N_{394} or N_{395}.

Quantitation of deamidation

MassHunter BioConfirm software enables an analyst to quantify the extent of PTMs on all the residues with a modification across a series of samples. It calculates the %peak area (or %peak height as an option) of the modified peptide relative to the sum of modified and unmodified forms. It also summarizes the comparison results in a table **(Figure 5)**. This Results Compare table enables an analyst to have an overview of the PTM quantitation results for all the modified residues across a series of samples. In addition to the Results Compare table, BioConfirm

Figure 4: Comparison of MS/MS spectra between wild type and the double-deamidated form (N_{389}, N_{394}, RT = 30.97 minutes) of the peptide GFYPSDIAVEWESNGQPENNYK using MassHunter BioConfirm software. The fragment ions with a differential feature for PTM localization assignment were zoomed in for detailed comparison.

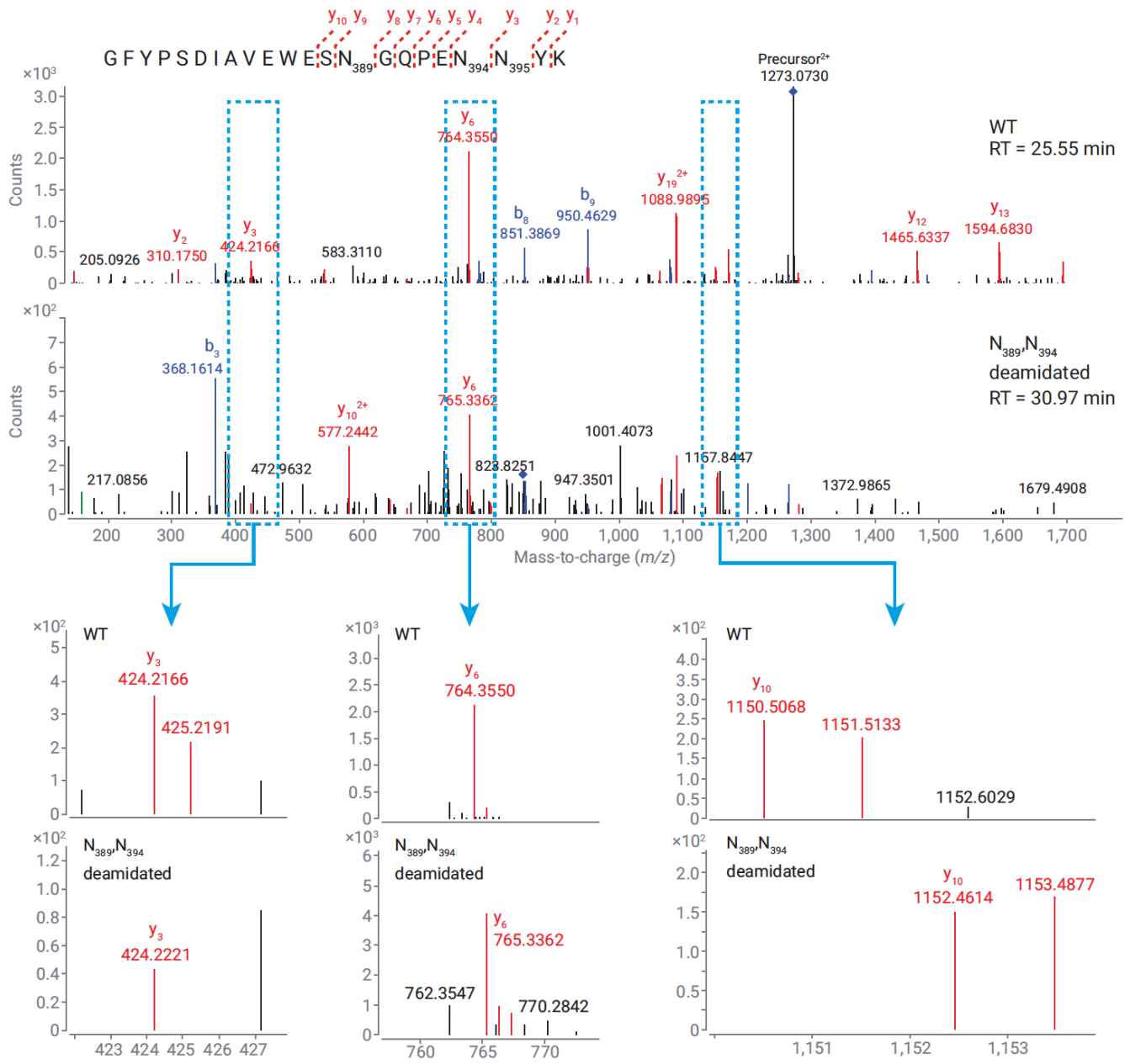


Figure 5: Results Compare table for PTM quantitation, showing a quantitative comparison of Asn deamidation in mAb1 heavy chain and light chain in high-pH conditions over a time course.

