

## Monitoring Intact Glycoprofiles and Spent Media Metabolites in Samples from Sartorius Ambr 250 High Throughput Bioreactor System to Support Upstream Process Development

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### Abstract

This application note details the use of the BioAccord™ LC-MS for the direct analysis of intact monoclonal antibody (mAb) and cell culture media (CCM) nutrient and metabolite profiles in spent media. The cell culture samples were retrieved from Ambr® 250 High Throughput system during a media and feed optimization study. Sample preparation including dilution and protein A purification were automated using the Andrew+™ Pipetting Robot and OneLab™ automated workflows. Major glycoform product quality attributes and detailed metabolite profiles were reported for each sample, thus providing a comprehensive snapshot of the bioreactors not available through conventional analyzers.

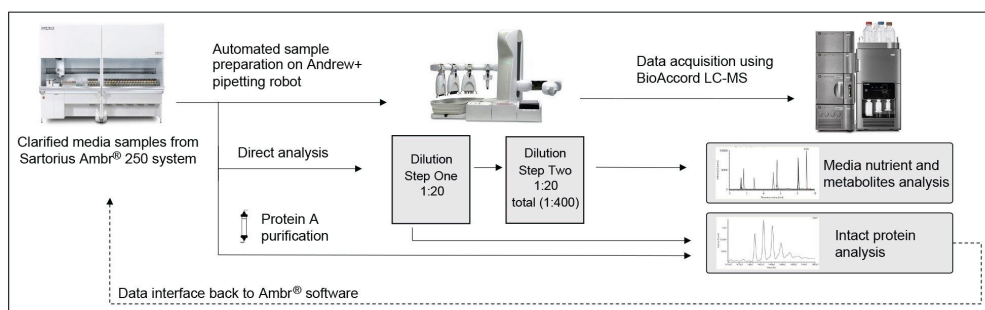
### Benefits

- Single analytical platform for rapid intact glycan distribution determination and cell culture media nutrient and metabolites monitoring
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- Automated and rapid one-step sample preparation for both intact mAb and cell culture media metabolite LC-MS analysis
- A single compliant-ready informatics package supporting data acquisition, data review, elucidation of unknowns, multivariate data analysis, and customized reporting

## Introduction

During the cultivation process in protein therapeutics production, critical quality attributes (CQAs) such as protein concentration denoted IgG titer (g/L) and post translational modification (PTMs) of the protein expressed as %modification are routinely monitored. Nutrients and metabolites, which are important for protein production, cell viability, and growth, can also be routinely monitored to provide guidance and understanding of the critical process parameters (CPPs). The analysis of CQA and CPP are typically run in separate LC-MS analyses with different analytical instruments. This application note demonstrates the advantage of synchronizing these two types of analyses through shared, rapid, and simple sample preparation using Andrew+ Pipetting Robot, and simplified analysis using the BioAccord LC-MS System. An overview of the analysis process is shown in Figure 1. Examples of data obtained for samples from an optimization experiment using the Sartorius Ambr 250 High Throughput multiparallel bioreactor platform are summarized. Detailed description for each of the workflow can be found in previous published Waters application notes.<sup>1,2</sup>



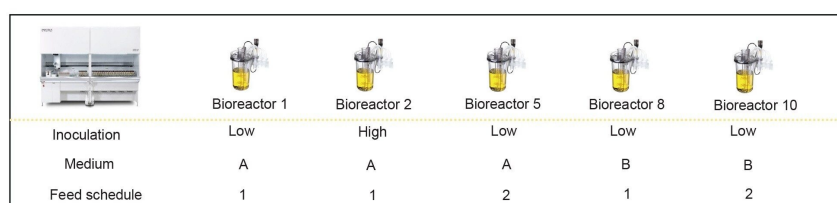
*Figure 1. A schematic illustration of automated sample preparation and BioAccord LC-MS analysis of intact protein and cell culture media nutrient and metabolites analysis in spent media from bioreactors.*

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## Experimental

### Sample Preparation

A process optimization experiment using a CHO cell line for mAb production was carried out on the Ambr 250 High Throughput multi-parallel bioreactor system (Sartorius, USA). Five bioreactors were monitored in parallel where starting media, feed, and inoculation density were varied and samples were taken at cultivation days 4, 6, 8, 10, and 12 (Figure 2).



