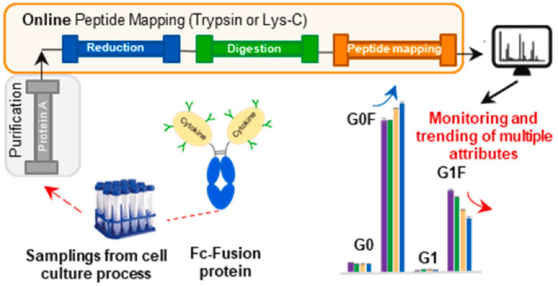
Online multi-dimensional LC/MS: the next generation PAT tool for real-time monitoring of antibody quality attributes in biopharmaceutical processes

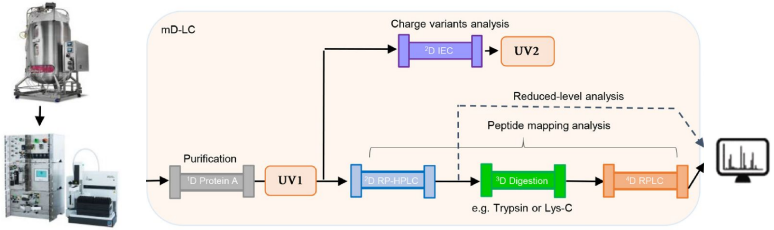
Julien Camperi and Cinzia Stella

MAM Consortium, June 3rd 2022

Outline

- **Current state** and **challenges** related to online monitoring of product quality attributes
- Overview of a multidimensional LC/MS workflow for the monitoring of antibody minor variants over the cell culture process: **proof of concept study**
- Overview of a multidimensional LC/MS workflow **directly connected to the bioreactor**
- Conclusion and **looking ahead**

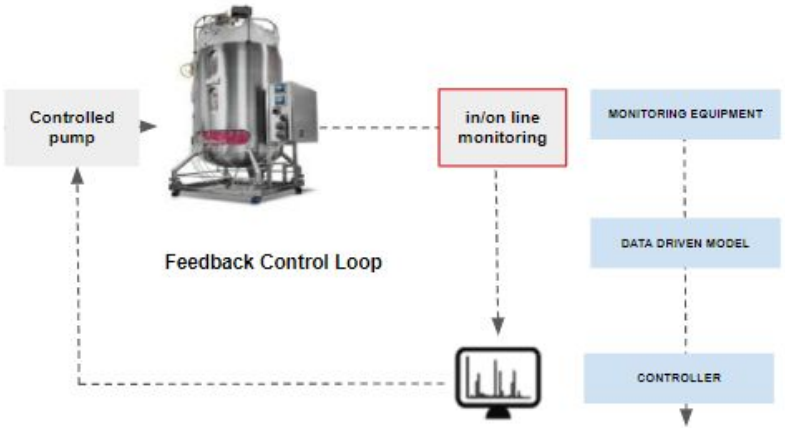




Introduction

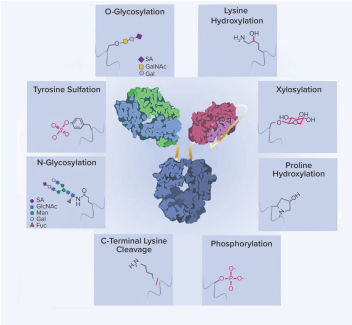
- **Online monitoring of critical quality attributes** (CQAs) can provide the basis for advanced processing of therapeutics production (real time monitoring, feedback control and real-time release testing)

- In 2021 the **FDA published a guidance document:** *"Q13 Continuous Manufacturing of Drug Substances and Drug Products",* where it **specifically supports the development and implementation of innovative online analytical technologies**

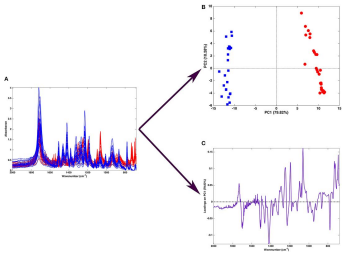


Current State and challenges

- PAT technologies have been mainly focused on **tools in the form of sensors of spectroscopic probes.**



- Most PAT tools are focused on improving the means of cell culture process performance and parameters (e.g. online glucose, pH, etc..), but **few can directly measure the product quality attributes** such as oxidation, deamidation, glycosylation.



- Spectroscopic techniques **often require the use of predictive chemometric models,** often product-specific and typically require large data sets to be validated, and need to be re-optimized and/or re-validated when the manufacturing process conditions change.

HPLC based MAM methods

- **HPLC has been the main workhorse** in the biopharmaceutical industry, from research and development (R&D) to quality control (QC) laboratories.

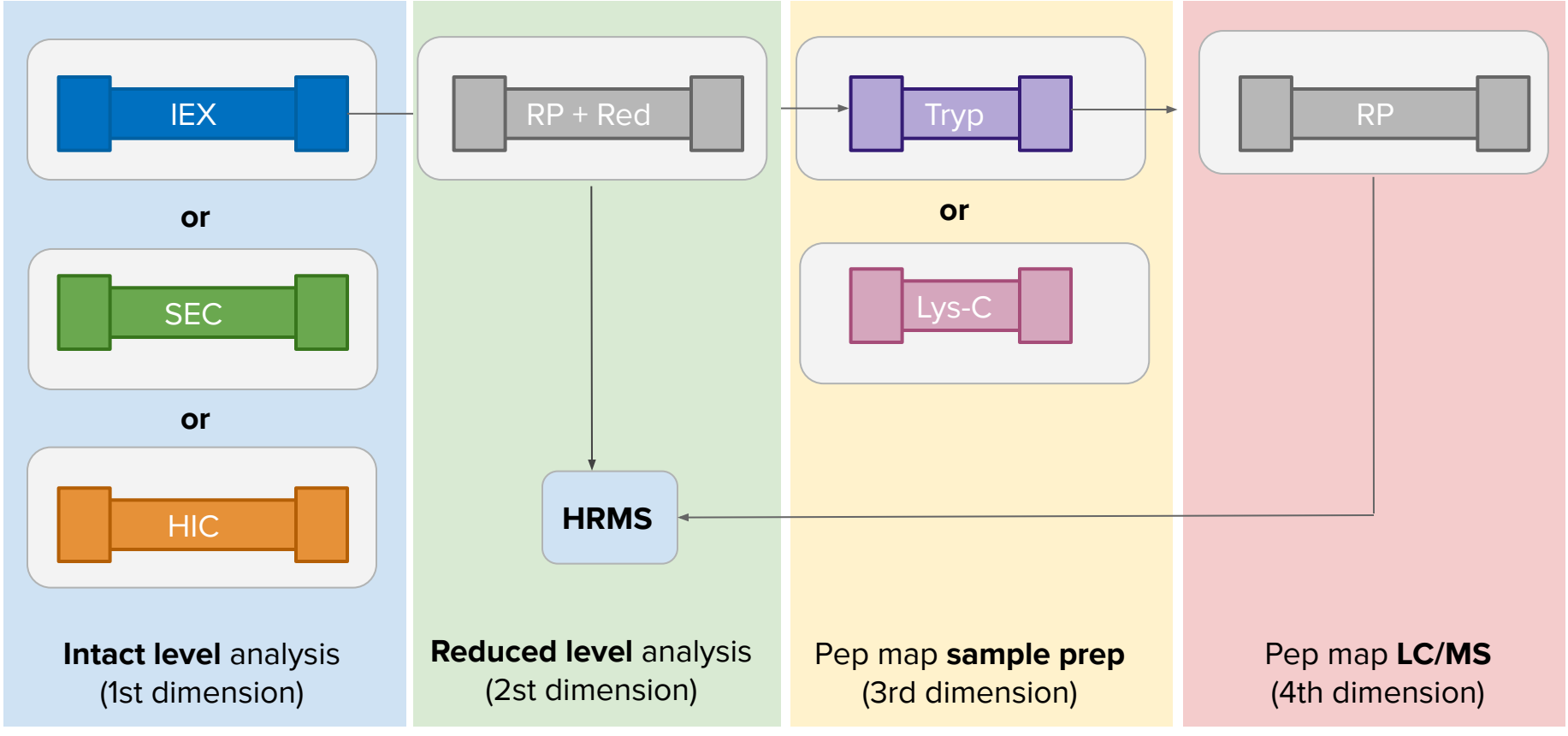
- The peptide mapping-based **MAM approach has the potential to replace conventional methods** for product release, due to its capacity to simultaneously monitor several CQAs.