Results Compare

Intact Protein | Protein Digest | Released Glycans

Sequence/Mass: IgG1_ mAb_HC

Location	Modification	mAb1-deamide-0-day-r001.d %Quant (Area)	mAb1-deamide-3-day-r001.d %Quant (Area)	mAb1-deamide-6-day-r001.d %Quant (Area)	mAb1-deamide-13-day-r001.d %Quant (Area)
N104 [A]	Deamidation N	97.7	53.22	18.19	23.41
N206 [A]	Deamidation N	7.25		2.84	0.28
N208 [A]	Deamidation N	7.25	15	6.49	7.96
N213 [A]	Deamidation N		0.37		
N281 [A]	Deamidation N	0.93	19.38	2.95	14.37
N291 [A]	Deamidation N	1.13	2.57	2.81	0.82
N320 [A]	Deamidation N	0.66	0.9	24.16	38.89
N366 [A]	Deamidation N	20.25	27.82	22.82	25.86
N389 [A]	Deamidation N	18.27	28.27	31.22	39.73
N394 [A]	Deamidation N		0.32	0.77	2.69
N395 [A]	Deamidation N		0.2	0.02	0.2
N426 [A]	Deamidation N	78.89	79.32	5.09	91.17
N439 [A]	Deamidation N	89.32	8.73	87.36	7.88
N55 [A]	Deamidation N	14.31	21.51	20.65	22.88
N74 [A]	Deamidation N	9.91	10.65	15.17	21.8
N77 [A]	Deamidation N	0.67	0.75	0.56	
N84 [A]	Deamidation N	1.74	3.19	2.69	3.69

Sequence/Mass: IgG1_ mAb_LC

Location	Modification	mAb1-deamide-0-day-r001.d %Quant (Area)	mAb1-deamide-3-day-r001.d %Quant (Area)	mAb1-deamide-6-day-r001.d %Quant (Area)	mAb1-deamide-13-day-r001.d %Quant (Area)
N142 [B]	Deamidation N	2.07	2.19	3.89	2.25
N143 [B]	Deamidation N	0.19	1.69		0.46
N157 [B]	Deamidation N	0.53			
N163 [B]	Deamidation N	0.21		0.44	0.16
N215 [B]	Deamidation N			0.28	
N58 [B]	Deamidation N	0.12	0.71	0.29	0.58

also allows visualization of the quantitation result with a histogram. To display the histogram for each modified residue, the user simply clicks the row containing the residue of interest in the Results Compare table.

Figure 6 shows the histograms of quantitation for the three Asn deamidations (N_{389}, N_{394}, and N_{395}) on mAb1 heavy chain in high-pH conditions during a time course using BioConfirm software. Two technical replicates were performed for each time point. As observed, N_{389} has the highest deamidation level among the three residues during the stress study. This observation is consistent with the work of other groups, which shows that Gly on the C-terminus of Asn has a higher deamidation rate compared to other residues. A large hydrophobic residue containing aromatic

Figure 6: Histograms of quantitation for the three Asn deamidations (N_{389}, N_{394}, and N_{395}) on mAb1 heavy chain in high-pH conditions during a time course using MassHunter BioConfirm software. Two technical replicates were performed for each time point.