	Bioreactor 1	Bioreactor 2	Bioreactor 5	Bioreactor 8	Bioreactor 10
Inoculation	Low	High	Low	Low	Low
Medium	A	A	A	B	B
Feed schedule	1	1	2	1	2

*Figure 2. Summary of experimental conditions of samples collected in the intact and culture media monitoring study.*

The cell culture media samples were immediately centrifuged and filtered using a 0.22 µm syringe filter (Sartorius Minisart® PES 15 mm p/n: 1776D-Q). The clarified samples were serially diluted at 1:20 (V/V) for intact mass analysis and further diluted at 1:20 (1:400 (V/V) in total) for spent media analysis into a 350 µL 96 well plate using the Andrew+ Pipetting Robot (Waters Corporation, USA). The diluent used was 0.1% formic acid (FA) containing 0.1 µM 3-chlorotyrosine as an internal standard. The initial 1:20 (V/V) diluted samples were analyzed using the intact protein analysis workflow. The subsequent 1:400 diluted samples were analyzed using the cell culture media screening workflow. For protein A purified samples, an additional 120 µL of sample was transferred to an Andrew+ Pipetting Robot and loaded onto Protein A resin which had been washed and conditioned. Samples were washed, then eluted with 100 mM glycine using the Andrew Extraction+ device.<sup>3</sup> The sample was further diluted 1:20 (V/V) prior to analysis.

### Method Conditions

LC-MS parameters described in the following tables employed the same mobile phases to run both intact and cell culture media reversed phase methods. This greatly simplified daily operation and maintenance of the LC-

MS system.

## LC-MS Conditions for Intact Protein Analysis

LC-MS system	BioAccord™ LC-MS system with ACQUITY Premier BSM				
Column(s)	ACQUITY Premier® Protein BEH C <sub>4</sub> 300 Å 1.7 µm, 2.1 × 50 mm (P/N 176005107)				
Column temp.	80 °C				
Sample temp.	10 °C				
Injection volume	2 µL				
TUV wavelength	280 nm				
Flow rate	0.4 mL/min				
Mobile phase A	0.1% FA in H <sub>2</sub> O				
Mobile phase B	90% ACN/10% IPA/0.1% FA				
Gradient table	<b>Time (min)</b>	<b>Flow (mL/min)</b>	<b>%A</b>	<b>%B</b>	<b>Curve</b>
	0	0.4	95	5	6
	1.0	0.4	95	5	6
	3.5	0.4	15	85	6
	3.7	0.4	5	95	6
	4.3	0.4	5	95	6
	4.5	0.4	95	5	6
	5.0	0.4	95	5	6
Ionization mode	Full scan				
Mass range	High (400–7000 <i>m/z</i> )				
Polarity	Positive				
Cone voltage	70 V				
Capillary voltage	1.5 kV				
Scan rate	5 Hz				
Desolvation temp.	550 °C				
Intelligent data capture	Off				
Lockmass correction mode	Standard				
MS Event table	0–0.8 min, 3.5 min divert to waste				
LC-MS software	waters_connect 3.1 or higher				
Informatics	Intact Mass App and UNIFI App				