- However, the application **of MAM, focusing on in-process samples analysis,** in particular during **scale-up and technology transfer has been lacking, due to time consuming sample prep.**

Multidimensional LC/MS

Combining sample preparation with multi-level analysis

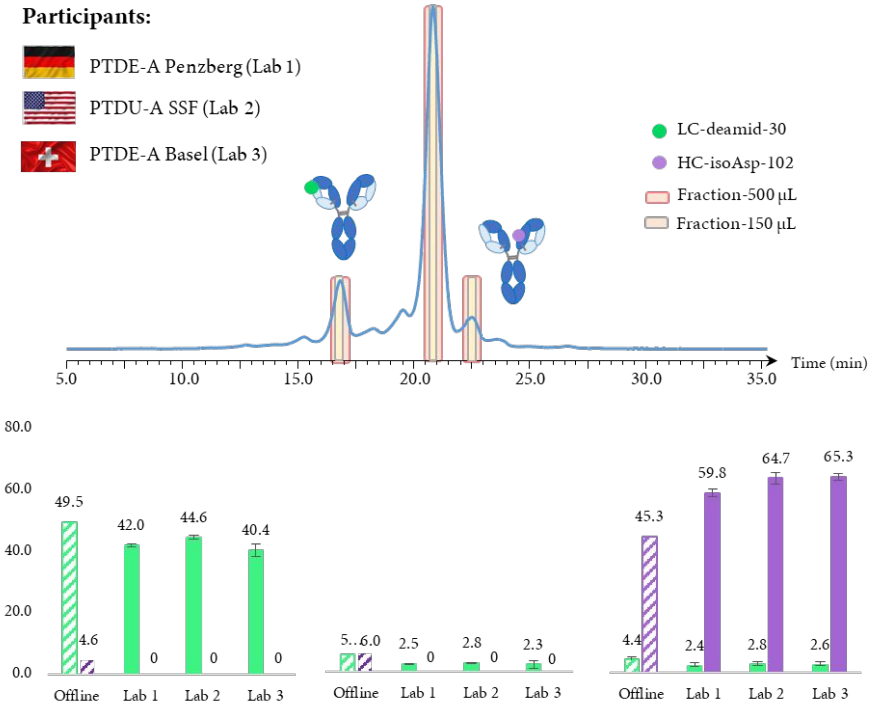
1 FTE 1 system



Multidimensional LC/MS

Global alignment at Roche-Genentech

- **Herceptin™ (150 µg)** – Fractionation of **3 identical IEX peaks**



Summary of all PTMs

	Laboratory 1	Laboratory 2	Laboratory 3
CEX fraction	Acid, main, basic	Acid, main, basic	Acid, main, basic)
	(n= 3, mean ± S.D)	(n= 3, mean ± S.D)	(n= 3, mean ± S.D)
LC-MS peptide mapping (%)			
PTMs			
LC-Asn-30			
LC-deamid-30	**42.0** (± 0.4), 2.5 (± 0.2), 2.4 (± 0.3)	**44.6** (± 0.7), 2.9 (± 0.2), 2.8 (± 0.2)	**40.4** (± 2.0), 2.5 (± 1.1), 2.4 (± 0.8)
LC-succ-30	0.6 (± 0.2), 0.5 (± 0.1), 0.45 (± 0.1)	0.7 (± 0.1) , 0.9 (± 0.4), 0.9 (± 0.1)	1.0 (± 0.7), 1.3 (± 0.4), 1.0 (± 0.2)
HC-Asn-55			
HC-deamid-55	0.5 (± 0.1), 0.3 (± 0.1), 0.1 (± 0.1)	0.8 (± 0.1), 0.7 (± 0.1), 0.7 (± 0.1)	0.8 (± 0.1), 0.7 (± 0.1), 0.8 (± 0.2)
HC-succ-55	1.3 (± 0.3), 1.4 (± 0.3), 1.4 (± 0.4)	1.8 (± 0.1), 1.7 (± 0.1), 1.6 (± 0.3)	1.3 (± 0.1), 1.4 (± 0.1), 1.4 (± 0.3)
HC-Asp-102			
HC-isoAsp-102	0.0, 0.0, **59.8** (± 1.2)	0.0, 0.0, **64.7** (± 1.9)	0.0, 0.0, **65.2** (± 1.2)
HC-succ-102	1.8 (± 0.1), 2.0 (± 0.1), 1.2 (± 0.2)	1.7 (± 0.2), 1.5 (± 0.2), 1.7 (± 0.2)	2.0 (± 0.1), 2.3 (± 0.1), 1.6 (± 0.3)
HC-Asn-387, 392, 393			
LC-deamid-387, 392, 393	0.5 (± 0.1), 0.5 (± 0.2), 0.4 (± 0.1)	0.9 (± 0.1), 0.8 (± 0.1), 0.8 (± 0.1)	0.6 (± 0.1), 0.6 (± 0.1), 0.7 (± 0.1)
LC-succ-387, 392, 393	1.8 (± 0.2), 1.8 (± 0.2), 1.5 (± 0.1)	1.8 (± 0.4), 2.4 (± 0.5), 1.6 (± 0.3)	1.9 (± 0.1), 2.2 (± 0.4), 2.0 (± 0.3)
HC-Met-255			
HC-Met-ox-255	2.8 (± 0.1), 2.0 (± 0.2), 2.3 (± 0.1)	3.0 (± 0.1), 2.9 (± 0.1), 2.7 (± 0.4)	2.6 (± 0.1), 2.7 (± 0.3), 2.5 (± 0.6)
HC-Met-431			
HC-Met-ox-431	3.3 (± 0.2) , 2.0 (± 0.6) , 2.3 (± 0.1)	3.7 (± 1.0) ,3.1(± 0.6) , 2.0 (± 0.4)	2.1 (± 0.1) , 2.4 (± 0.2) , 2.2 (± 0.3)

- ✓ **Reliability/performance** of the mD-LC systems
- ✓ **Different homemade** mD-LC-MS systems provide **similar results**

*Camperi J et al. "Inter-laboratory study to evaluate the performance of automated online characterization of antibody charge variants by multi-dimensional LC-MS/MS". **Talanta**. 2021 Nov 1;234:122628.*

Increasing number of publications on this approach

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3D-LC-MS with ^{2}D Multimethod Option for Fully Automated Assessment of Multiple Attributes of Monoclonal Antibodies Directly from Cell Culture Supernatants

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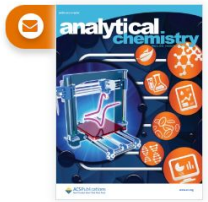
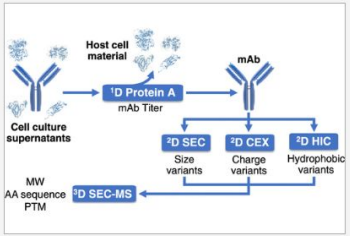


Analytical Chemistry

Abstract

Fully automated analysis of multiple structural attributes of monoclonal antibodies (mAbs) using three-dimensional liquid chromatography-mass spectrometry (3D-LC-MS) is described. The analyzer combines Protein A affinity chromatography in the first dimension (^{1}D) with a multimethod option in the second dimension (^{2}D) (choice between size exclusion (SEC), cation exchange (CEX), and hydrophobic interaction chromatography (HIC)) and desalting SEC-MS in the third dimension (^{3}D). This innovative 3D-LC-MS setup allows simultaneous and sequential assessment of mAb titer, size/charge/hydrophobic variants, molecular weight (MW), amino acid (AA) sequence, and post-translational modifications (PTMs) directly from cell culture supernatants. The reported methodology that finds multiple uses throughout the biopharmaceutical development trajectory was successfully challenged by the analysis of different trastuzumab and tocilizumab samples originating from biosimilar development programs.