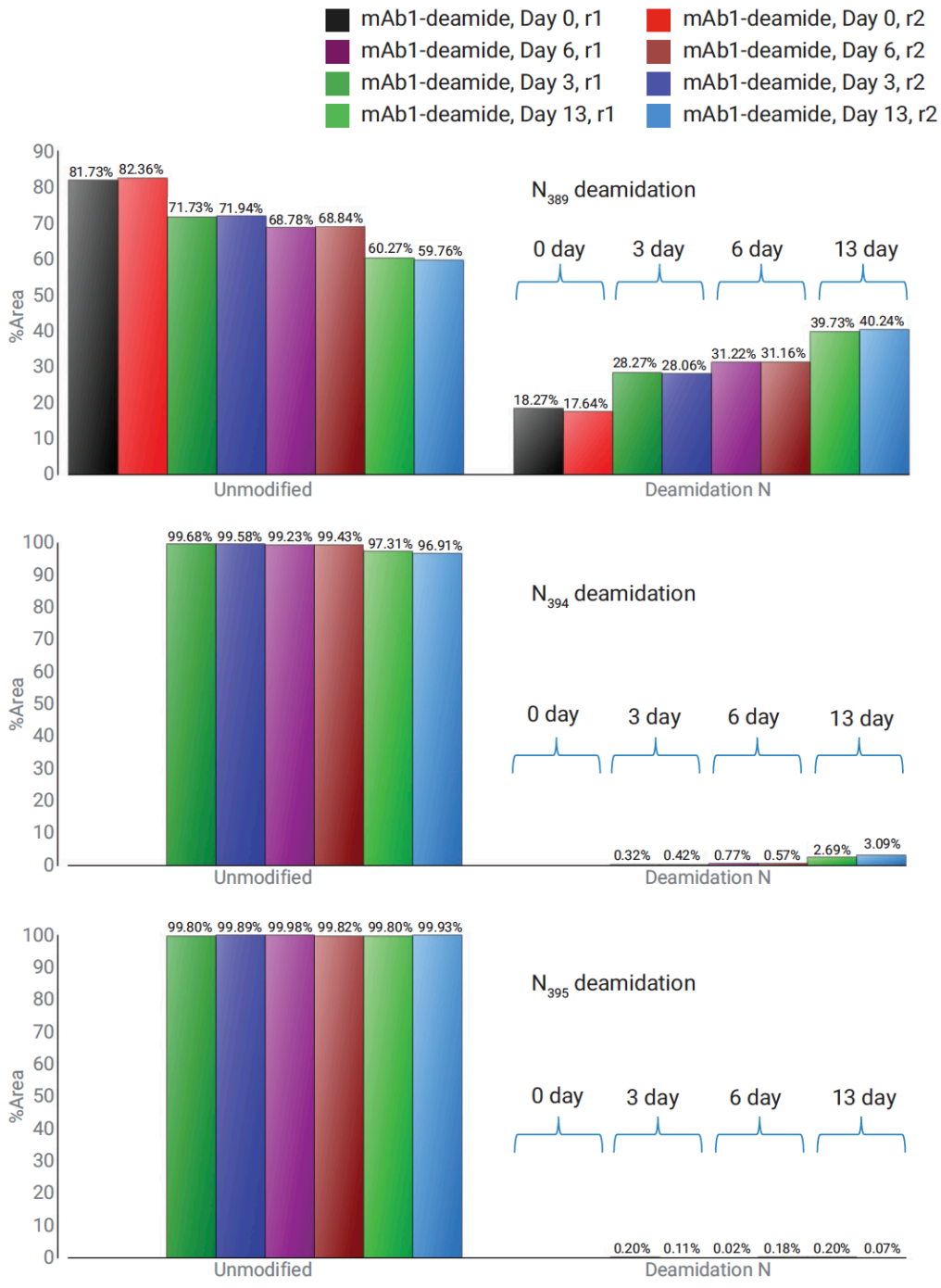
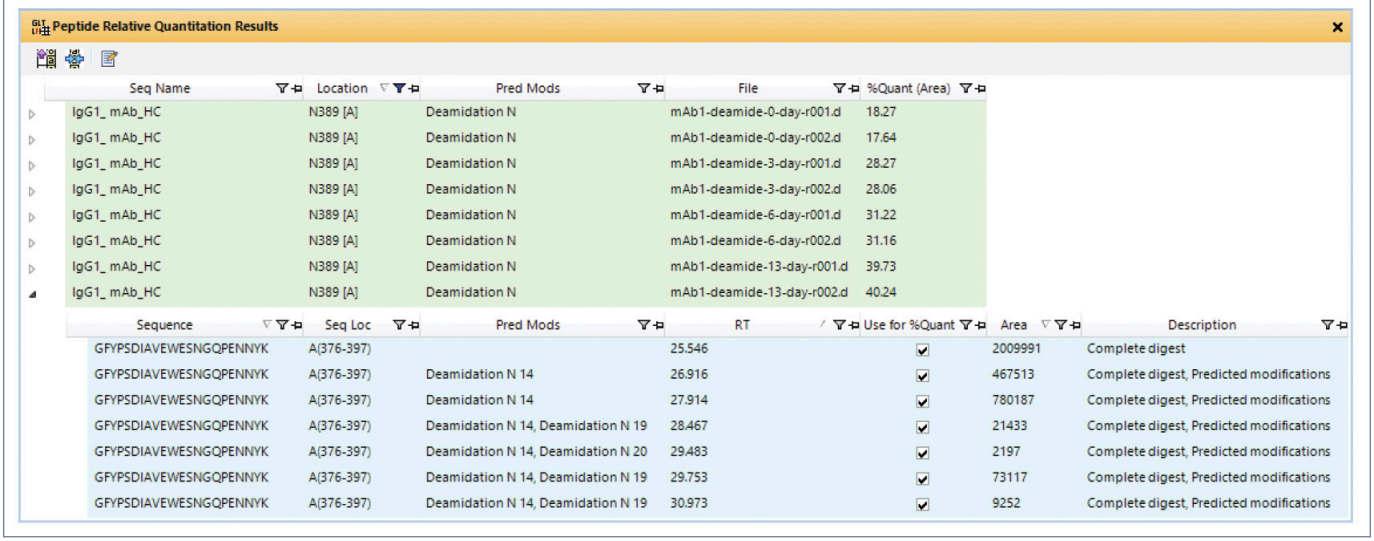


