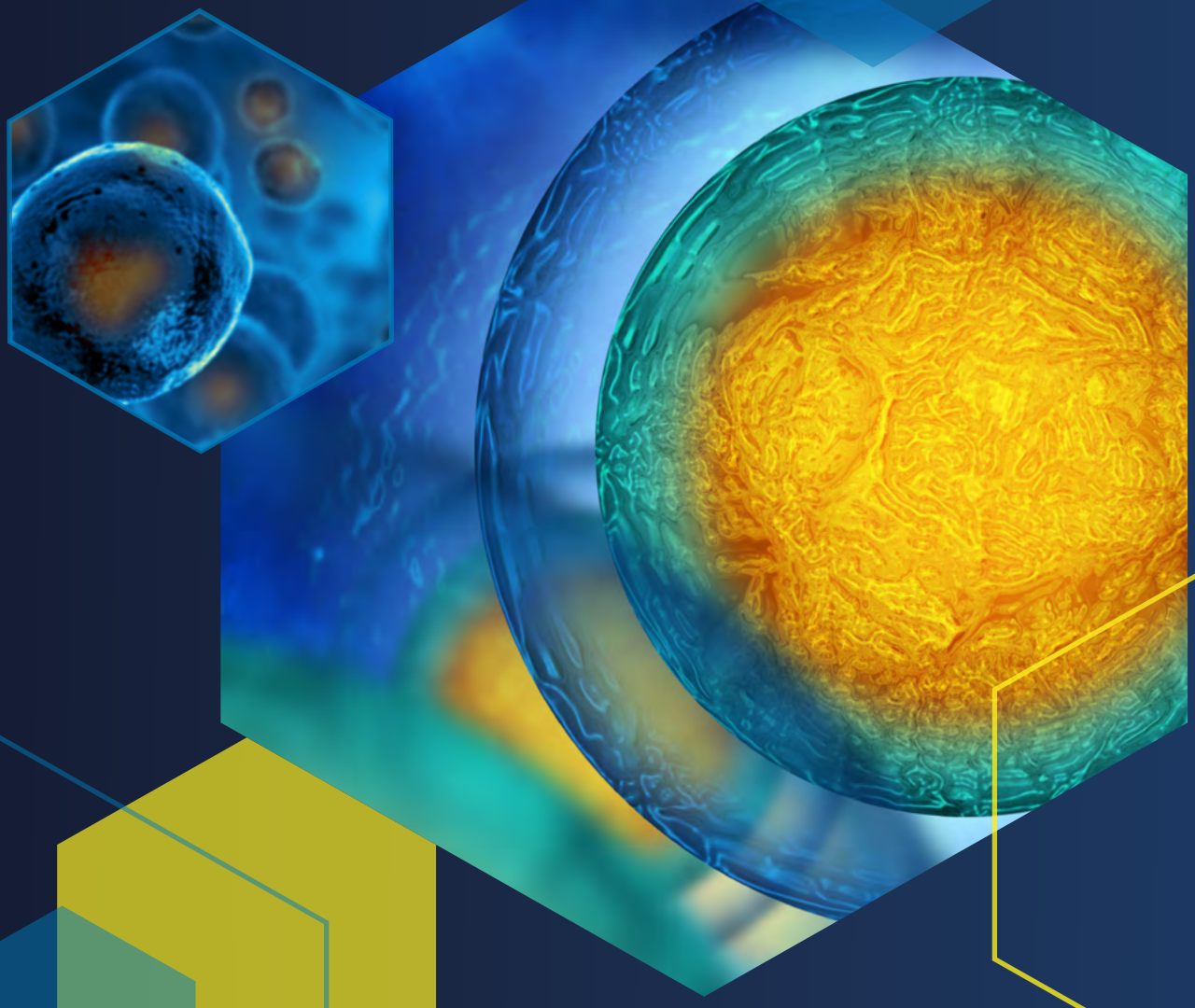
[APPLICATION NOTEBOOK]

At-line Solutions for Upstream Bioprocessing

Application Notes



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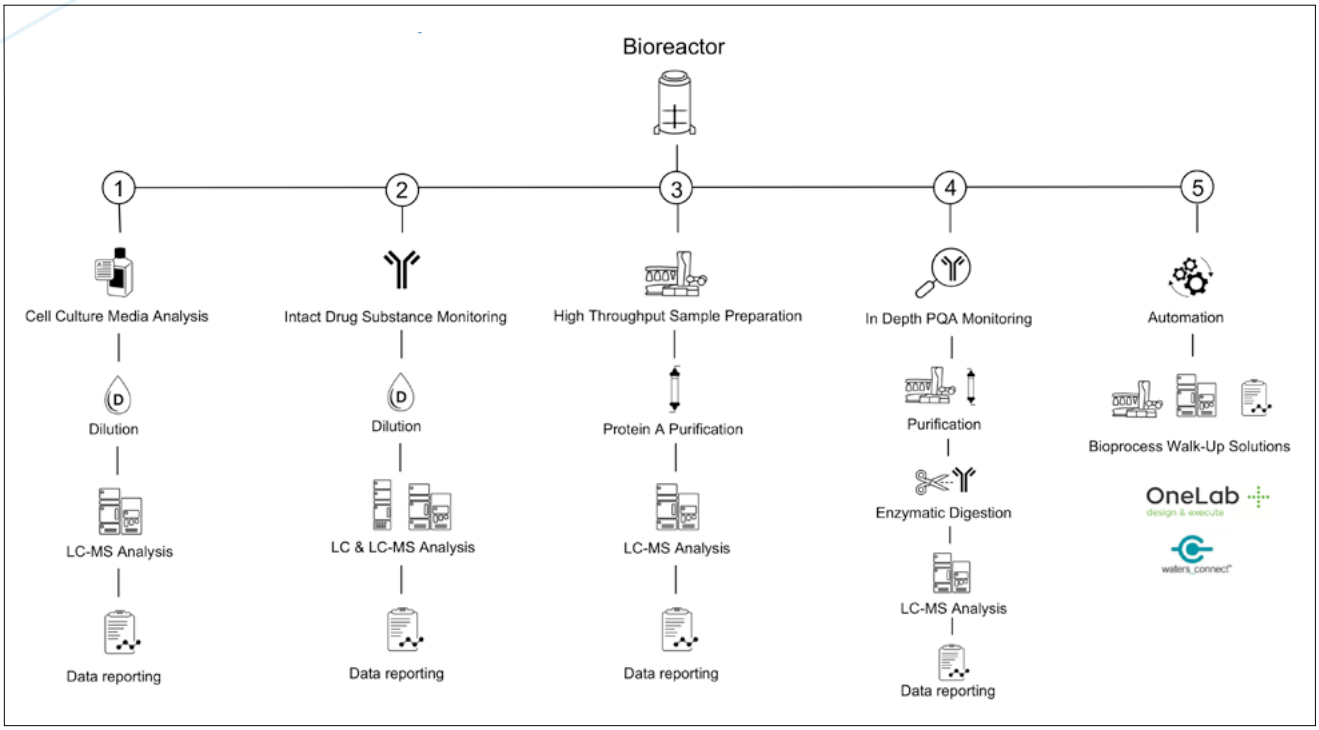
Introduction

Bioprocessing harnesses the power of living cells to manufacture valuable products such as biological drugs for clinical use. The introduction and use of biologics has revolutionized biotherapeutics to offer patients newer, more efficient, and safer treatment options. Therapeutic classes include recombinant proteins, monoclonal antibodies (mAbs), vaccines, gene therapies and beyond. The bioprocess development and manufacturing of next generation biotherapeutic modalities require the implementation of advanced process analytical technologies (PAT), such as liquid chromatography (LC) and mass spectrometry (MS), to obtain further comprehensive data on critical product quality attributes and process parameters to increase process understanding, robustness and throughput while simultaneously reducing developing timelines and costs. At the same time, the analytical LC-MS workflows and solutions need to be robust, rapid, and easy to use to enable non-MS experts to adopt them at point-of-need in their setting.

This notebook compiles selective recent application notes generated by the Discovery & Development Scientific Team at Waters. They illustrate key workflows and methods for process and product attribute monitoring in upstream cell line and process development applications using Waters Andrew+™ Pipetting Robot for sample preparation, the BioAccord™ LC-MS System for analysis and waters_connect™ and OneLab™softwares for streamlined data analytics and automation. The notebook is divided into five sections:

Overview of Waters at-line solutions for upstream bioprocessing



1. **Cell Culture Media Analysis -** Cells are the living factories to produce biologics and it is therefore fundamental to have a deep understanding of the cell metabolism and nutrient requirements to optimize the culturing conditions and process outcome. In this section we demonstrate the power of using high performing analytics (UPLC and Tof based high-resolution mass detection) in combination with a comprehensive cell culture media component library (200+ components) to be able to perform both absolute and relative monitoring of cell nutrients and metabolites. Deploying the easy-to-use BioAccord LC-MS System, with the comprehensive cell culture media (mammalian, bacterial and cell & gene therapy) library will give a fuller view of the cell media composition and changes. Another benefit of using at-line LC-MS is the possibility to identify and add unknowns that have important impact on the process. The embedded Umetrics® EZ-info solution for multivariate data analytics (MVDA) facilitates within and between batch comparisons, design-of-experiment (DoE) media feed strategies and to use this data in a quality-by-design (QBD) approach.
2. **Intact Drug Substance Monitoring –** Monitoring the intact drug substance gives rapid and vital insights on the consistency and quality of the process and product formation during cell culturing. The ability to use the BioAccord LC-MS System for direct analysis of crude bioreactor samples gives crucial information on the drug substance in near real time. Analyzing the intact mass is a quick and often sufficient analysis to enable early detection of deviations and admits for timely corrective actions to ensure overall integrity of the process and drug substance. Adding the high resolving and accurate Tof mass detection facilitates multiple product quality attribute monitoring in one and the same analysis. The most abundant glycan profiles, identity and purity are obtained within minutes allowing for high throughput screening of multi parallel bioreactor samples utilized in upstream cell line and process development. Fast size exclusion chromatography (SEC) of samples directly retrieved from the cell culturing without tedious off-line sample preparation give an understanding of product purity in the bioreactor.
3. **High Throughput Sample Preparation –** Sample preparation is often needed for many downstream analytical applications, and it enables more in-depth characterization of drug product quality attributes. High throughput sample preparation using the Andrew Plus pipetting robot facilitates rapid and efficient processing of many samples, as often is the case in upstream cell line and process development. It also enhances the analytical workflow efficiency and reproducibility by minimizing manual intervention and risk of errors. Using the Andrew Plus pipetting robot with streamlined ready to use protocols and workflows improves the data accuracy and reliability and significantly reduces the time required for sample preparation. The Andrew Plus pipetting robot and the OneLab control software have been designed with user friendliness in mind with visual and easily adapted workflows that can be modified to suit your specific needs.
4. **In Depth Product Quality Attribute Monitoring –** More thorough product quality attribute (PQA) analysis is performed to obtain deeper insights on the drug product quality on subunit or molecular level. This requires adequate sample preparation and the use of site-specific degradation enzymes such as Waters RapiZyme™ Trypsin for rapid and reproducible sample preparation for peptide multi attribute monitoring (MAM) or Genovis SmartEnzymes, such as FabRICATOR® for subunit analysis. The most comprehensive glycan profiling will be obtained using Waters automated GlycoWorks™ RapiFluor-MS™ sample preparation protocol in combination with the BioAccord LC-MS System. The Andrew Plus pipetting robot and the OneLab software contain many optimized sample preparation and enzymatic digestion protocols and will give you more reliability, control, and throughput in the analysis. The BioAccord LC-MS System is an ideal mass analyzer for in depth PQA analysis with very high accuracy, ease of use and the fit for purpose and compliant ready waters_connect informatics platform enable between instruments and labs method transfer with retained data integrity.

5. **Automation –** The last section is bringing it all together with automated sample tracking, preparation and analysis using the bioprocess walk-up solutions. The walk-up solution is combining the Andrew Plus pipetting robot and the BioAccord LC-MS System to automate the measurement of cell culture media and product quality data at point of need. The OneLab software provides integrated end-to-end workflows for intact mass and spent media analysis for non-LC-MS experts to generate high quality process and product data in their own lab. The walk-up solution is also integrated with the Sartorius Ambr® 15 and Ambr® 250 High Throughput (HT) multi parallel bioreactor systems to offer connectivity between the BioAccord and the Ambr systems for sample tracking, preparation, and analysis. The automated bioprocess walk-up solutions maximize the ease of use, efficiency, quality assurance and control and minimize human intervention and risk of errors. These advantages contribute to more reliable and cost-effective biopharmaceutical process development and manufacturing, enabling faster delivery of new treatments to the patients.

We hope that you will find good use of the applications and workflows described in this notebook. We also encourage you to visit our website: www.waters.com/bioprocessing or contact your local Waters office to find out how you can accelerate your process development with your own quality data.

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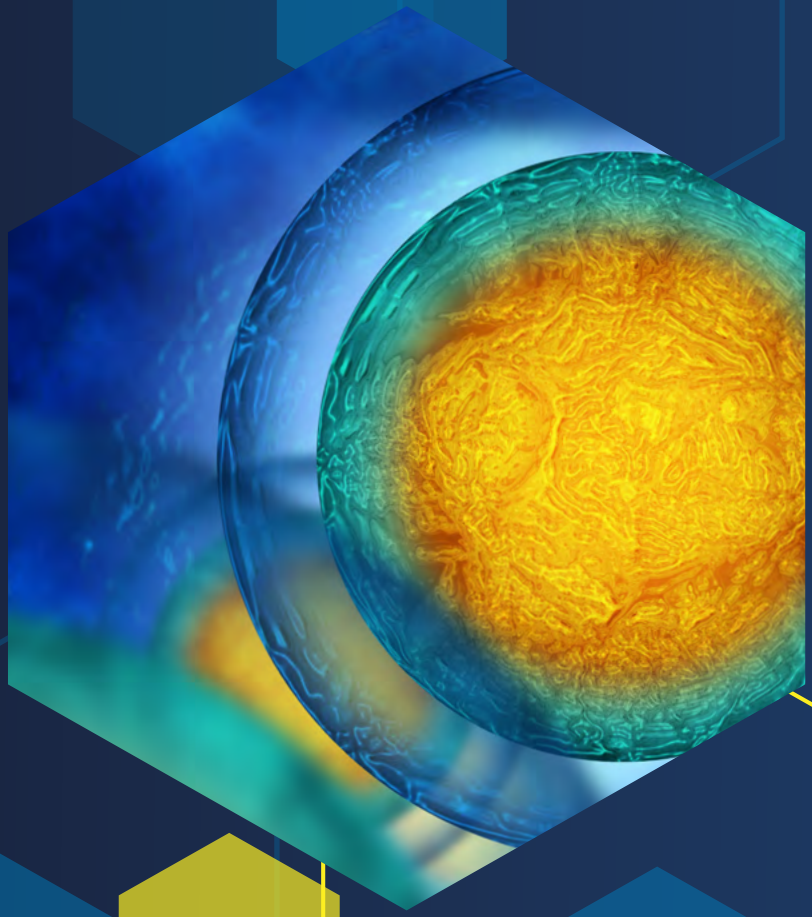

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Application Note

Monitoring Nutrients and Metabolites in Spent Cell Culture Media for Bioprocess Development Using the BioAccord LC-MS System With ACQUITY Premier

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Waters Corporation

Abstract

In biotherapeutics process development, cell culture media solution provides essential building blocks and nutrients for cell health and biotherapeutics production. Monitoring the feed components and metabolites can provide information on cell growth, biotherapeutic titer, and product quality. The BioAccord LC-MS System has been used to support product quality analysis such as intact protein analysis, peptide MAM, released glycan, and oligonucleotide mass confirmation. In this application the BioAccord System is utilized for the monitoring of the nutrients and metabolites in cell culture media. The method package includes a comprehensive reversed-phase LC-MS method, a 200+ compound library; a simple, stepwise workflow for data review including trend plots; a suite of tools for unknown screening; multivariate data analysis tools; and reporting template. The method has been applied in spent media analysis for clone selection and process optimization.

Benefits

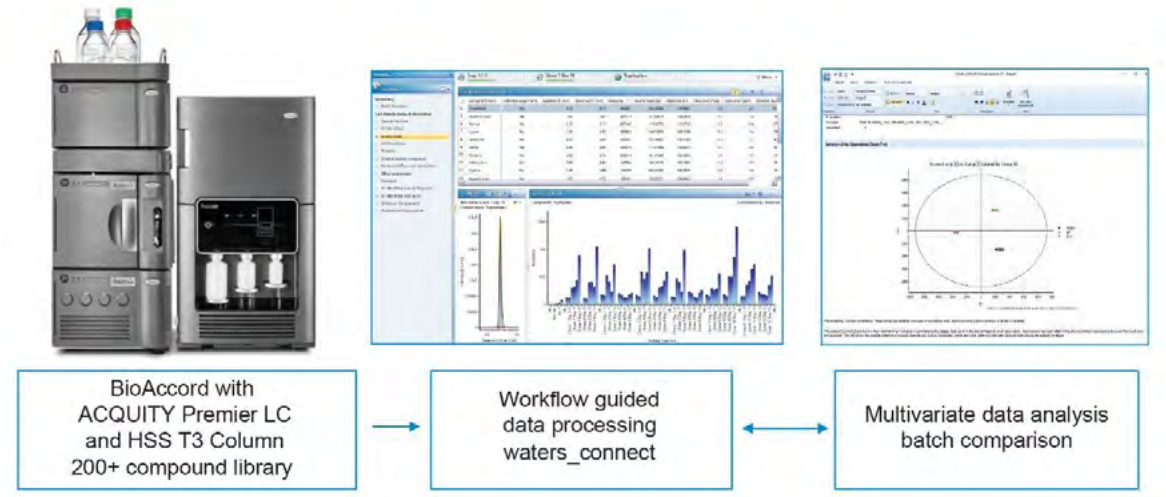
- Excellent chromatographic performance delivered by ACQUITY Premier Technology implemented for both the column and UPLC system hardware

- Rich full scan HRMS data using the simple-to-use BioAccord LC-MS System
- Inclusion of 200+ compound library, comprising amino acids, vitamins, nucleic acids/bases, nucleotides, metabolites, and others compounds of interest in bioprocessing development
- Streamlined function and class-based workflow to facilitate stepwise data review
- A single compliant-ready informatics package supporting data acquisition, data review, elucidation of unknowns, report template, and multivariate data analysis

Introduction

In biotherapeutics process development, cell culture media solution provides essential building blocks and nutrients for cell health and biotherapeutics production. The media solution is a complex mixture containing 100s of compounds, including starting chemicals and metabolites formed during the production cycle. Composition of the media component is constantly changing during the cell growth cycle. Monitoring the feed components and metabolites and ensuring they remain in optimal ranges have been shown to have impact on both cell growth, biotherapeutic titer, and product quality.[1] Routine monitoring of selective key feed components has been a part of bioprocess workflows. The desire to holistically monitor all feed components and metabolites present in the media is growing in the biomanufacturing industry for bioprocess development and optimization.

This application note describes a detailed reversed-phase LC-MS method and a comprehensive workflow for the monitoring of compounds in cell culture media (Scheme 1). The system is based on BioAccord with ACQUITY Premier Technology, a compact, self-optimizing LC-HRMS platform that broadens the accessibility to this technology and generates the same high-quality results whether you are an LC-MS expert or a new user. HRMS offers rich full scan datasets for analyte investigation and ease in method development since optimization of precursor to product transition needed in triple quadrupole MS^2 is not required. The ACQUITY Premier LC comprises low adsorption UPLC systems and columns based on MaxPeak High Performance Surfaces (HPS).[3] Recent literature has shown the ACQUITY Premier LC System is beneficial in improved peak symmetry and increased signal for many compound classes in the cell culture media such as TCA acids,[4] vitamins,[5] nucleic acids/bases, and nucleotides.[6] The current workflow includes 200+ compound library, a stepwise guide for data review, structural elucidation of unknowns, multivariate data analysis/batch comparison, and report. Detailed information for each of the workflow steps and examples of cell culture media analysis are described.



Scheme 1. A schematic illustration of BioAccord/waters_connect based workflow for cell culture media analysis.

Experimental

Sample Description

Commercial media solutions were prepared by diluting 1:100 (V/V) with H_2O. Spent media solutions were prepared by diluting 1:200 (V/V) with either H_2O or H_2O containing 0.1% formic acid.

LC Conditions

LC-MS system:	BioAccord LC-MS System with ACQUITY Premier BSM
Column(s):	ACQUITY Premier HSS T3 2.1 x 150 mm (P/N 186009469)
Column temp.:	40 °C
Sample temp.:	6 °C
Injection volume:	2 µL
Flow rate:	0.25 mL/min
Mobile phase A:	H_2O/0.1% FA
Mobile phase B:	90%ACN/10%IPA/0.1%FA

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
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

MS Conditions

LC-MS system:	BioAccord LC-MS System with ACQUITY Premier BSM	
Ionization mode:	Full scan or Full scan with fragmentation	
Acquisition range:	Low (50–2000 *m/z*)	
Polarity	Positive	
	Capillary voltage:	1 kV
	Cone voltage:	20 V
	Fragmentation cone voltage	40–60 V
	Negative	
	Capillary voltage:	0.8 kV
	Cone voltage:	15 V
	Fragmentation cone voltage	50–70 V
Scan rate	5 Hz	
Desolvation temperature	550 °C	
Intelligent data capture	On	
Dynamic lock mass correction	On	

Data Management

LC-MS software: waters_connect

Informatics: waters_connect base kit with UNIFI 1.9 SR13

Results and Discussion

General Method Discussion

The application on cell culture media compounds was developed using BioAccord LC-MS with ACQUITY Premier LC and HSS T3 Column. The system has excellent chromatographic performance with narrow and symmetrical peaks across diverse classes of compounds. Figure 1 is an overlaid extracted ion chromatogram (XIC) of compounds in the method under positive ionization mode of data acquisition. XIC chromatograms of representative individual compounds are available in the Appendix. Figure 1 also shows excellent baseline resolution between isobaric compound pairs, leucine/isoleucine, and citric acid/isocitric acid. Samples were run

with either full scan or full scan with fragmentation mode of data acquisition. Full scan was recommended for general monitoring, especially when a large number of samples are analyzed. Full scan with fragmentation is preferred to aid compound elucidation and reduce false positives in external ChemSpider database searches.

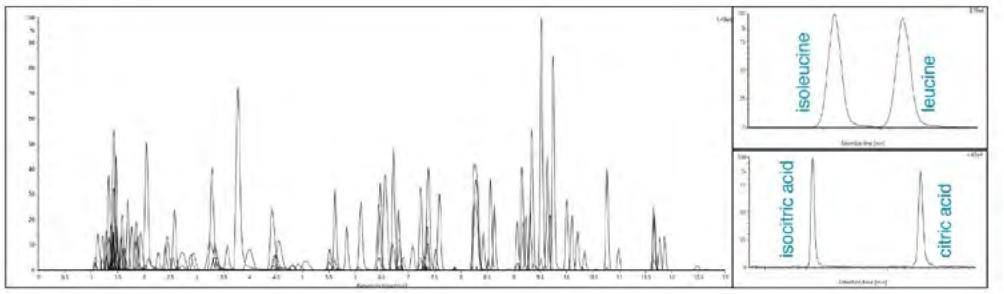


Figure 1. Overlaid extracted ion chromatogram (XIC) of general compound coverage, isobaric pair isoleucine/leucine, and isocritic acid/citric acid.

System performance was assessed based on 6 replicate injections of a basal cell culture media solution, IMDM (ext. Sigma Aldrich) at 1:100 diluted concentration. Reproducibility in retention time, mass error, and peak response are summarized for representative compounds across elution time window (Table 1). Results show excellent reproducibility in retention time (<0.02 min shift), mass error (<5 ppm), and response (<5%), all of which are independent of compound elution time. The system also produces excellent sensitivity ranging from sub ng/mL to ng/mL detection limit. The good sensitivity and reproducibility along with other attributes make the BioAccord System a suitable platform for the cell culture media compound monitoring.

Compound	Approx. conc. (µg/mL)	Neutral mass	r.t. (min)		Mass error (ppm)		Response	
			Avg	Stdev	Avg	Stdev	Avg	%RSD
Arginine	0.84	174.112	1.31	0.00	-1.3	2.0	1.1E+05	1.5
Cystine	0.91	240.024	1.36	0.00	-2.1	2.7	5.5E+04	2.9
Threonine	0.95	119.058	1.46	0.00	-0.7	0.8	5.0E+04	2.1
Valine	0.94	117.079	2.41	0.01	-0.2	1.7	1.2E+05	1.4
Nicotinamide	0.04	122.048	3.66	0.02	-3.2	1.6	7.1E+03	4.6
Isoleucine	1.05	131.095	5.21	0.02	-1.1	0.7	2.6E+05	1.3
Phenylalanine	0.66	165.079	8.10	0.00	-0.6	1.7	2.5E+05	1.8
Pantothenic acid	0.04	219.111	8.74	0.00	0.2	2.3	7.9E+03	3.5
Tryptophan	0.16	204.090	9.08	0.01	-0.9	1.7	7.0E+04	2.4
Folic acid	0.04	441.140	9.10	0.00	-0.4	1.9	1.3E+04	0.7

Table 1. Summary of reproducibility data for representative compounds based on 6 replicate injections. Sample was prepared by diluting neat media with H_2O 1:100 (V/V). The concentration was approximate concentration derived from vendor's product brochure.

Compound library

A UNIFI library containing 200+ compounds was created to facilitate their identification and tracking in analyses. Retention time and fragmentation data for each compound were derived and confirmed with authentic compound standards. Each compound was tagged in the library according to the following subclasses: amino acid, amino acid derivatives, organic acids, vitamins, nucleic acids/bases and nucleotides, and others. Each compound was also tagged with preferred mode of ionization, ESI^+ or ESI^-. The tagging information can be used to retrieve the subclass of compounds during library searching, compound importing into analysis method, and/or creation of filters during workflow step constructions (see below). The distribution of compound classes and example of library entry is shown in Figure 2.

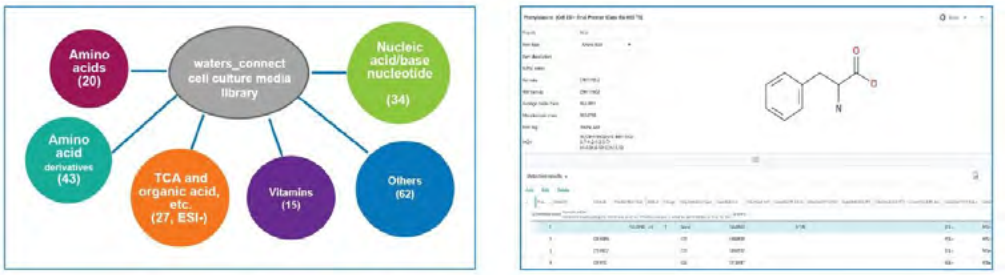


Figure 2. Compound classes included in the scientific library (left), and an example of compound display in the library (right).

Workflow guided data review

A bioprocess development project typically generates many cell culture media samples for analysis. In each sample, there are potentially greater than 100 compounds that bioprocessing engineers would like to acquire information for review. It is critical to have a streamlined data review process to readily extract the information needed and expedite the process. The workflow functionality available in UNIFI Informatics System is designed to provide a straightforward and stepwise review of the processed data based on function, class, and/or other criteria. A default data review workflow for cell culture media analysis is shown in Figure 3. Several key steps covering sample run list, compound classes, and cross sample comparisons were created to demonstrate how the information can be gathered and displayed in UNIFI. Compound review can be managed via trend plot across injections, graphic overlays, and/or tabulated information. The workflow can also be customized, and specific steps can be added easily to cover compounds of interest, critical pairs, and/or specific transformation pathways. In the default workflow, an example of choline pathway is included where three related compounds, choline, choline phosphate, and choline glycerophosphate are displayed. Changes of these compounds along with the incubation time and across bioreactors are readily displayed in the overlaid trend plot. The "unknown compound" step is a collection of all compounds that are not identified. It helps to aggregate data for compounds that merit further investigation/elucidation. The workflow can be exported or imported for sharing with other waters_connect platforms/data processing workstations.

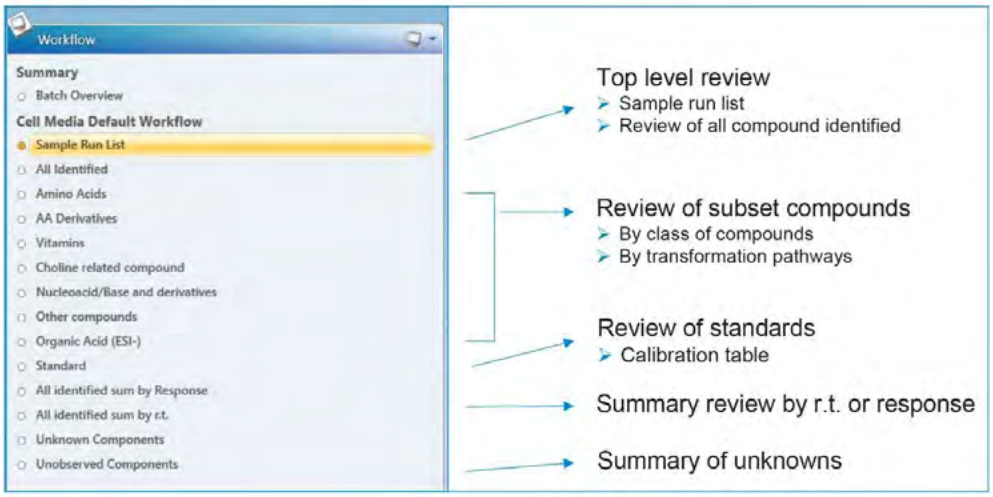


Figure 3. Default workflow for cell culture media data review.

Graphic Display showing the results from cell culture media analysis

Figure 4 is an overlaid chromatogram of commercially available basal cell culture media solution, DMEM (ext. Sigma Aldrich). DMEM contains amino acid and vitamins at concentration ranging from µg/mL to 100's µg/mL. For this analysis the media sample was diluted 100x with H_2O and 2 µL was injected. Excellent chromatographic performance was obtained on the system where all compounds were detected. This sample is very useful for the optimization of LC-MS separation conditions.

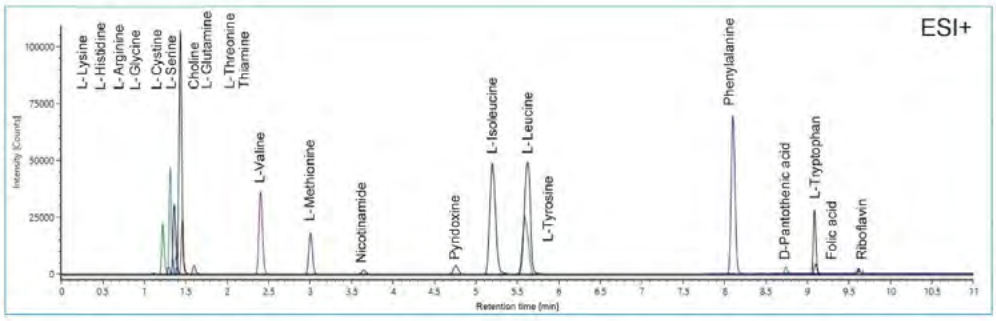


Figure 4. Overlaid chromatogram of basal cell culture media DMEM under ESI$^+$ ionization conditions.

For the analysis of spent media solutions from bioreactors in upstream process development, it is useful to track the abundance changes of specific component(s) in the media over incubation time and/or different bioreactors. A sample sets from 12 bioreactors sampled at 6 time points up to 12 days were analyzed by the present method. Figure 5 shows the overall trending plot of choline from all bioreactors and all sampling dates. The bar chart of each grouped trend plot below represents time course of one bioreactor. Using this view, changes of cell culture media components during the process development can be readily observed.

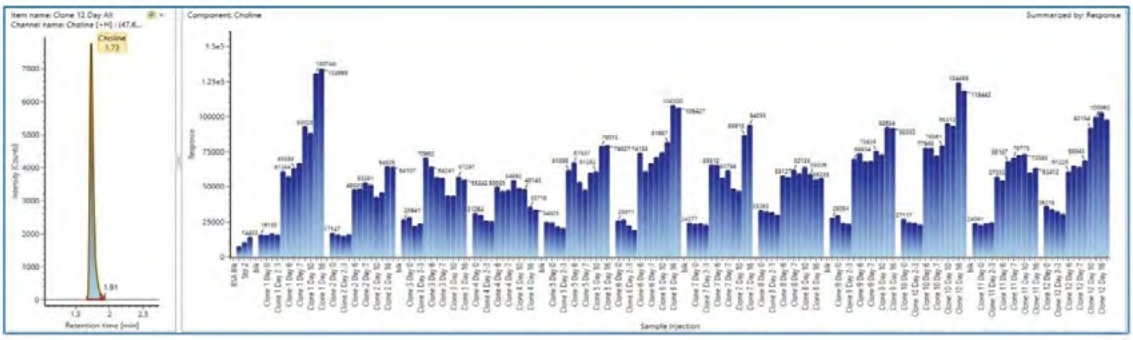


Figure 5. An example of abundance changes of choline in spent cell culture media is shown by the trending plot bar chart for 12 different bioreactors across multiple days.

Elucidation of unknowns

Cell culture nutrient metabolism during bioprocessing is a complex process. Metabolites formed during biotherapeutics production but not identified by the library match may still be of interest, especially if they correlate with titer, product quality attributes or processing parameters. An example of structural elucidation for an unknown compound in spent cell culture media is shown in Figure 6. For this example, the most abundant unknown compound was detected at *m/z* of 427.0950. A search of the ChemSpider database returned several hits, including a compound of interest, cysteine-glutathione disulfide (Figure 6). Identity of the compound was subsequently confirmed using an authentic standard of cysteine-glutathione disulfide. This compound was then added to the component table in the analysis method for future application. Upon data reprocessing after inclusion of the compound, the results revealed its abundance changes in different bioreactors throughout the time course. Lastly, it is recommended that acquisition with "full scan with fragmentation" should be performed to collect both precursor as well as fragmentation data. Fragmentation data collected this way will aid in identifying and reducing false positives in database searching.

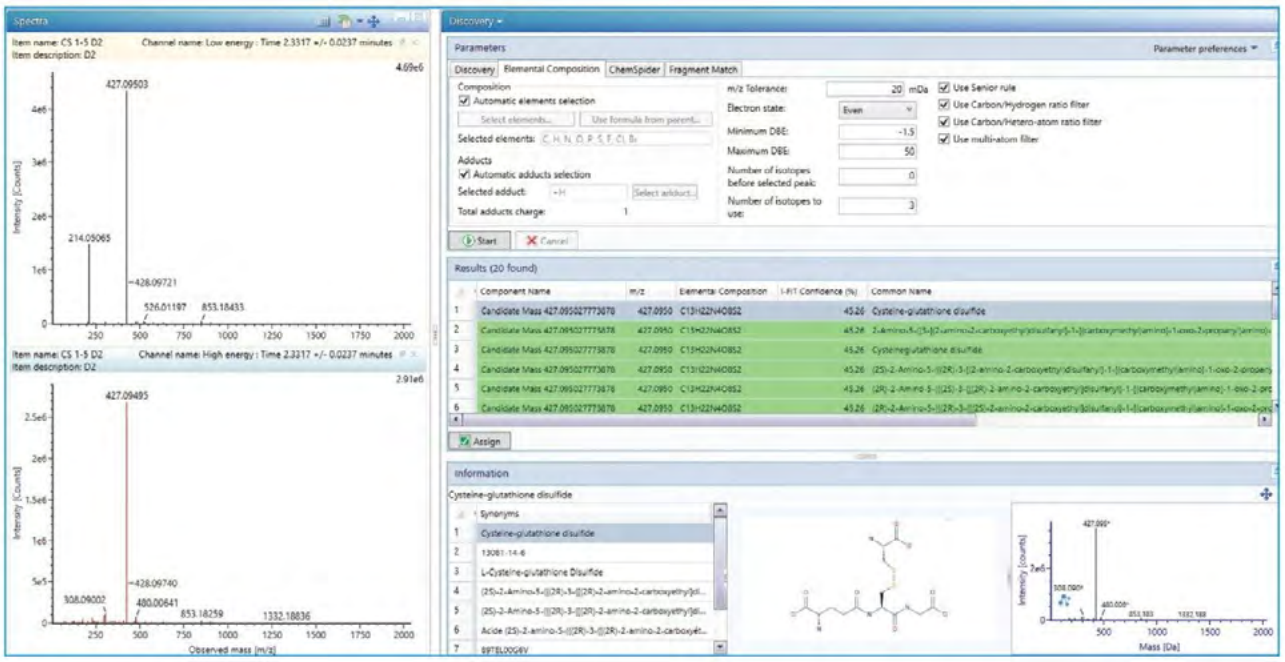


Figure 6. A screen capture of compound elucidation method setup displaying low and high energy fragmentation spectra of 427.0950 m/z unknown, and returns from ChemSpider database search.

Multivariate data analysis to reveal the difference among cell culture media samples

Additional multivariate data analysis tools based on Umetrics EZ-info software are available in UNIFI Informatics System for data analysis. EZ-info offers a powerful way to analyze and perform batch comparison of large number of datasets. For example, datasets for comparing multiple bioreactors with same/different cell lines, for comparing media components at the early incubation time to those in late incubation time in the same bioreactor, or for comparing different bioreactors at the same incubation timepoint, and many other comparisons. Figure 7 shows two example outputs after multivariate data analysis of samples from 12 bioreactors sampled for many days. After initial compound selection according to mass range, retention time and response, the selected compounds were automatically transferred to EZ-info. The PCA plot provided an overall view of differences between different bioreactors and different date of media sampling. The score plot or S-plot can be generated to visualize the differences between a pair of datasets, in this case, day 0 and day 16 from all bioreactors. The down regulated compounds on day 16 can then be selected and sent back to UNIFI for compound identification. This

data processing approach allows users to quickly locate the biomarkers that are associated with the difference between samples.

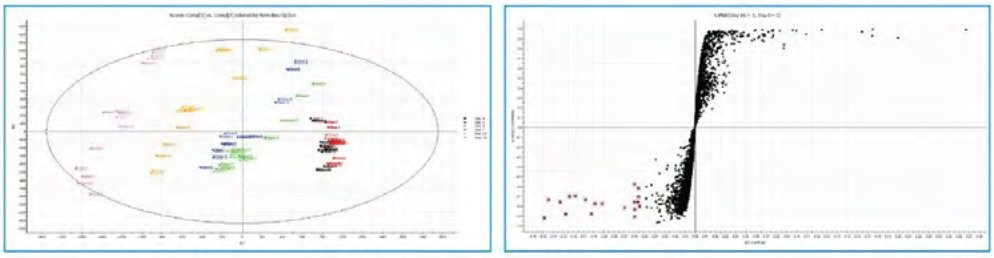


Figure 7. Example outputs of multivariate data analysis. (left) a PCA plot, colored by date of sampling from different bioreactors. (right) S-Plot of day 0 and day 16 cell culture media. Down regulated compounds (markers) in Day 16 are highlighed in red.

Reporting

A default report template is provided to offer a convenient way to summarize and present the analysis results of the cell culture media analysis. The report templates dramatically reduce the time needed to communicate the analytical results with collaborators or samples sources. The report consists of a data export in tabulated (.xls) format and a text/graphic export in PDF/XPS format. The text/graphic report contains information including filename, LC-MS method details, sample run list, bar or line chart plot either as single compound or overlay of multiple compounds, and others. Company logo image can also be added. The report can be customized or edited via user-friendly report template tools. A new report template can be generated through modification of existing report or adding additional report object.

Conclusion

A comprehensive LC-MS workflow based on the BioAccord System and UNIFI Informatics Platform has been developed for non-targeted cell culture media analysis. The BioAccord LC-MS System offers the benefits of easy setup, long-term performance stability and simplicity of operation. This platform allows bioprocessing engineers with limited LC-MS experience to quickly and easily run and process large numbers of cell culture media

samples and provide them with rich, insightful information on both feed additives and metabolites formed during the bioprocess development. The described methodology is developed based on reversed-phase separation principles to cover the majority of components encountered in cell culture media. The MaxPeak HPS Technology, which is implemented on Waters ACQUITY Premier Products (LC systems and columns), has provided improved chromatographic performance for a range of compound classes. In addition, the inclusion of Premier technologies and the compound library into the analysis method results in a high-performance platform and an end-to-end workflow to cover the key steps required in cell culture media analysis, facilitating the adoption of LC-MS technologies for bioprocessing development. The method was successfully demonstrated for the analysis of both starting media solutions and spent media samples from bioreactors in upstream process development and optimization.