[Link to publication]

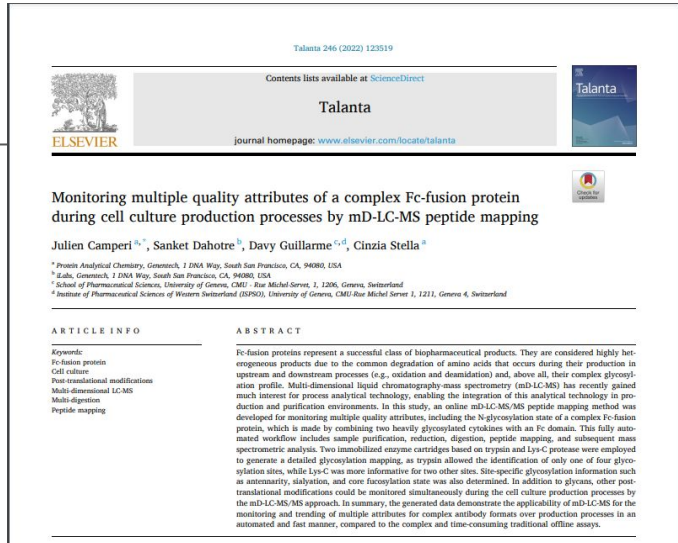
Multi-Attribute Monitoring and the Multi-Attribute Method: A Powerful Double Act for Supporting Biopharmaceutical Manufacturing

April 1, 2022
Craig Jakes, Sara Carillo, Silvia Millán Martín, Jonathan Bones
LCGC Supplements, Recent Developments in Biopharmaceutical Analysis, Volume 40, Issue s4
Pages: 23–25,34

[Link to publication]

Overview of a multidimensional LC/MS workflow for the monitoring of antibody minor variants over the cell culture process: **proof of concept study**

Link to paper



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Contents lists available at ScienceDirect

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Monitoring multiple quality attributes of a complex Fc-fusion protein during cell culture production processes by mD-LC-MS peptide mapping

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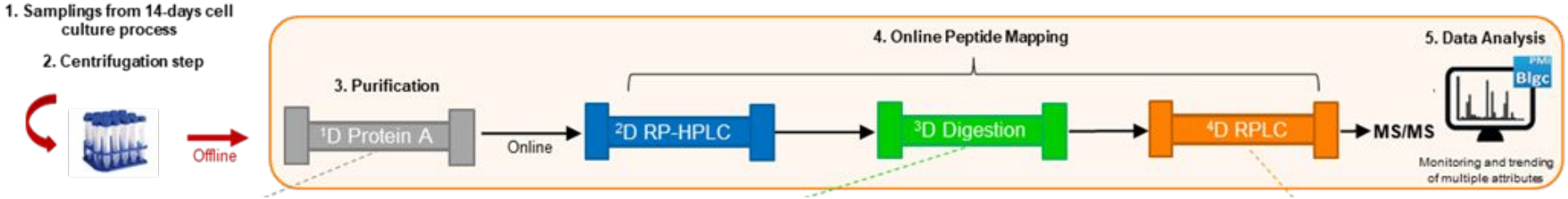
ABSTRACT

Fc-fusion proteins represent a successful class of biopharmaceutical products. They are considered highly heterogeneous products due to the common degradation of amino acids that occurs during their production in upstream and downstream processes (e.g., oxidation and deamidation) and, above all, their complex glycosylation profile. Multi-dimensional liquid chromatography-mass spectrometry (mD-LC-MS) has recently gained much interest for process analytical technology, enabling the integration of this analytical technology in production and purification environments. In this study, an online mD-LC-MS/MS peptide mapping method was developed for monitoring multiple quality attributes, including the N-glycosylation state of a complex Fc-fusion protein, which is made by combining two heavily glycosylated cytokines with an Fc domain. This fully automated workflow includes sample purification, reduction, digestion, peptide mapping, and subsequent mass spectrometric analysis. Two immobilized enzyme cartridges based on trypsin and Lys-C protease were employed to generate a detailed glycosylation mapping, as trypsin allowed the identification of only one of four glycosylation sites, while Lys-C was more informative for two other sites. Site-specific glycosylation information such as antennarity, sialyation, and core fucosylation state was also determined. In addition to glycans, other post-translational modifications could be monitored simultaneously during the cell culture production processes by the mD-LC-MS/MS approach. In summary, the generated data demonstrate the applicability of mD-LC-MS for the monitoring and trending of multiple attributes for complex antibody formats over production processes in an automated and fast manner, compared to the complex and time-consuming traditional offline assays.

mD-LC/MS workflow for the online monitoring of antibody minor variants over the cell culture production processes



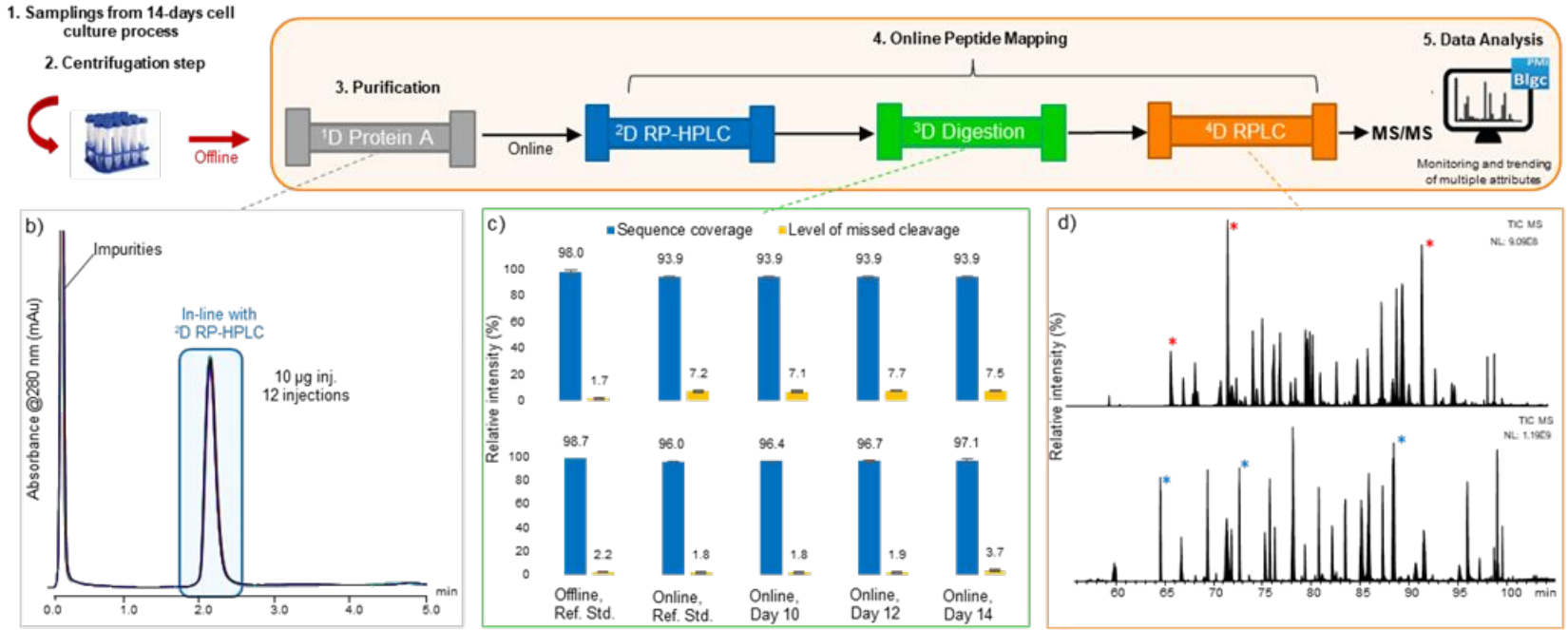
In-process cell culture samples collected over the 14-day process.