## LC-MS Conditions for Media Nutrients and Metabolites Analysis

LC-MS system	BioAccord LC-MS system with ACQUITY Premier BSM				
Column(s)	ACQUITY Premier HSS T3 Column 1.8 $\mu$ m, 2.1 $\times$ 150 mm (P/N 186009469)				
Column temp.	40 $^{\circ}$ C				
Sample temp.	10 $^{\circ}$ C				
Injection volume	2 $\mu$ L				
Flow rate	0.25 mL/min				
Mobile phase A	0.1% FA in H <sub>2</sub> O				
Mobile phase B	90%ACN/10%IPA/0.1%FA				
Gradient table	<b>Time (min)</b>	<b>Flow (mL/min)</b>	<b>%A</b>	<b>%B</b>	<b>Curve</b>
	0	0.25	100	0	6
	1.5	0.25	100	0	6
	6	0.25	95	5	6
	9	0.25	60	40	6
	14	0.25	5	95	6
	17	0.25	5	95	6
	17.1	0.25	100	0	6
	20	0.25	100	0	6
Mode:	Full scan*				
Mass range	Small molecules (50–800 <i>m/z</i> )				
Scan rate	5 Hz				
Polarity	Positive				
	Cone voltage	20 V			
	Fragmentation cone voltage	60–80 V			
	Capillary voltage	1.00 kV			
Polarity	Negative				
	Cone voltage	15 V			
	Fragmentation cone voltage	50–70 V			
	Capillary Voltage	0.8 kV			
Desolvation temp.	550 $^{\circ}$ C				
Intelligent data capture	On				
Lockmass correction mode	Standard				
Acquisition time window	Start time = 0 min, End time = 14 min				
MS event table	0–0.8 min, and 14 min divert to waste				
LC-MS software	waters_connect 3.1 or higher				
Informatics	UNIFI – Accurate mass screening using the cell culture media screening workflow				

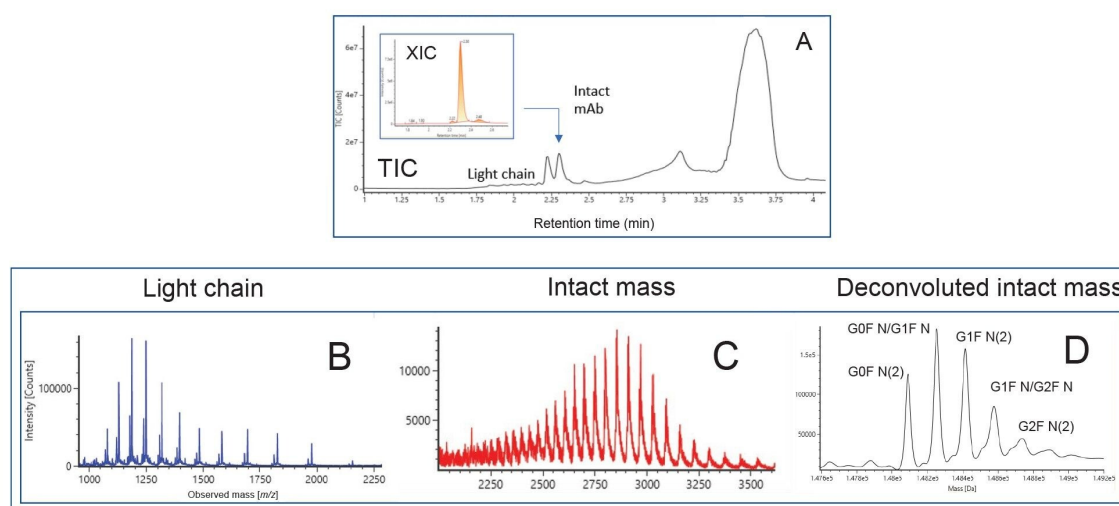
\*Full Scan with fragmentation mode is also available, useful when characterizing new campaigns.

## Results and Discussion

### Part I. Intact Mass Analysis

## 1. Determination of Glycan Distribution from Clarified Cell Culture Media

Protein modification and glycan distribution were determined from direct analysis without any prior affinity based sample purification using a high throughput LC-MS method. The organic mobile phase used contained 10% IPA, the same mobile phase used in cell culture media metabolites analysis, thus simplifying mobile phase preparations when both intact mass and media analysis are carried out on the same system. Chromatographic comparison of mobile phases containing either 100% ACN or 90%ACN/10%IPA showed similar results (see Appendix). Figure 3A shows the observed total ion chromatogram (TIC) of a representative media sample using intact analysis method, displaying light chain, intact mAb, and broad matrix peaks. The extracted ion chromatogram (XIC) showed good peak characteristics of intact mAb with minimal interference of the late eluting matrix with observed  $m/z < 1500$  Da (Figure 3A). Mass spectra in Figures B-D are consistent with charge envelopes typically observed for light chain and intact mAb.