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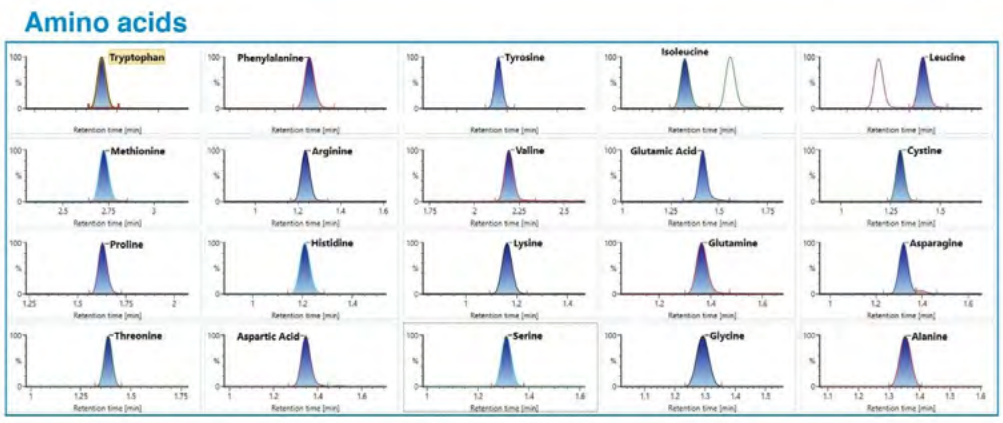
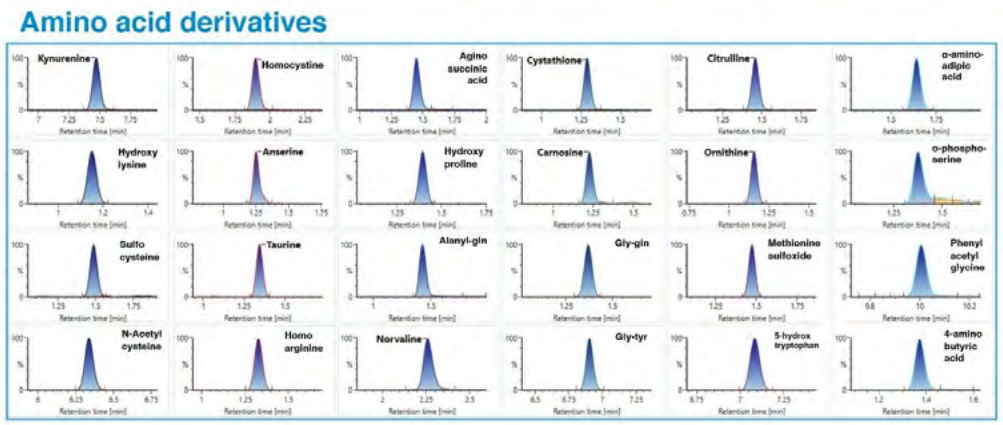
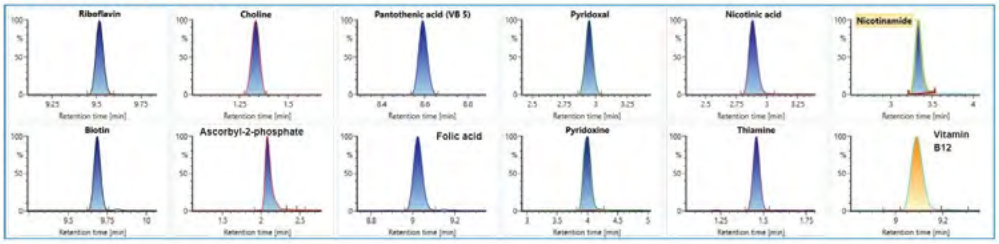
Appendix

Example extracted ion chromatograms (XIC) of compounds in the library.

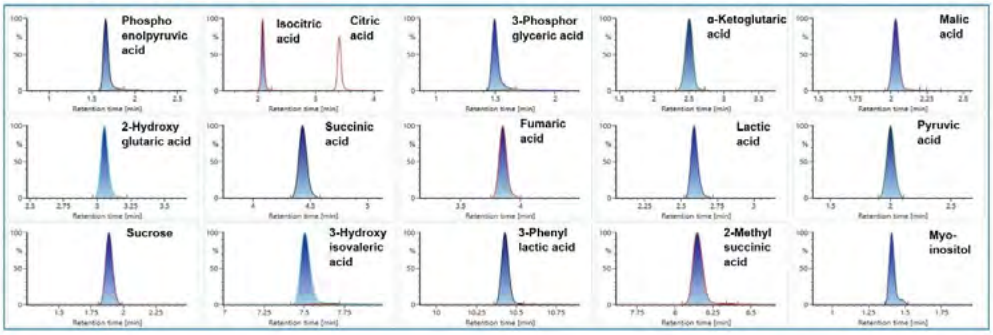




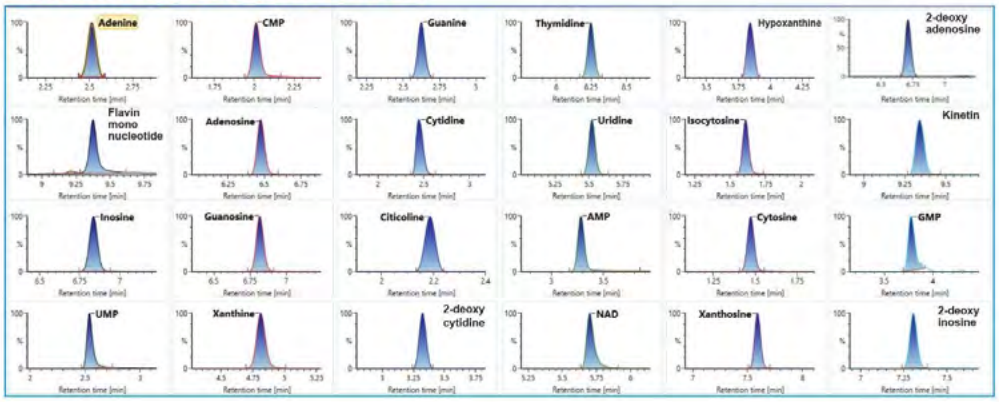
Vitamins (ESI+)



TCA and Organic acids



Nucleic acid/base, Nucleotides



Featured Products

BioAccord LC-MS System with ACQUITY Premier <https://www.waters.com/waters/nav.htm?cid=135087537>

UNIFI Scientific Information System <https://www.waters.com/134801648>

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720007359, September 2021

Waters™

Application Note

Cell Culture Media Monitoring in Cell and Gene Therapy Using the Bioaccord™ LC-MS System

Yun Wang Alelyunas, Mark D. Wrona, Ying Qing Yu

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

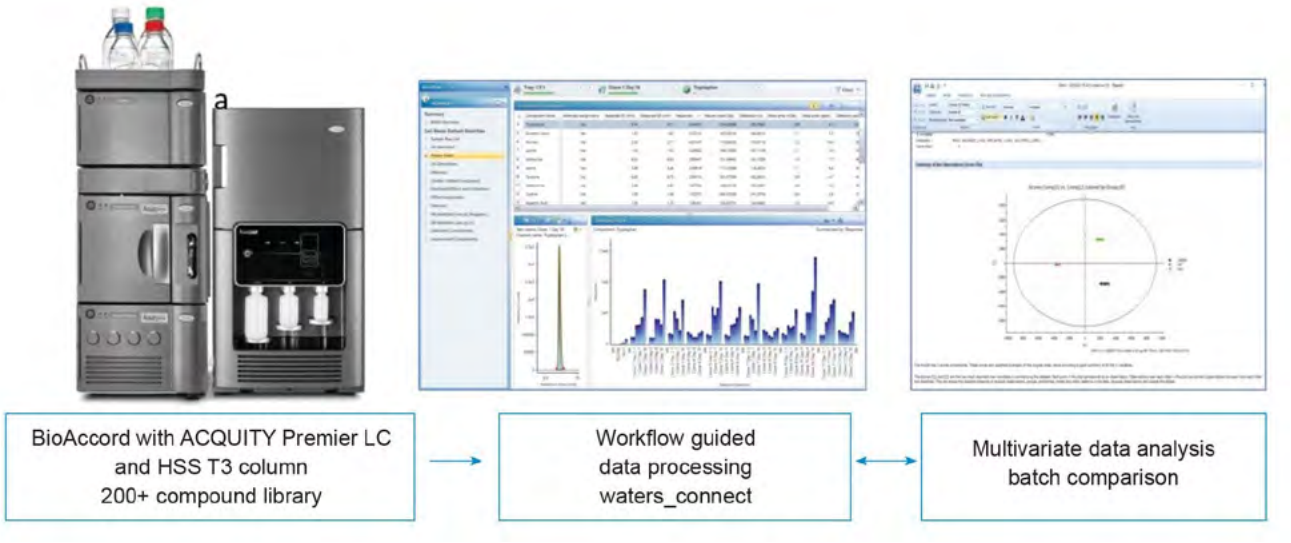
The HEK293 cell line is commonly used in the production of viral vectors and requires the use of cell culture media tailored for health and growth of these cell lines. Here, we demonstrate the use of the BioAccord Liquid Chromatography-Mass Spectrometry (LC-MS) System for the analysis of HEK293 viral vector media. Results show that the media contains typical classes of cell culture media compounds such as amino acids and vitamins. It also contains a lower amount of nucleic acids and nucleosides. In conclusion, the cell culture media method and workflow developed for protein production can be readily applied to viral vector based gene therapy.

Benefits

- Easy-to-use LC-MS platform for rapid and comprehensive monitoring of cell culture media used in viral vector based gene therapy
- One HRMS platform supporting media monitoring process analytics as well as product quality analysis in protein and viral vector production[3]

Introduction

In viral vector based gene therapy, modified viruses are used as drug-delivery vehicles to introduce specific DNA sequences into cells. The HEK293 cell line, from immortalized human embryonic kidney cells, is commonly used in the production of viral vectors such as adenoviral (AV), adeno-associated viral (AAV), and retroviral vectors.[1] In this technology brief, methodology developed for cell culture media analysis based on the BioAccord high resolution mass spectrometry (HRMS) platform is applied for HEK 293 viral vector media analysis. Scheme 1 is a general description of the method package; further details are given in a previously published Waters application note.[2]



Scheme 1. A schematic illustration of BioAccord/waters_connect™ based workflow for media analysis (adopted from a Waters Appnote)[2].

Results and Discussion

HEK293 viral vector media was purchased from MilliporeSigma (p/n: 14385C). Since media composition was not disclosed in the product, the media was diluted at ratios of 1:100, 1:200, 1:500, 1:1000, and 1:2000 to facilitate positive compound identification, and help in identifying the best dilution ratio for routine monitoring. The diluent used was H_2O containing 0.1% formic acid and 0.1 µM 3-chlorotyrosine as an internal standard. Basal media solutions, DMEM and IMDM at 1:100 dilution were also prepared. The solutions were subjected to LC-MS analysis using BioAccord as described previously.[2] A 17 amino acids calibration solution at concentrations from 0.01 to 10 µM were injected at the beginning and end of the sample analysis. A mass range of 50–800 *m/z* was used for data acquisition.

An overlaid extracted ion chromatogram (XIC) for the major compounds observed is shown in Figure 1 and representative trend bar plots for each compound class are shown in Figure 2. Results show that three major classes of compounds were detected in HEK293 media, amino acids, vitamins, and several nucleic acids and nucleosides. The most abundant compound class is amino acids, followed by vitamins. Nucleic acids and nucleosides are the least abundant compounds. The rapidly metabolizing amino acid, glutamine, was included in its stable dipeptide form as alanyl-glutamine. Compared to basal media DMEM and IMDM, HEK 293 viral vector media has in general a higher concentration of amino acids and vitamins. Nucleic acids and nucleosides are not typically present in basal media. Excellent LC-MS reproducibility for the top 35 compounds was observed at 1:200 and 1:500 dilution ratio is tabulated in Table 1. Based on the response observed at multiple dilutions, a dilution ratio of 1:500 is recommended for routine monitoring of the media components in a viral vector production process.

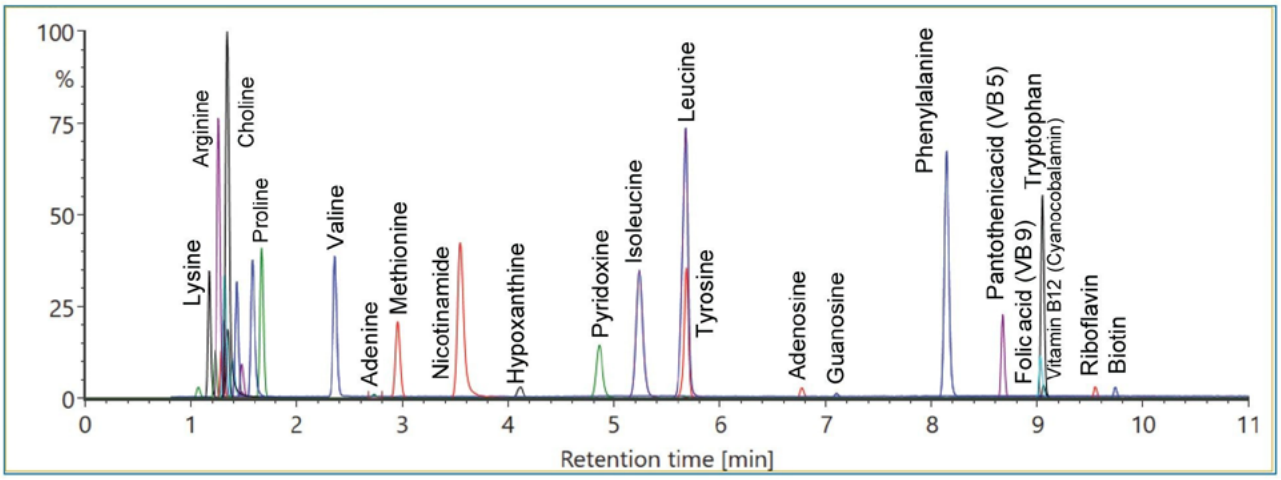


Figure 1. Overlaid XIC of the top 34 compounds observed in HEK 293 media under positive ion electrospray (ESI+) conditions. For display purposes, amino acids are extracted from the 1:2000 diluted sample, Vitamins and other compounds are extracted from the 1:100 diluted sample. The vitamin, myo-inositol is observed in negative ion mode (ES-) (data not shown).

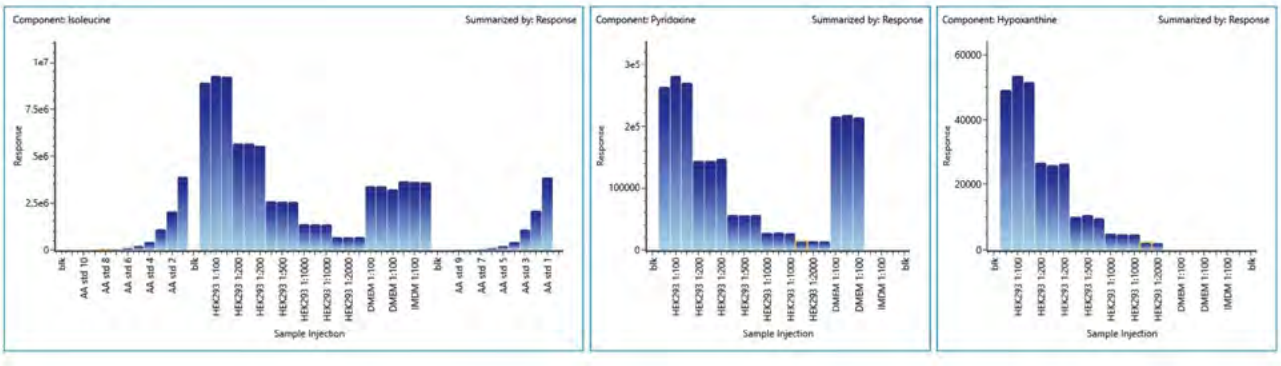


Figure 2. Representative bar trend plots of compound response (triplicate injection/sample). (A) amino acid example, isoleucine, showing standard calibration curve at the beginning and end of injection. Isoleucine is present in standard and all media samples. (B) vitamin example, pyridoxine, showing the compound is present in HEK293 media and DMEM media, and not present in IMDM media as expected. (C) nucleobase example, hypoxanthine, showing the compound is present in HEK293, and not in DMEM and IMDM media.

Compound	observed r.t (min)	Average response at 1:500 dilution ratio	%RSD at 1:500 dilution ratio	%RSD at 1:200 dilution ratio
Alanine	1.36	1.3E+04	2.2	1.7
Alanyl-Glutamine	1.48	4.1E+05	1.1	0.2
Arginine	1.26	2.2E+06	0.8	1.0
Asparagine	1.32	8.3E+05	0.7	1.0
Aspartic Acid	1.35	4.8E+05	1.2	0.9
Cystine	1.29	3.8E+05	0.7	1.1
Glutamic Acid	1.43	9.9E+05	0.5	0.5
Glycine	1.29	2.4E+03	6.1	4.4
Histidine	1.23	2.8E+05	0.2	0.4
Isoleucine	5.23	2.6E+06	0.5	1.2
Leucine	5.67	4.0E+06	0.6	2.7
Lysine	1.17	7.7E+05	1.5	0.6
Methionine	2.95	1.1E+06	0.6	0.9
Phenylalanine	8.14	3.5E+06	0.9	0.5
Proline	1.67	1.2E+06	1.3	2.5
Serine	1.31	5.3E+05	0.2	0.8
Threonine	1.39	2.8E+05	1.1	1.2
Tryptophan	9.05	2.0E+06	1.1	0.2
Tyrosine	5.68	1.2E+06	1.2	4.5
Valine	2.36	1.3E+06	1.4	2.4
Biotin	9.74	5.5E+03	4.3	0.8
Choline	1.34	3.1E+05	0.8	0.5
Folic acid (VB 9)	9.03	2.2E+04	1.9	0.7
Glucose/Myo-inositil	1.51	5.1E+04	1.3	0.9
Nicotinamide	3.55	1.6E+05	1.8	0.5
Pantothenic acid (VB 5)	8.67	4.0E+04	1.9	1.0
Pyridoxine	4.86	5.7E+04	0.5	1.3
Riboflavin	9.55	5.3E+03	0.7	1.3
Thiamine	1.58	9.8E+04	1.9	2.0
Vitamin B12 (Cyanocobalamin)	9.06	6.9E+03	3.9	1.0
Adenine	2.73	2.5E+03	12.4	2.9
Adenosine	6.78	6.9E+03	5.8	3.6
Cytidine	2.92	3.0E+03	5.4	4.1
Guanosine	7.11	3.2E+03	6.1	3.3
Hypoxanthine	4.11	1.0E+04	5.4	1.4

Table 1. Summary of response and reproducibility for the top 35 compounds based on three replicate injections. The table is ordered according to compound class and compound name.

Conclusion

A cell culture media method developed using the BioAccord LC-MS System has been employed for the analysis of HEK293 viral vector media. The analysis revealed HEK293 contains more than 35 compounds with compound classes including amino acids, vitamins, and nucleic acid and nucleosides that can be easily detected and monitored. The data suggests that in addition to cell culture and microbial media monitoring in protein production, the cell culture media analysis methodology is applicable for general media monitoring in gene therapy.

References

1. Tan E. *et al*. "HEK293 cell line as a platform to produce recombinant proteins and viral vectors:, Front Bioeng. Biotechnol. 2021, 9 796991.
2. Alelyunas YW, Wrona MD, Chen W, 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 September.
3. Zhang X, Koza SM, Yu YQ, Chen W. Optimizing Adeno-Associated Virus (AAV) Capsid Protein Analysis Using UPLC and UPLC-MS, Waters Application note 720006869, May 2020.

Featured Products

BioAccord LC-MS System for Biopharmaceuticals <

https://www.waters.com/waters/nav.htm?cid=135005818>

ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

720007705, September 2022

Waters™

Application Note

Quantification of Underivatized Amino Acids in Cell Culture Media Using the BioAccord™ LC-MS System

Yun Wang Alelyunas, Mark D. Wrona, Ying Qing Yu

Waters Corporation

Abstract

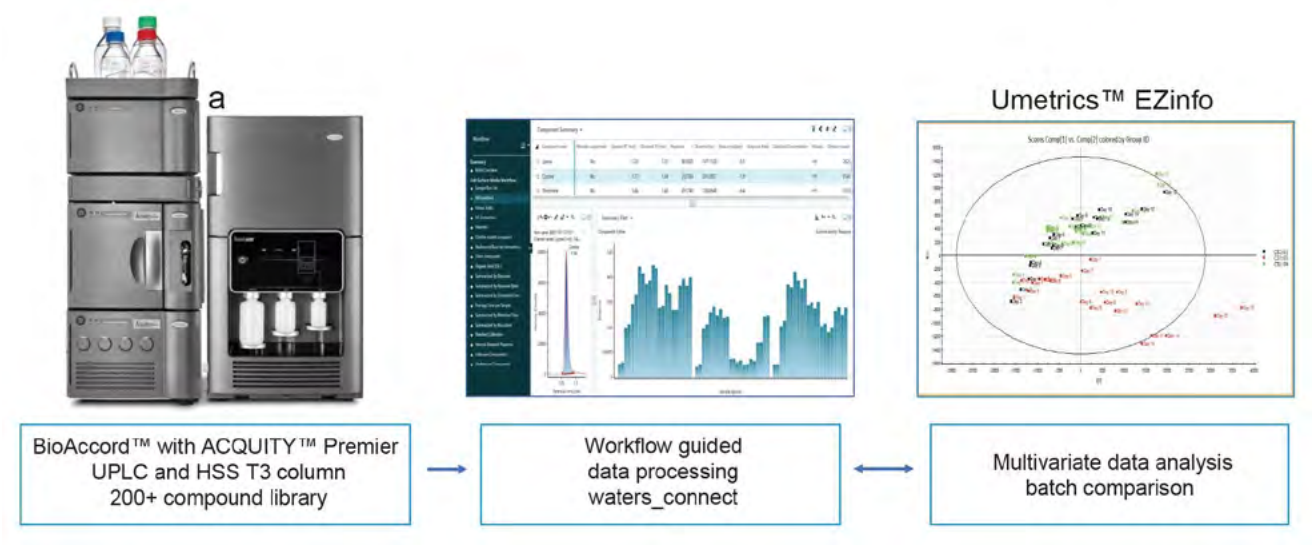
Accurate measurement of cell culture media components in bioprocessing is important for raw material testing (batch-to-batch and multiple vendor sourcing), media development, and spent media monitoring of metabolites in flux during the cultivation process. Here, we demonstrate the advantages and capabilities using the BioAccord LC-MS System for routine quantification of amino acids (AA) in cell culture media under underivatized conditions. Results show the method is capable of quantifying AAs with linearity range of three orders of magnitude (0.01 µM to 10 µM) with high accuracy and excellent precision. The use of an unlabeled compound as the internal standard is effective for response normalization. The data suggest that the method is suitable for the quality control of amino acids in raw media as well as their consumption rates in spent media.

Benefits

- Quantitative and qualitive monitoring of cell culture media using the simple-to-use BioAccord LC-MS System
- A single compliant-ready informatics package supporting data acquisition, data review, elucidation of unknowns, report template, and multivariate data analysis

Introduction

A reversed phase liquid chromatography and mass spectrometry (LC-MS) method with workflow using ACQUITY™ Premier HSS T3 Column on the BioAccord™ LC-MS System have been developed for the cell culture media analysis (Scheme 1).[1] The Premier system and column incorporate MaxPeak™ High Performance Surfaces Technology in the hardware design. The methodology has been applied to cell culture media monitoring in antibody production,[1] microbial fermentation,[2] and cell and gene therapy.[3] Monitoring changes of the media components in different bioreactors and over time during the cultivation process have shown value for media development and process optimization.[4] Among more than 200 compounds included in the method library, the reversed phase method offers direct, underivatized analysis of amino acids (AAs), with no sample preparation involving derivatization required. In all media analyzed so far, AAs are the most abundant compounds as they are essential building blocks in protein biotherapeutics production. Their monitoring of feed stock and spent media during cultivation process to ensure they are in optimum range is important for therapeutic titer and product quality. This application note focuses on underivatized amino acid quantitation using reversed phase chromatography.



Scheme 1. A schematic illustration of BioAccord System/waters_connect™ based workflow for cell culture media analysis.

Experimental

Sample and Standard Preparation

An external calibration solution was prepared by serially diluting a 17 amino acids stock solution to concentrations ranging from 0.01 to 10 µM. The diluent used above was 1:1000 diluted Earle's balanced salt (EBS) stock (MilliporeSigma p/n: E2888), using 0.1% formic acid (FA) in H_2O containing 0.1 µM 3-chloro-tyrosine as an internal standard. The use of EBS was to mimic general salt conditions in spent media.

Samples used in internal standard studies were commercial media solution IMDM (MilliporeSigma p/n: I3390) and stable isotope labeled (SIL) amino acid standard mixture (Waters p/n: 186009051 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009051-kairos-amino-acid-internal-standard-set-100.html>). The SIL mixture was used as the internal standard (IS) and spiked into 1:100 diluted IMDM 0.1% FA solution to give a final SIL concentration of 5 µM.

LC Conditions

LC-MS system	BioAccord LC-MS system with ACQUITY Premier BSM
Column(s)	ACQUITY Premier HSS T3 2.1 x 150 mm (P/N 186009469)
Column temp.	40 °C
Sample temp.	6 °C
Injection volume	2 µL
Flow rate	0.25 mL/min
Mobile phase A	0.1% FA in H_2O
Mobile phase B	90%ACN/10%IPA/0.1%FA

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
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

MS Conditions

LC-MS system	BioAccord LC-MS system with ACQUITY Premier BSM	
Ionization mode	Full scan	
Acquisition range	Small molecules (50–800 *m/z*)	
Polarity	Positive	
	Capillary voltage:	1 kV
	Cone voltage:	20 V
Scan rate	5 Hz	
Desolvation temperature	550 °C	
Intelligent data capture	On	
Lock mass correction	Standard	
Informatics	waters_connect 3.1, cell culture media screening workflow	

Results and Discussion

Standard Calibration Curve

Quantitative responses of amino acids using the cell culture media method and BioAccord LC-MS platform were collected using a 17 AA mixture. The calibration standard solutions were prepared using EBS, a balanced salt solution for media preparation, and injected at the beginning and end of a quality control (QC) sample set. The linear response based on log-log linear curve fitting was obtained for each of the amino acids. An example of the

calibration data is shown in Figure 1, displaying chromatograms, summary response plots, and calibration curves of the two isobaric compounds isoleucine and leucine. For these two compounds, excellent baseline chromatographic separation and reproducible response were observed (Table 1). The observed linear range was 0.01–10 µM or 3-orders of dynamic range with R^2 = 0.9996. Calibration curves and linear ranges for the rest of AAs are summarized in Figure 2 and Table 1. Data showed that the majority of the compounds displayed linear range of 0.01–10 µM or three orders of dynamic range with R^2 > 0.996. The exceptions are alanine and glycine with lower sensitivity, and threonine with narrower linear range.

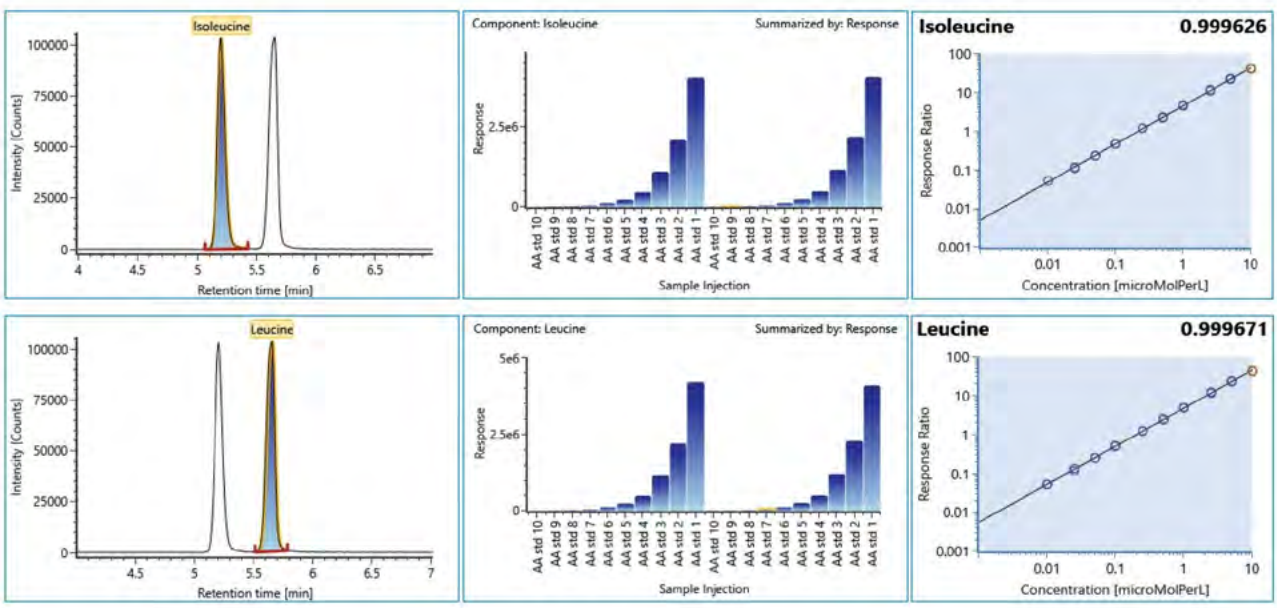


Figure 1. Chromatogram, response bar plots, calibration curve for leucine and isoleucine.

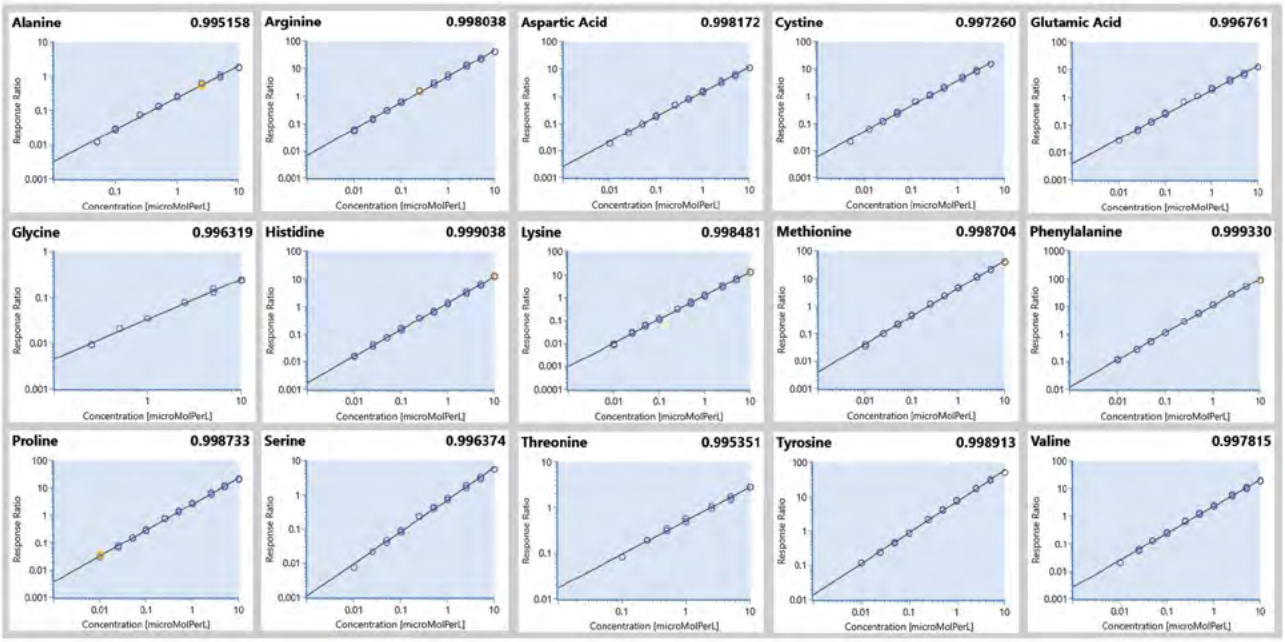


Figure 2. Standard calibration curve of amino acids. Log-log linear calibration was used for curve fitting.

Method accuracy and precision were determined for two QC samples, a low QC at 0.05 µM and a high QC at 5 µM. Data based on triplicate injections are summarized in Table 2. In all, excellent accuracy of 85–115% and reproducibility of <4% at high QC and <15% at low QC are observed. In all, the data suggest that the reversed phase method could be used for quantitative determination of amino acids in cell culture media either for quality control of raw media and/or quantitative monitoring in spent media during cultivation process.

Component name	Neutral mass (Da)	Expected RT (min)	Mass error (ppm)*	Linear range (µM)	R^2	Low QC at 0.05 µM (n=3)		High QC at 5 µM (n=3)	
						%Accuracy	%Precision	%Accuracy	%Precision
Alanine	89.0477	1.42	1.7	0.05–10	0.9951	91	9.2	93	2.2
Arginine	174.1117	1.31	-0.4	0.01–10	0.9980	95	8.5	89	2.7
Aspartic acid	133.0375	1.42	0.1	0.01–10	0.9981	95	8.8	91	1.6
Cystine	240.0239	1.37	-1.7	0.005–5	0.9973	92	11.5	89	1.8
Glutamic acid	147.0532	1.50	0.4	0.01–10	0.9968	92	5.8	88	1.8
Glycine	75.0320	1.36	3.2	0.25–10	0.9963	n/a	n/a	98	3.3
Histidine	155.0695	1.28	1.1	0.01–10	0.9990	96	5.5	95	1.0
Isoleucine	131.0946	5.22	3.4	0.01–10	0.9996	98	7.4	100	1.6
Leucine	131.0946	5.64	3.4	0.01–10	0.9997	97	3.2	101	0.9
Lysine	146.1055	1.23	0.8	0.01–10	0.9985	98	4.1	91	1.1
Methionine	149.0511	3.01	1.2	0.01–10	0.9987	107	4.3	97	3.5
Phenylalanine	165.0790	8.11	1.9	0.01–10	0.9993	95	1.2	102	1.7
Proline	115.0633	1.73	1.5	0.01–10	0.9987	92	7.5	93	0.6
Serine	105.0426	1.38	1.2	0.01–10	0.9964	92	13.6	89	0.5
Threonine	119.0582	1.46	0.6	0.1–10	0.9954	n/a	n/a	87	0.6
Tyrosine	181.0739	5.64	2.2	0.01–10	0.9989	89	1.3	96	1.6
Valine	117.0790	2.39	1.9	0.01–10	0.9978	99	11.7	92	1.5

*based on sample concentration at 5 µM for all amino acids except cystine at 2.5 µM.
n/a: below detection limit

Table 1. Summary of lineary, precision and accurary of QC samples from three replicate injections.

Internal Standard Considerations

In quantitative bioanalysis, internal standard (IS) is added to the sample for signal normalization to ensure the highest reproducibility and accuracy. The use of the SIL version of the same analyte is generally desired. An example using IS correction is shown in Figure 3, displaying trend plots from 100 repeated injections of leucine containing SIL-leucine as the IS. An individual plot of leucine or SIL-leucine showed response drift at the early stage of the injections. By using the SIL correction, variability was significantly reduced, resulting in better accuracy and signal reproducibility.

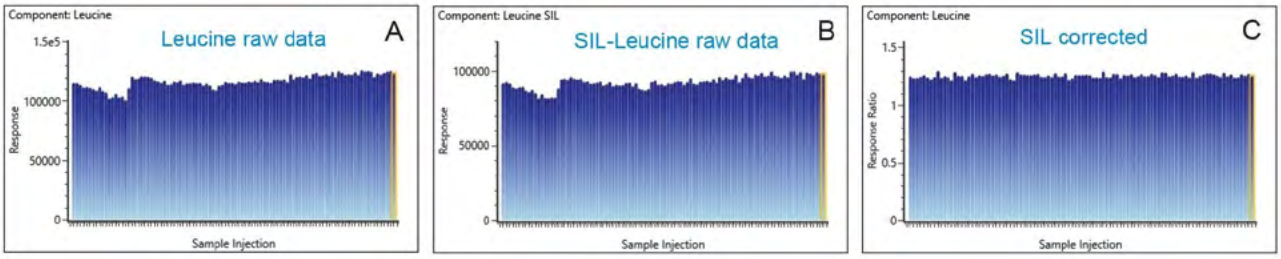


Figure 3. Bar trend plot of raw response from 100 injections. (A) leucine, (b) stable isotope labeled leucine, and (c) response ratio of leucine/SIL-leucine. The plot shows signal variation in raw response is corrected by employing an internal standard.

In this study, response reproducibility based on single IS using different compound is examined. The sample used was basal media mixture IMDM spiked with SIL amino acid mixture and injected 100 times or over 35 hours of data acquisition. Reproducibility of the 100 injections was calculated as follows: (1) no IS, (2) use early eluting compound SIL-proline (Pro) as the IS, (3) use mid eluting SIL-leucine (Leu) as the IS, or (4) use late eluting SIL-phenylalanine (Phe) as the IS.

Figure 4 is a summary plot of %RSD calculated for the 100 injections using above IS corrections. The results showed that when the response was not corrected, the %RSD is ~5%. All method of IS corrections resulted in a reduced %RSD to <3% with the best reproducibility obtained when the corresponding SIL compound was used. These data suggest that while compound specific SIL produced the best reproducibility as expected, using a structurally different compound as the IS can be a practical approach for improved reproducibility. In current raw and spent media analysis, 3-chlorotyrosine has been successfully used as the internal standard.[2–4]

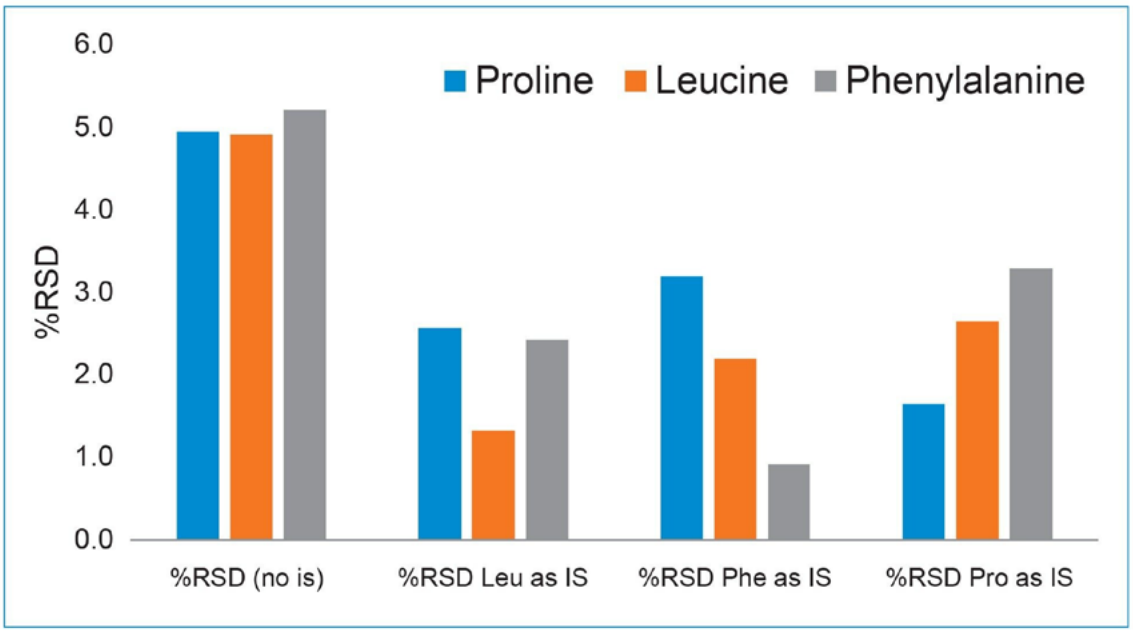


Figure 4. Summary plot of %RSD using different compound as the internal standard. Blue bar: proline (r.t. = 1.73 min), orange bar: leucine (r.t. = 5.64 min), and gray bar phenylalanine (r.t. = 8.11 min).

Conclusion

- Underivatized amino acids were successfully quantified using the cell culture media method and the BioAccord LC-MS System
- Excellent accuracy and reproducibility are obtained for all amino acids at relevant bioprocessing levels
- Investigation of internal standard suggests the use of a single compound as the internal standard is effective in obtaining good reproducibility
- When standard calibration solutions are available, the method can be potentially deployed in quality control of raw material testing in addition to spent cell culture media monitoring supporting process development

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1. 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, September 2021.
2. YW Alelyunas, MD Wrona, YQ Yu, Monitoring Nutrients and Metabolites in Microbial Culture Media using the BioAccord LC-MS System with ACQUITY Premier, Waters Application note, 720007485, January 2022.
3. YW Alelyunas, MD Wrona, YQ Yu, Cell Culture Media Monitoring in Cell and Gene Therapy Using the BioAccord LC-MS System, Waters Application note, 720007705, September 2022.
4. YW Alelyunas, C Prochaska, C Kukla, MD Wrona, YQ Yu, In-Process Media Monitoring Using the BioAccord™ LC-MS for the Automated High Throughput Multi-Parallel Ambr®15 Microbioreactor System, Waters application note, 720007581, April 2022.