Figure 7: Screenshot of a portion of the Peptide Relative Quantitation Results table from MassHunter BioConfirm.



Peptide Relative Quantitation Results

Seq Name	Location	Pred Mods	File	%Quant (Area)
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-0-day-r001.d	18.27
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-0-day-r002.d	17.64
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-3-day-r001.d	28.27
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-3-day-r002.d	28.06
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-6-day-r001.d	31.22
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-6-day-r002.d	31.16
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-13-day-r001.d	39.73
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-13-day-r002.d	40.24

Sequence	Seq Loc	Pred Mods	RT	Use for %Quant	Area	Description
GFYPSDIAVEWESNGQPENNYK	A(376-397)		25.546	☑	2009991	Complete digest
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14	26.916	☑	467513	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14	27.914	☑	780187	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	28.467	☑	21433	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 20	29.483	☑	2197	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	29.753	☑	73117	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	30.973	☑	9252	Complete digest, Predicted modifications

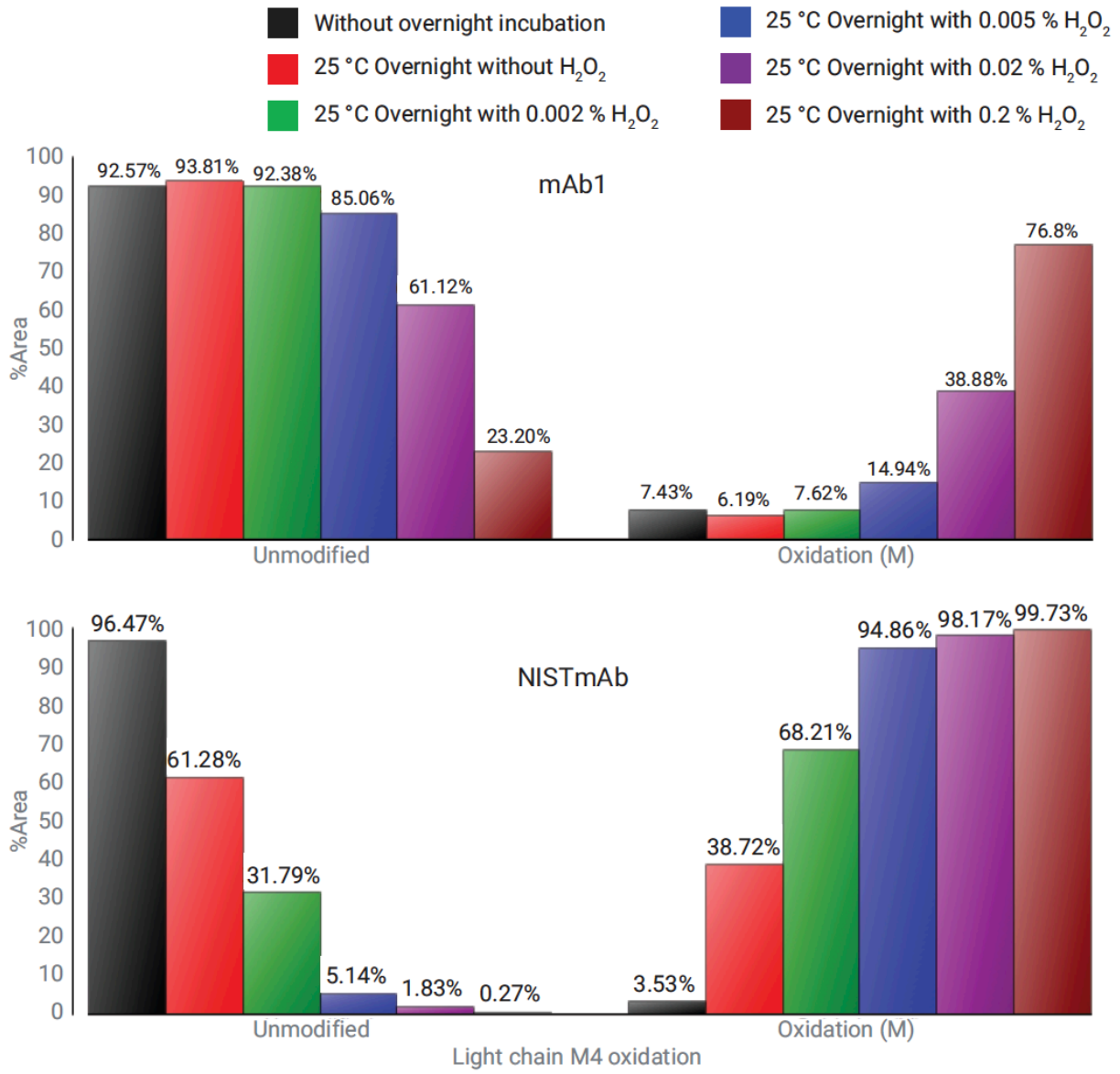
rings on its C-terminus also generally correlates with very slow deamidation[7,8]. Since the assignment of deamidation localization on N_{394} or N_{395} is sometimes ambiguous, it is reasonable to consider combining the deamidation quantitation on these two sites for data analysis. In a peptide-mapping workflow