*Figure 3. (A) Representative MS spectrum of direct intact mass analysis from cell culture media sample. The inserted figure showed XIC for intact mAb with  $m/z$  2000–4000 Da. (B) observed MS spectrum of light chain, (C) observed MS spectrum of intact mAb, and (D) deconvoluted spectrum of intact mAb, labelled with major glycoforms.*

The average mass of the molecule was obtained through MaxEnt1 deconvolution as shown in Figure 3D. From the deconvoluted spectrum glycan modifications were identified based on user input of the expected intact mAb mass ( $m/z$ ) and a list of potential modifications. For the mAb produced in this study, five major glycoforms were

detected: G0F N(2), G0F N/G1F N, G1F N(2), G1F N/G2F N, and G2F N(2). Figure 4 displays the overlaid bar graph of individual %glycan for each sample. This information was exported back to Ambr® software for rapid product quality assessment of mAb product using the data interface. An example of data display in Ambr software is shown in Appendix B.

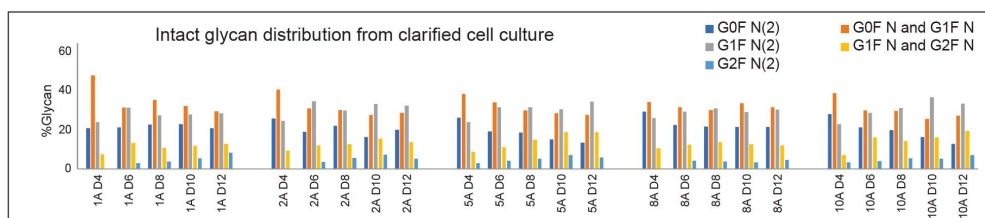


Figure 4. %Glycan determination from clarified cell culture media sample, showing overlaid bar plot of each of the glycan as a function of bioreactor and over time.

## 2. Determination of Glycan Distribution from Protein A Purified Sample

Protein modification and glycan distribution were also determined for samples prepared through protein A purification. Protein A purification process removed matrix ions and majority of process related impurities to produce "pure" mAb for analysis. The resulting total ion chromatogram showed a single large mAb peak in both MS and UV chromatograms (Figure 5). Protein modification and glycan distribution were determined based on MaxEnt1 deconvolution. The %glycan distribution shown in Figure 5 are similar to those determined from clarified sample without protein A purification (Figure 4). These data suggest that glycan profiles can be directly determined using clarified media samples for an improved process efficiency by simplifying and reducing sample preparation cost and time.

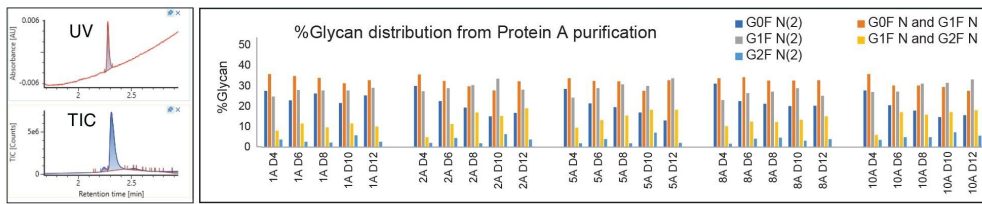


Figure 5.(left) Representative UV and MS TIC chromatogram of media sample post protein A purification. (right) %Glycan determination from protein A purified sample, showing overlaid bar plot of each of the glycan as a function of bioreactor and over time.