Acknowledgements

The authors would like to acknowledge our colleagues Magnus Wetterhall and Steve Preece for reviewing the note.

Featured Products

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

ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

720007766, October 2022

Waters™

Application Note

Introducing a Rapid Throughput LC-MS Method for Cell Culture Media Nutrient and Metabolite Analysis Supporting Upstream Bioprocessing

Yun Wang Alelyunas, Josh Gray, Mark D. Wrona, Patrick Boyce

Waters Corporation

Abstract

A nine-minute rapid and direct liquid chromatography and mass spectrometry (LC-MS) analysis method for cell culture media (CCM) nutrient and metabolite covering 220+ compounds is described. The method is based on reversed phase chromatography where amino acids are directly detected without derivatization. This rapid method reduces turn-around time for monitoring and decision making using previously established user-friendly data review workflows and access to multivariate data analytics (MVDA). The method has been applied for qualitative and quantitative determination of cell culture media nutrient and metabolite analysis in commercial cell culture media and spent media from process optimization in cNISTmAb production using NISTCHO cells. Monitoring of glucose directly from media using this method was also investigated and described.

Benefits

- Rapid nine-minute analysis for cell culture media nutrient and metabolite analysis with 220+ compound coverage in the provided library

- Superior chromatographic separation using ACQUITY™ Premier based technology and HSS™ T3 bonding chemistry offering robust repeatability and extended column lifetime
- LC-MS analysis using negative electrospray ionization (ESI-) mode of acquisition provides info of key nutrients and metabolites, such as glucose, lactate, glutamine, and glutamic acid
- Complete and comprehensive workflow for sample preparation, analysis, and reporting, offering deeper understanding of media used in incubation

Introduction

Correct composition and concentration of critical components in culture media are vital for optimal cell growth, quality, and yield in biotherapeutic cell culturing. Timely in-process monitoring of these compounds during incubation optimization is highly desirable. Recently, we described a LC-MS method and workflow for cell culture media (CCM) analysis based on the BioAccord™ LC-MS System.[1] In this application note, we describe an updated method, where the acquisition time is significantly reduced from 20 minutes to nine minutes while maintaining method robustness, data integrity, quality, and compound coverage. Figure 1 shows a schematic representation of the analysis method, from sample preparation using Andrew+™ Pipetting Robot, LC-MS analysis using BioAccord, to data review and report using waters_connect informatics™. This short run time and rapid analysis enables bioprocess engineers to quickly monitor metabolite changes and support faster decision making.

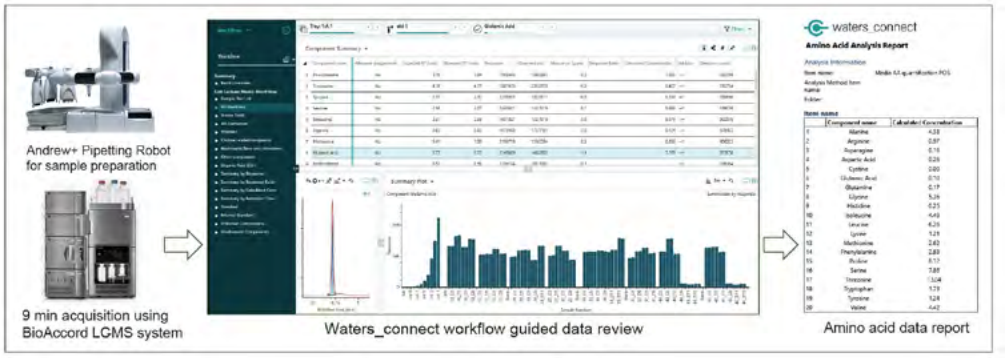

Figure 1. Schematic overview of sample preparation, LC-MS analysis, and report for cell culture media nutrient and metabolite analysis based on Andrew+ Pipette Robot and BioAccord LC-MS platform.

Experimental

Sample Preparation

Commercial media solutions were purchased from Millipore Sigma according to Table 1. Spent media samples were generated by Waters™ Immerse Delaware center where a fed-batch bioprocess experiment was carried out using NISTCHO cell line to produce cNISTmAb product in CHO Fed-batch medium (entry 5, Table 1). NISTCHO cell line (RGTM 10971 NISTCHO Test material) was obtained from nist.gov (https://www.nist.gov/programs-projects/nistcho <https://www.nist.gov/programs-projects/nistcho>). All sample preparation from clarified media solution and standards were performed using the Andrew+ Pipetting Robot. The spent media samples were diluted with 1:400 (V:V) using 0.1%FA containing 0.1 µM stable isotope labeled Tyrosine (Tyrosine $^{13}C_9{}^{15}N$) as the internal standard. Standard calibration solutions were prepared by serial dilution of Waters amino acid cell culture standards (entry 1, Table 1) from 10 µM to 0.01 µM using 0.1%FA solution containing 1:400 diluted Earl's Balanced Salt solution (Millipore Sigma E2888) and 0.1 µM Tyrosine SIL.

Method Conditions

LC-MS System	BioAccord LC-MS system with ACQUITY Premier BSM	
Sample prep system:	Andrew+ Pipetting Robot	
LC conditions:	ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 × 100 mm (P/N 186009468, or with guard P/N 186009471)	
	Mobile phase	(A) 0.1% FA in H_2O (B) 90%ACN/ 10%IPA/ 0.1%FA
	Injection volume	2 µL
	Run time	9 min, gradient elution
MS conditions:	Acquisition mode	Full scan or full scan with fragmentation, ESI+ or ESI-
	Mass Range	Small molecules (50–800 *m/z*)
	Scan rate	5 Hz
	Lockmass correction mode	Standard
LC-MS software:	waters_connect 3.1 or higher, preinstalled with UNIFI	

Results and Discussion

Description of the Rapid Throughput LC-MS Method

A rapid throughput nine-minute method for cell culture nutrient and metabolite analysis is described. The nine-minute method run time represents 50 percent reduction compared to the previously published 20-minute methods while maintaining the same compound coverage.[1] The method employs the ACQUITY Premier HSS T3 Column with dimensions of 2.1 x 100 mm and detected using BioAccord LC-MS System. Similar to the 20-minute method, amino acids are detected directly without derivatization. For spent media monitoring, a simple 1:100 to 1:400 (V:V) dilution of clarified media samples using the 0.1% formic acid aqueous mobile phase is the only sample preparation needed. The dilution ratio is determined by the concentration/fortification of media formulations. An automated and downloadable sample preparation protocol based on Andrew+ Pipetting Robot for the dilution is used. The compound library has been updated to include 220+ compounds, representing the most comprehensive coverage for cell culture media analysis currently available. The available workflow for data processing using waters_connect, including easy data review, elucidation of unknown compounds in the spent media, and MVDA using EZInfo, is the same as has been described previously in the 20-minute method.[1] The method's broad applicability was assessed by analyzing representative commercial media solutions, including Waters amino acid cell culture standard solution, DMEM, IMDM, CHO Fed-batch media, HEK293 viral vector media, and microbial growth media (see Table 1). For all media samples, the nine-minute method produced the same detection results as for the 20-minute method. Extracted ion chromatogram (XIC) of the 26 amino acids in

Waters amino acid cell culture standard kit is summarized in Figure 2. In all, the method produces sharp and symmetrical peaks as well as baseline separation of the isobaric compound pairs; isoleucine/leucine and 2-aminobutyric acid/4-aminobutyric acid.

Entry	Source	Name
1	Waters 186009300	Amino acid cell culture standard kit - has 26 amino acids
2	Millipore Sigma M4530	Medium 199
3	Millipore Sigma D6046	Dulbecco's Modified Eagle's Medium - low glucose
4	Millipore Sigma I3390	Iscove's Modified Dulbecco's Medium
5	Millipore Sigma 14366C	EX-CELL Advanced CHO Fed-batch Medium
6	Millipore Sigma 14385c	EX-CELL Advanced CD HEK293 Viral Vector Media
7	Millipore Sigma L2542	LB Broth (Miller), Liquid microbial growth media
8	Millipore Sigma T5574	Terrific broth, Liquid microbial growth media

Table 1. Summary of commercial solutions analyzed using the nine-minute method.

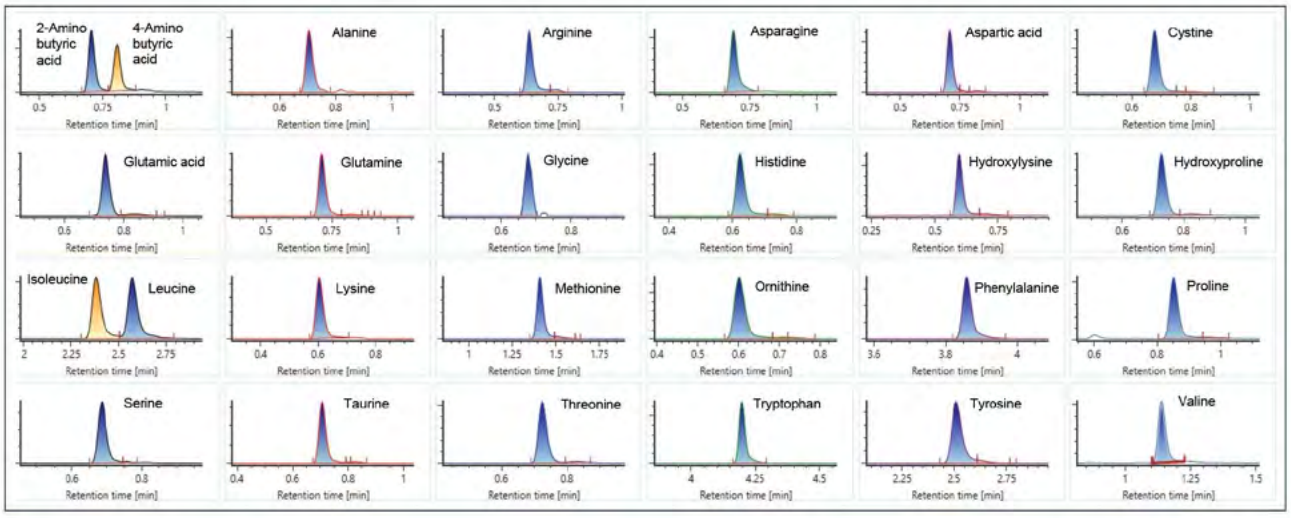

Figure 2. Extracted ion chromatogram (XIC) of 26 compounds in the amino acid cell culture standard kits. Two isobaric compound pairs, isoleucine/leucine and 2-amino/4-amino butyric acid are baseline separated.

The method performance is determined using Waters amino acid cell culture kit (p/n: 186009300 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009300-amino-acid-cell-culture-standard-kit.html>) containing 26 amino acids. A separate calibration solution of glucose was also prepared.

Extracted ion chromatogram for each of the amino acid is shown in Figure 2. Method linearity was determined using linear regression with $1/x^2$ fitting and a 20% deviaton exclusion criteria. Method precision and accuracy were determined based on 6 replicate injections at two concentrations, 0.5 µM and 2.5 µM respectively. Data summarized in Table 2 showed excellent reproducibility and accuracy for all compounds measured and comparable to the 20-minute method.[2] These results suggest that the rapid throughput method can provide both quantitative and qualititive results. In the absence of standard, the relative reponse can be highly informative for analyzing changes in media nutrients and metabolites in process optimization experiment and subsquent MVDA analytical approaches.

Entry	Component name	Expected RT (min)	Neutral mass (Da)	Range (uM)	R^2	QC conc = 0.5 µM (n=6)		QC conc = 2.5 µM (n=6)	
						%Accuracy	%Precision	%Accuracy	%Precision
1	4-Aminobutyric acid	0.62	103.0633	0.025-10	0.99	94	3.6	98	1.7
2	Alanine	0.67	89.0477	0.05-10	0.99	108	4.3	103	2.3
3	Arginine	0.63	174.1117	0.01-5	0.99	92	5.6	107	1.4
4	Asparagine	0.68	132.0535	0.025-10	0.99	98	3.3	97	2.0
5	Aspartic Acid	0.66	133.0375	0.25-10	0.98	101	5.6	108	5.1
6	Cystine	0.64	240.0239	0.01-5	0.99	92	5.3	96	1.7
7	Glutamic Acid	0.75	147.0532	0.01-10	0.99	97	1.6	103	2.8
8	Glutamine	0.72	146.0691	0.01-10	0.99	98	3.6	105	1.4
9	Glycine	0.64	75.0320	0.5-10	0.99	113	5.5	108	4.0
10	Histidine	0.62	155.0695	0.1-10	0.99	96	5.9	102	1.6
11	Hydroxylysine	0.57	162.1004	0.25-10	0.99	107	3.1	102	2.6
12	Hydroxyproline	0.62	131.0582	0.025-10	0.99	96	4.4	104	2.5
13	Isoleucine	2.37	131.0946	0.01-10	0.99	90	3.7	104	2.0
14	Leucine	2.56	131.0946	0.025-5	0.99	89	3.8	108	3.9
15	Lysine	0.58	146.1055	0.025-10	0.99	95	3.0	106	2.5
16	Methionine	1.41	149.0511	0.01-5	0.99	91	3.5	107	2.7
17	Ornithine	0.58	132.0899	0.025-10	0.99	99	5.1	104	2.8
18	Phenylalanine	3.79	165.0790	0.01-10	0.99	91	5.4	103	3.3
19	Proline	0.83	115.0633	0.05-10	0.99	97	3.6	111	2.8
20	Serine	0.65	105.0426	0.05-10	0.99	98	4.3	100	2.0
21	Taurine	0.66	125.0147	0.05-2.5	0.99	116	9.0	95	3.6
22	Threonine	0.69	119.05824	0.025-10	0.99	100	3.6	100	2.3
23	Tryptophan	4.18	204.08988	0.01-10	0.99	88	2.8	103	2.3
24	Tyrosine	2.57	181.07389	0.025-10	0.99	92	3.9	103	2.1
25	Valine	1.12	117.07898	0.025-10	0.99	93	2.7	110	11.8
26	Glucose	0.76	215.0323 [+Cl]	0.1-5	0.99	118	2.3	105	2.3

Table 2. Summary of linear range, accuracy, and precision of amino acids and glucose quantification using the nine-minute method. Amino acid standard solution used in the analysis is the amino acid cell culture standard kit (p/n: 186009300). The linear range is derived from linear regression with $1/x^2$ fitting, 20% deviation was used as the exclusion criteria. Amino acids data were acquired using ESI+ and glucose using ESI- mode of acquisition.

Application to Spent Media Analysis

The method has been applied to spent media analysis of a CHO cell cultivation to produce cNISTmAb. Spent media solutions under different glucose feeding conditions were sampled during a 14-day incubation. After centrifugation, the supernatant solutions were diluted 1:400 with 0.1%FA and analyzed using both the nine-minute fast method and the 20-minute method. Figure 3 shows trending plots of MS response versus sampling day of different reaction flasks and comparing two analysis methods. Three representative amino acids are included in Figure 3, aspartic acid as an example of early chromatographically eluting compound, leucine as an example of mid eluting compound, and phenylalanine as an example of late eluting compound. Results showed excellent correlation where same trends were obtained using either method, indicating high quality data obtained in the 20-minute method is maintained using the rapid nine-minute method.

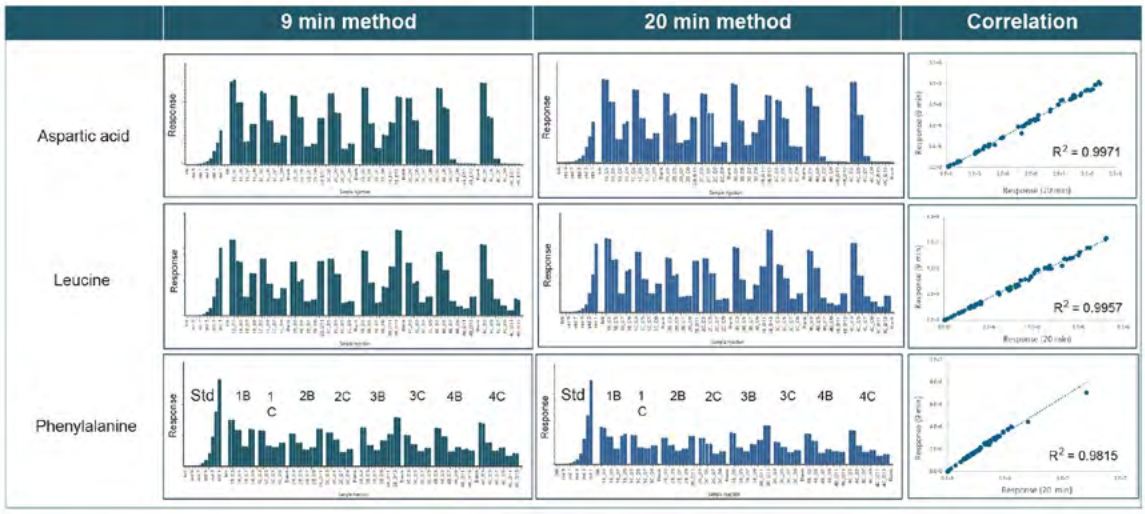

Figure 3. Trending plot of compound showing observed response as function of incubation condition and sampling time. Green graphs on the left are data from nine-minute method, blue graphs in the middle are from 20-minute method. On the right is correlation plot of response obtained from nine-minute method vs 20-minute method. All spent media samples were injected in duplicate.

Figure 4 is a plot of glucose reponse versus sampling time overlaying different glucose feeding conditions and comparing nine-minute (A) with 20-minute method (B). Figure 4 also included glucose data obtained from Nova Flex2 (C), a popular cell culture analyzer. In the experimental design, four glucose feeding conditions were tested. Flasks 1, 2, and 3 used reduced glucose feeding conditions. In flask 4, glucose were fed to maintain a constant 6 g/L concentration throughout the incubation. Results showed that at reduced glucose feeding conditions (flask 1–3), glucose was rapidly consumed and depleted during the exponential cell growth period (5–7 days); in comparison, glucose concentration was maintained during the same period using the maintanance dose conditions (greenline, flask 4). Results of cell viability and protein titer showed high cell viability and higher protein titer using the maintenance dose (see Waters application note for intact protein analysis in cell culture media using BioAccord[3]). Figure 4 shows that data from nine-minute method is comparable and of similar high quality to 20-minute LC-MS method, and there is good correlation between data obtained using either LC-MS method with the Nova Flex2 analyzer. In addition to glucose which was detected under negative ionization conditions, several amino acids including glutamine and glutamic acid are also detectable. Hence when monitoring key nutrient and metabolite, such as glucose, lactate, glutamine, and glutamic acids, is desired, results can be obtained from a single injection under negative ionization conditions.

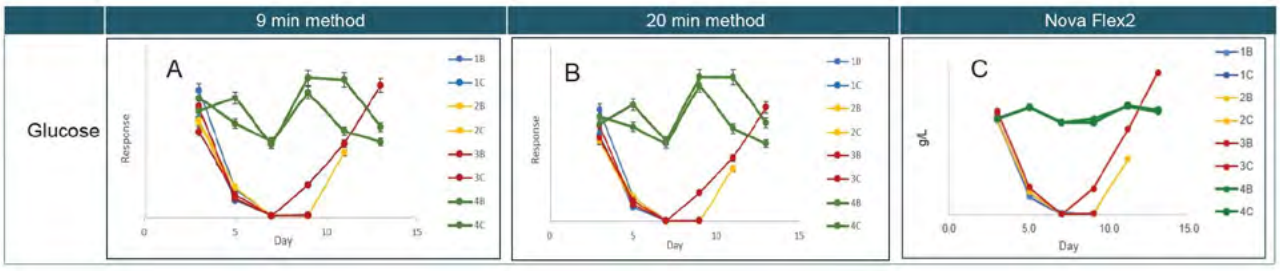

Figure 4. Plots of glucose response versus incubation time overlaying all feeding conditions. (A) data from nine-minute method, (B) data from 20-minute method, and (C) data from Nova Flex2 analyzer. Four glucose feeding conditions, represented by different colors, were used using four separate flasks in duplicate. Error bars in the graph represent duplicate measurements. Glucose chlorine adduct (+Cl) in LC-MS was used for its selective detection.

Conclusion

A rapid throughput method is described for cell culture media nutrient and metabolite monitoring. The method consists of a rapid nine-minute data acquisition, 220+ compound library screen, ease of data review, data report along with compound elucidation and batch comparison (MVDA) capabilities. Reducing the analysis time to nine-minute allows rapid data generation in bioprocess laboratories. The method uses less than 10 µL of spent media sample and employs a simple dilution of clarified spent media sampled from bioreactors using aqueous mobile phase. Automated sample preparation of the media and standard solutions are readily carried out using the Andrew+ Pipetting Robot. Extensive data comparison of the rapid nine-minute method with the previously published 20-minute method suggests the same high-quality data and method robustness are obtained even when the analysis time is shortened by 50%. In conclusion, combination of the rapid throughput method with available waters bioprocess walk-up solution can enable analytical scientist and bioprocess engineers to obtain high quality data easily and routinely for upstream bioprocess optimization.[4] The full featured BioAccord LC-MS System provides routine monitoring as well as in-depth analysis of media samples for fully understanding your process.

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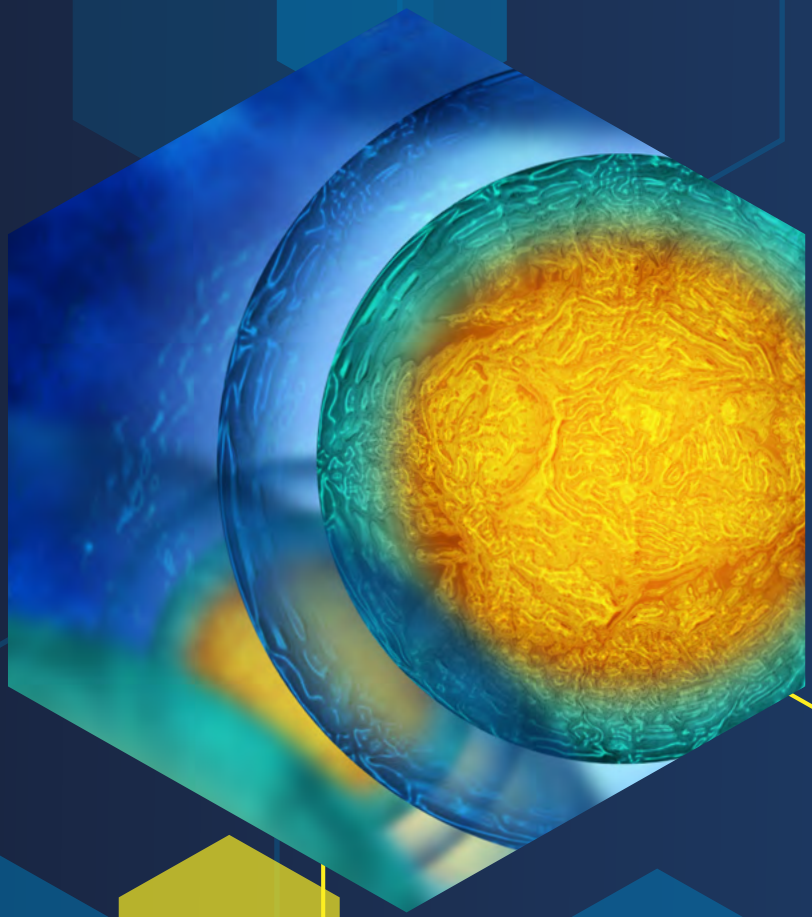

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Application Note

Direct and Rapid SEC Analysis of Monoclonal Antibody Titer and Aggregation in Cell-Culture

Stephan M. Koza, Ying Qing Yu

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

A rapid 2.4 minute size-exclusion chromatography (SEC) method using intrinsic protein fluorescence detection (SEC-FLR) for the direct analysis of monoclonal antibody (mAb) titer and aggregation (high molecular weight species, HMWS) in clarified cell-culture samples is highlighted. The proposed method employs a previously described 200 mM ammonium acetate (AMA) mobile phase that can be more efficiently deployed on an LC system dedicated for LC-MS analysis and was found to support lengthy column lifetimes when analyzing cell-culture samples. The use of FLR detection enables a cell-culture sample injection volume reduction of over 10-fold versus using UV absorbance at 280 nm (A280). As a result, both mAb titer and aggregation in cell-culture can be readily tracked for sample loads of 50 ng or more (0.1 µL sample at 0.5 µg/µL). Alternatively, when using A280 detection titer can be determined at this low sample load but not HMWS levels.

This high-throughput (HT), low sample load SEC method uses an XBridge™ Premier Protein SEC (250 Å, 2.5 µm, 4.6 x 150 mm) Column at a flow rate of 1.0 mL/min. Method performance and robustness were evaluated using an ACQUITY™ Premier UPLC System

Benefits

- Rapid (2.4 minute) SEC-FLR analyses of mAb titer and HMWS
- Direct injection of cell-culture samples without Protein A purification
- Extended column lifetimes projected at over 1000 analyses of mAb CHO cell-culture samples with injected volumes of 0.1 µL or less of cell-culture per analysis

Introduction

A method for the direct SEC analysis of mAb titer and HMWS in clarified cell-culture was developed. Typically, mAb HMWS levels in cell-culture samples are assessed by SEC after Protein A affinity purification. A version of this approach using a 2D-LC Protein A and SEC method to determine both titer and aggregation levels has been reported.[1] One of the challenges of Protein A purification for this application is the potential of altering HMWS levels via the Protein A elution conditions. One approach to potentially circumvent this is the direct SEC analysis of mAb in cell-culture samples.[2] This mode of analysis is often possible due to the overwhelming abundance and size of mAbs and their aggregates in comparison to individual host-cell proteins, however, column fouling by the cell-culture sample is an obstacle.

The genesis of this work was an earlier report, in which an Waters ACQUITY Premier Protein SEC 250 Å, 1.7 µm Column had been demonstrated to provide HT SEC analyses of Protein A purified mAb samples using an AMA mobile phase compatible with LC-MS instruments.[3] Of note in this previous work was the long-term performance of the SEC column when subjected to a broad range of partially purified cell-culture samples. It was speculated that the anti-microbial properties of 200 mM AMA may have provided some benefit.

Building on that observation, the direct SEC analysis of mAb in cell-culture was considered. While these samples, when obtained should not be microbiologically contaminated, they are far more prone to contamination during handling and storage due to their nutrient-rich composition. Another factor to consider, is the potential to introduce particulates and other components in clarified and filtered conditioned media cell culture samples that can foul an SEC column. Therefore, the volume injected onto the column was minimized by using intrinsic protein FLR as a detection method and an SEC column exhibiting minimal secondary interactions and larger packed particles. Accordingly, an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column was used.

This column uses hydrophilic MaxPeak™ HPS hardware (column body, end fittings, and frits) that is packed with high-coverage hydroxy-terminated polyethylene oxide (BEH-PEO) bonded ethylene bridged-hybrid particles. This results in an SEC column with low levels of binding between the column and the protein size variants being separated, which further enables the analysis of low protein loads with a wide range of mobile phase compositions. Method development, performance, and robustness studies were executed using an ACQUITY Premier UPLC System to further reduce potential protein-metal interactions.

Experimental

Sample Description

mAbs trastuzumab (Herceptin™) and trastuzumab-anns (Kanjinti™) were analyzed past expiry. Samples were diluted into phosphate buffered saline (PBS) or non-transfected CHO-cell media (NTM) to indicated concentrations. NTM was prepared by Syd Labs, Inc. using non-transfected CHO-K1 cells in a spinner flask where spent media was collected from the flask on days 2 through 15 (~90% average cell viability), pooled, and 0.2 µm filtered.

MS Conditions

LC system:	ACQUITY Premier UPLC with Quaternary Solvent Manager (QSM) and CH-A column heater
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 280 nm and ACQUITY UPLC FLR Detector (ex: 280 nm, em: 350 nm). Autozero programmed at 0.6 min. for 1.0 mL/min flow rate method
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)

Column(s):	XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column plus mAb Size Variant Standard (p/n: 176004779)
Column temperature.:	25 ˚C
Sample tempertaure.:	6 ˚C
Injection volume:	0.1 µL or 1.0 µL
Flow rate:	1.0 mL/min (run time=2.4 min), or as indicated,
Mobile phase:	ammonium acetate, LC-MS grade (Supelco LiChropur™, eluent additive for LC-MS, 73594), 0.1 µm sterile filtered, 200 mM

Data Management

Chromatography software:	Empower™ 3 (FR 4)

Results and Discussion

Method Development

The primary goal of this study was to develop a reliable HT non-denaturing SEC method for the analysis of mAb titer and HMWS in clarified CHO cell-culture media samples without prior treatment other than sample dilution. This target was met using an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column, and a 200 mM AMA mobile phase at a flow rate of 1.0 mL/min which delivered an effective separation with an analysis time of 2.4 minutes and peak detection via intrinsic protein FLR. Also, in order to start the next analysis before the prior

analysis was fully completed a detector autozero event was added at 0.6 min. In combination, when using an injection volume of 0.1 µL, the method was demonstrated to effectively monitor dimeric HMWS (HMWS1) at a 1% or higher level in a cell-culture sample with a mAb titer of 0.5 mg/mL.

The mAb monomer is the predominate component detected by A280 at its respective SEC retention time in cell-culture samples. This is clearly demonstrated when comparing the chromatograms of a purified mAb (1.0 mg/mL diluted in PBS) and of a non-transfected media (NTM) sample (Figure 1). We compared A280, which is the protein absorbance band primarily from tryptophan and tyrosine residues, and UV absorbance at 260 nm (A260), which is the peak absorbance band of DNA and RNA, of these two samples. The A280 is approximately two-fold higher than A260 for the purified mAb monomer and HMWS1 as would be predicted. However, for the NTM sample A280 and A260 nm are comparable (Figure 1). This result is consistent with A260 being partly due to the presence of low levels of DNA or RNA in the NTM sample.

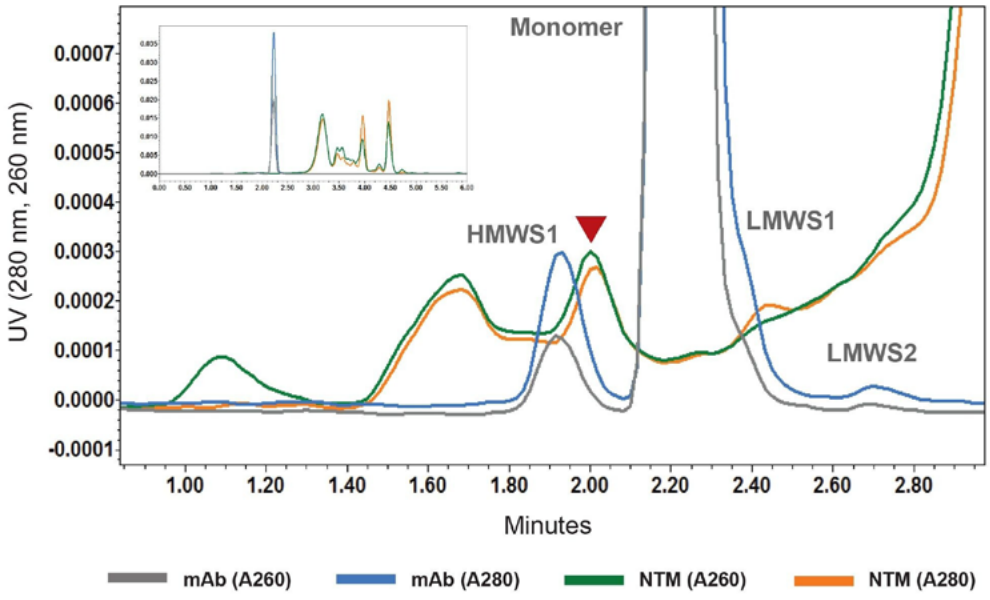

Figure 1. Shown are the SEC-UV 280 nm and 260 nm chromatograms for a purified mAb (trastuzumab-anns) sample diluted to 1 mg/mL in PBS (mAb) and for the non-transfected cell-culture sample (NTM). An XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm was used at a flow rate of 0.5 mL/min with a 200 mM AMA mobile. Injection volumes were 1 µL. Additional conditions are provided in the text. These data were collected on an ACQUITY Premier QSM UPLC.

The use of intrinsic protein FLR detection, which primarily detects tryptophan residues with high quantum yield, eliminates interference from DNA and RNA, and also provides over 10-fold greater signal-to-noise (S/N) for the detection of mAb HMWS (Figure 2) versus A280. This greater S/N will allow for the analysis of smaller sample volumes as demonstrated by the 0.1 µL injection used for FLR detection (Figure 2) versus 1.0 µL for A280. Also, in this example, a component in NTM that interferes with the measurement of dimeric HMWS1 when using A280 detection is not as prominent when FLR detection is used.

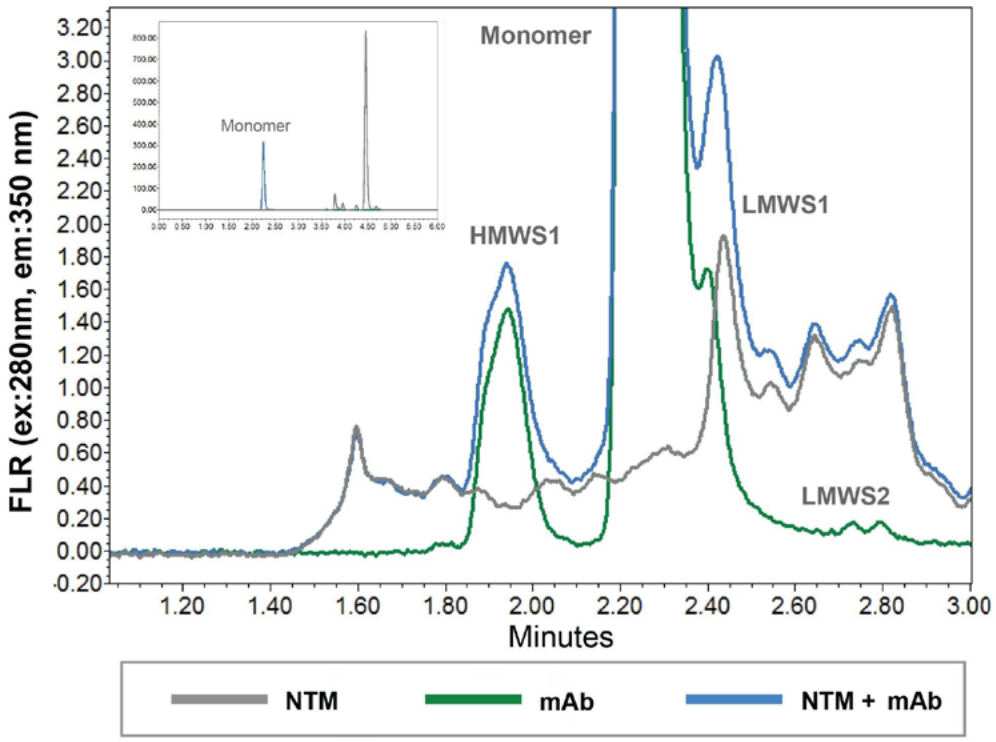

Figure 2. The SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample diluted to 1 mg/mL in PBS (mAb) and in NTM (NTM+mAb), in addition to the un-spiked NTM sample (NTM) are presented. Additional conditions are provided in the text and in Figure 1. Injection volumes were 0.1 µL. These data were collected on an ACQUITY Premier QSM UPLC.

Method Evaluation

Titers ranging from 0.25 mg/mL to 4.0 mg/mL of mAb in cell-culture samples were capably determined using either FLR or A280 detection. Serial dilutions of mAb into PBS and NTM were prepared and 0.1 µL injection volumes were made for A280 and FLR detection. In addition, 1.0 µL injection volumes of the PBS samples were evaluated using A280. The chromatograms for the 0.1 µL injection volume NTM and PBS diluted samples are shown in Figure 3. A drop-baseline integration was used for mAb samples diluted in PBS while a tangential-skim integration was used for mAb samples diluted in NTM. The mAb titers in the NTM samples were then calculated using the PBS calibration curves and then correlated to the targeted values (Figure 4). Both determinations of titer in the spiked NTM samples exhibited a minimal bias based on the slopes of the correlation plots and minimal overall deviations from the expected values (≤3.2%). This result demonstrates that direct mAb titer determinations in cell-culture could be determined using either A280 or FLR detection and injection volumes as small as 0.1 µL.

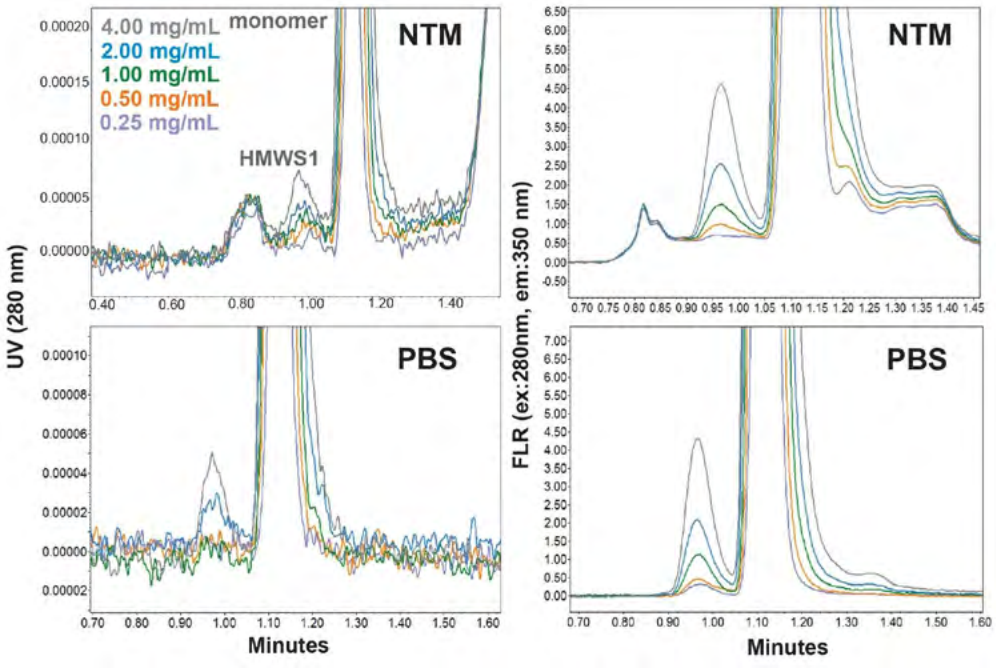

Figure 3. SEC-UV and SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample serially diluted to 4.0, 2.0, 1.0, 0.5, and 0.25 mg/mL in PBS and in NTM are overlayed in the figures. The flow rate was 1.0 mL/min (2.4 min analysis time) and injection volumes were 0.1 µL. Additional conditions are provided in the text and in Figure 1. These data were collected on an ACQUITY Premier QSM UPLC.

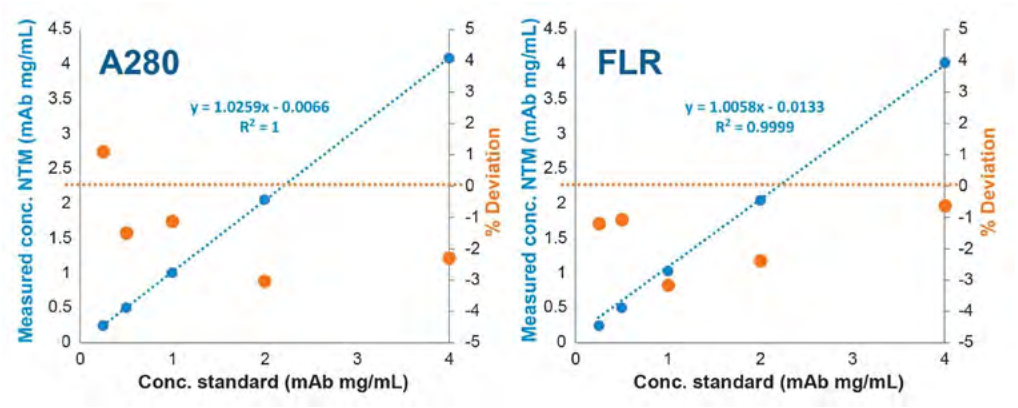

Figure 4. SEC-UV and SEC-FLR quantitative titer capabilities are demonstrated for the cell-culture samples correlated to the predicted values (left y-axis). Predicted values were based on a calibration curve generated using mAb drug product diluted in PBS. The SEC-UV and SEC-FLR chromatograms presented in Figure 3. Shown on the secondary (right) Y-axis are the relative percent deviations from the predicted values where the orange dashed line represents no deviation. See the text for further discussion and information.