The supernatants were injected onto the mD-LC/MS system combining:

- **Protein A** for purification step
- **Online digestion** (tryptic and Lys-C)
- **Peptide mapping** analysis by RPLC-MS/MS

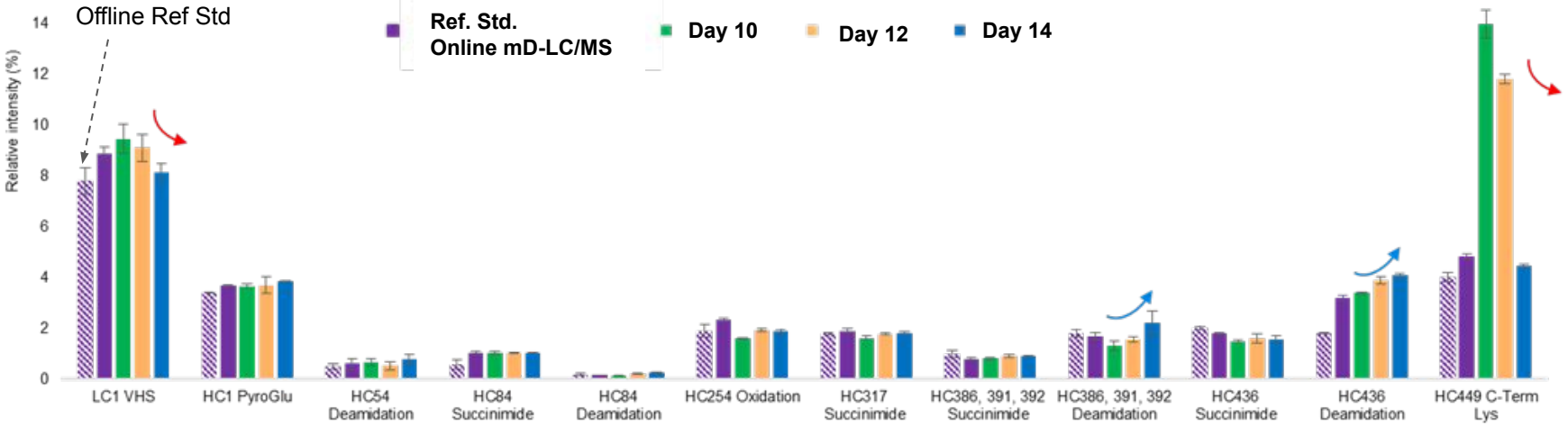
Each sample was analyzed in triplicate.

mD-LC/MS workflow for the online monitoring of antibody minor variants over the cell culture production processes



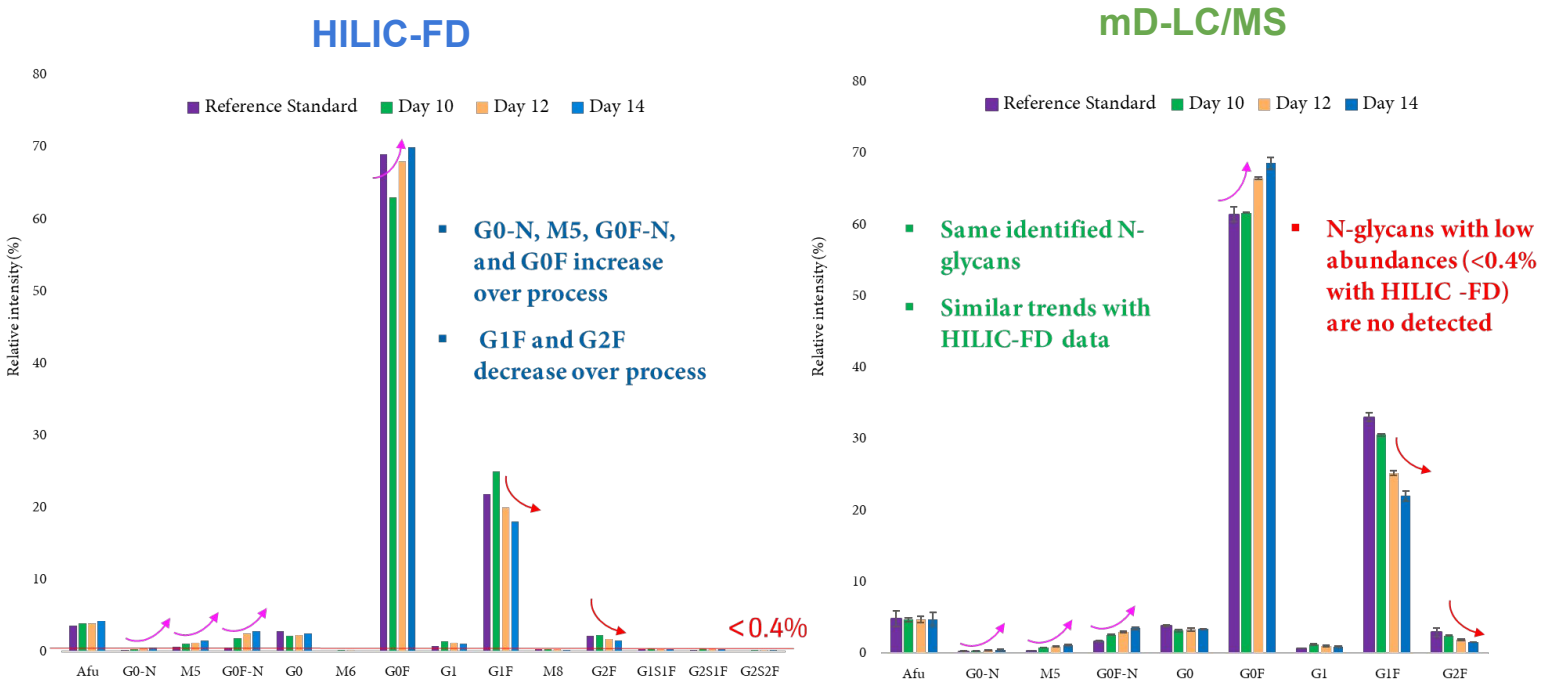
- **High sequence coverage** was obtained for both tryptic and Lys-C digests: **94%** and **96%**, respectively
- RSD values for the three selected unmodified peptides: 0.4 % (retention time) and 13% (peak area)

Comparison of PTMs with traditional offline assays (mAb)



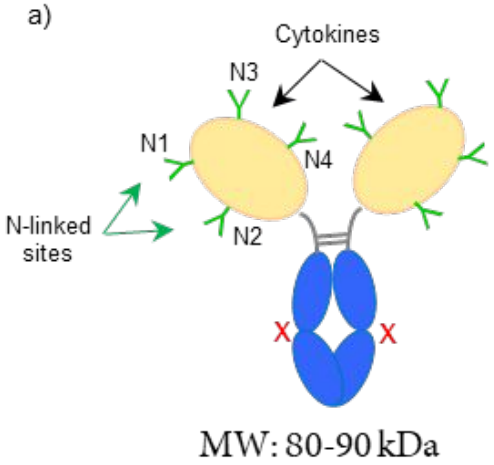
- **PTM levels** (n=3) of deamidation, succinimide formation, C-terminal lysine clipping, N-terminal glutamine cyclization, and the valine-histidine-serine (VHS) *vs* respective offline data obtained on the Ref Std: No significant differences
- **RSD < 8%** with the online approach
- VHS and C-term Lys decrease over the 14 days
- Deamidation slightly increase