### 3. Correlation of MS/UV Response with Cedex Bio HT Measurement

The observed UV and MS response were correlated with mAb titer determined using Cedex Bio HT instrument. UV response was calculated based on the peak area at 280 nm wavelength. MS response was calculated by summarizing the XIC signals from nine most abundant charge states in the observed spectrum. A description of intact protein quantification can be found in Waters application note.<sup>4</sup> Figure 6 showed the trend plots of UV and MS responses as a function of bioreactor and sampling days. Similar data trending of UV and MS plots suggest both responses may be used for titer estimation when standard was available. Correlation with the titer measurement produced correlation coefficient of  $R^2=0.954$  based on MS response and  $R^2=0.902$  based on UV absorbance. The slightly better correlation coefficient afforded from MS data appeared to be the results of added specificity from MS selectivity to minimize matrix and other interferences especially at lower titer. Overall, UV and MS response provided by the BioAccord displayed high correlation levels with measured IgG titer and can be part of glycoprofiling analysis output. The rapid analysis conditions suggest the LC-MS method could support throughput needed in process monitoring.



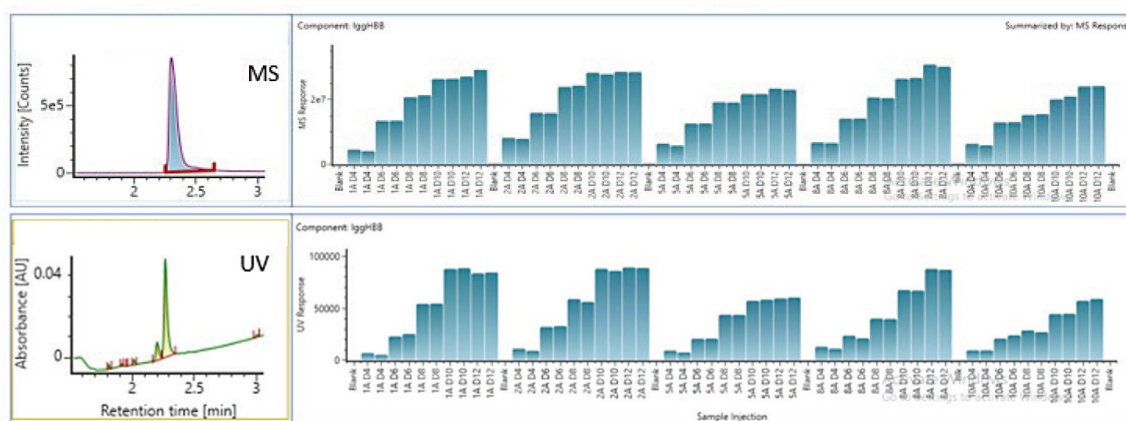


Figure 6. Overlaid bar plot of MS response or UV absorbance across bioreactors and overtime. Each sample was analyzed in duplicate with an overall reproducibility <5%.

## Part II. Cell Culture Media Nutrients and Metabolites Analysis

Bioreactor samples were also subjected to the spent media analysis workflow. A detailed discussion of the workflow has been described previously.<sup>2</sup> In this analysis, a recently introduced small mol mass acquisition range of 50–800  $m/z$  was employed which enabled a 5–10 times enhancement in detector sensitivity for both positive and negative acquisitions over previous methods. In all, ~100 nutrients and metabolites were detected.

Compound distribution identified in these samples are segmented by compound class is shown in Figure 7.

Examples of media changes as a function of bioreactor and day are shown in the trending plots in Figure 8. For

this study, all amino acids were calibrated using an AA cell culture standard solution Waters p/n: [186009300 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009300-amino-acid-cell-culture-standard-kit.html >](https://www.waters.com/nextgen/global/shop/standards--reagents/186009300-amino-acid-cell-culture-standard-kit.html) ), thus determined amino acids concentration are reported in mM (tryptophan example shown

in Figure 8 top). For all other detected compounds, relative response is provided and can be used to view trends (Figure 8 bottom). Optionally, additional components may be quantified by generating the appropriate standard curve. All data can be exported for additional reporting.