We next evaluated the determination of dimeric HMWS1 in NTM and PBS from the above experimental data (Figure 3 and Figure 5). Multimeric HMWS (HMWS2) was not present at appreciable levels in the mAb sample used and with a 0.1 µL injection volume HMWS1 was not quantifiable when using A280 detection. In comparing the quantification of HMWS1 for the PBS samples the average relative abundance, excluding the 0.25 mg/mL sample, of HMWS1 was 0.93% when measured by A280 (1.0 µL injection volume, chromatograms not shown) and 0.92% by FLR. A significant positive deviation from this average was noted for the HMWS1 determinations of the 0.25 mg/mL sample, which may be in part an artifact of integration and indicate the limit of quantification (LOQ) of the method.

The relative abundances of HMWS1 in the NTM samples were consistent at a mAb concentration of 0.5 mg/mL and greater for FLR, but are also lower than those observed for the PBS samples. This is consistent with the use of a tangential-skim integration for the NTM samples versus drop-baseline integration for the PBS samples. Despite this biased result, these data demonstrate that FLR detection can detect real change in the amount of HMWS1 in a CHO cell-culture sample.

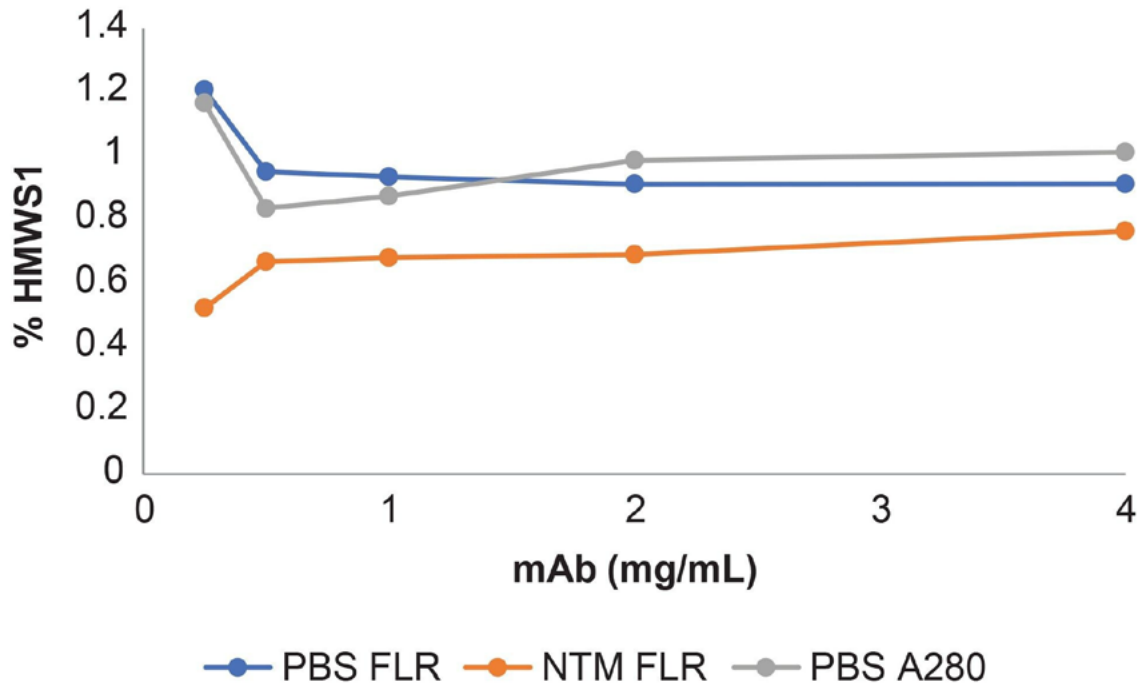

Figure 5. SEC-FLR quantitative determinations of HMWS1 are shown for the cell-culture samples (NTM FLR). Also plotted are the values observed for the PBS diluted mAb control using SEC-UV (1.0 µL injection volume) and SEC-FLR. The consistently lower HMWS1 abundance values are noted for the NTM FLR samples. See the text for further discussion and information.

The capacity of the SEC-FLR method to detect change in multimeric HMWS2 was also successfully demonstrated. For this study a concentrated mAb sample was stressed via overnight agitation at 45 °C. This sample was then diluted into PBS and NTM to 1.0 mg/mL and these two stressed samples were then diluted (1:3) with unstressed samples. (Figure 6). From these results we observe a reasonably linear response for HMWS2 for both the PBS and NTM diluted samples. However, the y-intercept is 0.5% for the NTM curve due to the co-elution of components in the NTM. The results for HMWS1 showed comparable response curves for the NTM and PBS samples consistent with previously described results.

Taken together, these results demonstrate that the proposed SEC method is capable of directly and reliably determining mAb titer in clarified cell-culture samples. In addition, substantial levels of dimeric HMWS1 (≥1%) and multimeric HMWS2 (≥1.5%) could also be readily observed. Detection limits will vary depending on cell-

culture conditions, mAb titer, and the extent of aggregation. These results were based on using mAb sample spiked into NTM with low viability (~90%), however, the veracity of the method could also be evaluated by removing mAb and aggregated mAb from cell-culture samples (***e.g.,*** Protein A capture) and then spiking the mAb depleted sample with purified mAb sample.

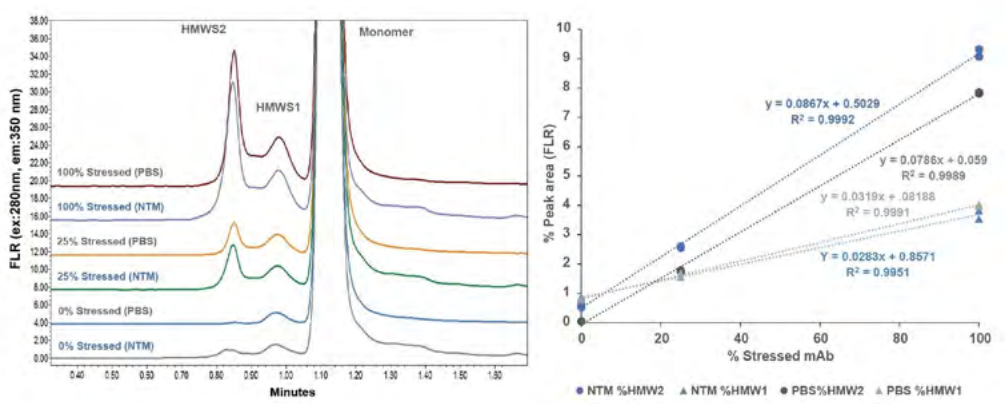

Figure 6. Overlaid are SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample that was stressed to induce aggregation. The stressed sample was subsequently diluted 1:3 with the unstressed sample (25% Stressed). Sample concentrations are 1.0 mg/mL and a dilution series in both PBS and NTM are overlayed. These data were collected on an ACQUITY Premier QSM UPLC. Quantitative determinations of HMWS1 and HMWS2 are plotted for the NTM and PBS diluted samples. See the text for further discussion and information.

Method Reliability

Column performance was assessed for over 500 consecutive analyses of NTM spiked with 1.0 mg/mL mAb. The sample was centrifuged at 10^3 X g for three minutes within 24-hours of analysis and held at 6–8 °C in the autosampler. The injection volume was 0.1 µL and the flow rate was 1.0 mL/min. The primary concerns with injecting cell-culture samples are the potential of injecting particulates that can disrupt flow patterns through the packed bed resulting in loss of resolution and chemical contamination of the column. For this study a guard column was not deployed to better assess the impact on analytical column performance. Injections of pure mAb diluted in PBS were performed intermittently to assess column performance (Figure 7). Over the course of the study we observed a slow yet steady loss of resolution (USP HH) between HMWS1 and the monomer. It is projected that less than baseline resolution (Rs <1.5) would be observed after over 1000 analyses.

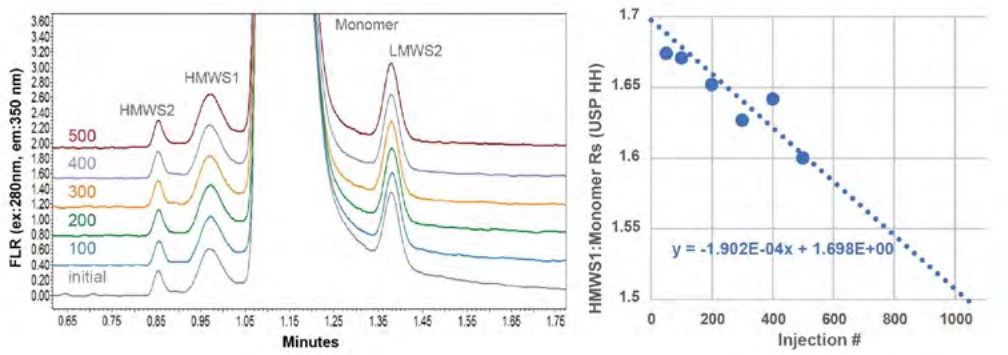

Figure 7. SEC-FLR column lifetime timepoint assessments using a purified mAb (trastuzumab) sample. This sample was analyzed well past expiry and exhibits significant levels (~ 1 %) of both HMWS and LMWS size variants. These data were collected on an ACQUITY Premier QSM UPLC. Determinations of USP (HH) Resolution (Rs) between HMWS1 and the monomer are plotted for the timepoints. See the text for further discussion and information.

One path to potentially improving column longevity is to inject a lower volume of media sample. Since 0.1 µL is the smallest injection volume specified for the LC system used, NTM samples containing 1.0 mg/mL mAb were serially diluted (1:1) with PBS and then analyzed using only FLR detection (Figure 8). Data evaluation was executed similarly to the approach previously described (see Figure 4). The determinations of titer and HMWS1 abundance in the PBS diluted spiked NTM samples were consistent down to a 4-fold dilution. However, for a cell-culture sample with a mAb titer of 1.0 mg/mL and HMWS1 at a 1% level, a 1:1 dilution is conservatively recommended, which should result in a HMWS1 peak above the LOQ (S/N=14). This dilution reduces the amount of cell-culture injected per analysis two-fold which should significantly reduce the loss of column performance due to cell-culture components. The extent of cell-culture sample dilution could certainly be increased for samples with higher titers or higher levels of HMWS. In addition, while not assessed in this work, incorporating a guard-column could also be used to reduce column fouling.

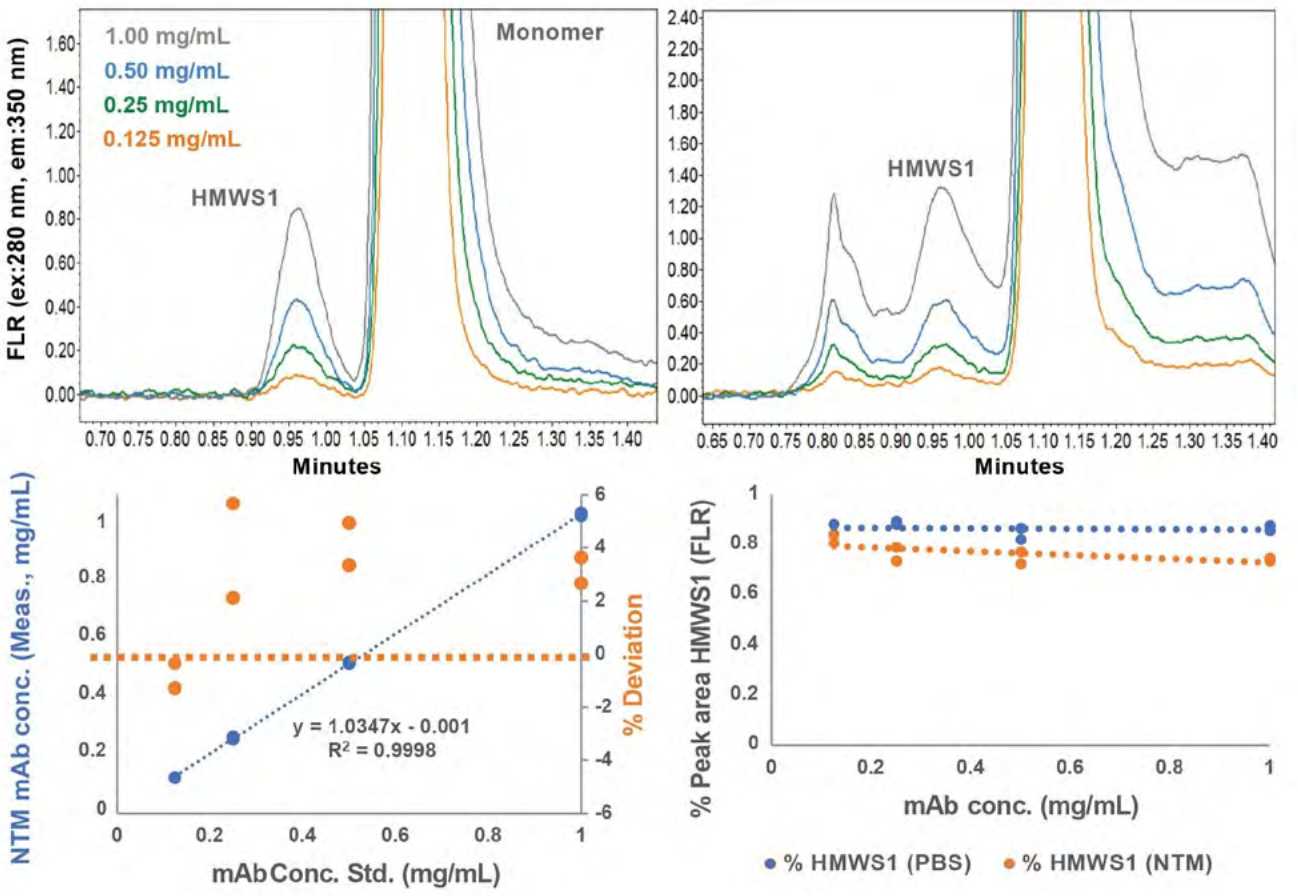

Figure 8. SEC-FLR chromatograms for a purified mAb (trastuzumab-anns) sample initially diluted to 1.0 mg/mL in PBS and in NTM, and then each serially diluted with PBS to concentrations of 0.5, 0.25, and 0.125 mg/mL are overlayed in the figures. LC conditions and data analysis are as described in Figures 3 through 5, and in the text.

Conclusion

A high-throughput (2.4 minute2.4-minute run time) SEC method using an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm Column, 200 mM AMA mobile phase at a flow rate of 1.0 mL/min, and intrinsic protein FLR detection for the simultaneous titer and aggregation (HMWS) analysis of monoclonal antibodies in clarified cell-culture without prior protein purification is described. This method was developed with the intent of potentially supporting cell-line and cell-culture optimization experiments. However, it could also be deployed to support purification process development, for example, an initial product capture step where media components are still present in some samples.

In summary, the sensitivity and resolution of this method is enabled through the use of intrinsic FLR detection and an LC system and SEC column that exhibits negligible levels of protein-surface interactions. This results in a method capable of effectively monitoring mAb titer and significant increases in HMWS (≥1% to 1.5% level). In addition, by significantly reducing the volume of cell-culture sample injected to 0.05 µL to 0.10 µL on a 4.6 mm ID column and using a mobile phase with anti-microbial properties, useful column lifetimes may be extended to over 1000 analyses without the use of a guard column and potentially longer if a guard is used. It should be noted that for this evaluation, the mobile phase was 0.1 µm sterile filtered, and the samples were 0.2 µm filtered and also centrifuged prior to analysis.

Acknowledgements

We would like to thank Albert W. Jiang, Alireza Aghayee, and Xiao Dong for their efforts and thoughts that eventually led to this project.

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Application Note

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

Yun Wang Alelyunas, Charles Prochaska, Clint Kukla, Caitlin Dunning, Caitlin Hanna, Mark D. Wrona

Waters Corporation, Sartorius Stedim

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

- 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.[1,2]

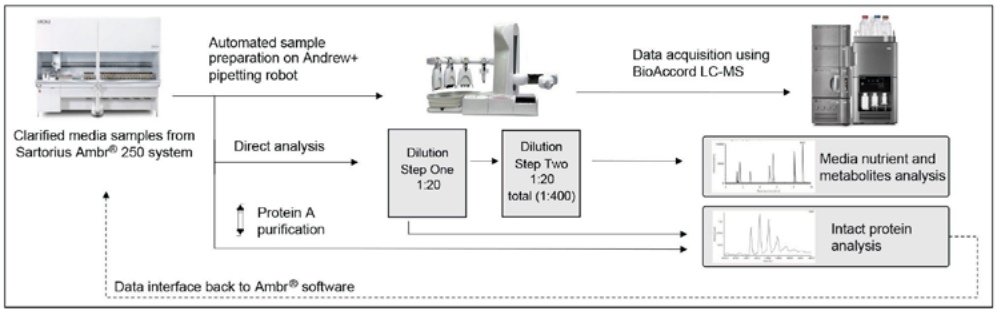

Figure 1. A schematic illustration of automated sample preparation and BioAccord LC-MS analysis of intact protein and cell culture media nutrient and metabolites analysis in spent media from bioreactors.

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).

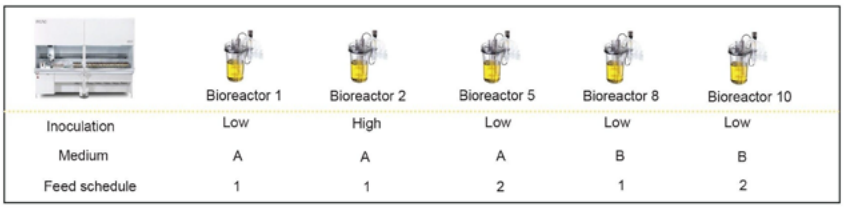

Figure 2. Summary of experimental conditions of samples collected in the intact and culture media monitoring study.

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.[3] 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_4 300 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_2O				
Mobile phase B	90% ACN/10% IPA/0.1% FA				
Gradient table	**Time (min)**	**Flow (mL/min)**	**%A**	**%B**	**Curve**
	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 *m/z*)				
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 µm, 2.1 × 150 mm (P/N 186009469)				
Column temp.	40 °C				
Sample temp.	10 °C				
Injection volume	2 µL				
Flow rate	0.25 mL/min				
Mobile phase A	0.1% FA in H_2O				
Mobile phase B	90%ACN/10%IPA/0.1%FA				
Gradient table	**Time (min)**	**Flow (mL/min)**	**%A**	**%B**	**Curve**
	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 *m/z*)				
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 °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

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.

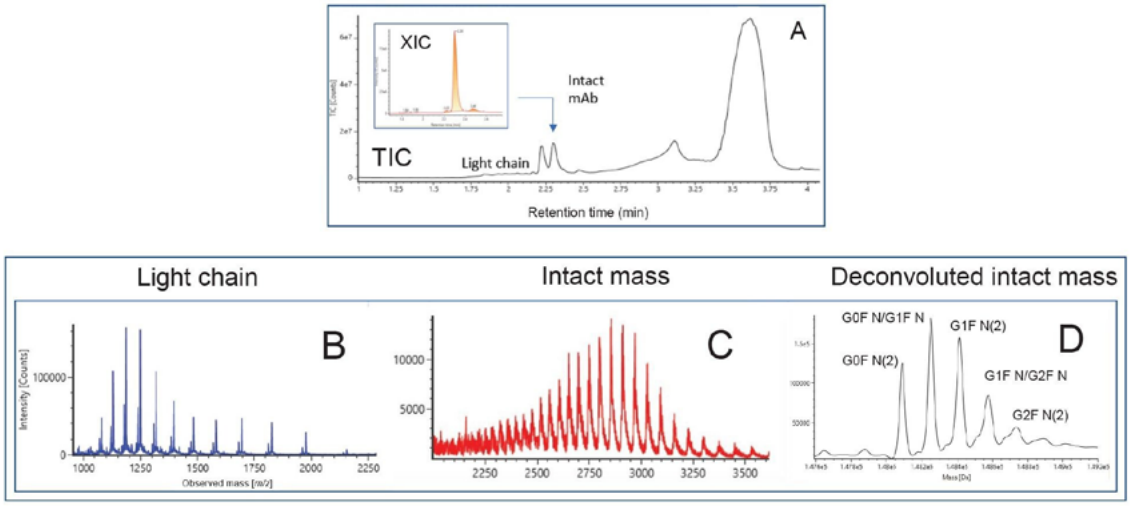

Figure 3. (A) Representative MS spectrum of direct intact mass analysis from cell culture media sample. The inserted figure showed XIC for intact mAb with m/z 2000–4000 Da. (B) observed MS spectrum of light chain, (C) observed MS spectrum of intact mAb, and (D) deconvoluted spectrum of intact mAb, labelled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

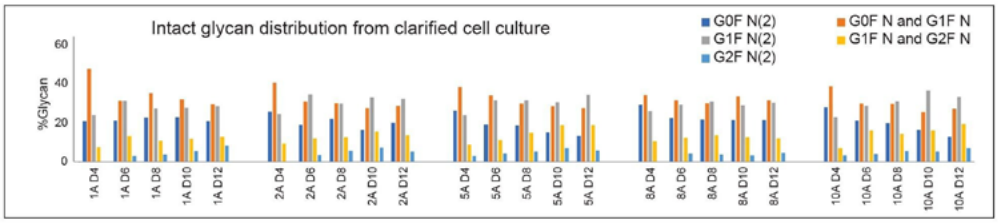

Figure 4. %Glycan determination from clarified cell culture media sample, showing overlaid bar plot of each of the glycan as a function of bioreactor and over time.

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 impuries 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 clarifed 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.

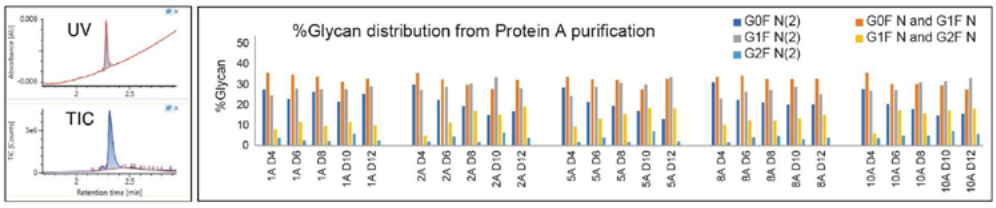

Figure 5.(left) Representative UV and MS TIC chromatogram of media sample post protein A purification. (right) %Glycan determination from protein A purified sample, showing overlaid bar plot of each of the glycan as a function of bioreactor and over time.

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 abunband charge states in the observed spectrum. A description of intact protein quantification can be found in Waters application note.[4] 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 coefficent 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.

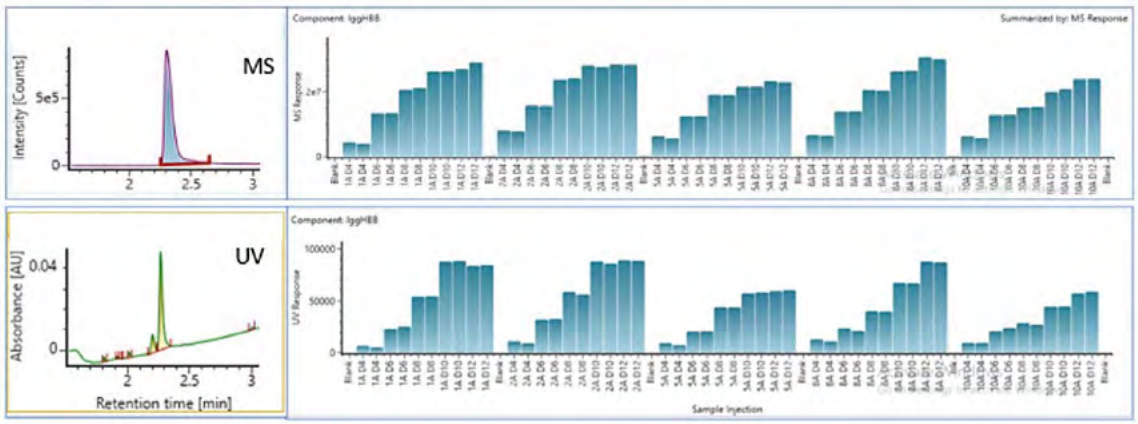

Figure 6. Overlaid bar plot of MS response or UV absorbance across bioreactors and overtime. Each sample was analyzed in dupiicate with an overall reproducibility <5%.

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.[2] 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 acquisitons 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>), 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.

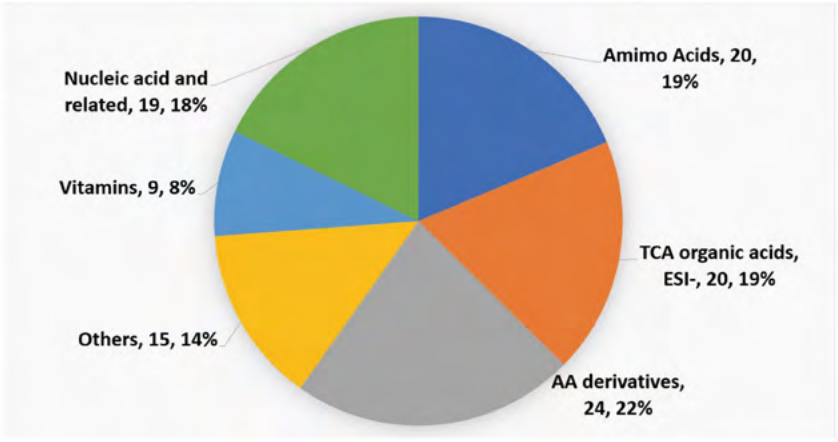

Figure 7. Observed compounds in the spent media, represented as %distribution and number of compounds detected, grouped by compound class.

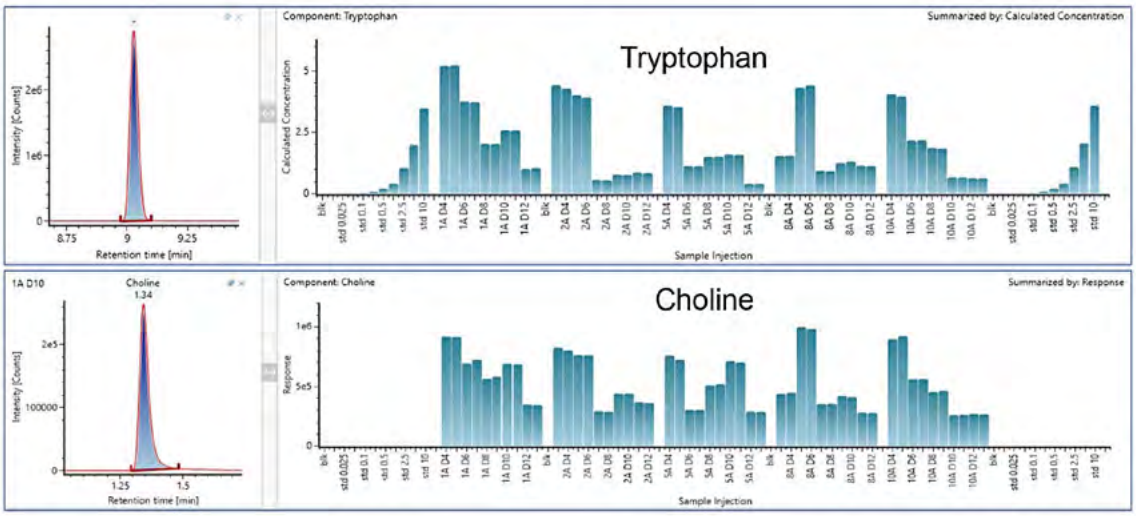

Figure 8. Representative trend plots of metabolites displaying bioreactor sampling over time. Representative results for Tryptophan (absolute concentrations) and Choline (relative trends) shown.

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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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.

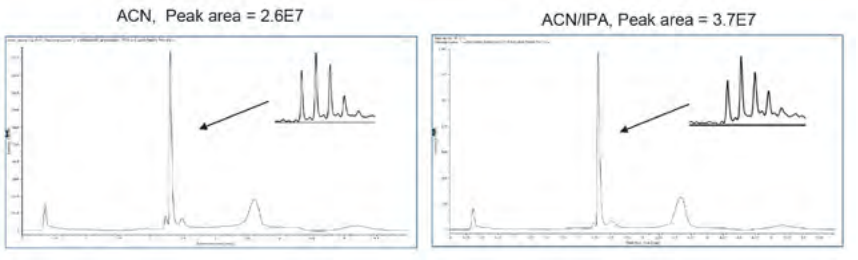

Figure A. Extracted ion chromatogram of intact mAb analysis of a clarified cell culture media sample, comparing using 100% ACN (left) with 90%ACN/10%IPA (right) as the organic mobile phase B.

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).

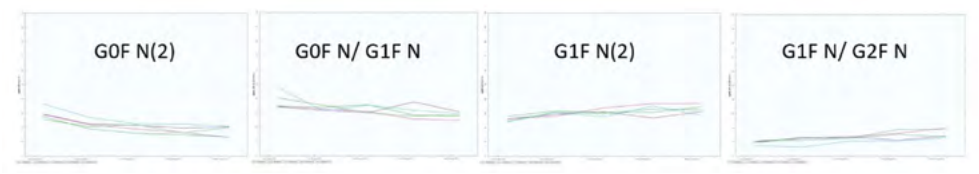

Figure B. Data interface display of overlaid plots of major %glycan modifications as a function of bioreactor and over time.

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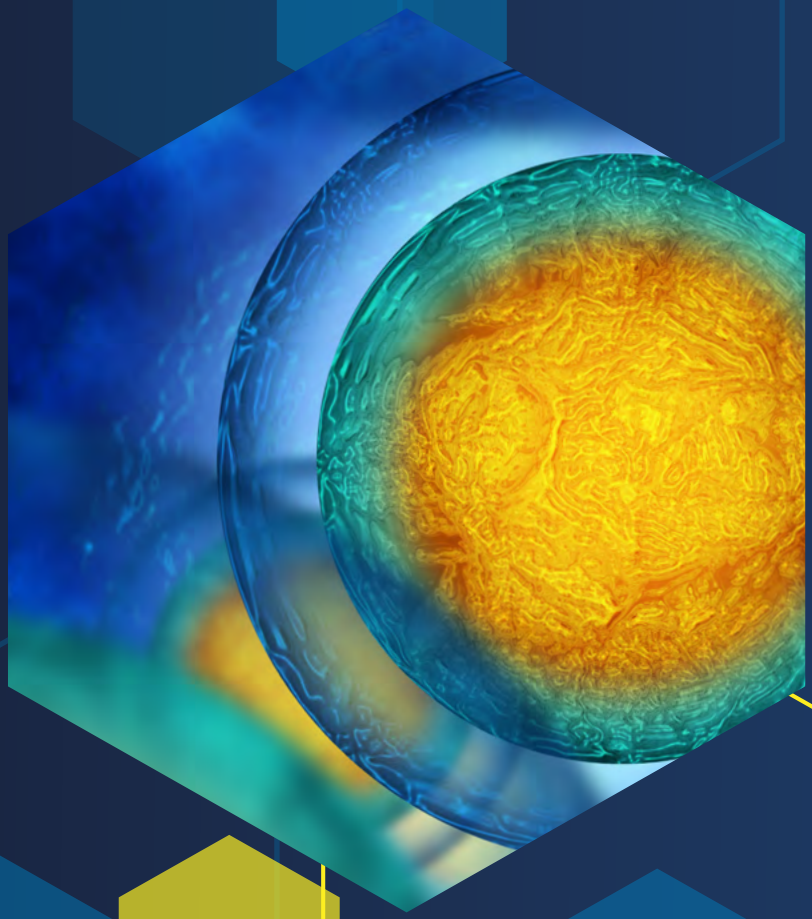

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Application Note

Automated High-Throughput Analytical-Scale Monoclonal Antibody Purification Using Production-Scale Protein A Affinity Chromatography Resin

Stephan M. Koza, Caitlin M. Hanna, Albert H. W. Jiang, Ying Qing Yu

Waters Corporation

Abstract

An automated analytical-scale Protein A affinity based purification of monoclonal antibody (mAb) from Chinese hamster ovarian (CHO) cell conditioned media is described. This cost effective procedure was demonstrated to purify between 120 µg to 240 µg of mAb using the analyst's choice of process-scale Protein A resin, a 96-well 0.2 µm filtration plate, and an orbital plate shaker. While the multiple pipetting steps (12 steps/sample) of the purification method can be performed manually, the demands on the analyst's time, tediousness of the purification, and potential for errors, are greatly reduced when using the Andrew+ robotic platform, taking approximately 1 hr for 48 samples. In addition to deployment of a developed and vetted purification protocol, the intuitive OneLab visual programming interface for the Andrew+ robot can facilitate the optimization and evaluation of this and similar plate-based procedures.

The finalized procedure yields a purified and neutralized mAb sample with a concentration of 1.02 µg/µL or greater when loading 120 µg (≥85% recovery in 100 µL) and 2.27 µg/µL when loading 240 µg (≥94% recovery in 100 µL). An assessment of this procedure as a sample pretreatment for size variant analysis (size-exclusion chromatography, SEC) and released N-glycan profiling using LC-MS is also presented.

Benefits

- High throughput, automated analytical-scale (120 µg to 240 µg) Protein A affinity batch purification of mAb from cell culture with high sample recovery (≥85%)
- Use of the analyst's choice of process-scale Protein A or other affinity chromatography resin (Protein L or G)
- Preparation time of approximately 1 hr for 48 samples
- Effective removal of host cell proteins and other interferences for SEC and released N-Glycan analysis

Introduction

The high-throughput analytical-scale purification of therapeutic recombinant monoclonal antibodies (mAb) from cell culture samples can be essential to support the development of their cell culture processes.[1] Reliable and reproducible purification can add to the success of analytical methods such as size-exclusion chromatography (SEC), released N-glycan analysis, peptide mapping, and other methods where the conditioned media components, host-cell proteins, and nucleic acids may interfere with sample preparation or the analysis.

Numerous approaches and formats for the analytical-scale purification of mAbs have been developed in the preceding decades using Protein A affinity capture. The primary goals of this Protein A purification study was to develop an automated method that minimizes mAb mass requirements and maximizes recovery along with final mAb concentration, all while using the analyst's choice of process-scale Protein A resin.

Experimental

Preparations of mAb (trastuzumab) were obtained from various sources and diluted into phosphate buffered saline (PBS) or non-transfected conditioned (14-day) CHO cell media (NTM) to indicated concentrations. NTM was prepared with the assistance of Syd Labs, Inc. using non-transfected CHO-K1 cells in a spinner flask. Spent media was collected from the flask on days 2 through 15 (~90% average cell viability), pooled, and 0.2 µm filtered.

Protein A

Robotic system:	Andrew+ Pipeting Robot with Extraction+ Module
Filter plate:	Pall™ AcroPrep™ Advance 96-well Filter Plates - 350 µL, 0.2 µm Supor™ membrane (Product ID: 8019)
Collection plate:	Waters QuanRecovery™ 700 µL 96-well plate (p/n: 186009184)
Protein A Resin:	Cytiva MabSelect™ (p/n: 17519901), slurries are ~25% (1:1, PBS:50%). for 50% resin, centrifuge at 1000 g for 3 minutes and replace supernatant with volume of 400 mM NaCl, 20% ethanol equal to resin volume.
PBS:	phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4
NB:	neutralization buffer: 1M Tris, pH 7.5
EB:	elution buffer: 100 mM glycine, pH 3.0
Orbital shaker:	Eppendorf ThermoMixer® C (8 °C)
Software:	OneLab (Andew Alliance/Waters)

SEC

LC system:	ACQUITY Premier UPLC with Binary Management (BSM or QSM) with CH-A column heater or a BioAccordTM LC-MS (ESI-ToF) system

Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 280 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Column(s):	ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm, Column plus mAb Size Variant Standard (p/n: 176004783)
Column temp.:	25 ˚C
Sample temp.:	6 ˚C
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Mobile phase:	ammonium acetate, LC-MS grade (Supelco LiChropur™, eluent additive for LC-MS, 73594), 0.1 µm sterile filtered, 200 mM or as indicated
Chromatography software:	Empower™ 3 (FR 4)

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS

Sample collection:	Waters QuanRecovery™ 700 µL 96-well plate p/n: 186009184
Column:	ACQUITY UPLC Glycan BEH™ Amide Column p/n: 186004742 (1.7 µm, 2.1 mm x 150 mm, 130 Å)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	15 µL
Mobile phase A:	50 mM Ammonium Formate, pH 4.4 (LC-MS grade, p/n: 186007081)
Mobile phase B:	Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.0	25	75	Initial
3.50	1.0	42	58	6
3.55	1.0	60	40	6
3.75	1.0	60	40	6
3.80	1.0	25	75	6
5.00	1.0	25	75	6

ACQUITY RDa Detector Settings

Mass range:	400–7000 *m/z*
Mode:	ESI+
Sample rate:	10 Hz
Cone voltage:	45
Desolvation temperature:	300
Capillary voltage:	1.50 kV
Informatics:	Accurate Mass Screening using a glycan database

Data Management

Chromatography software:	waters_connect

Results and Discussion

Method Development

An automated mAb purification method was successfully adapted from previously described filter plate based approaches for Protein A affinity and other modes of chromatography.[1–3] An outline of the basic protocol used is shown in Figure 1. This relatively simple procedure requires 12 pipetting steps and four incubation steps per sample. In brief, the optimization of the presented automated mAb Protein A purification included evaluations of binding and elution mechanics, volumes, and times, in addition to elution buffer pH.

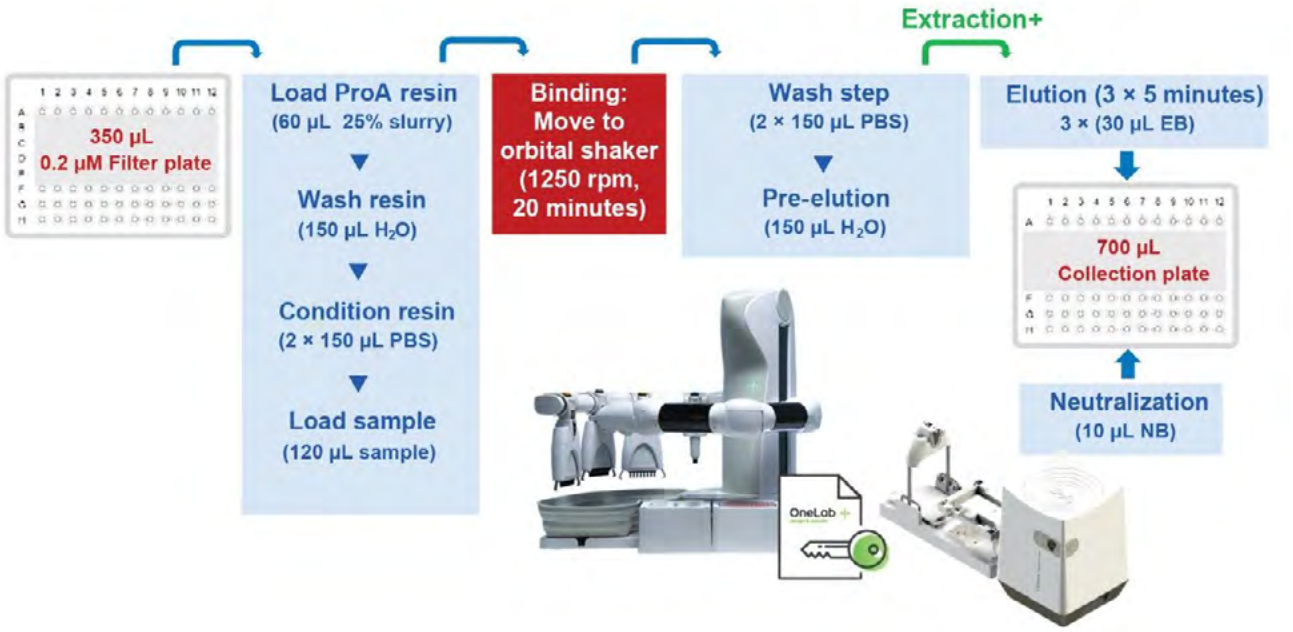

Figure 1. Shown is a diagram of the automated analytical-scale Protein A affinity-based purification of monoclonal antibody from CHO cell conditioned media using the Andrew+ robotic platform with the Extraction+ device and the OneLab visual programming interface. Elution buffer (EB) and neutralization buffer (NB) are defined in the experimental section.

It is critical that the Protein A resin is suspended during the loading step for effective binding. To accomplish this, the use of an orbital plate mixer (1250 RPM for 20 minutes, at 8 °C) produced greater mAb recoveries than repetitive pipet aspiration and dispensing (up to 2 minutes per loading step). When using the current Andrew+ robotic platform, transfer of the filter plate to and from an orbital shaker for the 20-minute binding step is the only action that the analyst needs to perform.