Comparability with traditional offline assays (mAb N-glycopeptides)

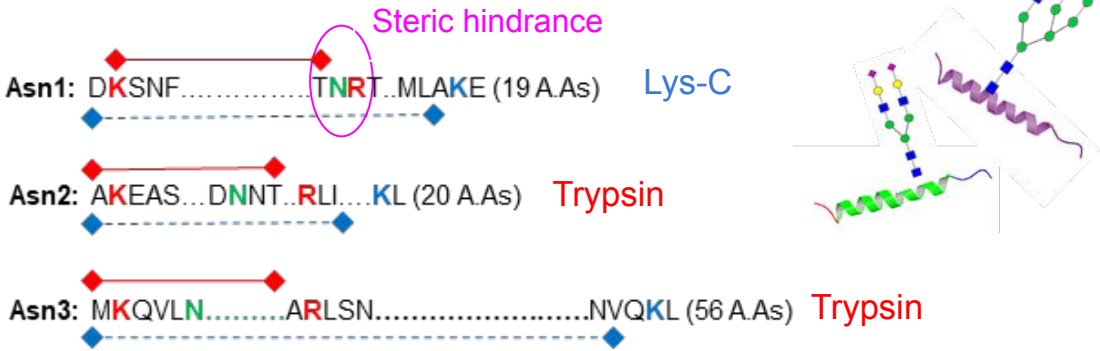


- A total of **nine N-glycans were identified by both approaches at similar levels,** with RSD < 11% for the online approach
- **Low (< 0.4%) level glycans** (G1S1F, G2S1F, G2S2F and M8) were only detected with the **HILIC method**
- Overall **good correlation** between the two assays

Use of mD-LC/MS for the analysis of a highly glycosylated Fc-fusion protein

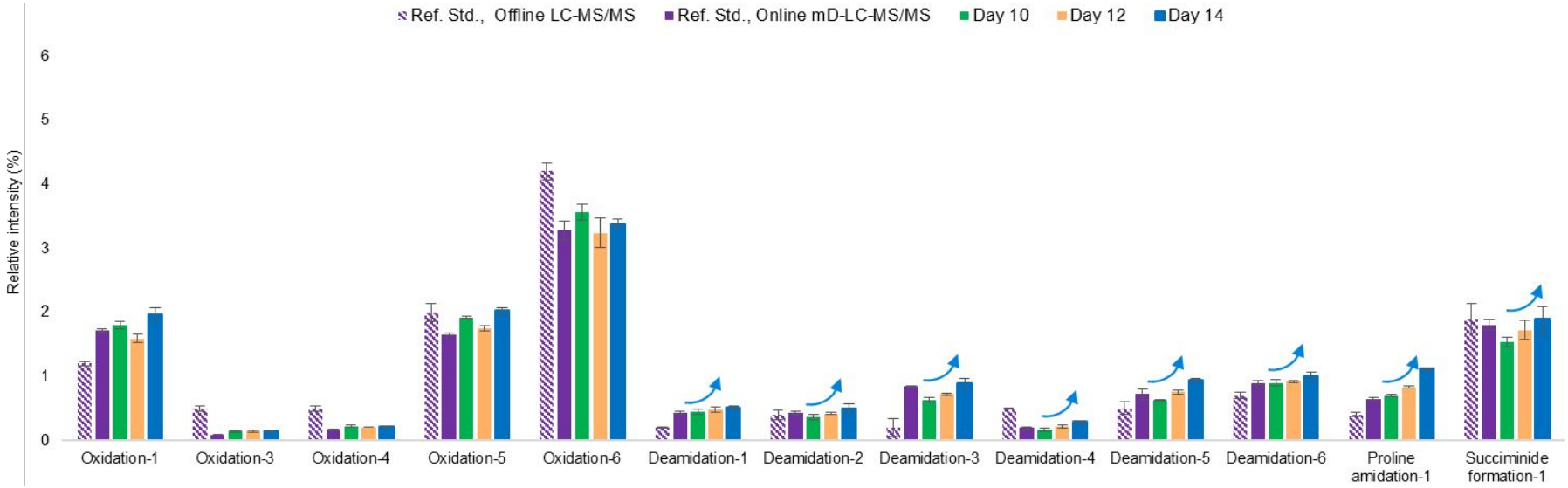


- **Two highly glycosylated cytokine ligands** attached to a Fc domain
- **Four N-glycan linked sites** were identified per cytokine
- The sequence of peptides having one N-linked site from Trypsin or Lys-C is represented:



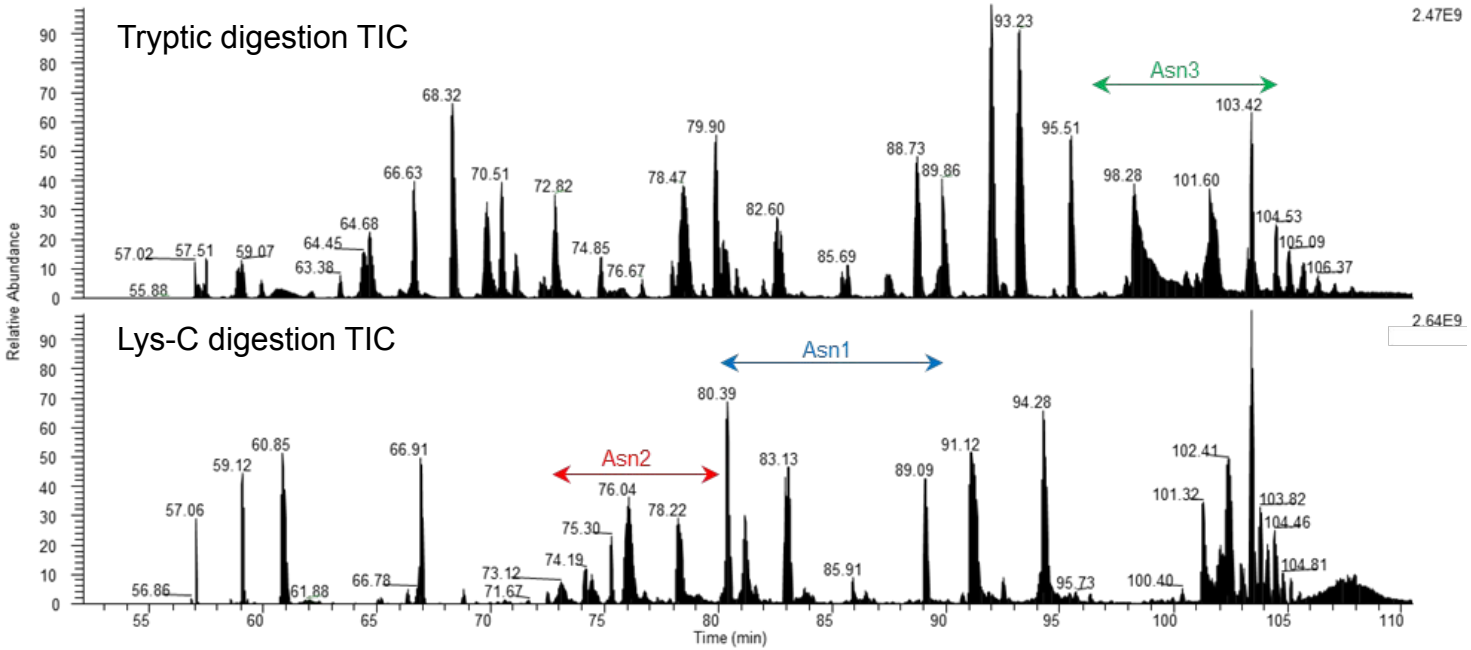
Complementarity of Trypsin and Lys-C proteases for the mapping glycosylation of the Fc-fusion protein