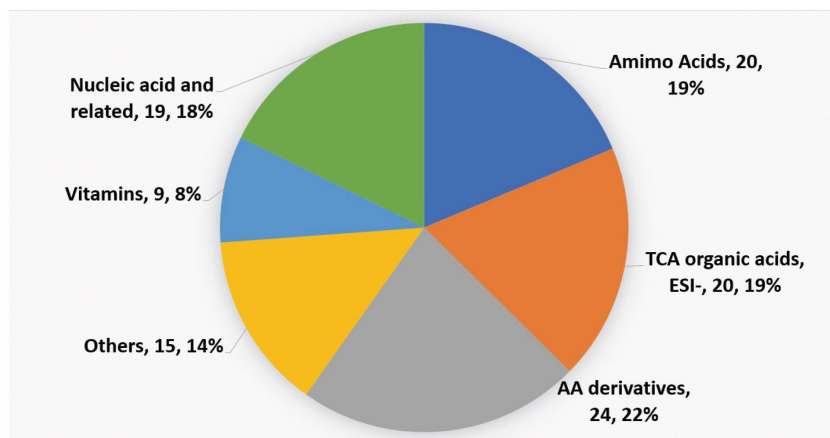


Figure 7. Observed compounds in the spent media, represented as %distribution and number of compounds detected, grouped by compound class.

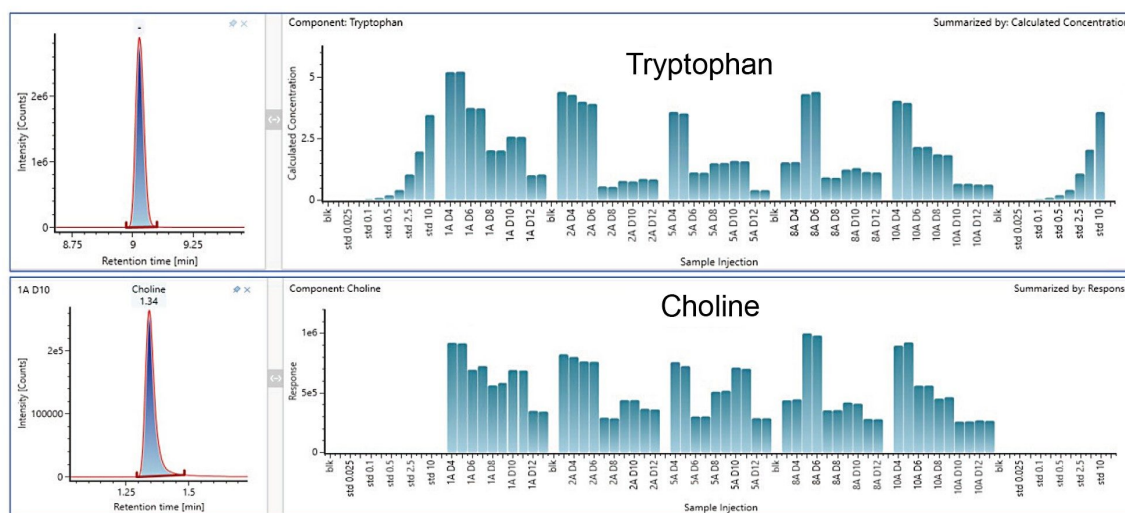


Figure 8. Representative trend plots of metabolites displaying bioreactor sampling over time. Representative results for Tryptophan (absolute concentrations) and Choline (relative trends) shown.

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## Conclusion

Automated sample preparation and LC-MS based analytical methods for intact protein, titer and cell culture nutrient and metabolites in spent media were described. The combination of BioAccord LC-MS System and Andrew+ Pipetting Robot provides the capability to process PD samples from Ambr 250 High Throughput multi-parallel bioreactor system quickly to provide high quality results. Highlights and capabilities include:

- Automated sample preparation combining both intact mAb and spent media components analysis using the Andrew+ Pipetting Robot
- Intact mAb analysis for IgG titer and glycoprofiling either directly from clarified cell culture samples or from protein A purified samples
- Direct media nutrients and metabolites analyses performed on clarified cell culture media sampled from bioreactors
- Connectivity by waters\_connect data Interface for a seamless data transfer of glycoprofiling results back to Ambr software
- The compact and user friendly BioAccord LC-MS System produced excellent data quality and was sufficient for throughput needs

The BioAccord LC-MS System offers process development labs the capability to add high quality process monitoring and product quality control assays to support process optimization in a timely manner.