Elution buffer (glycine) pH is also a key factor in mAb recovery from Protein A. Here, we observed that while at pH values below 3.0 an increase in mAb recovery was observed, there was also a concomitant increase in artifactual multimeric aggregation (high molecular weight species, HMWS). As a result, in the final method 100 mM glycine, pH 3.0 was used for elution which could be effectively neutralized with 1.0 M TRIS, pH 7.5 added at a 1:9 ratio to the eluted sample. The volumes of buffer delivered for the equilibration, wash, and elution steps were also optimized. This process was greatly facilitated by the ease with which multiple volumes for a pipetting step can be programmed within the graphical OneLab interface.

Method Evaluation

At a targeted sample titer of 1.0 µg/µL and load volume of 120 µL, the goal for this purification procedure of producing 100 µL of purified mAb sample at a concentration of 1.0 µg/µL or greater was achieved in day-to-day replicate experiments (Experiments B & C, Figure 2). The recoveries for these samples were 87%. At a lower titer of 0.5 µg/µL, and with a 120 µL load, we observed only 70% recovery, however when two separate 120 µL loadings (20 minutes each) of the 0.5 µg/µL sample were performed the recovery increased to 85% and the final concentration of the purified mAb sample was 1.02 µg/µL (Experiments A and D, Figure 2). The greater percentage of mAb lost for the lower loads indicates that unspecific losses due to the filter plate or Protein A resin are likely occurring. Phase ratios ($Volume_{sample}/Volume_{resin}$) of 8 or 4 were used for these experiments as indicated and a previously described high-throughput SEC method was used to monitor the results of the Protein A purifications.[4]

The proposed method was also shown to be capable of purifying 240 µg of mAb on 15 µL of Protein A resin (Experiment E, Figure 2). Two experiments were conducted. One in which two 120 µL load steps at 1.0 µg/µL (20 minutes each, phase ratio=8) were performed, and a second in which the amount of Protein A resin and the concentration of the mAb was doubled (Experiments E and F, Figure 2). For the latter experiment the phase ratio is reduced two-fold to 4. Experiments doubling the volume of the sample load could not be accommodated by the 350 µL filter plate being used when placed on the orbital shaker. Both experiments resulted in recoveries of 95% or more, however, the purified sample is approximately two-fold higher in concentration when loading 240 µg of mAb on 15 µL of Protein A resin. This further demonstrates the utility of multiple binding steps in the event that the mAb titer of the cell culture is significantly lower than the desired concentration of the purified sample. Although not executed in this study, these data suggest that up to 480 µg mAb could be purified when using a higher sample concentration, or more binding steps, and 30 µL of resin per well.

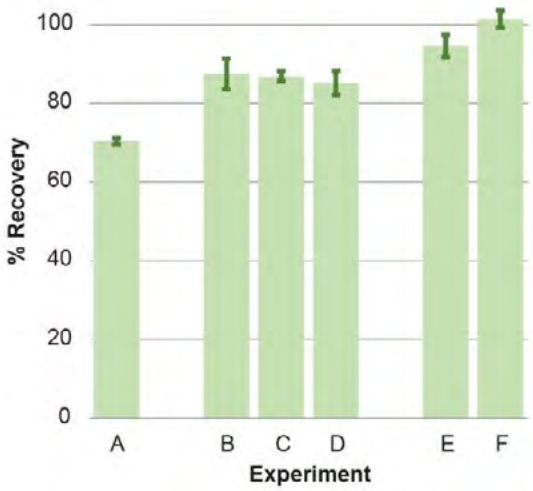

Chart ID	A	B	C	D	E	F
Initial mAb conc. (µg/µL)	0.50	1.00	1.00	0.50	1.00	2.00
mAb Volume (µL)	120	120	120	2 × 120	2 × 120	120
mAb Load (µg)	60	120	120	120	240	240
ProA media volume (µL of 25% slurry)	60	60	60	60	60	120
Phase ratio	8	8	8	8	8	4
Elution volume (3 X µL)	30	30	30	30	30	60
Neutralization volume (µL)	10	10	10	10	10	20
% Recovery	70.4	87.5	86.9	85.2	94.6	101.4
Purified mAb conc (µg/µL)	0.42	1.05	1.03	1.02	2.27	1.22

Figure 2. Loading study experiments and recoveries are presented. For sample loads of 120 µg or more 85% recovery or higher was observed. Details are provided in text. Error bars represent the range of values obtained (n=2). These data were collected on an ACQUITY Premier BSM UPLC.

In an expanded reproducibility study, the targeted mAb purification (120 µL at 1.0 µg/µL) achieved recoveries of 90% or greater. For this evaluation, the mAb sample was diluted with PBS to 1.0 µg/µL and a second sample was prepared by spiking mAb into NTM to the same concentration. Eight replicates of both were assessed based on total SEC peak areas (280 nm detection). Both samples resulted in comparable high sample recoveries with the PBS samples having a recovery of 94.4 ± 5.8% and the NTM samples having a recovery of 90.8 ± 5.4% (95% CI) with both resulting in a purified mAb concentration of greater than 1.09 µg/µL.

In addition to reliable recovery, the Protein A method also provided a relatively effective purification for mAb samples to be further analyzed for native size variants and released N-glycans. Due to a low average cell viability (90%) the NTM exhibited significant levels of components (host cell protein, DNA, *etc.*) that could interfere with both of these analyses as observed in the SEC chromatograms presented in Figure 3.

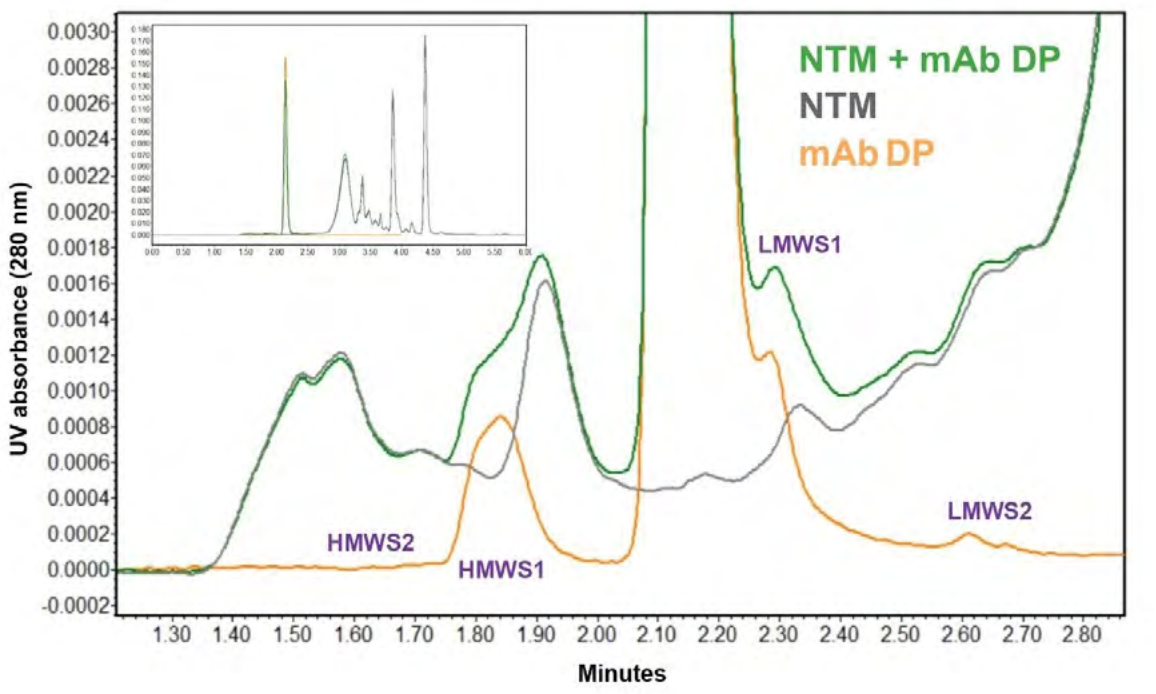

Figure 3. Shown are full-scale and zoomed views of SEC chromatograms of non-transfected CHO cell media both neat (NTM, gray) and spiked with mAb drug product (NTM+DP, green). A 1 µg/µL dilution of drug product (DP, orange) diluted in PBS is also shown. Additional details provided in the text. These data were collected on an ACQUITY Premier BSM UPLC.

SEC was used to evaluate the relative abundances of HMWS and low molecular weight size variants (LMWS) for the Protein A purified samples as compared to the original sample as a control (Figure 4). When comparing to the chromatograms presented in Figure 3, a significant removal of interfering components is achieved. However, it is noted that the Protein A purification procedure alters the mAb HMWS levels. Trace level amounts (<0.05%) of multimeric HMWS2 are artifactually generated while dimeric HMWS1 is partially recovered. Absolute HMWS1 size variant recoveries were estimated as 68% for the spiked NTM sample and 59% for the spiked PBS sample, assuming that additional HMWS1 forms were not also generated by the protein A purification process. Challenges with the quantitative recovery of HMWS mAb variants when using Protein A affinity chromatography purification, even when deploying a more precise LC-based methodology, have been previously reported.[6] Despite this bias, the Protein A method presented may still be able to provide valuable information with respect to the level of HMWS in conditioned media samples. Although out of the scope of this study, further optimization of the purification method may also increase the accuracy of the size variant assessment.

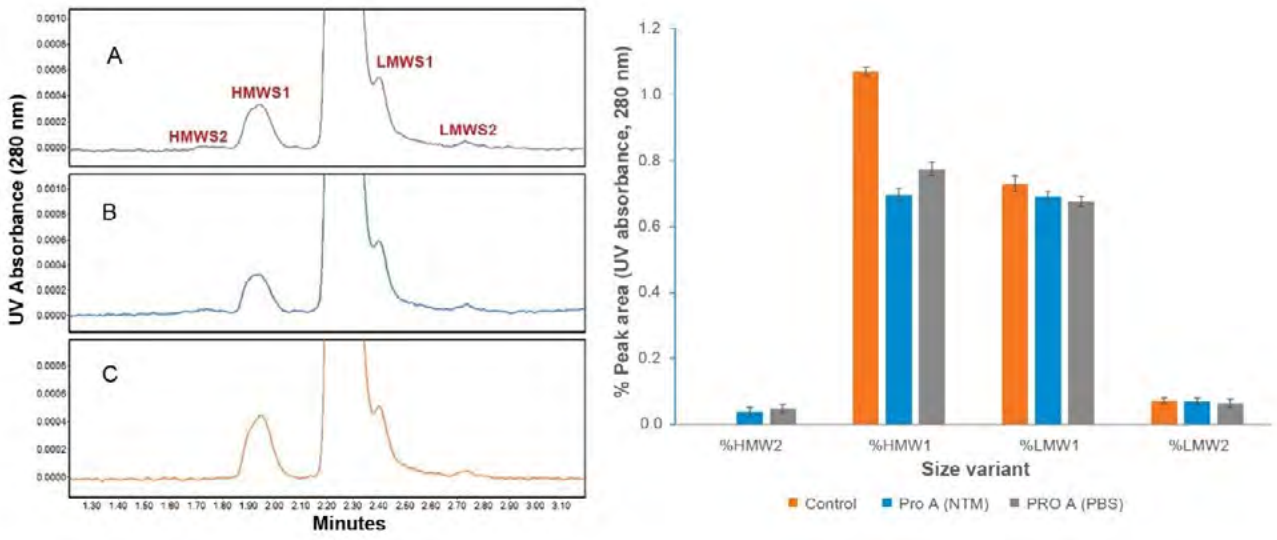

Figure 4. Shown is an evaluation the SEC-UV quantitative results for protein A purified mAb spiked into A; PBS (n=8, gray) and B; non-transfected CHO cell conditioned media (NTM, n=8, blue) are compared to C; a spike control (n=3, orange). Chromatographic conditions are provided in the text. Error bars represent the uncertainty (95% CI) of values obtained. These data were collected on an ACQUITY Premier BSM UPLC.

The effectiveness of the Protein A purification was also effective in conjunction with released N-glycan analysis. Over 6000 proteins and glycoproteins have been identified as part of the CHO cell proteome, many of which are not entirely removed even during the Protein A purification of mAb.[7] To address the utility of the proposed mAb purification for N-glycan analysis a comparison was made between mAb samples that were Protein A purified from NTM and PBS (Figure 5). These results were generated using a high-throughput LC-MS method with ESI-ToF detection as previously reported.[7] Consistent with the extent of overall purification observed by SEC, comparable results were observed for the major mAb glycoforms. Also noted were three trace level glycoforms, that may be of interest for mAb product development as they can impact product safety (FA2BG1) or efficacy (FA2G2S1 and M5). Of these, the only significant change observed was a measurable increase in the relative abundance of the high-mannose glycan (M5) from 0.38% to 0.58% for the mAb purified from NTM. This increase is likely due to low abundance co-purified HCP, and although not pursued in this work modifications to the volumes and solutions used in the Protein A washing step (Figure 1) may further improve HCP removal.

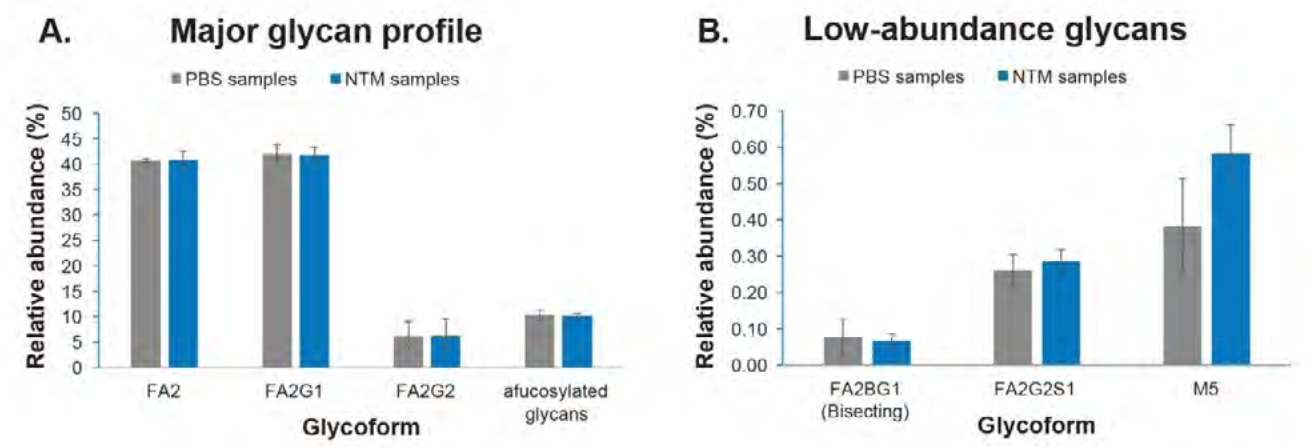

Figure 5. Comparison of UPLC-MS N-glycan profiles of mAb purified from PBS (gray) and NTM (blue). Error bars show the 95% confidence interval (n=4). The method enables comparison of N-glycan profiles between multiple sample types for high- and low-abundance glycans.

Conclusion

Taken together, these data demonstrate that the Andrew+ robotic platform can be effectively adapted to perform a filter-plate based Protein A affinity purification of 120 µg to 240 µg quantities of mAb from clarified cell culture samples with high recovery (>90%). Amounts of mAb as low as 60 µg can also be purified with lower recovery (70%). The method has a predicted upper purification limit of at least 480 µg when using MabSelect resin, however, this value may vary depending on the binding capacity of the manufacturing scale Protein A resin selected. The method produces a 0.2 µm filtered sample that can be concentrated up to 2.2 µg/mL depending on sample load.

The automated procedure performs 12 separate pipetting steps per sample and the only user action required is to move the filter plate to and from an orbital shaker for the 20-minute binding step. The method has a preparation time of approximately 1 hr for 48 samples, with 35 minutes attributable to the 20-minute binding step and three hold times of 5 minutes each during mAb elution. And finally, the effective removal of host cell proteins and other SEC and released N-Glycan analysis interferences along with minimal generation of artifactual aggregation was demonstrated.

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720007861, February 2023

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Application Note

Rapid Automated Antibody Purification Using Protein A-Coupled Magnetic Beads With Andrew+

Yamin Htet, Stephan M. Koza, Weibin Chen

Waters Corporation

For research use only. Not for use in diagnostic procedures.

Abstract

Monoclonal antibodies remain one of the rapid growing classes of biopharmaceuticals.[1] High-throughput small-scale analytical platforms are increasingly sought after within general research and to aid in the development of biopharmaceuticals when using miniaturized bioreactors.[1] In this regard, affinity purification is one of the most

widely employed purification strategies prior to analysis because high purity antibody samples are often required for further analysis.[2] As an easy-to-use separation medium, magnetic beads streamline the purification process without the need to use complex instrumentation.[2]

Currently, in the field of magnetic separation, many existing automated protocols still require several manual intervention steps.[3] A fully automated antibody purification protocol will increase throughput, save time, and increase the efficiency of analytical workflow in biotherapeutics.

Here, we present a rapid and fully automated antibody purification protocol that can isolate target antibody from cell culture media using Andrew+ pipetting robot. The protocol can be easily adjusted to accommodate samples with either high or low titer to yield purified antibodies at the desired concentration. In addition, samples of low titer can be concentrated to produce purified antibody at a higher concentration.

The rapid protocol is fully automated, reproducible, robust, and is highly adaptable for further analysis.

Benefits

- Fully automated Protein A magnetic beads purification protocol with Andrew+
- Rapid purification protocol (35 min for eight samples)
- Greater than 75% recovery for antibody loads of 20 µg to 180 µg and media sample titers ranging from 0.2 µg/µL to 1.5 µg/µL

Introduction

During process development, manufacturing, and quality control (QC) of therapeutic antibodies, purification is routinely carried out.[4] Affinity purification using Protein A is a widely used purification strategy due to the selectivity and high binding affinity of Protein A to IgG antibodies.[1] Similarly, magnetic beads are a commonly used medium for purification due to their ease of use.[1] Protein A-coupled magnetic beads combine the benefits of high selectivity of protein A and effective removal of the beads from the purified sample.[1]

Existing automated protocols using the magnetic separation method require several manual intervention steps such as adding magnetic beads to a 96-well plate, transferring the plate, adding buffers to the plates during washing and elution steps, and transferring the purified samples to a fresh plate.[3]

Here, we describe a fully automated antibody purification protocol using the Andrew+ pipetting robot (p/n: 176850100). The robot uses dominos, a microplate grabber (p/n: 186009776), and the connected devices such as 96-PCR Plate Magnet+ (p/n: 186009956), and Microplate Shaker+ (p/n: 186009594) and requires minimal manual intervention. The automation capabilities of Andrew+ allow for the flexibility of processing samples using either a single-channel or 8-channel pipetting depending on the throughput needs.

Experimental

Sample Description

Conditioned cell culture media samples, with CHO cells removed, were provided by a collaborator. Clarified samples were used without further centrifugation. Other reagents and chemicals used were shown in the table below.

Protocol specifications	Materials	Volume
Magnetic beads	Magne® Protein A Beads, Promega Corporation	50 µL
Cell culture media	Conditioned cell culture media with CHO cells removed	Varies
Equilibration buffer	1x Phosphate buffered saline (PBS), pH 7.4	3 x 150 µL
Binding buffer	1x PBS, pH 7.4	Varies
Wash buffers	1x PBS, pH 7.4 Water	2 x 150 µL 2 x 150 µL
Elution buffer	Glycine-HCl, 200 mM, pH 2.5	2 x 50 µL
Neutralization buffer	Tris-HCl, 2.0 M, pH 7.5	20 µL
Sample platform	twin.tec PCR Plate 96, skirted, green, Eppendorf (p/n: 951020443)	200 µL/well

Table 1. Reagents used for antibody purification protocol.

Method Conditions

LC system:	ACQUITY UPLC H-Class Bio System
Detection:	TUV detector 220 nm and 280 nm
Sample collection:	twin.tec PCR Plate 96, skirted, green, Eppendorf, (p/n: 951020443)
Column:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225)
Column temp.:	Off
Sample temp.:	4 °C
Needle placement:	5.0 mm (default is 2.0 mm)
Injection volume:	2 µL
Flow rate:	0.4 mL/min
Run time:	7 min
Mobile phase:	100 mM ammonium acetate, pH 5.25 (acetic acid)
Gradient:	Isocratic

Data Management

Chromatography software:	Empower 3

Instrumentation

To prevent a magnetic bead from accidentally getting into the LC system, two accomodations were put in place in the procedure. A magnet (96-Well Deep Bar Magnet Plate, Permagen, p/n: MSPU650) was placed under the 96-well PCR plate in the sample manager to fully capture any rogue beads in the final sample. The modified plate parameters for LC are shown in Figure 1. The needle placement in the LC method was changed to 5.0 mm from the default value of 2.0 mm to accommodate the change.

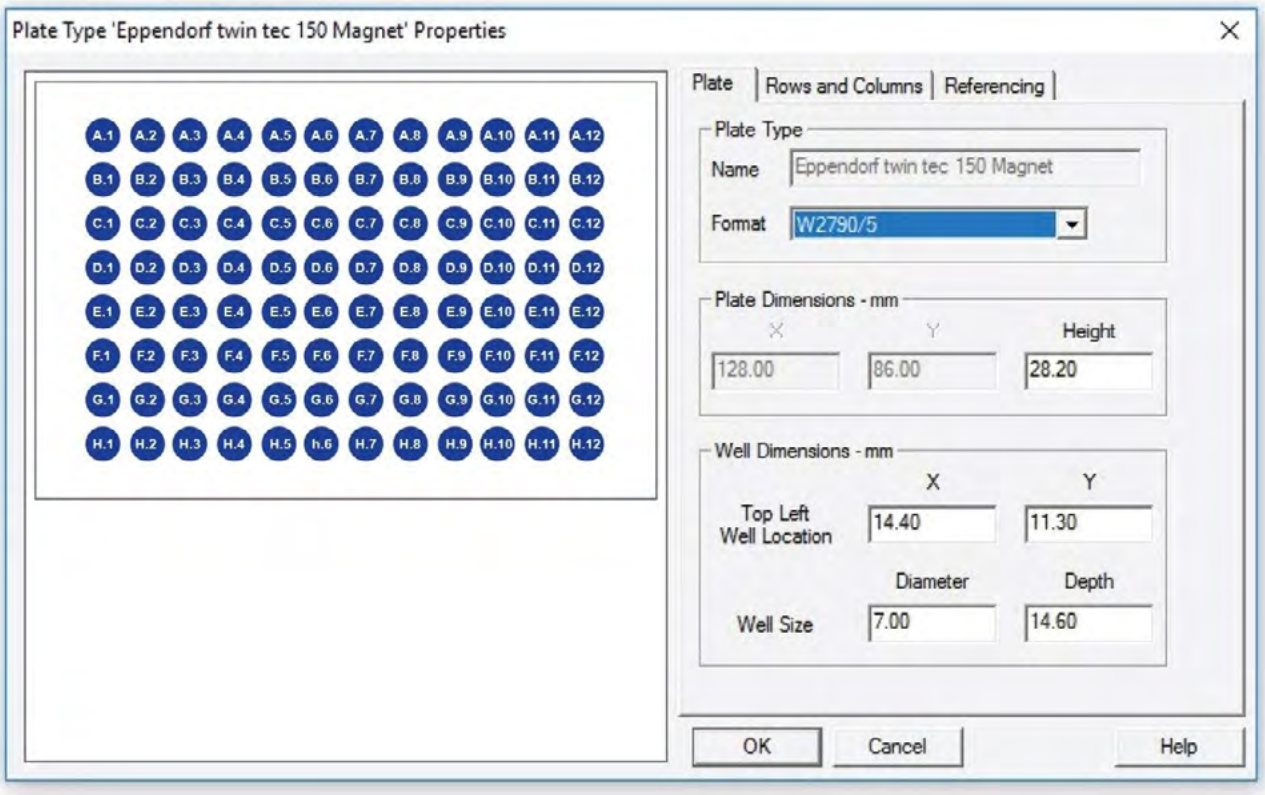


Figure 1. Plate parameters used in Empower to accommodate the 96-well PCR plate with a bar magnet underneath.

Results and Discussion

The general principle behind the antibody purification with the magnetic beads is illustrated in Figure 2.[5] Briefly, samples are first incubated with protein A-coupled magnetic beads in a binding buffer for a certain amount of time with gentle mixing. Then, impurities are washed away, and bound antibodies are eluted from the beads using an acidic elution buffer.

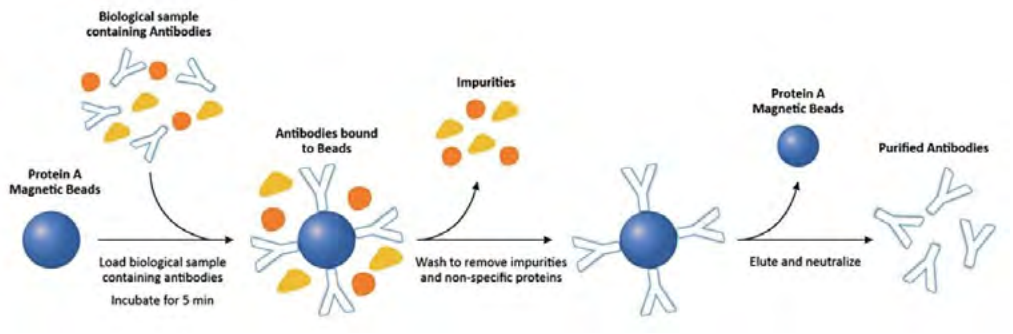


Figure 2. General protocol for antibody purification using Protein A-coupled magnetic beads.

Rapid automated antibody purification protocol is essential to the development of biotherapeutics

The general protocol is relatively simple yet consists of several equilibration/washing steps, and a manual execution of a routine protocol for multiple samples can be laborious and time consuming. Using our fully automated protocol, users can perform the antibody purification without any manual intervention step, increasing sample throughput. The flow diagram describing the steps performed during the automation experiments are shown in Figure 3. Three procedures are presented here to accommodate the varying titers and loads of antibody (low ≤50 µg, standard ≤75 µg, high >75 µg) in the cell culture media. Low load protocol has additional steps to load the samples multiple times so that more concentrated samples are eluted at the end. If the purified antibody of higher titer sample is not required for further analysis, the standard protocol (load 50 µg, 0.5 mg/mL) can also be used for low titer samples. High load protocol has a longer incubation time of 10 min, instead of the standard incubation time of 5 min. Samples of high titer may also be diluted with the binding buffer (1x PBS, pH 7.4) before loading, if necessary.

Automation capability of Andrew+

In the field of magnetic separation and protein purification, Andrew+ provides a streamlined fully automated protocol. In comparison, other magnetic bead handlers provide a semi-automated protocol that requires one to five manual intervention steps for a similar procedure.[3]

Rapid automated antibody purification protocol improves sample throughput

The rapid automated protocol presented here needs minimal manual interventions during the purification process and the total execution time for eight samples is 35 min. In comparison, the total run time needed to complete the protein purification on other similar device is one hour, 20 min when using the same type of magnetic beads from Promega.[6] Therefore, Andrew+ can effectively streamline the antibody purification with magnetic beads using the connected devices as shown in Figure 4.

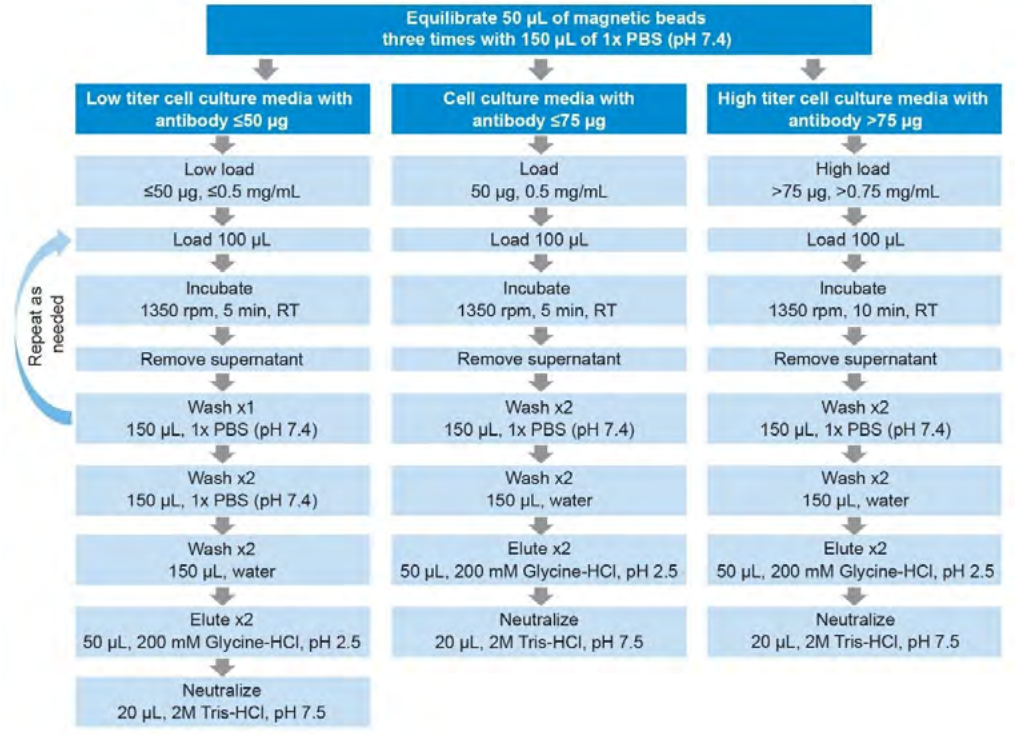
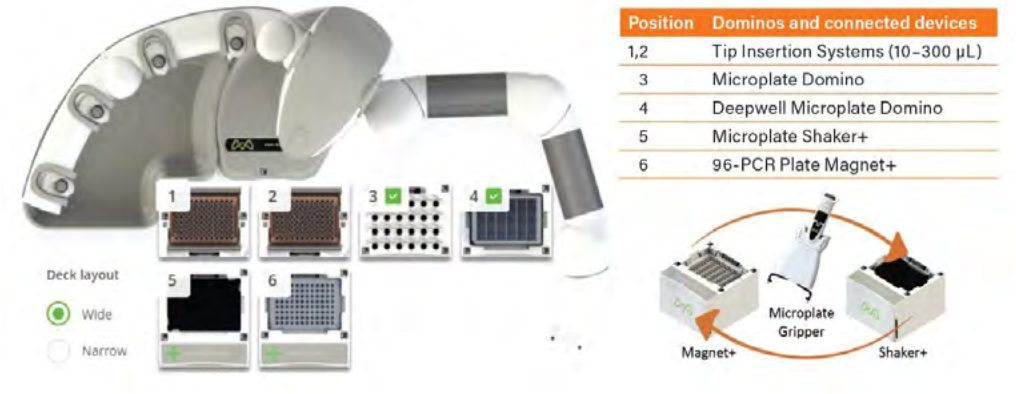


Figure 3. Flow diagram of automated antibody purification procedures. Three procedures were presented here to accommodate varying titers and loads of antibody (low ≤50 µg , standard ≤75 µg, high >75 µg) in cell culture media. RT: room temperature.



Position	Dominos and connected devices
1,2	Tip Insertion Systems (10–300 µL)
3	Microplate Domino
4	Deepwell Microplate Domino
5	Microplate Shaker+
6	96-PCR Plate Magnet+

Figure 4. Andrew+ Dominos and Connected Devices configuration for the rapid automated antibody purification eight-sample protocol. For a fully automated protocol, Andrew+ requires two connected devices (Magnet+ and Shaker+) and a microplate gripper or a microplate grabber. The execution time for eight samples is 35 min.

Automation development of antibody purification protocol

Automation development included the adjustments of pipetting settings, tip position, pipetting technique, and shaker speed to maximize recovery.

Pipetting settings/Handling liquid viscosity

Due to the low viscosity and high volatility of 20% ethanol solution, in which the magnetic beads are suspended, the pipette settings were adjusted for low viscosity mode with faster than normal speed of aspiration and dispensing. This will ensure to dispense a similar number of beads into all the wells. If using a different magnetic bead supplier, these settings may need to be adjusted.

For cell culture media samples, the pipette settings were adjusted to pipette each sample using high viscosity mode to assure slow aspiration and dispensing of the samples without vigorous stirring, which can introduce air bubbles to the protein solution.

Tip position

Regarding the pipette tip position, “With respect to liquid” option was used for all the equilibrating steps so as not to aspirate and discard the magnetic beads before purification. “With respect of bottom” option was used for all other steps to remove all the binding buffer completely before elution.

Pipetting technique/Mixing

Multiple aspirations are used to ensure thorough mixing and distribution of samples. Mixing the samples several times at the source will ensure the equal distribution of magnetic beads. Magnetic beads tend to settle, and the solution need to be mixed right before aspirating into the individual wells. Thus, repetitive mode of pipetting must be avoided. It is recommended to have 0.5–1.5 mL of magnetic beads slurry in the source tube to enable thorough mixing of the slurry. For 0.5–1.5 mL slurry, thorough mixing was obtained by using mixing volume of 300 µL.

Cell culture media samples also need to be mixed before aspiration and thus, a similar pipetting technique (aspirate three times at the source before transfer) was used.

Shaker speed and time

It is imperative that during the binding steps that the beads must remain in suspension for maximum binding, which is accomplished using the Microplate Shaker+ (Figure 4). The optimum speed and time were found to be 1350 rpm and 5 min. Sample shaker speed and time may need to be adjusted, if a different magnetic bead supplier is used. However, it is important that the shaking is not too vigorous, which can result in excessive sample foaming.

Determination of antibody recovery

Antibody recoveries from protein A purifications were determined based on the SEC peak area of the mAb monomer as measured for both the conditioned media and Protein A purified samples. While the co-elution of lower abundance host-cell proteins with the mAb in the SEC separation of the conditioned media is possible, the co-elution was considered to have a minimal effect on the results and would only result in an underestimation of antibody recovery for the Protein A purification. Figure 5 shows the size exclusion chromatograms of media before purification (Figure 5A, green trace), after purification (Figure 5C, red trace), and supernatant (Figure 5B, orange trace). Supernatant consists of the components in the media other than the antibody and it is the solution left over after the antibody is bound to the Protein A on the magnetic beads.

$$\%\text{Recovery} = 100 * (\text{Adjusted Area}_{\text{Purified}} / \text{Area}_{\text{ProteinA Load}})$$

$$\text{Adjusted Area}_{\text{Purified}}^{\#} = \text{Area}_{\text{Purified}} * (\text{Total Volume}_{\text{Purified}})/(\text{Total Volume}_{\text{ProteinA Load}})$$

$^{\#}$The peak area of the eluted protein ($\text{Area}_{\text{Purified}}$) was adjusted as shown in the second equation because the eluted volume of protein ($\text{Total Volume}_{\text{Purified}}$) is 120 µL whereas the loaded volume of protein ($\text{Total Volume}_{\text{ProteinA Load}}$) is only 100 µL.

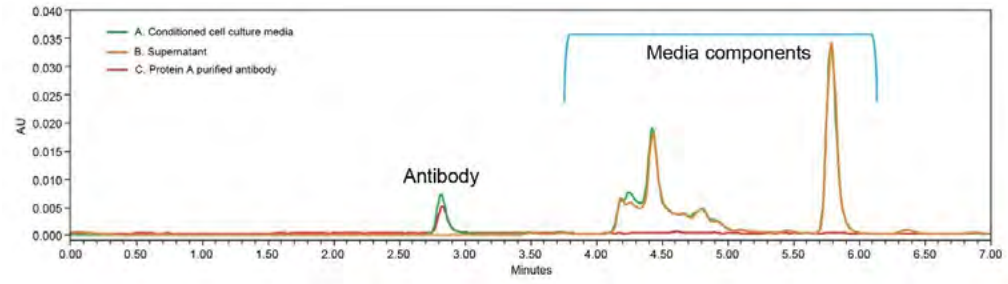


Figure 5. Size exclusion chromatograms of cell culture media samples before purification (A, green trace), after purification (C, red trace), and supernatant (B, orange trace).

Rapid automated antibody purification protocol is reproducible and robust

Using the rapid automated protocol, Andrew+ can achieve a high recovery of antibody with the recovery values generally greater than 75% for samples with a titer of approximately ≤0.75 mg/mL with a reliable reproducibility (%RSD ≤10%). Recovery obtained from the manual execution of the procedure and the automated procedure are comparable as shown in Figure 6.

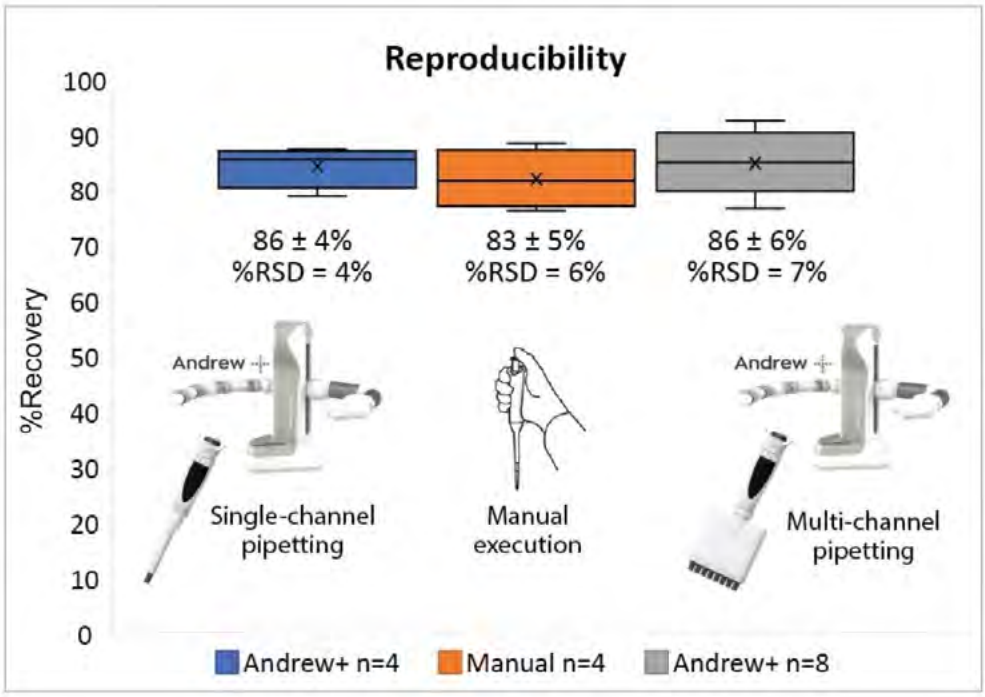


Figure 6. Comparison of the recoveries of antibody obtained from the manual execution (n=4) and the Andrew+ Pipetting Robot (n=4 and n=8). Markers (x) represent the average values.

The robustness of the automated protocol was demonstrated by varying the amount of Protein A magnetic beads used for the capture step (Figure 7A) and the pH of the elution buffer (Figure 7B). The use of magnetic beads requires pipetting the beads from a homogenous slurry. To evaluate the impact on antibody recovery by the number of beads used, the standard amount of the slurry used (50 µL) was varied by ±10%. These experiments with variations still produced satisfactory recovery of antibody (>50%, Figure 7A).

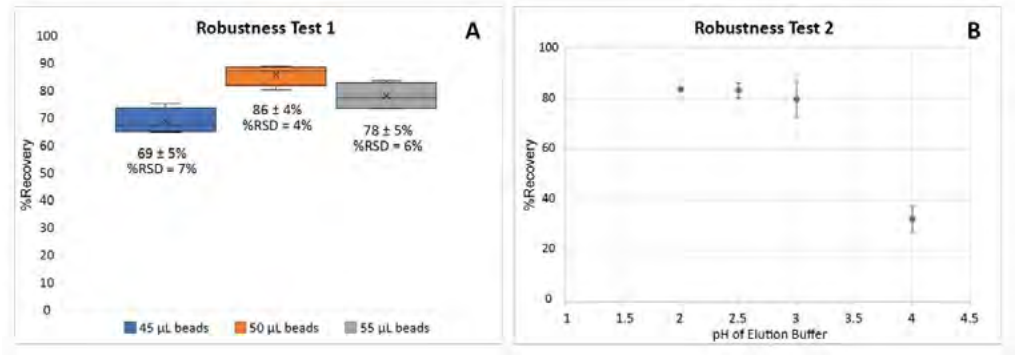


Figure 7. Robustness assessment of the automated protocol. Comparison of the recovery of antibody (A) with three different amounts of beads (45 µL, 50 µL [standard], 55 µL), n=4 and (B) with four different buffered pH levels (pH = 2, 2.5 [standard], 3, 4), n=2. Markers (x) represent the average values.

Another variable that may have a significant impact on antibody recovery is the pH of the elution buffer. Our standard protocol uses Glycine-HCl (0.2 M) at pH 2.5 as the elution buffer. To test the robustness, elution buffers with a pH ranging from 2 to 4 were used for elution (Figure 7B). Recovery was not affected when the pH of the elution buffer was between 2 and 3. However, a low recovery was observed with the elution buffer with pH 4. Therefore, the purification method is robust between pH 2 and 3.