In a peptide-mapping workflow, the therapeutic protein is first digested to produce peptide fragments, which sometimes generate incomplete digestion. Due to the complexity of the sample digest, an analyst should inspect the peptide forms used for PTM quantitation. BioConfirm software generates a table titled Peptide Relative Quantitation Results, listing all the modified residues and their corresponding peptides from each data file (**Figure 7**). Figure 7 shows the quantification of N_{389} deamidation on mAb heavy chain in the eight data files during the stress study. To show all the corresponding peptides identified in this data file, the subtable for the last data file is expanded. To automatically determine the peptide forms used for PTM quantitation, BioConfirm applies a series of rules related to protein digestion and PTM analysis. It also allows a user to adjust the peptide selection by clicking the Use for %Quant check box. The calculated %Quant, the Results Compare Table, and the histogram are immediately synchronized with updated quantitation results.

Quantitation of oxidation

The extent of Met oxidation in two mAbs (mAb1 and NISTmAb) was also evaluated under accelerated oxidation conditions. As an example, **Figure 8** shows the quantitative histograms of light chain Met 4 (M4) oxidation for mAb1 and NISTmAb in

Figure 8: Quantitative histograms of light chain M4 oxidation for mAb1 and NISTmAb under accelerated oxidation conditions using MassHunter BioConfirm software.



response to accelerated H_2O_2 exposure using BioConfirm software. As expected, both mAbs show increasing extent of oxidation at M4 with a different oxidation rate in response to accelerated H_2O_2 exposure.