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## References

1. H Shion, P Boyce, SJ Berger, YQ Yu. INTACT Mass™ - a Versatile waters\_connect™ Application for Rapid Mass Confirmation and Purity Assessment of Biotherapeutics. Waters Application Note. [720007547](#). 2022.
2. YW Alelyunas, MD Wrona, W Chen. Monitoring Nutrients and Metabolites in Spent Cell Culture Media for Bioprocess Development Using the BioAccord LC-MS System with ACQUITY Premier. Waters Application Note. [720007359](#). 2021.
3. SM Koza, CM Hanna AHW Jiang, YQ Yu. Automated High-Throughput Analytical-Scale Monoclonal Antibody

Purification Using Production-Scale Protein A Affinity Chromatography Resin. Waters Application Note. [720007861](#). 2023.

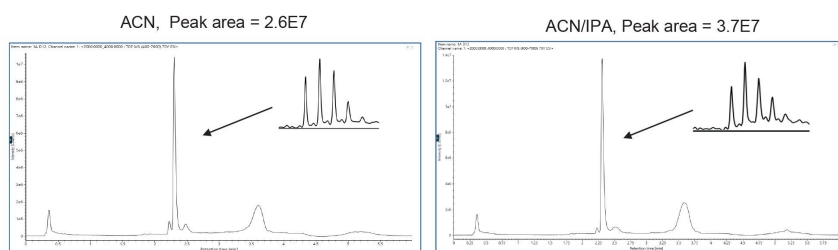
4. YW Alelyunas, H Shion, MD Wrona. High Sensitivity Intact Monoclonal Antibody (mAb) HRMS Quantification. Waters Application Note. [720006222](#). 2018.

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## Appendix

### A. Intact Mass Chromatographic Comparison Between Using 100% ACN vs 90%ACN/10%IPA

In the present intact mass method, LC-MS organic mobile phase B consisted of 90% acetonitrile (ACN) and 10% isopropanol alcohol (IPA), the same mobile phase as used for cell culture media nutrients and metabolite analysis. In traditional intact protein analysis, organic mobile phase B usually consists of 100% ACN only. The effect of adding 10% IPA into ACN for mobile phase B on separation was compared with those using 100% ACN. Results shown in Figure A suggest that the addition of 10% IPA had minimal effect on chromatographic performance and observed mass spectra. Comparison of peak areas suggested that the addition of IPA could be beneficial, leading to >30% increase in peak area for the sample analyzed. This enhancement could be the result of potential enhanced ionization efficiency due to the presence of IPA. In conclusion, 90%ACN/10%IPA was chosen for intact mass analysis to simplify daily operation and maintenance of the LC-MS system.



*Figure A. Extracted ion chromatogram of intact mAb analysis of a clarified cell culture media sample, comparing using 100% ACN (left) with 90%ACN/10%IPA (right) as the organic mobile phase B.*

## B. Example of Data Display in Ambr Software Using Data Interface Output

After data acquisition using Intact Mass App, the resulting glycoform data can be exported to Ambr software.

Figure B is one example showing the overlay of %glycoforms from different bioreactors as a function of incubation time (day).

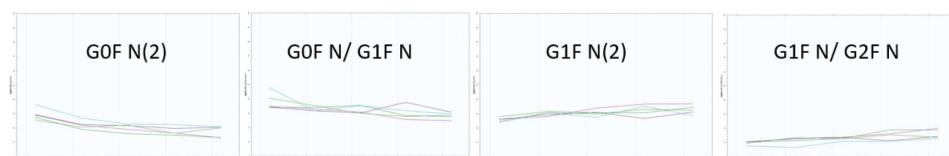


Figure B. Data interface display of overlaid plots of major %glycan modifications as a function of bioreactor and over time.

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## Featured Products

[ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>](https://www.waters.com/134613317)

[BioAccord LC-MS System for Biopharmaceuticals <https://www.waters.com/waters/nav.htm?cid=135005818>](https://www.waters.com/waters/nav.htm?cid=135005818)

[ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>](https://www.waters.com/514228)

[Screening Platform Solution with UNIFI <https://www.waters.com/134682903>](https://www.waters.com/134682903)

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720008042, September 2023



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