Rapid automated protocol can be used for both low and high titer samples

For samples with low titer (≤50 µg), multiple loadings can be performed to achieve a highly concentrated purified antibody. Three 100 µL loadings (5 min each) of cell culture media with a titer of 0.6 µg/µL (total of 180 µg) with an alternating washing step, yielded a comparable recovery of antibody (80%) to that of single loadings of media samples (Figure 8).

While the standard incubation time of 5 min was adequate for low titer samples, for samples with high titer (>75 µg), a longer incubation time of 10 min was necessary to obtain a high recovery. The purification of samples with a media load of 150 µg resulted in 65% recovery with a 5 min incubation. Prolonging the incubation time from 5 min to 10 min increased the yield from 65% to 87%, as shown in Figure 8. Alternatively, the samples of high titer can also be diluted with the binding buffer prior to loading.

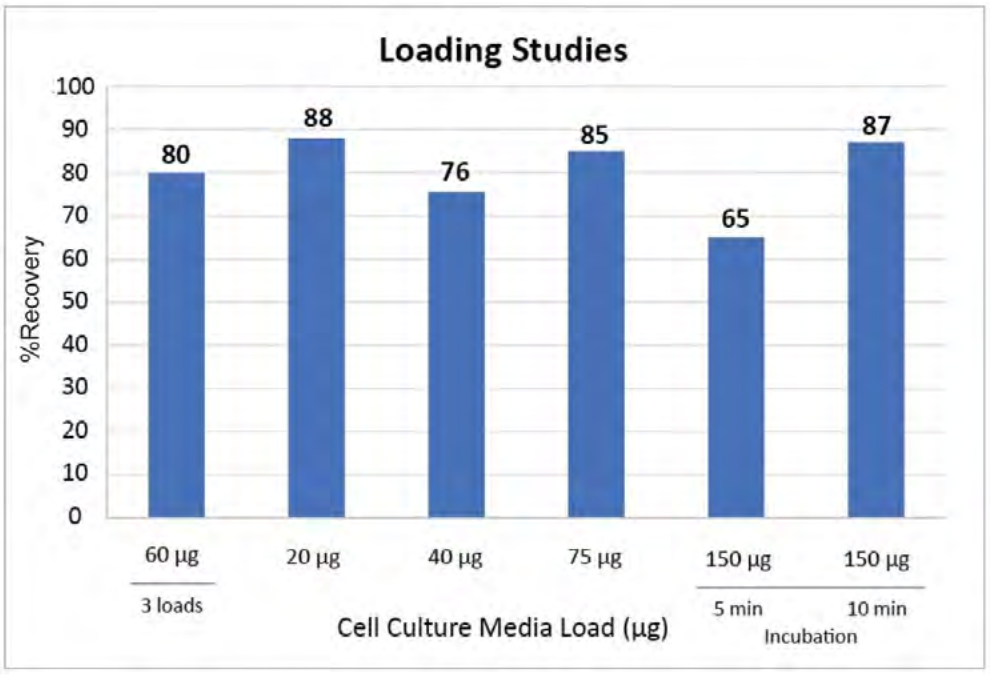


Figure 8. Antibody recovery from cell culture media samples with various loads (20 µg, 40 µg, 75 µg, 150 µg, 180 µg) of antibody. Multiple loadings were obtained by loading 60 µg three times with an intermediate washing step (n=2). Loading volumes were 100 µL. Titers for media samples ranged from 0.2 µg/µL to 1.5 µg/µL.

Conclusion

The rapid antibody purification protocol is reproducible, robust, and can extract antibodies in a high yield (>75% recovery) from cell culture media. The protocol is fully automated, requires no manual intervention, and increases throughput. The protocol can be easily adjusted for both high and low titer samples to yield the purified antibodies at the desired concentration. In addition, low titer samples can be concentrated to obtain pure antibody at a higher concentration.

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Acknowledgments

We thank our collaborators for their generous supply of cell culture media.

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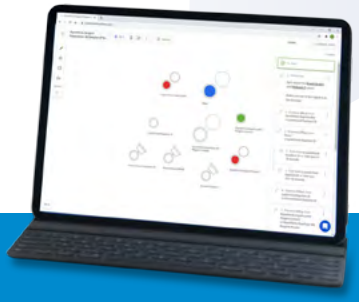

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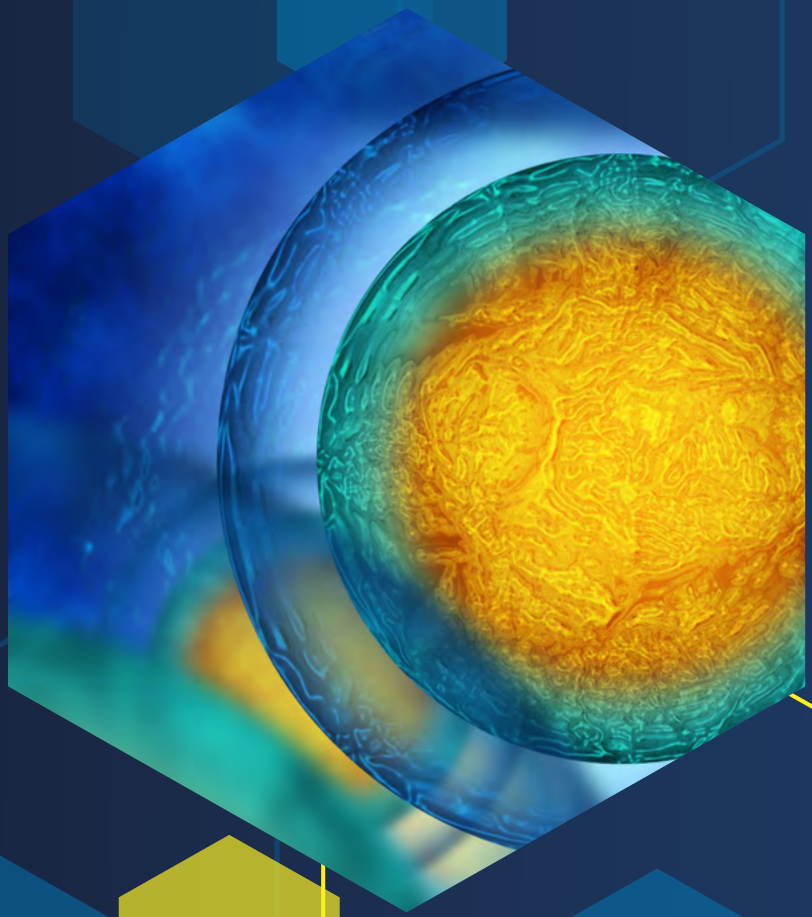

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Application Note

Automating Rapid High-Throughput LC-MS mAb Subunit Screening of Microbioreactor Cell Culture Samples

Alireza Aghayee, Yamin Htet, Stephan M. Koza, Lindsay Morrison, Henry Shion, Ying Qing Yu

Waters Corporation

Abstract

Monoclonal antibodies (mAbs) remain one of the fast-growing classes of biopharmaceuticals and are significantly improving the quality of life for patients all around the world. Discovery and development of successful mAb therapeutics requires sophisticated analytical technologies that can rapidly measure the critical product attributes that have profound impact on the safety and efficacy of these drugs. Therefore, high-throughput analytical platforms for the monitoring of proteins produced at the clone selection stage and during process development have been increasingly in demand.[1]

Due to their production in host cell cultures, large sizes, and heterogenous structures monitoring mAb production raises many analytical challenges. Selection of an appropriate clone typically requires parallel incubation of tens to hundreds of transfected lines, requiring many samples to be analyzed. The optimization of cell culture conditions typically also requires parallel cultures with analytics needed for each culture at least once per experiment. This requires high-throughput methods with fast sample preparation and robust analytical instrumentation, along with facile and straightforward data interpretation. As an integral part of the method, automated sample preparation protocols can increase the sample throughput and improve the efficiency of analytical workflows in biopharmaceutical development.[2]

Here, we present a fully automated workflow for sample preparation and LC-MS analysis of mAbs obtained directly from complex samples such as spent cell culture media including host cell protein. The method includes a mAb purification step using Protein A followed by FabRICATOR® (IdeS) digestion and subsequent DTT reduction to yield mAb subunits suitable for high-throughput LC-MS analysis using a Waters™ BioAccord™ LC-MS System.

Benefits

- Rapid mAb subunit analysis with fully automated Protein A and Ides digestion protocol with Andrew+™ Pipetting Robot
- High-Throughput LC-MS Analysis using BioAccord™ LC-MS System with waters_connect™ Informatics Solution/INTACT Mass™ App[3]
- Reduced subunits analyzed by reversed-phase UPLC™ using the BioResolve™ RP mAb Polyphenyl column for optimal peak shape and resolution with this solid core particle with a 450 Å pore size

Introduction

The development and manufacturing of therapeutic mAbs require close monitoring of multiple product quality attributes. N-glycosylation, oxidation, C-terminal lysine clipping, and glycation are some of the product quality attributes evaluated. Numerous samples are often required to be analyzed during clone selection and throughout process development to ensure a successful final product. This may create a bottleneck which can either slow down the development of new therapeutics or limit the extent of process optimization. Ultimately rapid LC-MS methods along with the automation of mundane sample preparation and sample analysis can help to relieve this bottleneck. We have already demonstrated that the analysis of mAbs at the intact level can be fast and be performed with minimum sample preparation.[4] However, intact mAbs analysis can limit the protein attributes that can be tracked without the use of high-resolution LC-MS instrumentation.

Rapid and high-throughput screening of mAbs in the form of subunits can potentially enhance development and improve overall quality of biopharmaceutical products, while reducing manufacturing costs. In this work, mAb

subunits (23–25 kDa in size) are generated using FabRICATOR (IdeS) protease to cleave antibody at a specific site below the hinge followed by DTT reduction of the disulfide bonds between the light chain and the heavy chain. Subunits are more homogeneous than the intact mAb and of low enough mass to allow for the acquisition of adequate-quality spectra with more moderately priced MS instruments while still providing considerably short analysis time and simplified data interpretation. Here, we present a fully automated workflow for purification and digestion of therapeutic mAbs harvested directly from cell culture media. This method is based on purification using magnetic protein A beads followed by a combined digestion/reduction. An Andrew+ Pipetting Robot was used to automate the entire sample preparation process which yielded subunits ready for analysis by Waters BioAccord LC-MS System. In order to develop a procedure more amenable for use with micro-bioreactors small volumes (20 µL to 100 µL) of the mAb media samples were evaluated for this study either directly (using unpurified media) or as Protein A purified samples for intact, subunit level analyses.[5]

Sample Description

Non-transfected Chinese hamster ovary (CHO) cells culture media was commissioned from Syd Labs, Inc.. Briefly, 6 x 10^6 CHO-K1 cells/mL per were seeded in flask on day 1 and were incubated in 120 mL culture media. On day 2, 100 mL of spent media was collected from flask and was 0.2 µm filtered. This was repeated every day to day 15. All collected media was pooled and stored at 4 °C. Cell viability and numbers were recorded accordingly throughout cell culture process (Figure 1a). Then, trastuzumab (T-mab) was added to collected cell culture to prepare samples at 0.5 µg/µL to create a mock media sample of known concentration (Figure 1b–c). The lower cell viability aliquots observed in the later days of this cell culture were included in the mock sample to provide a greater challenge for the sample purification step.

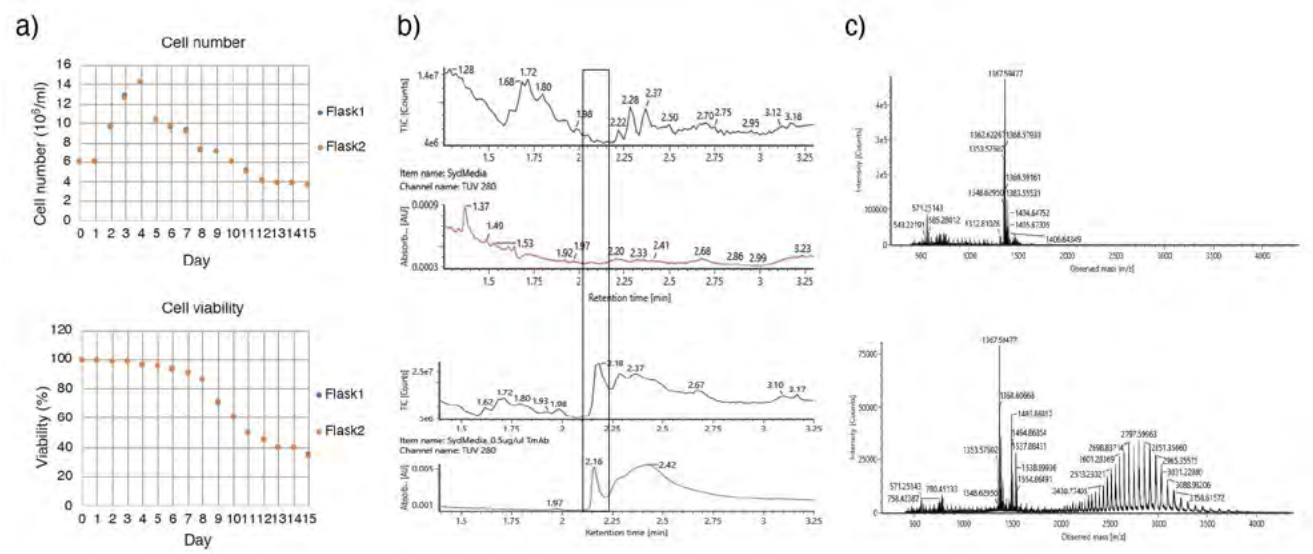


Figure 1. a) Cell count, and viability of non-transfected CHO cells cultured for 15 days. b–c) Direct LC-MS analysis (b: TIC traces, c: MS spectra) of CHO cell culture media (top) and after spiked addition of trastuzumab (bottom).

Sample Preparation

Using an Andrew+ Pipetting Robot, a fully automated workflow was developed for sample preparation. Purification of the mAb from cell culture media was performed by incubating 100 µL of sample (with the indicated concentration) with Magne® Protein A Beads (Promega, 50 µL slurry per sample) followed by capturing of the magnetic beads on the Andrew+ Robot in a 96-well plate format using the Magnet+ device. After washing, purified mAb was eluted in 50 µL Glycine-HCl (2M, pH 2.5) two times (100 µL total) and were added to 60 µL neutralization buffer comprised of MES (100 mM) and Tris-HCl (900 mM) at pH 7.5.

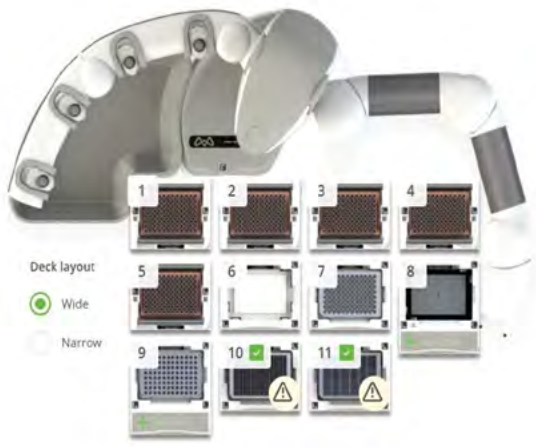
Afterwards, an aliquot of 20 µL purified mAb (with <0.5 µg/µL concentration) were digested and the disulfide bonds reduced to yield three mAb subunits (Fc/2, light chain and Fd') by adding 10 µL FabRICATOR (Ides) at 2 units/µL and 30 µL dithiothreitol (DTT) at 40 mM to each sample and incubating for 60 minutes at 37 °C. The detailed protocols for the Andrew+ robot can be downloaded from the OneLab Library (onelab.andrewalliance.com). All reagents and chemicals used were shown in the table below (Table 1).

Protocol specification	Materials	Volume
Cell culture media	Filtered CHO cells culture spent media	100 µL/well
Magnetic beads	Promega Magne™ protein A magnetic affinity beads	50 µL/well
Equilibration buffer	1x Phosphate buffer saline (PBS), pH 7.4	3 × 150 µL/well
Binding buffer	1x PBS pH 7.4	3 × 150 µL/well
Wash buffers	1x PBS pH 7.4 and water	3 × 150 µL/well
Elution buffer	Glycine-HCl, 200 mM, pH 2.5	2 × 50 µL/well
Neutralization buffer	MES 100 mM and Tris-HCl 900 mM pH 7.5	60 µL/well
Fabricator	Ides 2 unites/µL in water	15 µL/well
DTT in buffer	DTT in Guanidine-HCl 6 M and Tris-HCl 200 mM	35 µL/well
Sample platform	twin.tec PCR Plate 96, skirted, green, Eppendorf	200 µL/well

Table 1. List of all reagents and materials used for mAbs purification and digestion.

Andrew+ Automation

Andrew+ Pipetting Robot can provide a streamlined fully automated protocol for protein purification and digestion, as shown in (Figure 2). In comparison, other automated liquid handlers might provide a semi-automated protocol that requires one to five manual intervention steps for a similar procedure.[5]



Position	Dominos and connected devices
1,2,3,4,5	Tip insertion systems (10–300 µL)
6,7	Microplate domino
8	Plate heater-shaker+
9	96-PCR plate magnet+
10, 11	Deepwell microplate

Figure 2. Andrew+ Domino and connected device configuration for automated Protein A purification and subunit analysis of up to 48 samples.

LC-MS Analysis

The LC-MS analysis of the mAb subunits was performed using a 4.5 minute reversed-phase LC-MS method with 0.1% formic acid and acetonitrile mobile phase on a Waters BioAccord System according to the parameters in Table 2. All data was acquired and processed using UNIFI v2.1.2.14.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Detection:	TUV Detector
Sample collection:	twin.tec PCR Plate 96, skirted, green, Eppendorf, p/n: 951020443
Column:	BioResolve RP mAb Polyphenyl Column 450 Å, 2.7 µm, 2.1 mm x 100 mm p/n: 176004157
Column temp.:	80 °C
Sample temp.:	10 °C
Injection volume:	3 µL
Flow rate:	0.4 mL/min
Run time:	4.5 minutes
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile

Gradient Table

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
0	0.4	80	20	Initial
2	0.4	59	41	6
2.2	0.4	15	85	6
2.3	0.4	15	85	6
2.5	0.4	80	20	6
3.5	0.4	59	41	6
3.6	0.4	15	85	6
3.65	0.4	15	85	6
3.8	0.4	80	20	6
4.5	0.4	80	20	6

MS Conditions

MS system:	ACQUITY RDa™
Ionization mode:	ESI+
Acquisition range:	50–2000 *m/z*
Capillary voltage:	1.50 kV
Scan rate:	2 Hz
Cone voltage:	30 V
Lock-mass:	waters_connect Lockmass Kit (p/n: 186009298)

Data Management

Data acquisition and processing software:	waters_connect with INTACT Mass App

Results and Discussion

Fully automated sample preparation using low volumes (20–100 µL) of cell culture media for purification and digestion were performed in a 96-well plate format by the Andrew+ Pipetting Robot. Affinity purification of mAb from crude samples such as cell culture is a standard procedure during the process development and manufacturing of therapeutic antibodies. This is usually performed using an affinity ligand such as Protein A, which binds specifically to IgG. Protein A purification can be a tedious process as it involves numerous equilibrations and washing steps and uses a significant amount of an analyst's time (Figure 3a). At the intact level, both unpurified and purified samples generated comparable MS results. However, the Protein A purified samples additionally resulted in higher quality chromatograms that were very similar to those of the intact mAb in formulation buffer (Figure 3b).

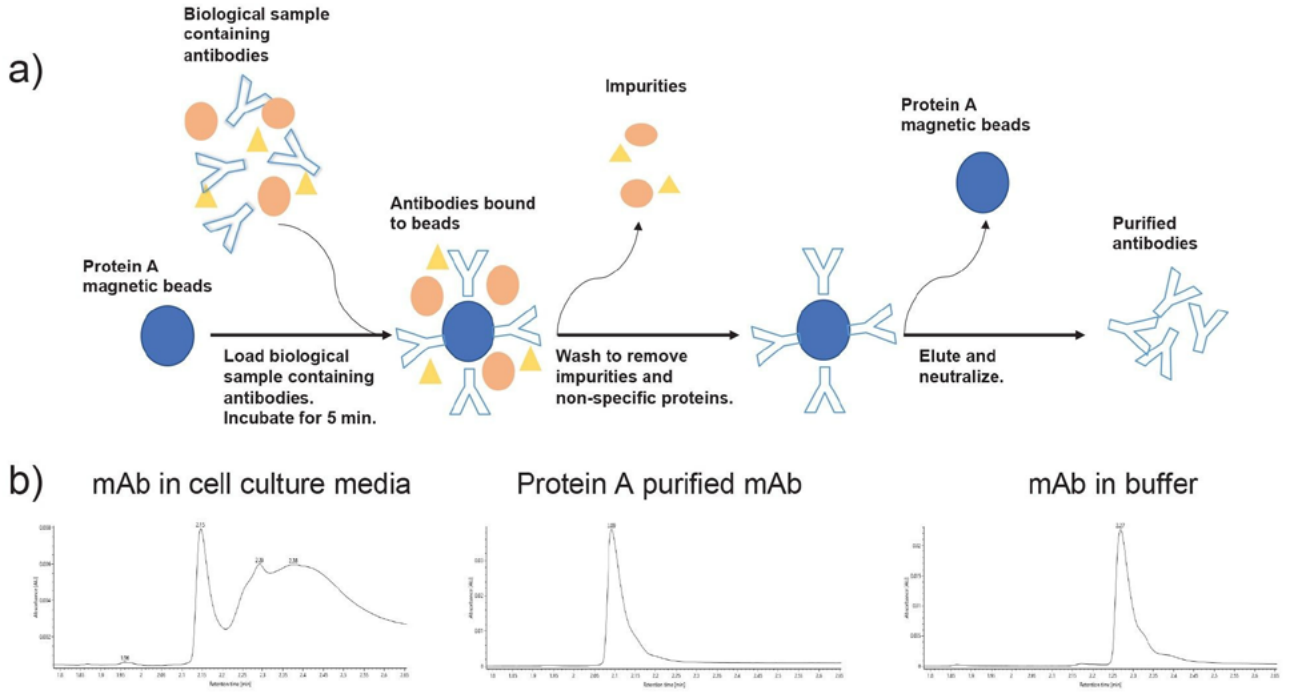


Figure 3. a) Schematic of antibody purification using Protein A-coupled magnetic beads. b) Chromatograms of mAb in cell culture media, after Protein A purification and mAb In buffer.

A highly reproducible, scalable, and fully automated 96-well plate protocol was designed to be used with cell culture samples using the Andrew+ Pipetting Robot. Samples were incubated with magnetic Protein A beads on the Shaker+ device followed by magnetic capture of the protein A beads using the Magnet+ device to allow for removal of the flow-through fractions. The magnetic Protein A beads with the captured mAb were washed, and purified mAb was eluted using a low pH wash. After neutralization of the pH, the pure mAb samples were digested using FabRICATOR (Ides) in the presence of DTT, a disulfide-bond reducing agent. This yielded reduced mAb subunits on a 96-well plate which were suitable for direct transfer to the autosampler of a BioAccord LC-MS System. Analysis of fractions demonstrated effective capture of the mAb from cell culture media, followed by elution of pure mAb and digestion into subunits using FabRICATOR (Ides) and DTT (Figure 4).

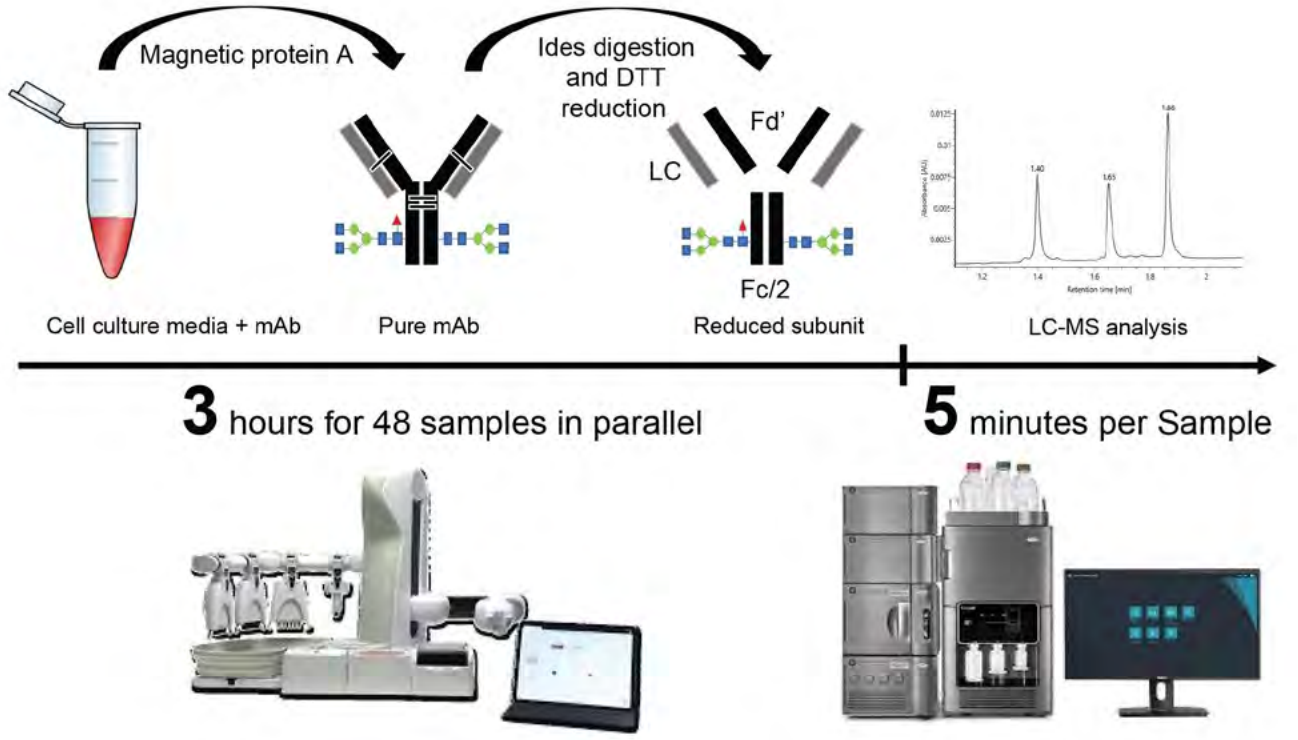


Figure 4. Workflow of automated antibody purification and digestion with Andrew+ and rapid LC-MS analysis using Waters BioAccord System.

Reduced subunits were separated by reversed-phase UPLC using a BioResolve RP mAb Polyphenyl Column on the BioAccord LC-MS. Waters mAb Subunit Standard (p/n: 186008927 < https://www.waters.com/nextgen/global/shop/standards--reagents/186008927-mab-subunit-standard.html>) was used to benchmark System performance. The mass accuracy of RDa Detector for the subunit analysis was determined to be within specification at <20 ppm. LC-MS of the purified samples yielded high-quality spectra of all three subunits (Figure 5 a–c), allowing for the relative quantification of different modifications such as Fc glycosylation. The direct LC-MS analysis of unpurified cell culture samples did not yield subunits suitable for LC-MS analysis (Figure 5d). This may be due to inefficient enzymatic reaction in unpurified cell culture media and presence of host cell proteins.

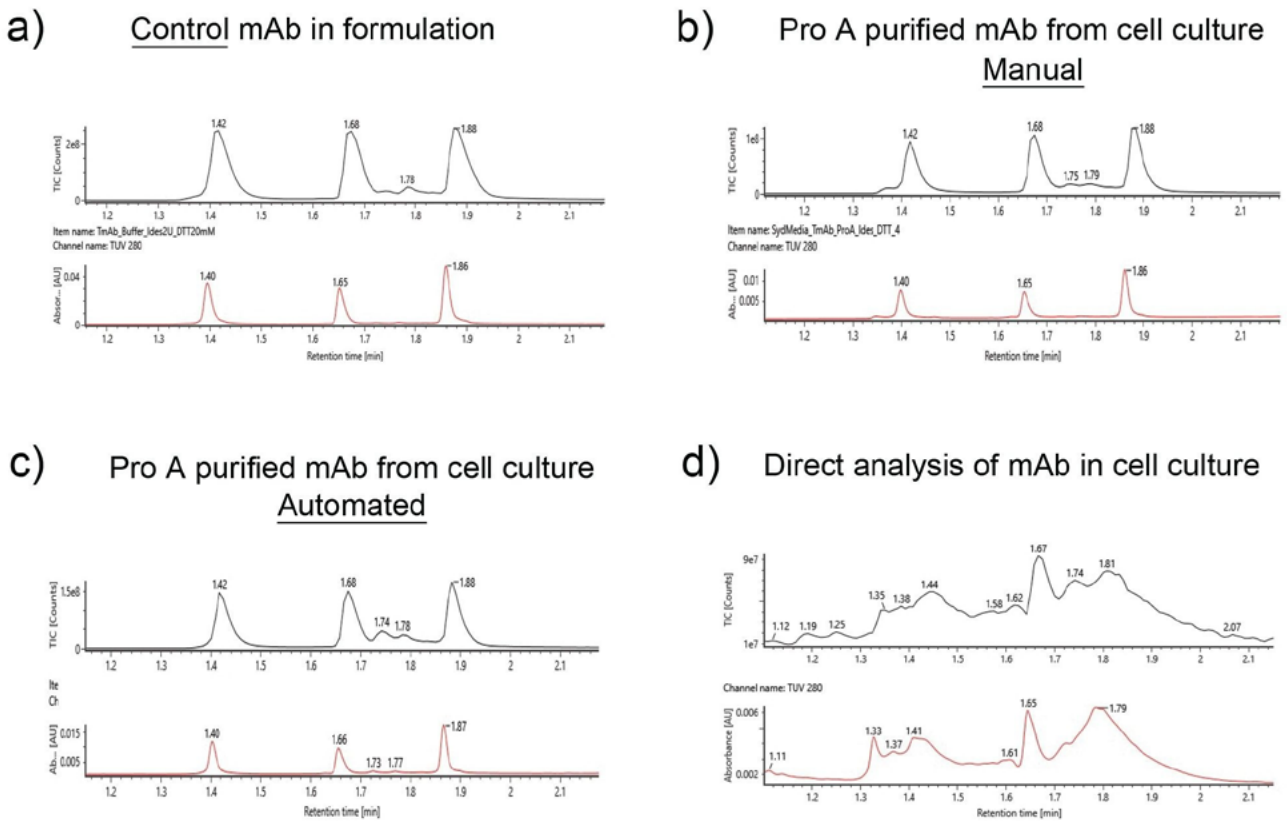


Figure 5. a-c) LC-MS analysis of subunits from purified and digested mAbs. d) unpurified and digested mAbs.

The LC-MS results of protein A purified and digested mAb generated by both manual and Andrew+ Robot were similar and all results were comparable to that of a control sample (Table 2). These subunit level analysis facilitated readily interpreted experiment results for LC, Fd', and Fc/2, and also provided information on mAb Fc glycosylation (Figure 6).

Subunits	Control average subunit mass (Da)	Manual average subunit mass (Da)	Andrew+ average subunit mass (Da)
LC	23443.26 ± 0.03	23443.18 ± 0.05	23443.19 ± 0.05
Fd'	25383.39 ± 0.07	25383.13 ± 0.09	25383.59 ± 0.07
Fc/2			
▪G0	25089.67 ± 0.08	25089.30 ± 0.16	25089.04± 0.13
▪G0F	25236.05 ± 0.05	25236.07 ± 0.11	25236.02 ± 0.09
▪G1F	25398.37 ± 0.06	25398.30 ± 0.12	25398.46 ± 0.08
▪G2F	25560.82 ± 0.12	25560.64 ± 0.26	25560.14 ± 0.26

Table 2. Subunits LC-MS analysis comparison between control (TmAb in Buffer, No Pro A, Digested), manual (TmAb in Cells Media, Pro A Purified, Digested), and automated (TmAb in Cells Media, Pro A Purified, Digested) protocols. N=8 for each condition.

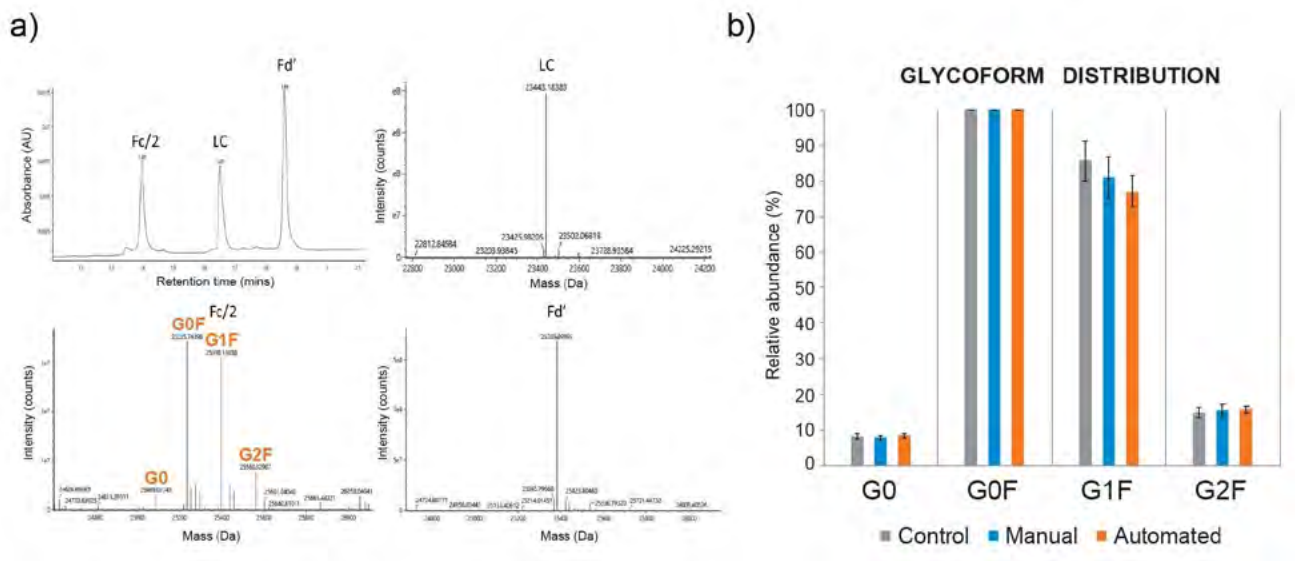


Figure 6. a) Subunit level analysis interpreted results for LC, Fd', and Fc/2 b) Fc N-glycosylation profile from deconvoluted mass spectrum of the Fc/2 fragment generated by manual (blue) and Andrew+ assisted (orange), compared to control (grey). N=8 for each condition.

Conclusion

Developing and implementing analytical methods to facilitate recombinant protein process development can be challenging as efficient and robust analytics are crucial. With effective automated sample preparation and LC-MS screening, we have shown mAbs can be analyzed directly from complex media for the determination of several important product quality attributes.

This fully automated combined protocol for purification and digestion takes approximately two hours for eight samples and three hours for 48 samples and can consistently and reliably generate results similar to the manual execution of the procedure. In addition, due to the low minimum amount of sample required and the scalability of the magnetic bead purification step, this procedure can be deployed for the preparation of limited sample amounts as low as 0.5 µg and up to 10 µg of mAb and is therefore amenable to be use with both microbioreactors and larger scale bioreactor setups.

Further more, the ease of use of the BioAccord LC-MS System with the waters_connect Informatics Solution/INTACT Mass App can improve analyst efficiency with automated data acquisition and processing for high-throughput mass confirmation of mAb subunits.

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Application Note

Similis Bio - Streamlined mAb Subunit LC-MS Workflow for Multiple Attribute Monitoring of Biosimilar mAb Candidates During Bioprocessing and Development

Samantha Ippoliti, Jared Young, Katy McNally, Caitlin Hanna, Ying Qing Yu, Bradley Prater, Mark D. Wrona, Emma Harry

Waters Corporation, Similis Bio

Abstract

This application note summarizes a collaboration between Similis Bio and Waters Corporation™ to demonstrate the feasibility of an automated Protein A purification and LC-MS mAb subunit analysis of bioreactor samples to screen biosimilar monoclonal antibody (mAb) candidates in comparison to the innovator to support process development. The method was demonstrated to have sufficient sensitivity and precision to quantitatively assess N-linked oligosaccharides at levels known to impact pharmacokinetics and effector function activity, in addition to other post-translational modifications (PTMs) such as C-terminal lysine variants and other subunit-specific (LC & Fd') modifications. Subunit mAb LC-MS monitoring methods such as this can be utilized to support mAb bioprocessing, development, and quality control within biopharmaceutical and biomanufacturing organizations.

Benefits

- Automated ProA purification of cell culture samples with subsequent FabRICATOR™ subunit digestion using the Andrew+™ Pipetting Robot

- Simplified LC-MS acquisition using BioAccord™ LC-MS System, a benchtop TOF MS designed for ease of operation for analysts of all levels of expertise
- Utilization of the waters_connect™ Intact Mass App for streamlined automated data acquisition and analysis with compliance-ready features to ensure data integrity
- Localization of Fab vs Fc modifications is possible using this mAb subunit LC-MS method
- Demonstrated orthogonality between mAb subunit LC-MS workflow and released N-glycan assay results

Introduction

The development of biosimilars has gained significant momentum in recent years due to their potential to provide more affordable and effective treatments for a range of diseases. However, the development of biosimilars is a complex and challenging process that requires extensive characterization and comparison to the reference product. Clone selection, early in the development lifecycle, has an outsize impact on the ability to match the quality target product profile for a given product. N-linked oligosaccharide profiles and common PTMs, such as C-terminal lysines, sidechain oxidation and glycation, may vary between clones as a result of expression dynamics and cellular stress responses. Therefore, the evaluation of these product quality attributes during clone selection can reduce subsequent process development costs by selecting a clone with a profile that closely matches the innovator. Additionally, design-of-experiment (DoE) studies to evaluate cell culture feed media and supplementation generate many samples that require testing across numerous analytical methods to guide process development. This creates significant analytical testing burdens and necessitates rapid turnaround times to enable iterative development workflows.

A rapid, automated at-line mAb subunit attribute monitoring workflow (Figure 1) was used to comparatively screen mAb biosimilar product quality attributes during clone selection and upstream process development. Biosimilar samples taken from Sartorius Ambr250™ High Throughput bioreactors were subjected to an automated Protein A (ProA) affinity capture and subunit enzymatic digestion with Genovis FabRICATOR using the Andrew+ Pipetting Robot. The purified and FabRICATOR-digested samples were then analyzed via intact mass analysis of the liberated subunits using the BioAccord LC-MS System. The Intact Mass App within waters_connect Informatics Platform was used to automatically acquire, process, and visualize data as each sample completed acquisition.

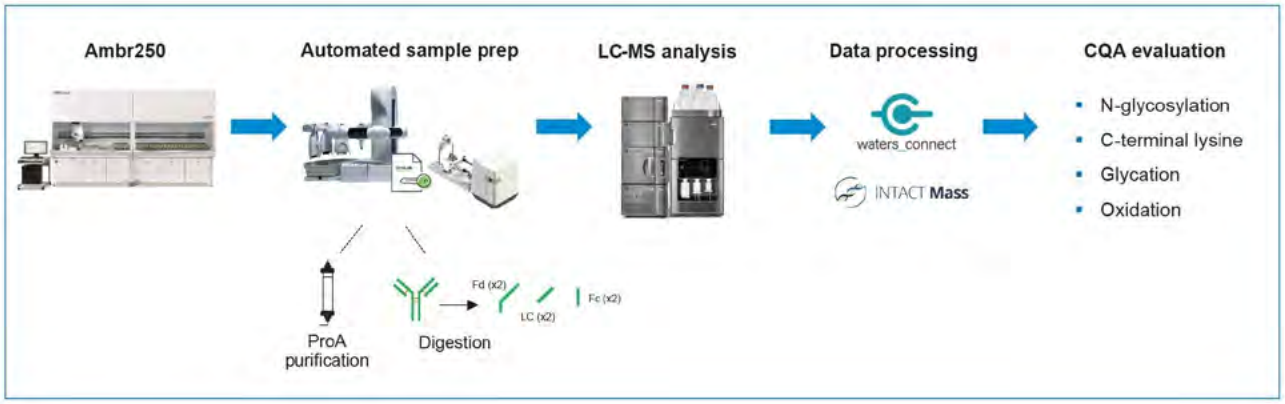

Figure 1. mAb Subunit MAM workflow, with automated sample preparation using Andrew+ Robotics Platform for ProA purification & FabRICATOR digestion, followed by LC-MS data acquisition and processing on the BioAccord LC-MS System with waters_connect informatics.