Quantification of PTM levels of the Fc-fusion protein



- PTMs were measured after **online tryptic digestion**
- The results show a **good correlation between the offline and online data** for the analysis of the reference standard, with RSD values lower than 7% for the online approach.
- Over the process, the **oxidation levels remained constant** and low, while **deamidation slightly increased**

Site specific N-glycosylation profile by mD-LC/MS



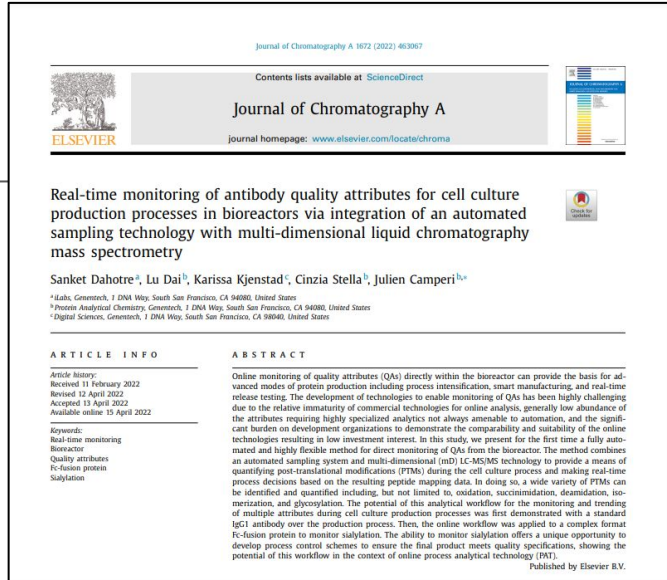
- The **Lys-C** peptide mapping provided information on the two N-linked sites at **Asn1 and Asn2**
- The **tryptic** peptide mapping provided information on the N-linked site at **Asn3**

Site specific N-glycosylation profile by mD-LC/MS

- **mD-LC/MS was able to provide site specific information** which would be lost upon releasing and pooling the N-linked glycans by traditional offline methods

Overview of a multidimensional LC/MS workflow **directly connected to the bioreactor**

Link to paper



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Real-time monitoring of antibody quality attributes for cell culture production processes in bioreactors via integration of an automated sampling technology with multi-dimensional liquid chromatography mass spectrometry

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ABSTRACT

Online monitoring of quality attributes (QAs) directly within the bioreactor can provide the basis for advanced modes of protein production including process intensification, smart manufacturing, and real-time release testing. The development of technologies to enable monitoring of QAs has been highly challenging due to the relative immaturity of commercial technologies for online analysis, generally low abundance of the attributes requiring highly specialized analytics not always amenable to automation, and the significant burden on development organizations to demonstrate the comparability and suitability of the online technologies resulting in low investment interest. In this study, we present for the first time a fully automated and highly flexible method for direct monitoring of QAs from the bioreactor. The method combines an automated sampling system and multi-dimensional (mD) LC-MS/MS technology to provide a means of quantifying post-translational modifications (PTMs) during the cell culture process and making real-time process decisions based on the resulting peptide mapping data. In doing so, a wide variety of PTMs can be identified and quantified including, but not limited to, oxidation, succinimidation, deamidation, isomerization, and glycosylation. The potential of this analytical workflow for the monitoring and trending of multiple attributes during cell culture production processes was first demonstrated with a standard IgG1 antibody over the production process. Then, the online workflow was applied to a complex format Fc-fusion protein to monitor sialylation. The ability to monitor sialylation offers a unique opportunity to develop process control schemes to ensure the final product meets quality specifications, showing the potential of this workflow in the context of online process analytical technology (PAT).

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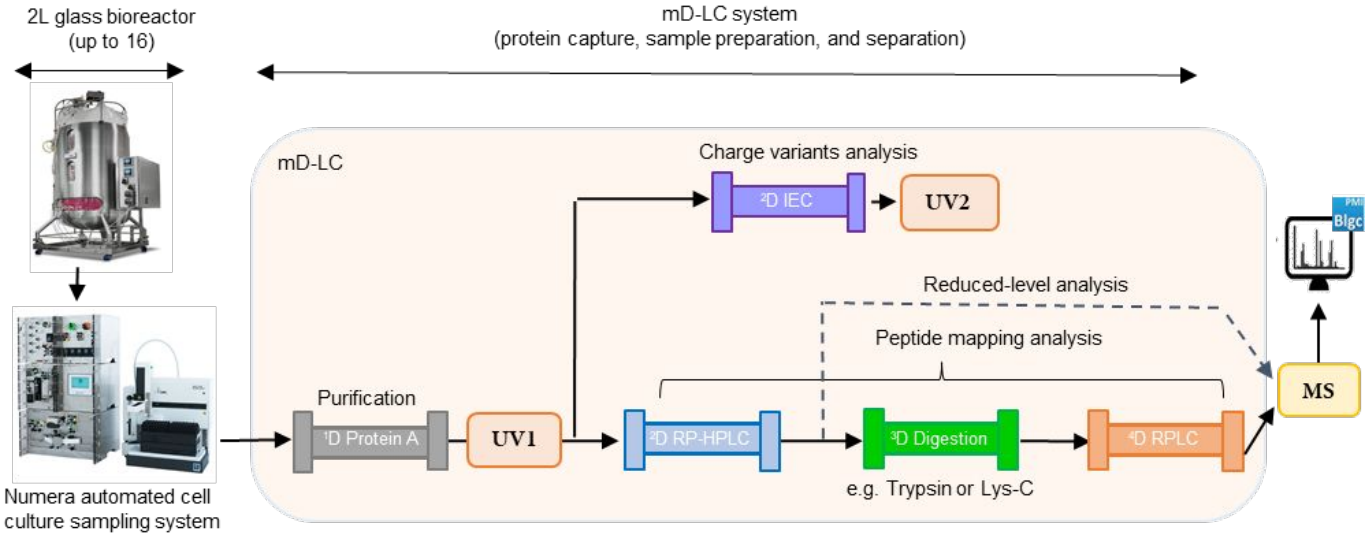
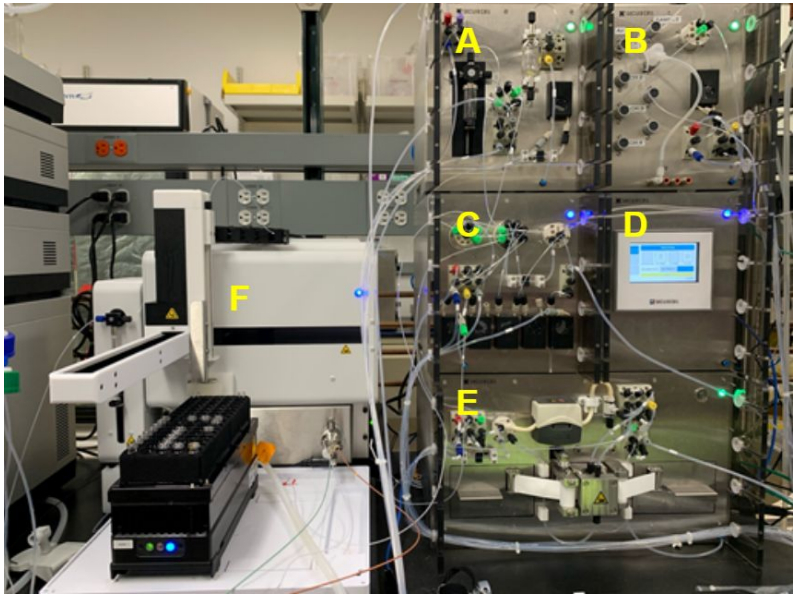
Online system for the real-time monitoring of antibody QAs at the manufacturing floor



mD-LC system allows to monitor charge variants and other PTMs at the **intact** and **peptide level** on the same instrument **without manual intervention** permits rapid and reliable characterization.