Conclusion

A complete workflow including automated sample preparation using an AssayMAP Bravo platform, LC separation with a

1290 Infinity II LC, data acquisition using 6545XT AdvanceBio LC/Q-TOF, and data analysis using MassHunter BioConfirm software has been demonstrated for the simultaneous identification and quantification of chemical-induced deamidation and oxidation on mAbs. The MassHunter BioConfirm 10.0 software is capable of:

- Automated data batch processing
- Peptide-spectrum matching with statistical score and FDR
- Linked navigation through the results table to the mass spectra and chromatograms
- Quantitation analysis of PTMs

A combination of these features enhances the workflow for peptide mapping and PTM quantitation during the development and manufacture of protein biotherapeutic drugs.

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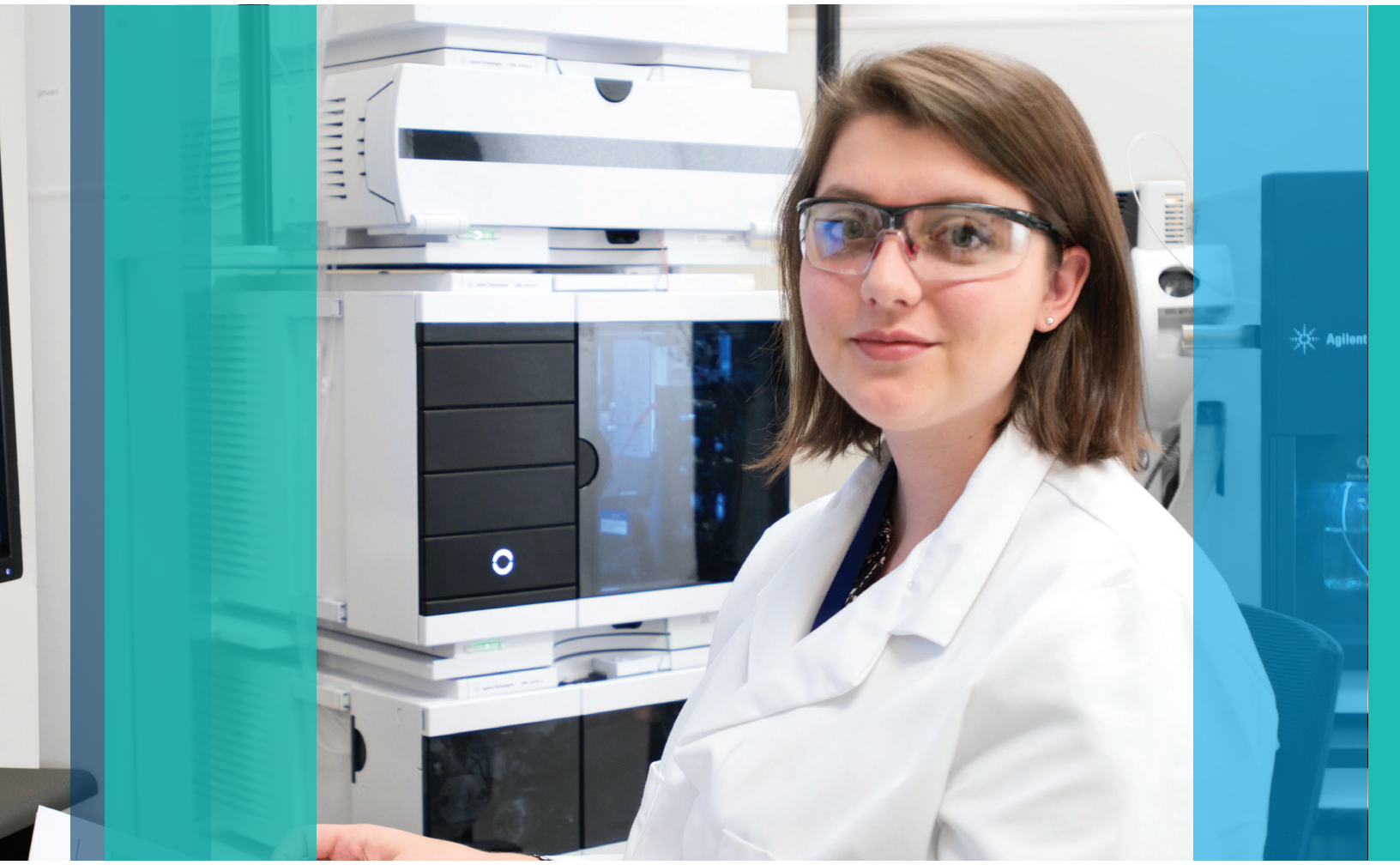

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