This higher-throughput method provided N-linked glycosylation profile, unprocessed C-terminal lysine abundance, glycation, and oxidation levels comparable to orthogonal analytical techniques for each quality attribute, while also providing significant benefits in terms of automation, scalability, and throughput. Method sensitivity for high mannose and afucosylated glycans are of particular interest to decision makers, as these species can have a significant impact on mAb pharmacokinetics (pK) and effector function activity at a low relative abundance. This mAb subunit LC-MS workflow is successful in meeting data sensitivity, precision, and reproducibility requirements for early biosimilar development. A similar mAb subunit LC-MS monitoring workflow has been recently published by Genovis, demonstrating that the BioAccord System is an ideal platform for routine and efficient product quality attribute monitoring at the mAb subunit level.[1]

Experimental

Sample Description (Automated Purification)

Cell culture samples were purified with an Andrew+ Robotics Platform (equipped with the Extraction+ module) workflow that was published recently.[2] Briefly, using a 0.2 µm filter plate, each well was supplied with ProA resin, washed, and the sample was loaded. Each sample was first washed, then eluted into a collection plate containing neutralization buffer (Fig. 2A). Two clones were used to produce distinct cell culture samples for the same product, called "Biosimilar" and "Biosimilar -Negative Control" below.

Sample Description (Subunit Digestion)

Using Andrew+ automation (including the Heater/Shaker+ module), 20 µL of the resulting ProA-purified sample (and innovator sample (commercial drug product)) at ~1 mg/mL, were transferred to a PCR plate for a 1-step subunit digestion with partial subunit reduction. 10 µL of a prepared master mix (Fig. 2B) (2 units/µL FabRICATOR enzyme, 50 mM Tris, 10 mM DTT, pH 7.5) was added to each sample well, and the plate was incubated at 37 °C for 30 minutes (Fig. 2C). Samples were diluted 5x (to 0.1 mg/mL) with 0.1% formic acid in water for LC-MS analysis (Fig. 2D).

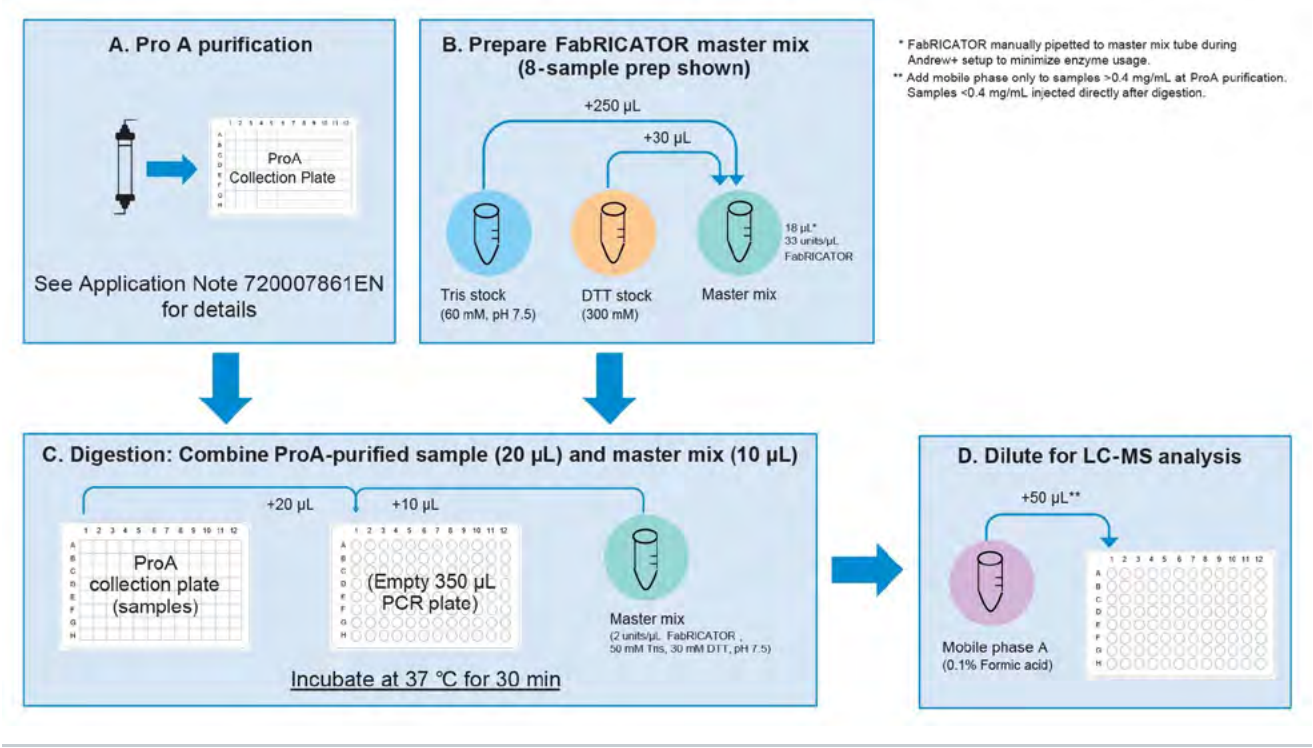

Figure 2. Subunit sample preparation using Andrew+ Pipetting Robot.

Protein A & FabRICATOR Sample Preparation

Robotics system:	Andrew+ Pipetting Robot with Extraction+ and Heater/Shaker+ Modules
Software:	OneLab (Andrew Alliance / Waters)
Filter plate:	Pall AcroPrep™ Advance 96-well Filter Plates- 350 µL, 0.2 µm Supor™ membrane (p/n: 8019)
Collection plate:	QuanRecovery™ 700 µL 96-well plate (p/n: 186009184)
Subunit digestion plate:	Eppendorf twin.tec™ PCR Plate 96 LoBind™ (***e.g.*** p/n: 0030129555)
Protein A resin:	Cytiva MabSelect™ (p/n: 17519901), slurries ~25% (1:1, PBS:50% resin). For 50% resin, centrifuge at 1000 x g for 3 mins and replace supernatant with volume of 400 mM NaCl, 20% ethanol equal to resin volume.
PBS:	Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4)
Neutralization buffer (NB):	1M Tris HCl, pH 7.5
Elution buffer (EB):	100 mM Glycine, pH 3.0
Orbital shaker:	Eppendorf Thermomixer® C (8 °C)
FabRICATOR enzyme:	Genovis (p/n: A0-FR1-020)
Dithiothreitol (DTT):	Pierce No-Weigh DTT (p/n: A39255)

LC Conditions

LC system:	ACQUITY™ Premier UPLC System
Detection:	ACQUITY UPLC TUV (280 nm)
Column(s):	BioResolve™ RP mAb Polyphenyl, 2.7 µm, 2.1 x 100 mm (p/n: 186008945)
Column tempertaure:	60 °C
Sample temperature:	6 °C
Injection:	0.5 µg FabRICATOR-digested mAb (5 µL injection of 0.1 mg/mL sample)
Flow rate:	0.3 mL/min
Mobile phase A:	0.1% Formic Acid in Water
Mobile phase B:	0.1% Formic Acid in Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.3	80	25	6
10.0	0.3	60	40	6
10.3	0.3	20	80	6
11.3	0.3	20	80	6
11.6	0.3	80	25	6
15.0	0.3	80	25	6

MS Conditions

MS system:	ACQUITY RDa™
Ionization mode:	ESI Positive
Acquisition range:	400–7000 *m/z* (High Mass)
Capillary voltage:	1.5 kV
Cone voltage:	50 V
Desolvation temperature:	550 °C

Data Management

Data was acquired and processed through the Intact Mass App (v 1.4.0.0) in waters_connect Informatics Platform (v 3.1).

Results and Discussion

mAb subunit LC-MS attribute monitoring workflows offer a compromise of shortened 15-minute sample analysis times while still providing a means monitoring vital localized product quality attributes of innovator and biosimilar mAbs.[3–7] Here we demonstrate an automated ProA purification and FabRICATOR subunit digestion of mAbs sampled at-line from Ambr250 bioreactors and analyzed via LC-MS. Using the Acquire and Process function within the Intact Mass App (Figure 3), the user can set up an analysis sequence, monitor real-time data readouts, and view deconvoluted/processed data as each injection completes data acquisition. The ability to view processed data while the instrument is running subsequent samples enables quicker decision-making which benefits the development process.

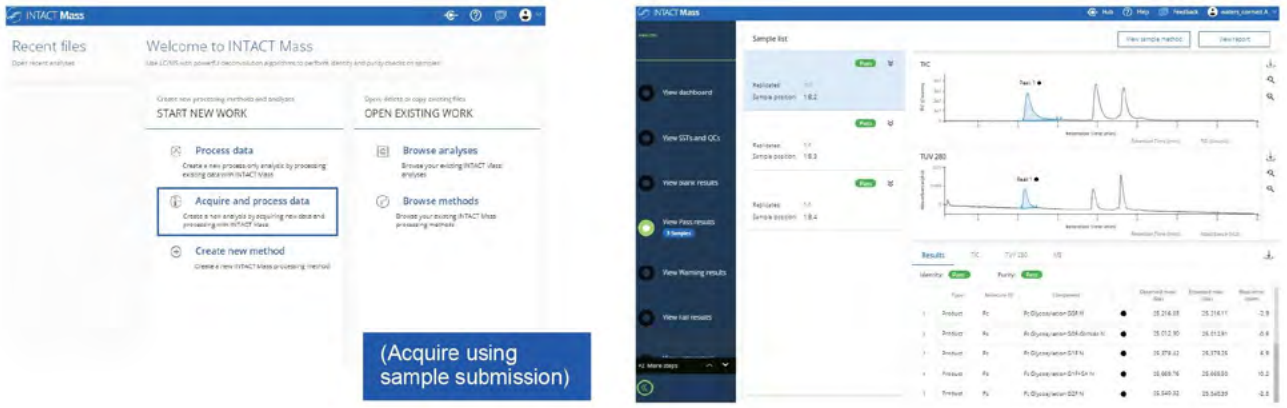

Figure 3. Integrated acquisition and data processing using the Intact Mass App within the waters_connect Informatics Platform. Data was acquired through Sample Submission App and automatically processed by Intact Mass App after each injection was acquired.

The primary aim of this study was to determine if the sensitivity and precision of the mAb subunit LC-MS attribute monitoring approach for Man5 and afucosylated N-glycosylation levels of biosimilar mAb samples reduces the need for other orthogonal assays. FabRICATOR digestion with reduction of inter-chain disulfide bonds produces three protein chains to monitor via RPLC-MS (Fc, LC, & Fd'), as shown (Figure 3 chromatogram), all having masses between 23–25 kDa. The N-glycosylation site is located on the Fc subunit. The MaxEnt1-deconvoluted spectra for the Innovator (Figure 4A, top), Biosimilar (Figure 4A middle), and a Biosimilar-Negative Control (Figure 4A bottom), the latter known to contain high levels of Man5. The Innovator and Biosimilar share relatively consistent N-glycoprofiles but differ significantly in the level of unprocessed C-terminal lysine present in the Biosimilar (0% and 15%, respectively in Figure 4B). The Biosimilar-Negative Control, as expected, is observed with roughly 10% Man5 species, while the Biosimilar sample contains lower levels similar to the Innovator (Figure 4B).

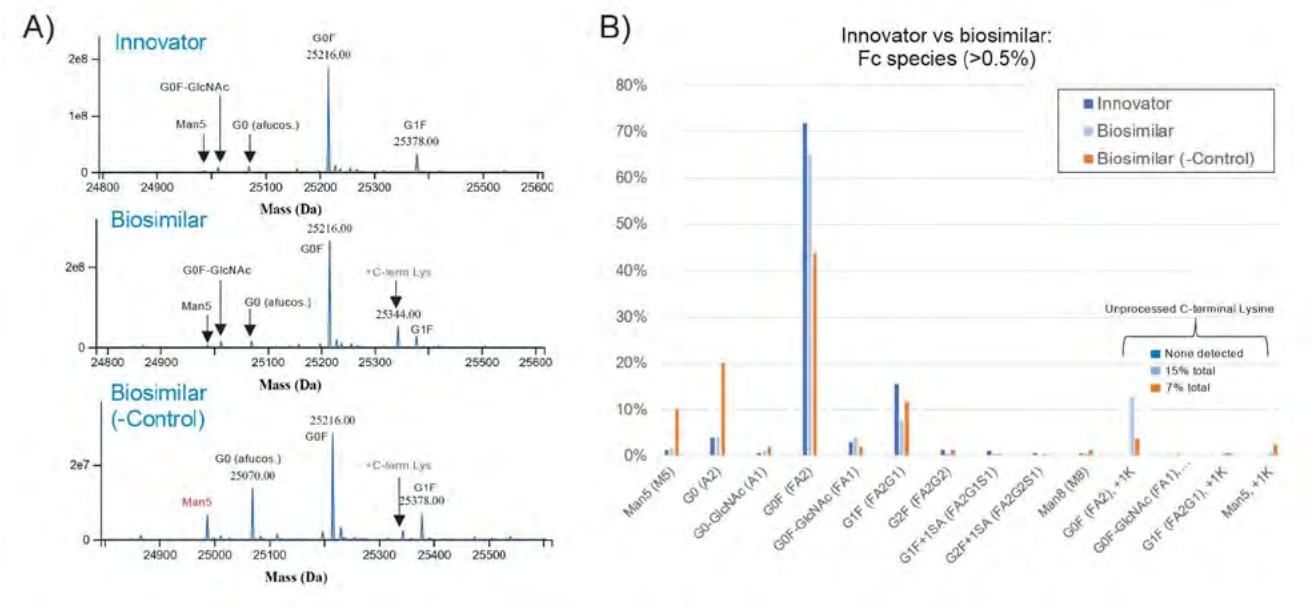

Figure 4. A) Deconvoluted spectra for Fc species generated by the Intact Mass App, comparing innovator, ProA purified biosimilar, and negative control biosimilar sample (containing high Man5 level); B) Relative quantitation of Fc species, including N-glycoforms and unprocessed C-terminal lysine.

The Similis Bio Standard Operating Procedure (SOP) for monitoring glycan levels has been a released N-glycan assay, where glycans that are enzymatically released from the mAbs are labelled with a fluorescent tag (RapiFluor-MS) and analyzed via HILIC-FLR-MS.[8] This method is very sensitive and robust, but it provides only a very targeted set of information limited to the N-glycans present in the sample. If a mAb subunit LC-MS method could produce comparable results to those from the released glycan assay, plus provide additional quality attribute information about the mAb, it could replace the current workflow and potentially additional assays. The LC-MS results for the top five abundant N-glycoforms* (Fig 5A) were compared to the released glycan assay results performed in-house at Similis Bio. The difference in relative percentage (Δ%) for each sample and species is plotted in Figure 5B. All values for relative percentage of these N-glycan species detected in LC-MS subunit analysis were within 2% of the reported value from released glycan. This demonstrates acceptable comparability of results between the two methods.

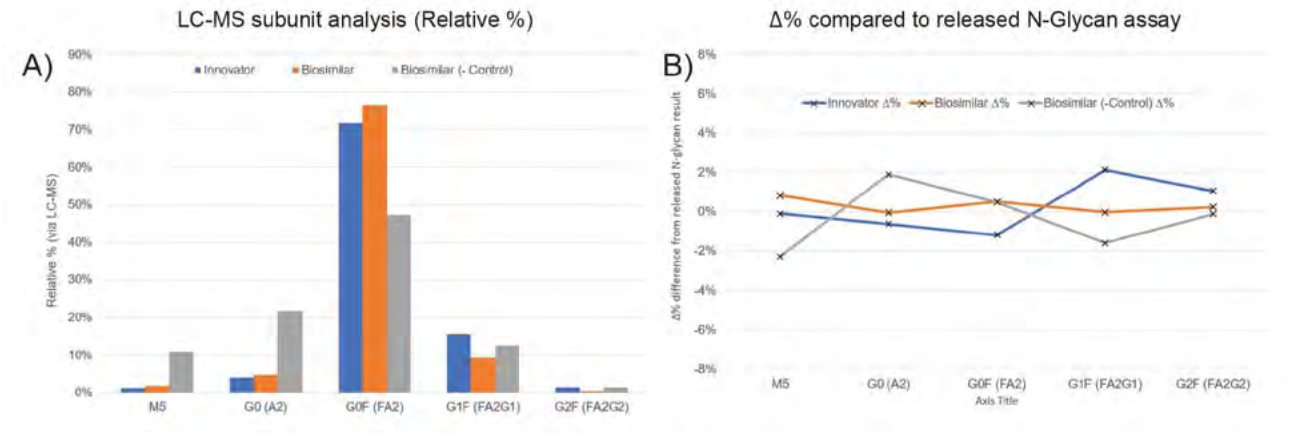

Figure 5. Comparison of Fc Subunit and released RFMS N-glycan results (Top 5 abundant species). Panel A: Relative % detected via LC-MS subunit method (adjusted for C-terminal lysine variants not observable in released N-glycan assay). Panel B: Difference (Δ%) between LC-MS & the released N-glycan assay results.

In addition to the N-glycoprofile, the mAb subunit LC-MS monitoring method provided insights into other quality attributes such as the unprocessed C-terminal lysine, Fd' & LC glycation, and oxidation. Similar efforts have been shown in subunit assays for process development and subsequently validated for mAb QC implementation.[9, 10]

These attributes would normally be assessed via orthogonal methods such as peptide mapping (Optical or LCMS) or using indirect optical LC and CE methods at the peptide, intact, or subunit level. C-terminal lysine, for instance, could be investigated by comparing the charge variant profiles for an untreated sample vs the same sample treated with carboxypeptidase B (CPB) enzyme (for removal of the remaining positively charged C-terminal lysine). In the charge profile, the unprocessed C-terminal lysine species usually appear as basic variants, which then decrease with CPB digestion. Logically, the difference in basic species between the samples should give a rough estimation of unprocessed C-terminal lysine present in the sample. This approach is generally very useful for mAbs with simple charge profiles, but as the complexity increases, the more ambiguous the analysis becomes. The demonstrated mAb subunit LC-MS method simplifies the analysis of this attribute.

Additionally, this mAb subunit LC-MS monitoring method can provide a view of potential modifications on the Fd' and LC chains, which contain the parts of the sequence responsible for binding, and therefore the activity, of the mAb drug product. These potential modifications include, but are not limited to, glycation and oxidation and/or unconverted N-terminal pyroglutamic acid (non-pyroQ). (Note, the oxidation and non-pyroQ modifications are not readily distinguished at subunit level by LC-MS.) In this case study, low levels of Fd' and LC

glycation (<2%) were detected for both the Innovator and Biosimilar samples. In the case of oxidation/non-pyroQ, there was no observable level for the LC in either Innovator or Biosimilar, but the Fd' species contained 10–15%. To distinguish the Fd' oxidation and/or non-pyroQ species, further investigation would be required.

To test the robustness of the mAb subunit LC-MS monitoring method, the same Innovator (drug product) sample was prepared at two different sites (manual vs automated FabRICATOR digestion), and the samples were run on a BioAccord System located at each of the labs. The results for all Fd', LC, & Fc species of >0.5% relative abundance (Fc species shown in Fig 6) were consistent (within 1.5%). The mAb subunit LC-MS method, performed with manual or automated sample preparation, is found to be robust and reliable across multiple laboratories.

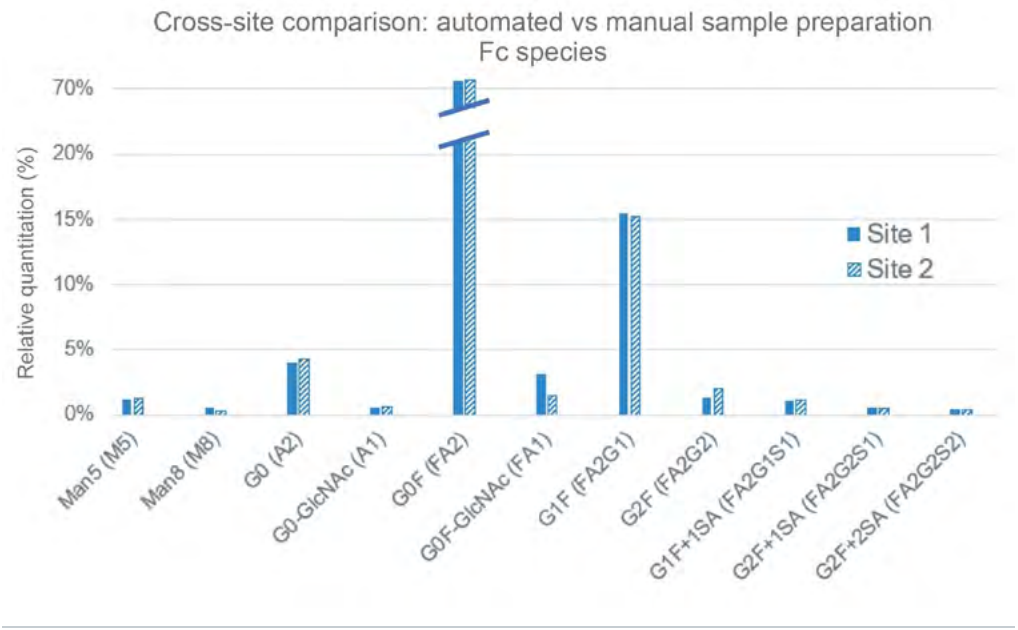

Figure 6. Comparable Fc N-glycosylation results generated in a cross-site comparison (Site 1=automated sample preparation, Site 2=manual sample preparation).

**LC-MS subunit N-glycoform relative percentages were adjusted for the comparison to the released glycan assay results by combining values with and without the terminal lysine residue. This was to exclude the response generated from C-terminal lysine-containing species, which would not be accounted for by released glycan assay.*

Conclusion

The collaborative effort between Similis Bio and Waters Corporation has demonstrated the utility of a workflow for monitoring of select product quality attributes of mAbs using subunit LC-MS analysis of an innovator and of biosimilar drug candidates sampled directly from a bioreactor. The at-line workflow consists of automated ProA purification and FabRICATOR digestion using the Andrew+ Robotics Platform, followed by LC-MS analysis using a BioAccord System. The Intact Mass App allows for seamless data acquisition, monitoring, and processing- all within the compliance-ready waters_connect Informatics Platform. The results generated using this mAb subunit LC-MS workflow are consistent with released glycan results for both Innovator and Biosimilar samples. The same workflow also provided insights into other quality attributes including the unprocessed C-terminal lysine, glycation, and oxidation, reducing the time and effort needed to acquire this information through orthogonal methods.

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720007997, August 2023

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Application Note

Automated High-Throughput LC-MS Focused Peptide Mapping of Monoclonal Antibodies in Microbioreactor Samples

Hua Yang, Stephan M. Koza, Ying Qing Yu

Waters Corporation

Abstract

To facilitate cell line selection and bioprocess optimization, an automated high-throughput (HT) sample preparation method and fast LC-MS peptide mapping for Critical Quality Attribute (CQA) peptide measurement was developed for low volume and low concentration microbioreactor samples. For this method, 30 µL of a 1 mg/mL sample of a monoclonal antibody (mAb) in neutralized Protein A affinity chromatography elution buffer was buffer exchanged and trypsin digested using Andrew+™ automation. The resulting peptides were separated on an ACQUITY™ Premier Peptide CSH™ C_{18} Column and detected by a BioAccord™ LC-MS System. Effective analysis was demonstrated for selected CQA peptides including N-glycosylated, deamidated, and oxidized peptides. The automated preparation of 48 samples takes 3.5 hours, with a two-hour digestion time, and LC-MS analysis time was ten minutes per sample. Besides Protein A purified mAb, this method could also be adapted to the analysis of other samples with limited amounts of protein.

Benefits

- Consistent results for the relative quantification of CQA peptides from 30 µg of mAb even at low concentrations

Benefits

- Consistent results for the relative quantification of CQA peptides from 30 µg of mAb even at low concentrations
- An automated trypsin digestion protocol with the Andrew+ robotic platform and using a new commercialized trypsin (RapiZyme™ Trypsin) that is highly resistant to autolysis
- High-throughput (HT) LC-MS analysis of CQA peptides using an ESI-ToF BioAccord LC-MS and automated data analysis using the waters_connect™ Peptide MAM application software

Introduction

Recombinant protein biopharmaceuticals, such as monoclonal antibodies (mAbs), have benefited patients for many years. Development and production of recombinant protein can be costly, and one potential bottleneck in developing an efficient process producing high quality product can be host cell-line selection and bioprocess optimization. To reduce the timeline for cell line selection and process optimization, microbioreactors have been used in early process development.[1] In recent years, product quality has been included in cell line selection criteria, driven in part by the regulatory demands of making biosimilars. While microbioreactors provide advantages of being able to approximate the production conditions of a large-scale bioreactor, the amount of sample taken from a microbioreactor can be analytically limiting. Another challenge is that analyzing CQAs of the sample such as site-specific modifications usually involve peptide mapping via LC-UV or LC-MS, which can be labor-intensive and low throughput.

This application note demonstrates an automated procedure using an Andrew+ robot (Andrew Alliance™) with minimum manual intervention for a CQA peptide mapping method to assist cell line selection and cell-culture optimization (Figure 1). Presented is a focused or targeted peptide mapping method for monitoring the abundances of selected CQA peptides and their modified forms. For demonstration purposes, 30 µg of 1 mg/mL mAb (infliximab) sample was reduced and trypsin digested, after which its CQA peptides were monitored using a ten minute long reversed-phase separation with MS detection. This method could also be adapted for use with mAb and other protein samples with concentrations lower than 1 mg/mL, due to a pre-concentration step in the sample preparation procedure.

Figure 1. Image of Andrew+ Pipetting Robot and BioAccord LC-MS System.

Experimental

Sample Description

Infliximab (10 mg/mL) was diluted into neutralized Protein A elution buffer which contains 100 mM glycine (pH 3) and 1 M Tris (pH 7.5) in a 5:1 v/v ratio. The final infliximab concentration was 1.0 mg/mL.

For the stressed sample experiments, infliximab (10 mg/mL) was incubated in 0.005% H_2O_2 and 50 mM sodium phosphate (pH 7.6) for two weeks at 37 °C to induce oxidation and deamidation. The stressed sample was also co-mixed with original unstressed sample in 1:1 volume ratio. All samples were then diluted in the above neutralized Protein A elution buffer to 1.0 mg/mL.

LC Conditions

LC system:	ACQUITY UPLC™ I-Class PLUS
Detection:	ACQUITY BioAccord MS System
Plates:	Acroprep™ Advance 350 µl Omega 10 k MWCO (p/n: PALL-8034) Eppendorf twin.tec® PCR Plate 96, skirted, 150 µL (p/n: 951020443) 6mm Pre-Slit Silicone/PTFE Cap Mat (Analytical Sales and services, p/n: 96727)
Column(s):	ACQUITY Premier Peptide CSH C_{18} 1.7 µm, 2.1 x 100 mm (p/n: 186009488)
Column temperature:	60 ˚C
Sample temperature:	10 ˚C
Injection volume:	5 µL, 10 µL
Flow rate:	0.2 mL/min, 0.4 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient Table (50-min gradient, 80-minute run time)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.2	99	1	Initial
1.0	0.2	99	1	6
51.0	0.2	65	35	6
57.0	0.2	15	85	6
61.0	0.2	15	85	6
66.0	0.2	99	1	6
90.0	0	99	1	11

Gradient Table (6-min gradient, 10-minute run time)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.4	99	1	Initial
1.0	0.4	99	1	6
7.0	0.4	70	30	6
7.8	0.4	15	85	6
8.3	0.4	15	85	6
8.8	0.4	99	1	6
14.0	0	99	1	11

ACQUITY RDa Detector Settings

Mode:	Full scan with fragmentation
Mass range:	50–2000 *m/z*
Polarity:	Positive
Sample rate:	5 Hz
Cone voltage:	30 V
Fragmentation cone voltage:	60 V – 120 V
Capillary voltage:	1.20 kV
Desolvation temperature:	350 °C

Data Management

LC-MS software: waters_connect

Results and Discussion

General Procedure

An automated high throughput (HT) peptide mapping method using LC-MS analysis to monitor CQA peptides was successfully developed for low volume and low concentration microbioreactor mAb samples. Peptide mapping has been used for multiple attribute monitoring (MAM) by pharmaceutical industry for many years.[2] It is typical to use 100 µg of protein or more in these digestion procedures because large amount of concentrated sample is often available for product characterization studies. However, in the current application, the samples that obtained from microbioreactors usually have low concentrations and volumes. To achieve an effective focused peptide mapping sample preparation, optimization of parameters including guanidine-HCl concentration, protein to enzyme ratio, digestion time was carried out (data not shown). It is important to note that the presented methods were not developed with the intent of comprehensively mapping the mAb, as such, significant under digestion is observed for these digest conditions. Also, the sample is only disulfide bond reduced without alkylation as part of this procedure.

Figure 2A shows the major steps for the automated focused peptide mapping procedure. Thirty µg of infliximab was diluted in neutralized Protein A elution buffer to 1.0 mg/mL (See EXPERIMENTAL for details). It is assumed that the mAb is affinity-purified with Protein A affinity chromatography resin.

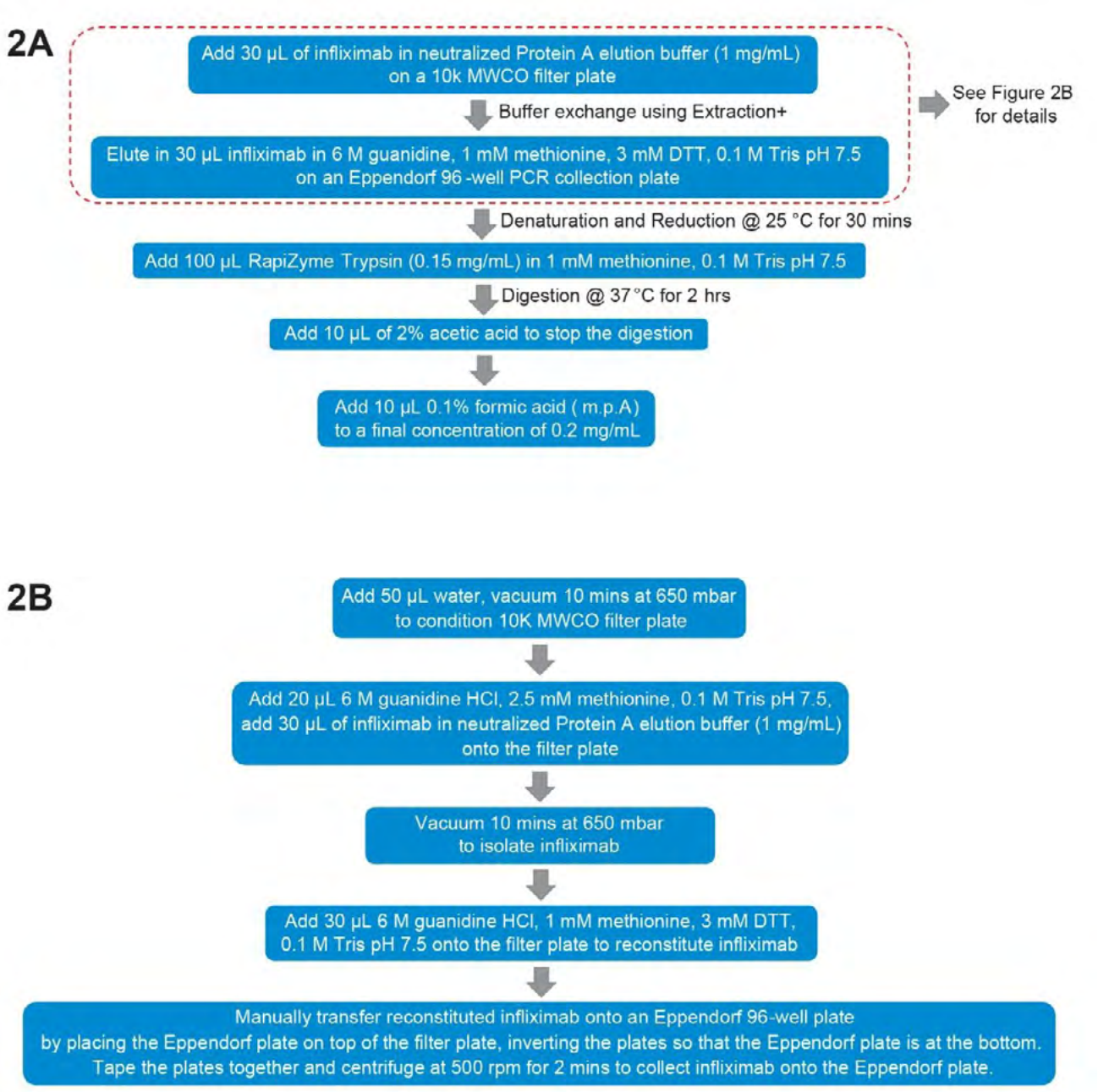

Figure 2. A. General digestion procedure using Andrew+ automation;

B. Detailed procedure of buffer exchange.

For this method, the first step is to buffer exchange the samples into the denaturation and reduction buffer (DRB, 6 M guanidine HCl, 1 mM methionine, 3 mM DTT, and 0.1 M Tris pH 7.5) as shown in Figure 2B. This is done

For this method, the first step is to buffer exchange the samples into the denaturation and reduction buffer (DRB, 6 M guanidine HCl, 1 mM methionine, 3 mM DTT, and 0.1 M Tris pH 7.5) as shown in Figure 2B. This is done using Extraction+ domino on the Andrew+ automation system and a 10 K MWCO filter plate to retain the sample. Before the samples are loaded, 50 µL water is added to the filter plate wells and vacuum applied for ten minutes at 650 mbar to drain the water through the filter. It was discovered that this conditioning step can expedite the buffer drainage in the next steps.[3] Then 20 µL of 6 M guanidine HCl, 2.5 mM methionine, 0.1 M Tris pH 7.5 solution and 30 µL of mAb (1.0 mg/mL) is added and vacuum is applied for 12 minutes at 650 mbar to drain the buffers while retaining the mAb. DRB (30 µL) is then added into the wells. To transfer samples from the filter plate to an Eppendorf 96-well PCR plate, the filter plate is inverted over the PCR collection plate and centrifuged at 500 RPM for two minutes. It is important to note that this inversion and centrifugation requires a user action and results in changing the well positions of the samples in a mirror image fashion.

After denaturation and reduction for 30 minutes at 25 °C, 100 µL 0.15 mg/mL RapiZyme Trypsin, a modified trypsin, in 1.0 mM methionine and 0.1 M Tris pH 7.5 is added to lower the guanidine-HCl concentration while digesting the mAb. The reaction mix is incubated at 37 °C for two hours, after which 10 µL of 2% acetic acid is added to stop the digestion. Finally, 0.10 % formic acid (mobile phase A) is added to dilute the reaction mix so that the final concentration of the digested protein is approximately 0.20 mg/mL.

A few points are worth noting for this procedure. First, the buffer exchange step can potentially pre-concentrate the sample if the sample concentration is low. In current experiments, for the purpose of demonstration, 30 µL sample was loaded and 30 µL was recovered. However, if the sample concentration is low, more volume can be loaded, and recovery can be done using less volume to concentrate the sample. Secondly, comparable results were obtained with and without alkylation. Therefore, alkylation step is not included in the procedure. Thirdly, the trypsin used in this procedure has an advantage of minimal low autolysis, so it can be used at high concentrations to speed up the digestion rate. Figure 3A and 3B shows the blank digest and the infliximab digest run on an ACQUITY Premier Peptide CSH C_{18}, 2.1 x 100 mm Column with a 50-min gradient, respectively. Note: An ACQUITY Premier Peptide CSH C_{18} Column was selected to help ensure column to column performance consistency by QC testing each batch of synthesized CSH C_{18} particles with a tryptic protein digest rejecting those batches that do not meet performance specifications. The blank digest does not show many interfering trypsin peaks even though the Rapizyme Trypsin amount is high with a protein to enzyme ratio of 2:1.

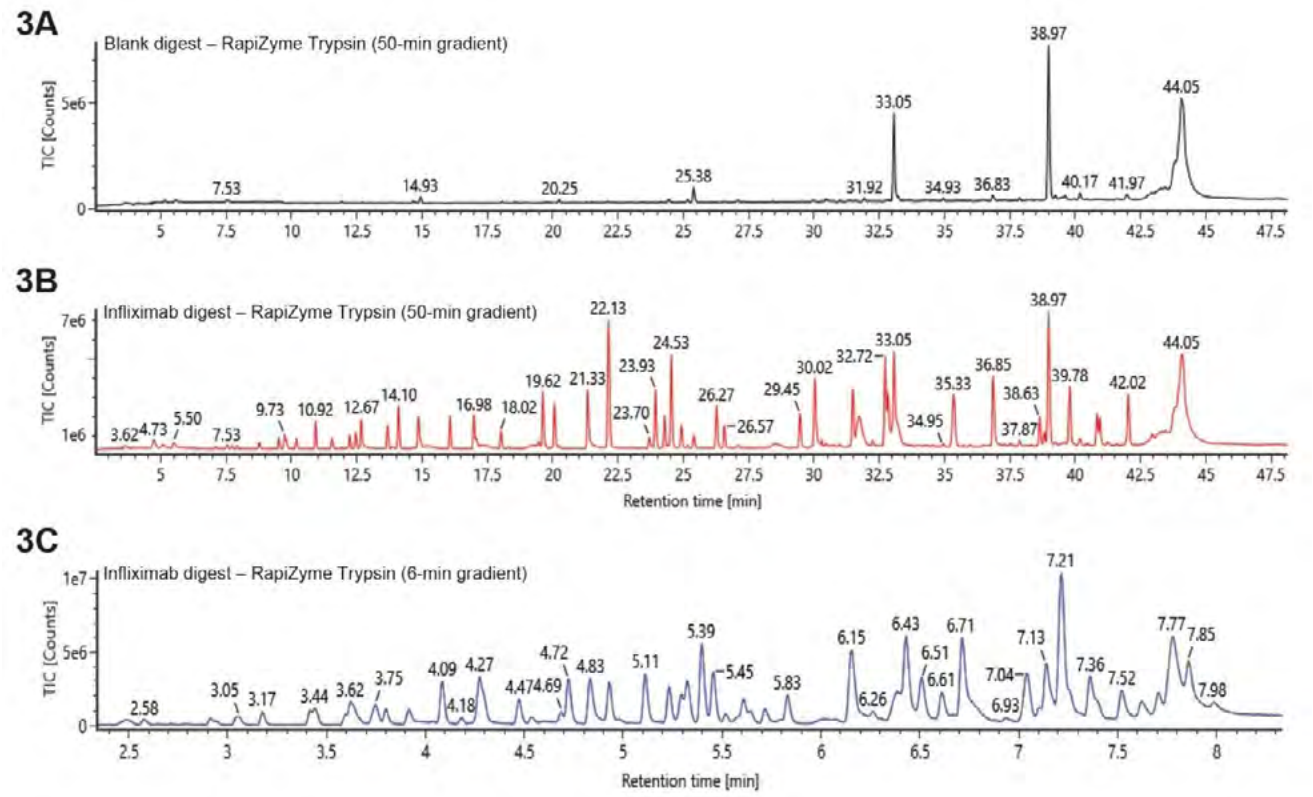

Figure 3. LC-MS chromatograms of infliximab CQA peptide mapping by RapiZyme Trypsin.

A. Blank digest;

B. infliximab digest;

C. infliximab digest.

For A and B, the gradient is 1–35%B in 50 mins, 0.2 mL/min. For C, the gradient is 1–30%B in 6 mins, 0.4 mL/min.

For high throughput analysis, the digest was run with a 6-min elution gradient. As predicted, the 6-min gradient (Figure 3C) resulted in lower chromatographic resolution than the 50-min gradient (Figure 3B). Since the goal of this method is to enable relative quantitation of CQA peptides, calculated using combined ions counts from observed charge states, instead of fully characterizing the protein, it was found that the 6-min gradient was adequate.

Figure 4 shows the layout of Andrew+ for this experiment. It takes <3.5 hours to digest 48 samples using Andrew+ automation and eliminates seven manual pipetting steps.