Overview of Numera Automated Sampling System



A	Dilution Module
B	Multiplexer Module
C	Routing Module
D	Control Module
E	Filtration Module
F	Liquid Handler

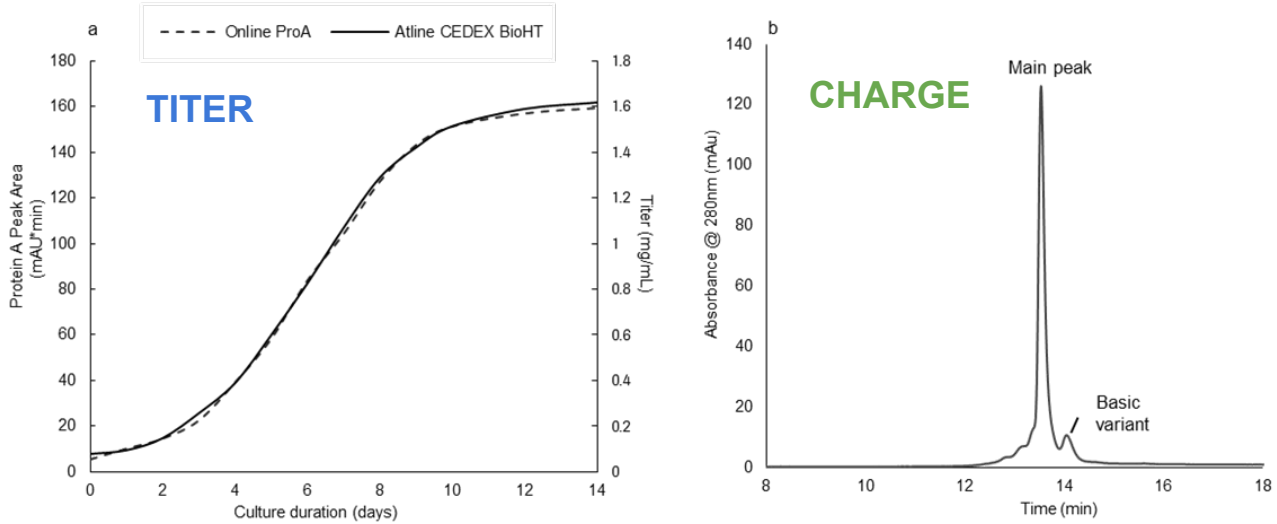
- The time from **sample draw to data output is about 2 hrs**
- Inputs regarding parameter specifications are placed into the Numera software to initiate the sample draw followed by mD-LC sample preparation and analysis

Comparison of Key Attributes for Commercially Available Autosamplers

Assessment Criteria	SegFlow by Flownamics	MAST by Bend Research	Numera by SecureCell
Contamination Rate	0%	0%	0%
Operational CHO Cell Density Upper Limit	6e7 cells/mL	>1e8 cells/mL	>1e8 cells/mL
Clogging and Carryover in flowpaths	Ceramic filter prone to clog, membrane has cracking issues	Cell-free system reported clogs and carryover	No reported issues
Cell-Free Sample Generation	Ceramic filter placed directly in reactor. Requires multiple headplate ports (one for cell-free and one for cell-containing). Clogs, but reusable.	ATF based. Very expensive, not robust. Vendor was seeking new solutions (Q2 2018).	Filter tape refreshed after each sample. No reported issues.
Rapid Sample Generation	<30min	<30min	15 min
Ease of Use	Single-use flow paths, simplest operationally	Complicated hardware, expensive, high maintenance	Single-use, minimal manual involvement.

Flexibility of the online mD-LC system

Significant flexibility to monitor process parameters and additional QAs such as titer determination or/and charge variants distribution



- **Online monitoring of titer** could enable a more streamlined process development or aid in the generation of large datasets to help with building models for spectroscopic evaluation
- **No manual intervention** permits rapid and reliable characterization.

Performance of the online peptide mapping workflow

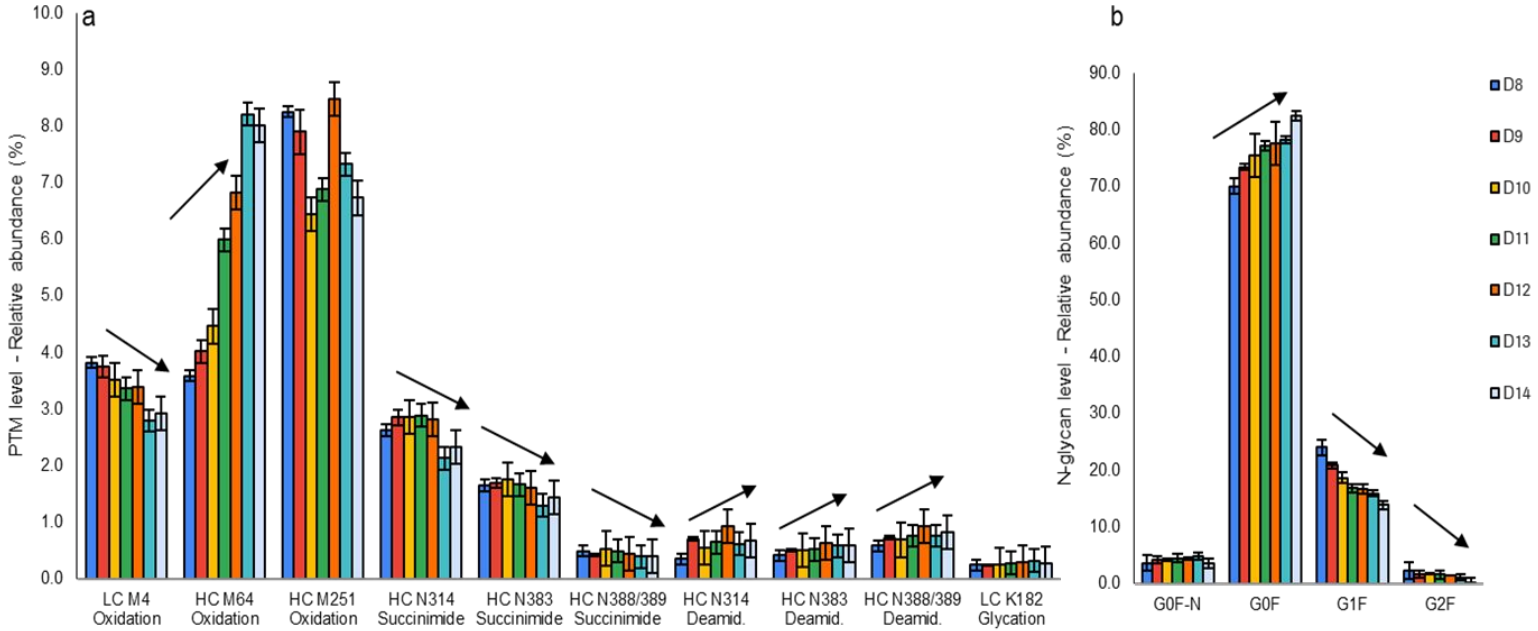
Digestion efficiency

Culture duration (Days)	Sequence coverage (%)	Missed cleavage (%)	Precursor m/z accuracy (ppm)	Fragment m/z accuracy (ppm)
8	LC:100; HC 96.8	5.5	2.7	3.0
9	LC:100; HC 96.8	5.9	2.3	3.1
10	LC:100; HC 96.8	6.4	2.4	3.3
11	LC:100; HC 96.8	6.9	2.2	3.6
12	LC:100; HC 96.8	7.7	2.2	3.3
13	LC:100; HC 96.8	8.4	2.6	4.0
14	LC:100; HC 96.8	9.2	2.4	3.8

- A **slight increase in the missed cleavage over the injections** is observed due to a higher amount of mAb injected onto the digestion cartridge.
- **High sequence coverage and missed cleavage levels remains below than 10%,** which is suitable for characterization of mAb variants.
- **High Mass Accuracy of the MS instrument** and consistency between multiple samples
- Critical aspect of real-time attribute monitoring based on peptide mapping data is the **daily performance of the MS.**

Monitoring and trending of PTMs of IgG1 antibody via online peptide mapping workflow

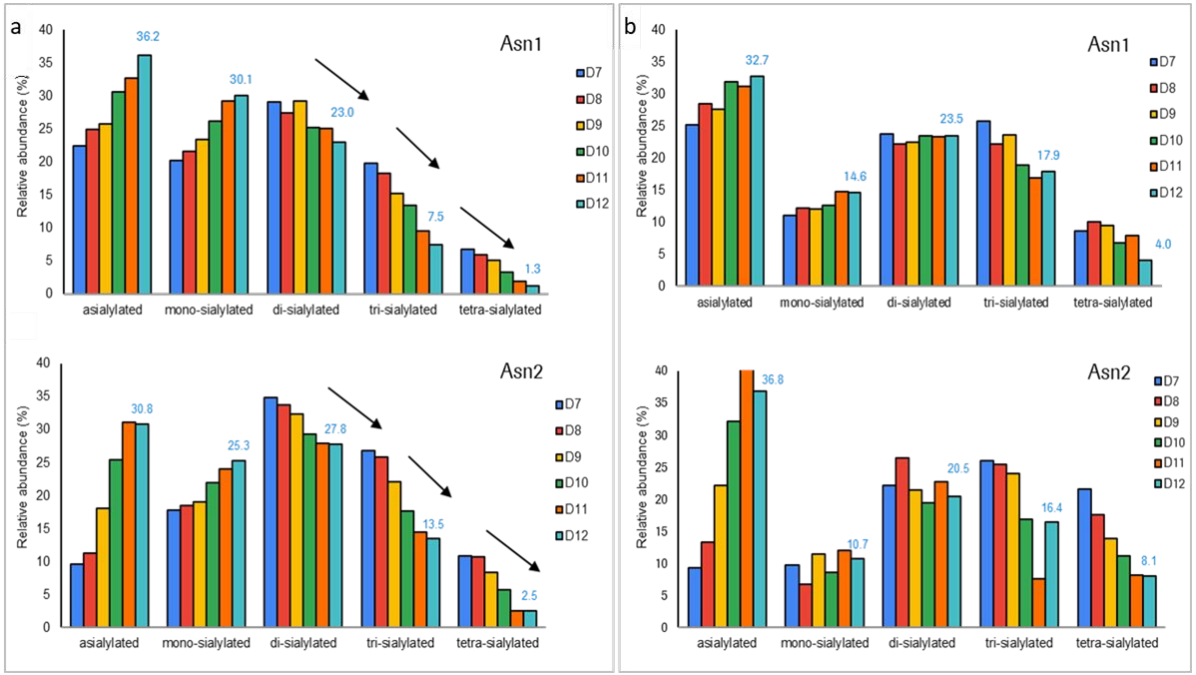
Day 8-14 PTMs trending



Considering the importance of glycans on product safety, efficacy, and clearance, the **capability to monitor these species in real time presents an opportunity to develop more robust processes and enable more consistent product quality**.

Online monitoring of sialic acid content of Asn1 and Asn2 sites for the Fc-fusion protein

Online system to monitor sialylation in real time directly at the bioreactor offers the **novel capability to make in-process decisions upon determination of the sialic acid concentration.**



- A clear d**ownward trend is observed for higher order sialylated species** (di-, tri, and tetra-sialylated)
- **Process conditions can easily be compared to determine the impact of inputs on quality outputs (**connected to up to 16 reactors).
- Culture 2 was supplemented with zinc at inoculation. **The addition of zinc is known to increase the total sialic acid concentration.**

Conclusions

- **Demonstrated capability of mD-LC/MS workflow for monitoring and trending** of multiple PTMs, for both standard mAbs and complex fusion proteins

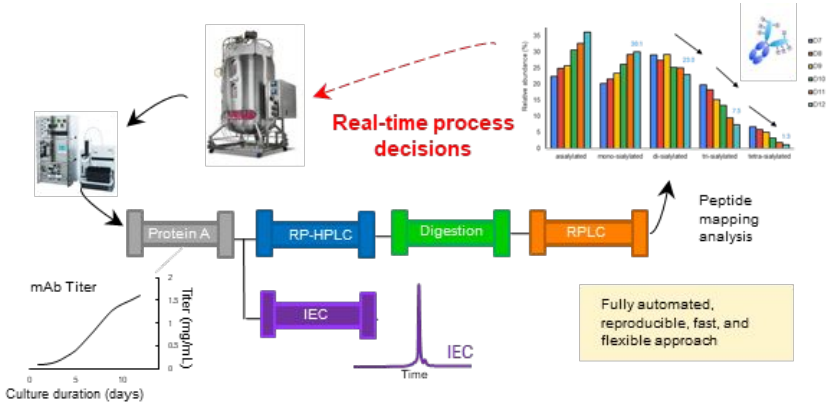
- **Multiple digestion enzymes can be used** on the same set-up, allowing complementarity of PTM characterization (including site specific N-glycosylation)

- The **connectivity with the bioreactor** was successfully demonstrated

Looking ahead

- **Online mD-LC/MS is a promising tool to enable advanced processing modes** including real time monitoring, feedback control, aimed at improving the process yield, as well as the product quality.

- **Online mD-LC/MS can also be used to complement probes and spectroscopic based measurements in the bioreactor**, to enhance the accuracy of the chemometric predictive models



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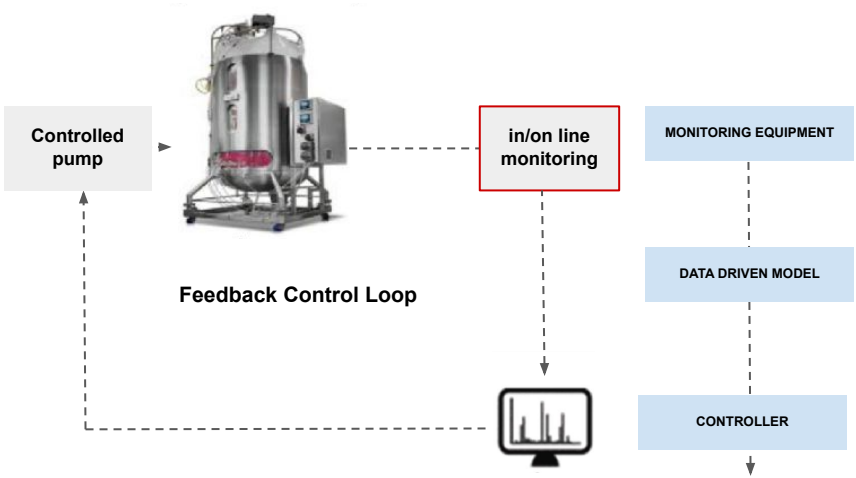
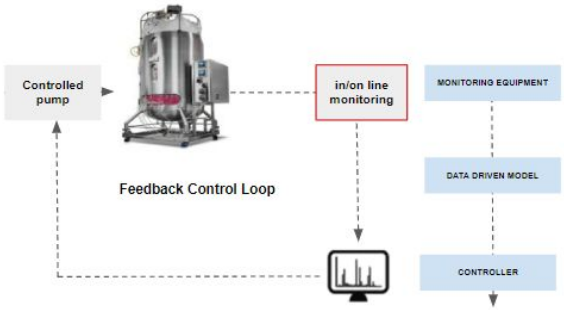

Controlled pump
in/on line monitoring
MONITORING EQUIPMENT
DATA DRIVEN MODEL
CONTROLLER
Feedback Control Loop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