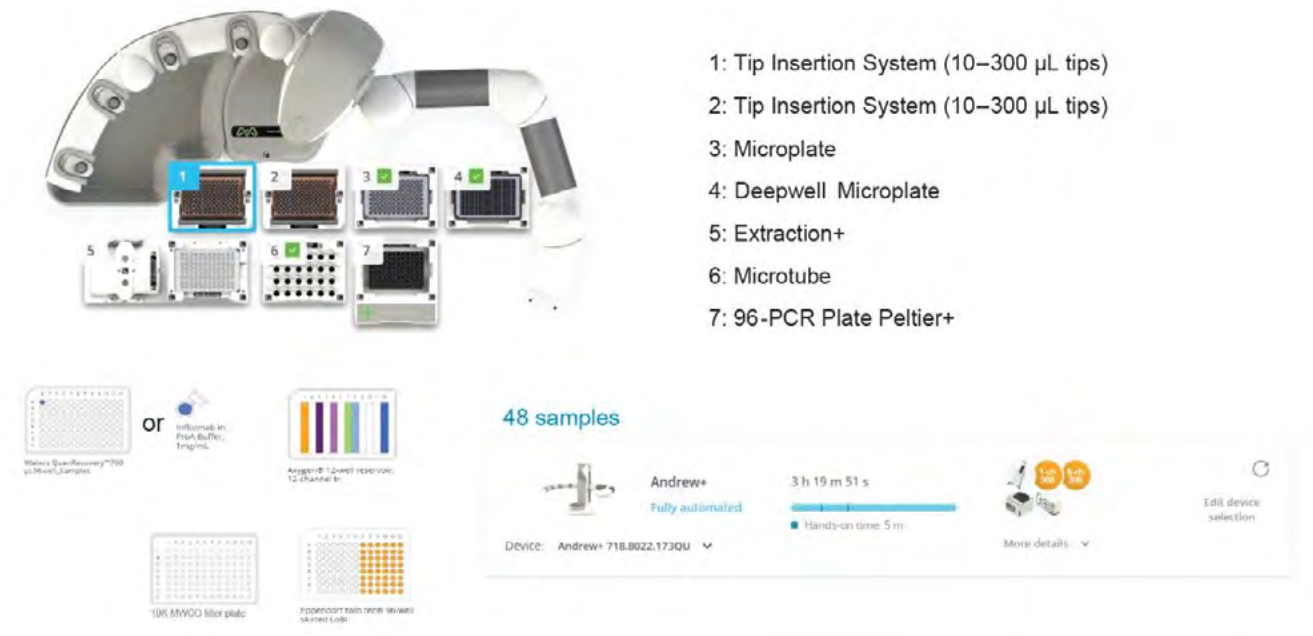

Figure 4. Andrew+ layout, dominos, and experimental time for digesting 48 samples.

Reproducibility

The automated sample preparation and LC-MS analysis demonstrated acceptable reproducibility for a range of CQA peptides. For this study, samples of infliximab (30 µL of 1.0 mg/mL) were digested using a 48-sample protocol. Of these 10 samples representing different positions on the plate were chosen for LC-MS analysis (Figure 5). Automatic data analysis was executed using the waters_connect informatics platform and the Peptide MAM application software. Table 1 and Figure 6 show the relative abundances of the CQA peptides that were evaluated, and the relative standard deviations of those measurements. An injection volume of 10 µL (2 µg mAb) was found to result in more consistent results than injecting 5 µL (1 µg). In this example, injecting more than 2 µg did not improve the reproducibility results further. For all 20 CQA peptides, %RSD of the percent modification was < 20% (n=10), and of the eight CQA peptides with a %RSD between 10% to 20%, seven were at levels of 1.66% or lower. The cause of slightly higher RSD% for oxidated peptides is under investigation. On the whole, these data demonstrate that this HT LC-MS method can provide consistent results of relative abundance of site-specific modifications of a protein using limited amounts (30 µg) sample.

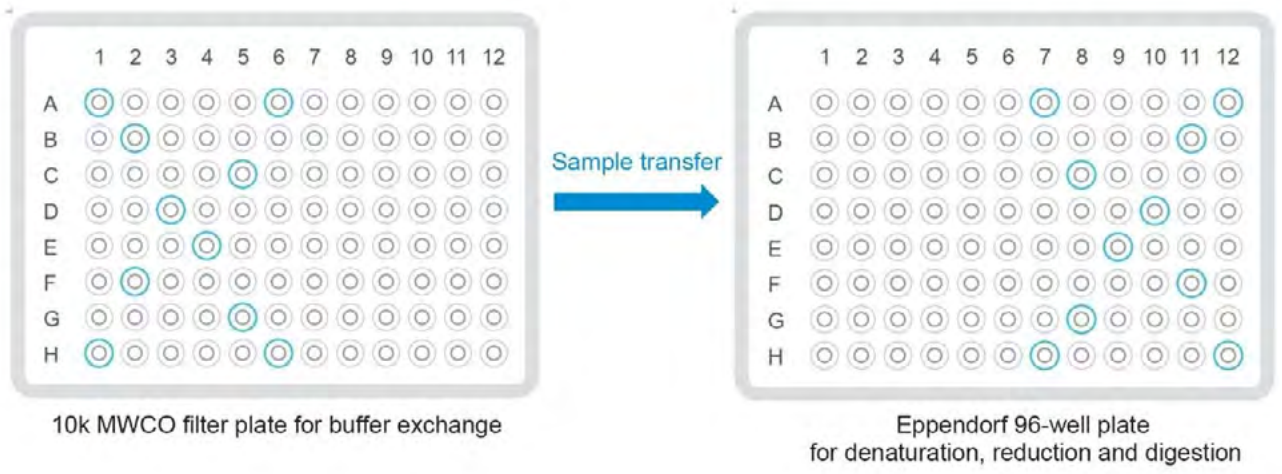

Figure 5. Positions of 10 representative wells for reproducibility study. Notice that the positions of the wells are mirror-images before and after buffer exchange.

Peptide number	Peptide name and modification	Average % modification	Standard deviation	%RSD
#1	HC:T2_LEESGGGLVQPGGSMK Oxidation M	0.66	0.13	19.47%
#2	HC:T3_LSCVASGFIFSNHWMNWVR Deamidation N	1.47	0.08	5.30%
#3	HC:T3_LSCVASGFIFSNHWMNWVR Oxidation M	0.69	0.06	8.87%
#4	HC:T3_LSCVASGFIFSNHWMNWVR Oxidation W, Oxidation M	0.50	0.07	14.94%
#5	HC:T7_SINSATHYAESVK Deamidation N	0.94	0.05	5.69%
#6	HC:T11_SAVYLQMTDLR Oxidation M	0.50	0.08	16.88%
#7	HC:T22_DTLMISR Oxidation M	1.66	0.27	16.04%
#8	HC:T24_FNWYVDGVEVHNAK Oxidation W	0.20	0.04	17.27%
#9	HC:T26_EEQYNSTYR G0F N	56.76	0.67	1.19%
#10	HC:T26_EEQYNSTYR G0F-GlcNAc N	3.91	0.43	10.90%
#11	HC:T26_EEQYNSTYR G1F N	31.28	0.62	1.98%
#12	HC:T26_EEQYNSTYR G2F N	3.48	0.28	8.08%
#13	HC:T26_EEQYNSTYR Man5 N	4.57	0.28	6.18%
#14	HC:T27_VVSVLTVLHQDWLNGK Deamidation N	0.55	0.03	5.33%
#15	HC:T37_NQVSLTCLVK Deamidation N	0.22	0.01	5.06%
#16	HC:T38_GFYPSDIAVEWESNGQPENNYK Deamidation N	3.87	0.14	3.58%
#17	HC:T42_WQQGNVFSCSVMHEALHNHTYQK Deamidation N	2.39	0.15	6.39%
#18	HC:T42_WQQGNVFSCSVMHEALHNHTYQK Oxidation M	1.19	0.24	19.82%
#19	HC:T43_SLSLSPG +Lysine C-TERM	53.37	0.18	0.34%
#20	LC:T6_YASESMSGIPSR Oxidation M	0.59	0.07	11.91%

Table 1. Average, standard deviation, and %RSD of relative abundances of infliximab CQA peptides (n=10).

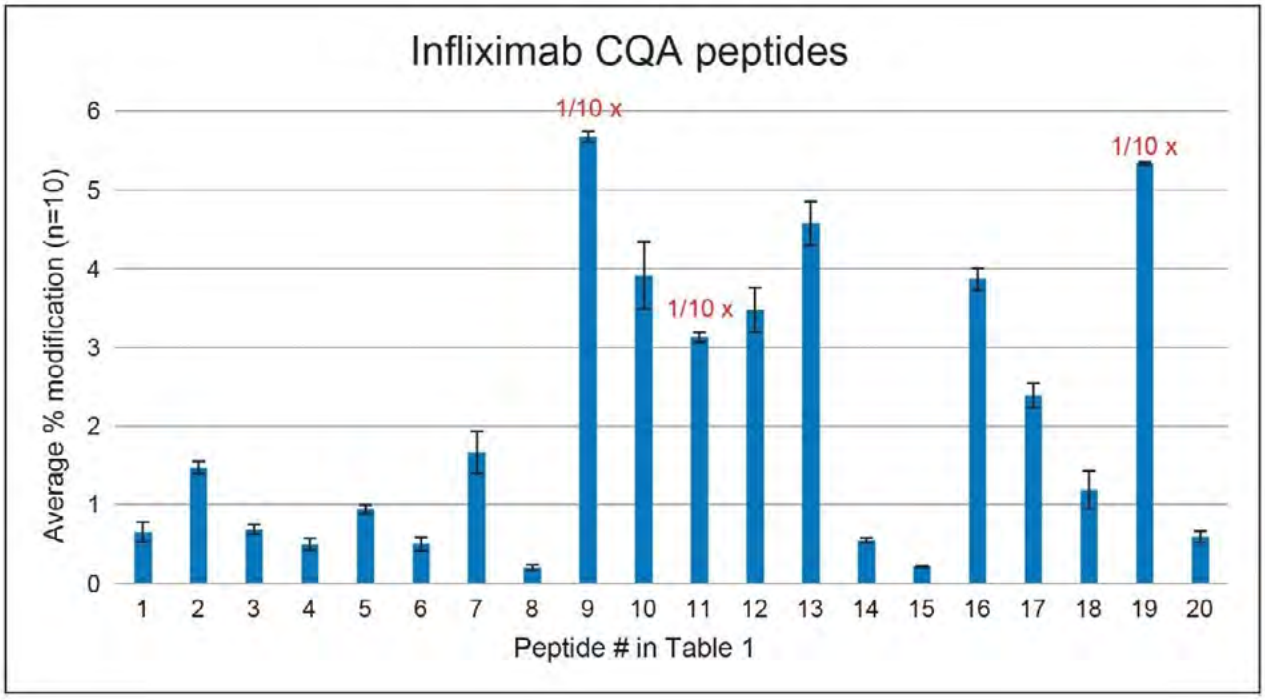

Figure 6. Average and standard deviation of relative abundances of infliximab CQA peptides (n=10). For peptide #9, #11 and #19, one tenth of the signal was plotted.

Stressed Sample Analysis

For cell line selection and process optimization, it is important that the method can detect changes of the percent modification of CQA peptides. For a fit for purpose demonstration, samples were stressed to increase oxidation and deamidation (please see EXPERIMENTAL for details). Stressed sample was also mixed with un-stressed sample (1:1 volume ratio). Figure 7A shows percent modification of several infliximab CQA peptides. The LC-MS data for the four oxidized and three deamidated CQA peptides evaluated were consistent with the 1:1 co-mixed sample having intermediate levels of degraded peptides. The sensitivity of this analysis to detect CQA changes is best exemplified in the results observed for the deamidated HC:T37 peptide NQVSLTCLVK (f in Figure 7A), which increased in abundance from 0.1% to 0.6%. The mass spectra of un-modified (top) and deamidated (bottom) form of this peptide are shown in Figure 7B. Based on the retention time, this deamidated form is likely aspartic acid that is converted from asparagine. Figure 7C shows results for the N-glycan modified and C-term heavy chain (HC) peptides. The HC C-terminal peptide can be present with or without a lysine residue at the C-terminus. As predicted, consistent percent modification was obtained for these stable peptide modifications. Overall, the results show that this automated HT CQA peptide mapping method is able to detect site-specific changes among different bioprocessing samples.

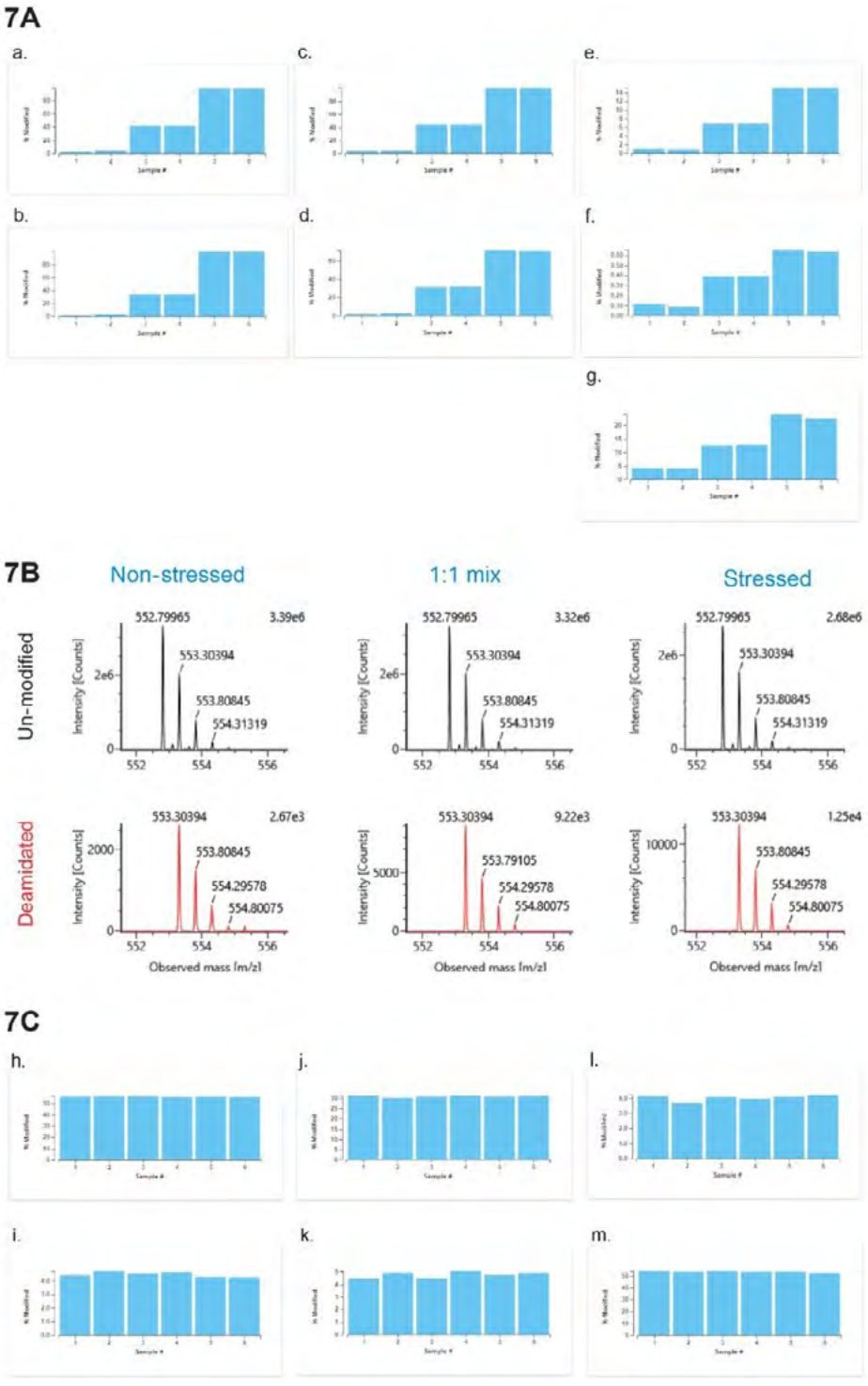

Figure 7. Percent modification of infliximab CQA peptides. #1 and #2 are un-stressed samples, #5 A. For CQA peptides that have oxidation or deamidation modification, the percent modification of the un-stressed and stressed sample 1:1 volume mix is approximately in the middle of the percent

modification of the un-stressed sample and the stressed sample. a. HC:T2 (LEES...GSMK) Oxidation M; b. LC:T6 (YASE...IPSR) Oxidation M; c. HC:T22 (DTLMISR) Oxidation M; d. HC:T42 (WQQG...TYQK) Oxidation M; e. HC:T7 (SINS...ESVK) Deamidation N; f. HC:T37 (NQVS...CLVK) Deamidation N; g. HC:T38 (GFYP...NNYK) Deamidation N.
B. Mass spectra of HC:T37 peptide NQVSLTCLVK (f in Figure 7A). Top: un-modified form (m/z: 552.81, doubly charged); Bottom: deamidated form (m/z: 553.30, doubly charged).
C. For CQA peptides that do not have oxidation or deamidation modification, the percent modification is consistent regardless of the stress state. h: HC:T26 (EEQYNSTYR) G0F N; i: HC:T26 (EEQYNSTYR) G0F-GlcNAc N; j: HC:T26 (EEQYNSTYR) G1F N; k: HC:T26 (EEQYNSTYR) Man5 N; l: HC:T26 (EEQYNSTYR) G2F N; m. HC:T43 (SLSLSPG) +Lysine C-TERM.

Conclusion

Consistent results for the relative quantification of CQA peptides using 30 µg of mAb sample were obtained using a method featuring an automated trypsin digestion protocol with the Andrew+ robotic platform and using a new trypsin, RapiZyme Trypsin, that is highly resistant to autolysis, and offers increased activity compared to other sequencing grade trypsin products.[4,5] This automated trypsin digest method is capable of generating 48 samples within four hours. In addition, the ESI-ToF BioAccord LC-MS method has a ten minutes total run time and automated LC-MS data processing is delivered by waters_connect using the Peptide MAM application software.

The general procedures outlined in this application note demonstrate the capabilities of the robotic platform and LC-MS used but can be readily optimized to meet the specific analytical requirements of other protein samples.

References

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2. Ren D., Pipes G.D., Liu D., Shih L-Y, Nichols A.C., Treuheit M.J., Brems D.N., Bondarenko P.V. An improved trypsin digestion method minimizes digestion-inducedmodifications on proteins. *Analytical Biochemistry* 392 (2009) 12–21.
3. Hanna C. M., Koza S. M., Yu Y. Q. Automated High-Throughput N-glycan Labelling and LC-MS Analysis for Protein A Purified Monoclonal Antibodies. Waters Application note. 720007854, February 2023.
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720007885, April 2023

Waters™

Application Note

Automated High-Throughput *N*-Glycan Labelling and LC-MS Analysis for Protein A Purified Monoclonal Antibodies

Caitlin M. Hanna, Stephan M. Koza, Ying Qing Yu

Waters Corporation

Abstract

N-Glycans are routinely monitored during the development of biotherapeutics because they can affect the safety and efficacy of therapeutic proteins. Waters™ automated GlycoWorks™ *RapiFluor*-MS™ sample preparation protocol is a rapid and robust method for analyzing the glycan profile of glycoproteins, but it is limited to samples prepared in a narrow concentration range in non-nucleophilic buffers. Here, we present a complementary sample preparation and analysis method that provides a reproducible profile of *N*-glycans released from Protein A-purified monoclonal antibodies (mAbs) using the Andrew+™ pipetting robot and the BioAccord™ LC-MS System. This automated diafiltration protocol can be readily adapted for samples prepared in any buffer at any concentration.

Benefits

- Rapid automated sample preparation of released and labeled *N*-glycans from 48 samples in under 3 hours followed by BioAccord LC-MS analysis of all samples in less than 4 hours
- Reproducible *N*-glycan profiles from mAbs in a wide range of sample concentrations and buffers
- A method for the high-throughput glycan critical quality attribute (CQA) assessment of affinity-purified bioreactor samples or downstream analytical samples

Introduction

N-linked glycosylation of biotherapeutics plays a key role in drug safety and efficacy due to its impact on immunogenicity, clearance rates, and effector function for antibodies.[1,2] As a result, glycosylation is closely monitored as a critical quality attribute (CQA) during drug development and production. *N*-Glycan analysis is particularly important in the development of biosimilars because the heterogeneity of different expression systems can render glycosylation difficult to control.[3] Rapid and robust methods for *N*-glycan analysis are therefore imperative for efficient development and approval of biotherapeutic drugs.

Glycosylation is typically assessed by releasing *N*-linked glycans from the protein backbone and labelling the released glycan with a chromophore for UV or fluorescence detection. Traditional methods for labelling released *N*-glycans are time-consuming, use hazardous labelling reagents, and produce labeled glycans with weak MS responses.[4] Waters GlycoWorks *RapiFluor*-MS *N*-Glycan labelling kit provides an alternative method capable of labelling released *N*-glycans within five minutes.[5] *RapiFluor*-MS label contains a quinoline fluorophore and a tertiary amine for enhanced fluorescence and MS detection. The rapid labelling is preceded by fast PNGase F deglycosylation and a quantitative HILIC-SPE cleanup procedure to facilitate immediate LC-FLR/MS analysis of released and labeled glycans.

The GlycoWorks *RapiFluor*-MS protocol has recently been adapted for automation using the Andrew+ pipetting robot and coupled to a five-minute UPLC-MS method to further increase sample throughput.[4] These high-throughput sample preparation and UPLC-MS methods were used to prepare and analyze released *N*-glycans from 48 infliximab (Remicade®) samples within eight hours. The analysis produced consistent results for critical and trace level high-mannose and sialylated glycoforms. This workflow is useful at various stages of drug development to improve throughput and productivity. However, its use for mAbs is limited to samples prepared within a narrow concentration range (0.5–3 mg/mL) in non-nucleophilic buffers due to the incompatibility of *RapiFluor*-MS with high concentrations of nucleophiles.[6]

The concentration and buffer limitations of the GlycoWorks *RapiFluor*-MS protocol can complicate its use in drug development. If, for example, Protein A affinity chromatography is used to purify a biotherapeutic mAb, buffer exchange is required prior to *N*-glycan analysis to ensure compatibility with *RapiFluor*-MS when nucleophiles

such as glycine and tris are used for elution and neutralization. To accommodate scenarios in which mAbs are prepared at low concentrations or in nucleophilic buffers, we incorporated a high-throughput diafiltration step into the automated GlycoWorks *RapiFluor*-MS protocol. The diafiltration step is compatible with both Vacuum+™ and Extraction+™ devices used with the Andrew+ liquid handler and can concentrate or buffer exchange up to 48 samples prior to *N*-glycan release and labelling.

After the automated sample preparation, a fast UPLC-MS method was optimized for high-throughput analysis using the BioAccord LC-MS System controlled by waters_connect™ informatics software. Figure 1 illustrates the entire analytical setup used for the high-throughput released glycan assay.

Figure 1. Workflow for the automated preparation of labeled N-glycans from samples at low concentration or in nucleophilic buffers using the Andrew+ pipetting robot and rapid sample analysis using a five-minute LC-MS method on the BioAccord LC-MS System.

Experimental

The GlycoWorks sample preparation protocol was adapted from the QC/Automation-friendly protocol in Application Note 720005506 to accommodate diafiltration and automation using the Andrew+ pipetting robot. Prior to automation, GlycoWorks reagents (p/n: 186008840 <https://www.waters.com/nextgen/global/shop/application-kits/186008840-glycoworks-rapid-deglycosylation-kit---4-x-24.html> and 186007989 <https://www.waters.com/nextgen/global/shop/application-kits/186007989-glycoworks-rapifluor-ms-label-96-sample.html>) were prepared as described below. Three vials of *Rapi*Gest™ Surfactant (10 mg) were reconstituted with 200 µL of GlycoWorks Rapid Buffer and 135 µL 18.2 MΩ water each,

combined, and diluted with 1 mL PBS (pH 7.4, 1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic, 155 mM sodium chloride; Thermo Fisher Scientific, p/n: 10010031). Four vials of GlycoWorks Rapid PNGase F enzyme were reconstituted with 270 µL 18.2 MΩ water each and combined. Three vials of GlycoWorks *RapiFluor*-MS (23 mg) were dissolved in 280 µL anhydrous DMSO each and combined. Additionally, 20 mL of 25 mM HEPES, 50 mM NaCl (pH 7.9) was prepared in 18.2 MΩ water. The Andrew+ pipetting robot equipped with Extraction+ was used to perform diafiltration, deglycosylation, labelling, and HILIC SPE cleanup (p/n: 186008747 <https://www.waters.com/nextgen/global/shop/application-kits/186008747-glycoworks-spe-reagents-automation.html> and 186002780 <https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186002780-glycoworks-hilic--elution-plate.html>). Diafiltration was carried out using a Pall AcroPrep™ Advance Omega 10 K MWCO Filter Plate. For this study, anhydrous DMSO (Thermo Fisher Scientific, p/n: D12345) was substituted for DMF in the GlycoWorks labelling protocol and ACS grade DMSO (Fisher Scientific, p/n: D128-500) was substituted for DMF in the cleanup protocol. DMSO was found to produce comparable results when used in place of DMF for these procedures in separate experiments (data not shown) while providing lower exposure risks.

The mAb used in this study was Kanjinti™ (trastuzumab-anns), a biosimilar of Herceptin™. Four samples of trastuzumab-anns were analyzed: 1 mg/mL prepared in PBS, 1 mg/mL prepared in 100 mM glycine, 100 mM tris buffer (pH 6.8) as a mock Protein A-purified sample, 1 mg/mL prepared in PBS and Protein A-purified, and 1 mg/mL spiked into clarified non-transfected CHO cell media (NTM) and Protein A-purified. The NTM was prepared by Syd Labs, Inc.. Briefly, 6 x 106 non-transfected CHO-K1 cells/mL were seeded in a spinner flask on day one and were incubated in 120 mL culture media. On days two through 15, 100 mL of spent media was collected from the flask and 0.2 µm filtered. All collected media, with an average cell viability of approximately 90%, was then pooled and stored at 4 °C.

All data were collected using the BioAccord LC-MS System under the control of waters_connect software. Data processing was performed using the embedded Accurate Mass Screening workflow in the software. A glycan database was used for glycan assignment; assignment was based on the molecular weight and retention time.

Reagent	Automated diafiltration protocol (new protocol)	QC/automation-friendly protocol (standard protocol)[7]
Diafiltration, deglycosylation, and labelling		
mAb	20 µL (1 mg/mL)	10 µL (1.5 mg/mL)
25 mM HEPES, 50 mM NaCl dilution buffer (pH 7.9)	200 µL	NA
*Rapi*Gest SF	20 µL (1:1 PBS:*Rapi*Gest SF)	10 µL
GlycoWorks Rapid PNGase F Enzyme	12 µL (5:1 GlycoWorks Rapid PNGase F Enzyme:H_2O)	10 µL
GlycoWorks *Rapi*Fluor-MS Solution in DMSO or DMF	10 µL in anhydrous DMSO (ThermoFisher Scientific, p/n D12345)	10 µL in anhydrous DMF (GlycoWorks Kit)
Cleanup		
Acetonitrile dilution	300 µL (2 × 150 µL)	300 µL (2 × 150 µL)
Water (condition)	200 µL	200 µL
Equilibration buffer (85:15 acetonitrile:water)	200 µL	200 µL
Wash buffer (1% formic acid in 9:1 acetonitrile:water)	4 × 290 µL	4 × 290 µL
Elution buffer (200 mM ammonium acetate in 95:5 acetonitrile:water)	90 µL (3 × 30 µL)	90 µL (3 × 30 µL)
Sample diluent (21:10 acetonitrile:DMSO or DMF)	310 µL (2 × 155 µL, DMSO, Fisher, p/n D128–500)	310 µL (2 × 155 µL, DMF)
Final volume	400 µL	400 µL

Table 1. Volumes for the automated and manual protocols used for this application note. Refer to experimental section above for more details on reagent preparation.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Sample collection:	Waters QuanRecovery™ 700 µL 96-well plate p/n: 186009184

Column:	ACQUITY UPLC Glycan BEH™ Amide Column p/n:186004742 (1.7 µm, 2.1 mm x 150 mm, 130 Å)
Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	15 µL
Mobile phase A:	50 mM Ammonium Formate, pH 4.4 (LC-MS grade, p/n:186007081)
Mobile phase B:	Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.0	25	75	Initial
3.50	1.0	42	58	6
3.55	1.0	60	40	6
3.75	1.0	60	40	6
3.80	1.0	25	75	6
5.00	1.0	25	75	6

ACQUITY RDa Detector Settings

Mass range:	400–7000 *m/z*
Mode:	ESI+

Sample rate:	10 Hz
Cone voltage:	45
Desolvation temperature:	300
Capillary voltage:	1.50 kV
Informatics:	Accurate Mass Screening using a glycan database

Data Management

Chromatography software: waters_connect

Results and Discussion

The Automated Diafiltration GlycoWorks RapiFluor-MS Protocol

Initially, attempts were made to release and label *N*-glycans directly off Protein A-bound mAbs. mAbs were bound to Protein A magnetic beads, washed, and treated with PNGase F enzyme to release *N*-glycans directly off the surface-bound mAb. However, automation of this method yielded inconsistent results with low recovery. Instead, automated diafiltration using a 96-well 10 K molecular weight cutoff (MWCO) plate was used to buffer exchange Protein A-purified mAbs. *N*-Glycan release and labelling was then performed following a modified GlycoWorks *RapiFluor*-MS protocol with high recovery and precision (Figure 2).

For this procedure, the Andrew+ pipetting robot equipped with Extraction+ is placed inside a chemical hood (dimensions: 1.83 m W x 0.85 m D; 1.28 sq. m.; Model: HBBV6, Lab Crafters Inc.). Due to space constraints within the hood, a two-step protocol is employed wherein Step 1 executes diafiltration, deglycosylation, and labelling and Step 2 executes the purification. Figure 2 displays a flow diagram outlining the automated diafiltration GlycoWorks *Rapi*Fluor-MS protocol.

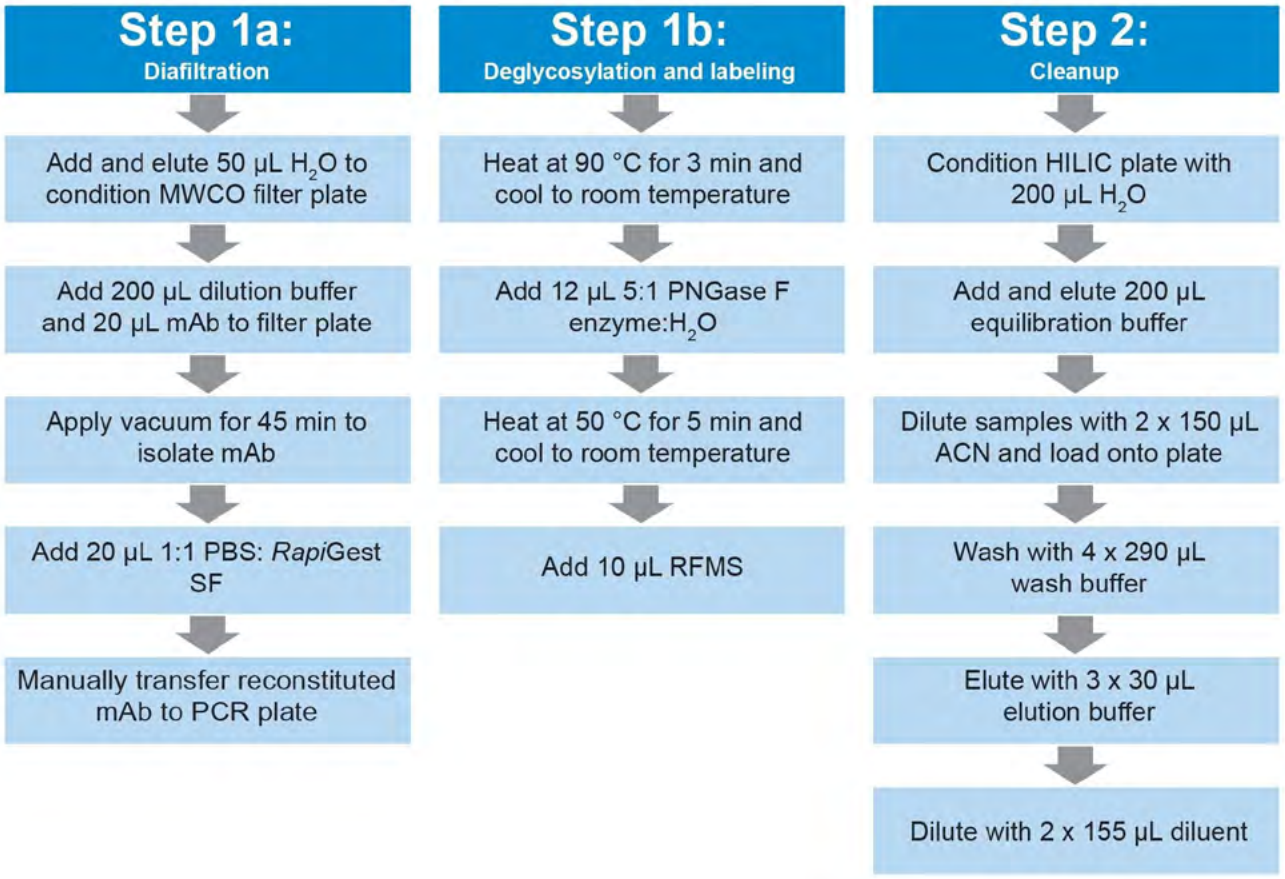

Figure 2. Flow diagram outlining the automated diafiltration GlycoWorks RapiFluor-MS protocol for rapid preparation of labeled N-glycans from mAb samples at low concentration or in nucleophilic buffer.

Step 1 begins with diafiltration; a HEPES dilution buffer is added to the MWCO plate followed by addition of mAb. In this procedure, the mAb load is increased relative to the standard QC/Automation-Friendly protocol to account for sample loss during diafiltration.[7] The volume of mAb can be adjusted to reach the desired sample load if lower or higher sample concentrations are used. Next, the Andrew+ pipetting robot isolates the sample on the MWCO plate via vacuum filtration and reconstitutes with a 1:1 PBS:*Rapi*Gest SF solution. Incorporation of PBS into the reconstitution solution is necessary to ensure complete sample digestion. Transfer between the MWCO plate and a PCR collection plate requires user intervention due to Andrew+ limitations. To circumvent arduous manual pipetting, the MWCO plate can be inverted over the PCR collection plate and centrifuged at 500 RPM for two minutes to transfer samples to the PCR collection plate for further workup. It is important to note that this centrifugation procedure will reverse the order of the samples in their respective columns.

Digestion proceeds with heating for three minutes at 90 °C to denature the mAb. *N*-Glycans are then released by adding 5:1 GlycoWorks Rapid PNGase F Enzyme:H_2O and heating for five minutes at 50 °C. This procedure uses an increased volume of diluted PNGase F enzyme to account for solvent loss during the uncapped heating steps (Table 1). Finally, *N*-glycans are labeled with *Rapi*Fluor-MS (RFMS) and purified in Step 2 following the standard QC/Automation-Friendly protocol using DMSO in place of DMF.

Comparison of the Automated Diafiltration Protocol and the Standard Manual Protocol

To assess the efficacy of the new automated diafiltration GlycoWorks *RapiFluor*-MS protocol, *N*-glycans from the mock Protein A-purified mAb were released, labeled, and analyzed via UPLC-FLR. This sample mimics the solution of a Protein A-purified mAb where both tris and glycine are nucleophilic and will compromise the labelling step if their concentrations are not significantly reduced prior to analysis. A representative chromatogram of trastuzumab-anns *N*-glycans obtained from this procedure is shown in Figure 3. For comparison, the chromatogram of *N*-glycans manually released and labeled from trastuzumab-anns prepared in PBS using the standard QC/Automation-Friendly protocol is also shown in Figure 3. The *N*-glycan profile obtained from the automated diafiltration protocol exhibits minimal deviation from the *N*-glycan profile obtained from the manual protocol.

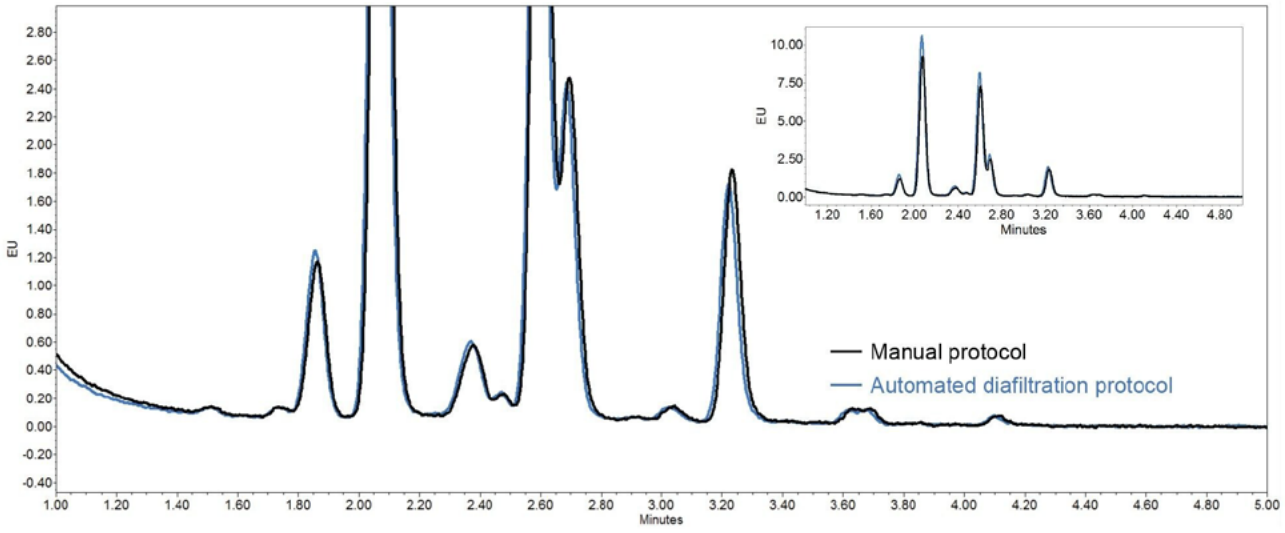

Figure 3. UPLC-FLR chromatograms of released and labeled N-glycans from trastuzumab-anns using the manual protocol (black trace) and the automated diafiltration protocol (blue trace). Zoomed chromatograms are y-axis normalized; full-scale chromatograms are absolute and shown in the inset.

In Figure 4, a comparison of the relative and total FLR peak abundances of selected *N*-glycans (FA2, FA2G1, FA2G2) released using the automated diafiltration protocol and the manual protocol (four samples were analyzed in each scenario) is shown. Both protocols yield comparable total and relative *N*-glycan abundances. The total *N*-glycan recoveries obtained from the manual protocol were higher than those obtained from the automated diafiltration protocol because the average recovery of the automated diafiltration protocol is 73%. Sample losses during the diafiltration process may be due to protein adsorption to the MWCO filter plate or incomplete liquid transfer during transfer to the PCR collection plate. As a result, a larger initial sample load is used in the automated diafiltration protocol (20 µg) relative to the manual protocol (15 µg). The final buffer exchanged sample prepared using the diafiltration protocol was comparable in concentration and produced comparable relative *N*-glycan abundances in comparison to the control sample prepared using the manual protocol.

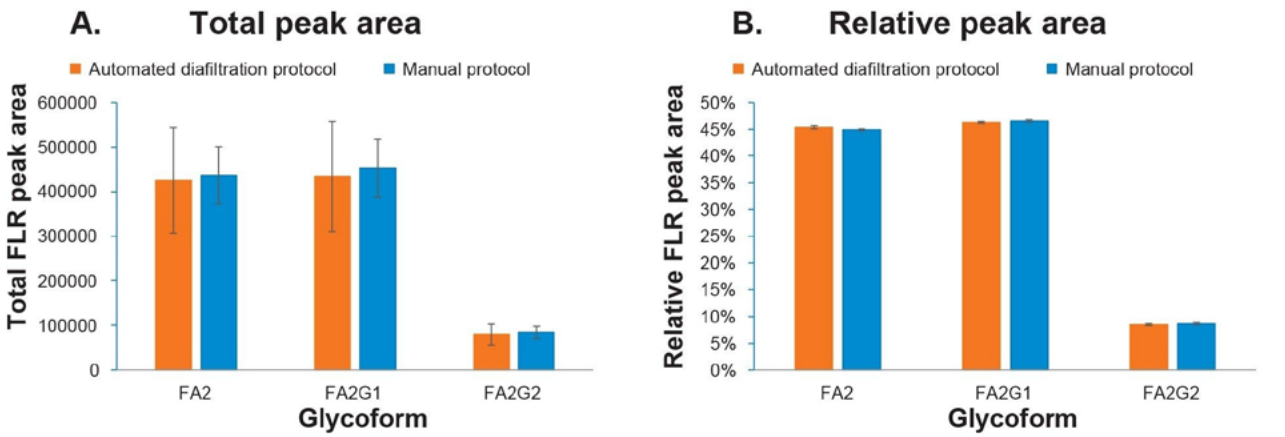

Figure 4. Comparison of UPLC-FLR relative and total N-glycan profiles of trastuzumab-anns using the automated diafiltration and manual protocols. Error bars show the 95% confidence interval (n=4). Both protocols yield comparable N-glycan profiles.

N-Glycan Analysis of Protein A-Purified mAb

The automated diafiltration protocol was used to release and label *N*-glycans from Protein A-purified trastuzumab-anns. Briefly, the 48-sample protocol was employed to release and label *N*-glycans and eight samples were selected for subsequent UPLC-MS analysis. Two sample types were used: mAb purified from PBS and mAb purified from NTM (non-transfected media). The released and labeled *N*-glycans were analyzed with a five-minute UPLC-MS method. Figure 5 displays the *N*-glycan profiles obtained from this procedure; both PBS and NTM samples yield similar chromatograms.

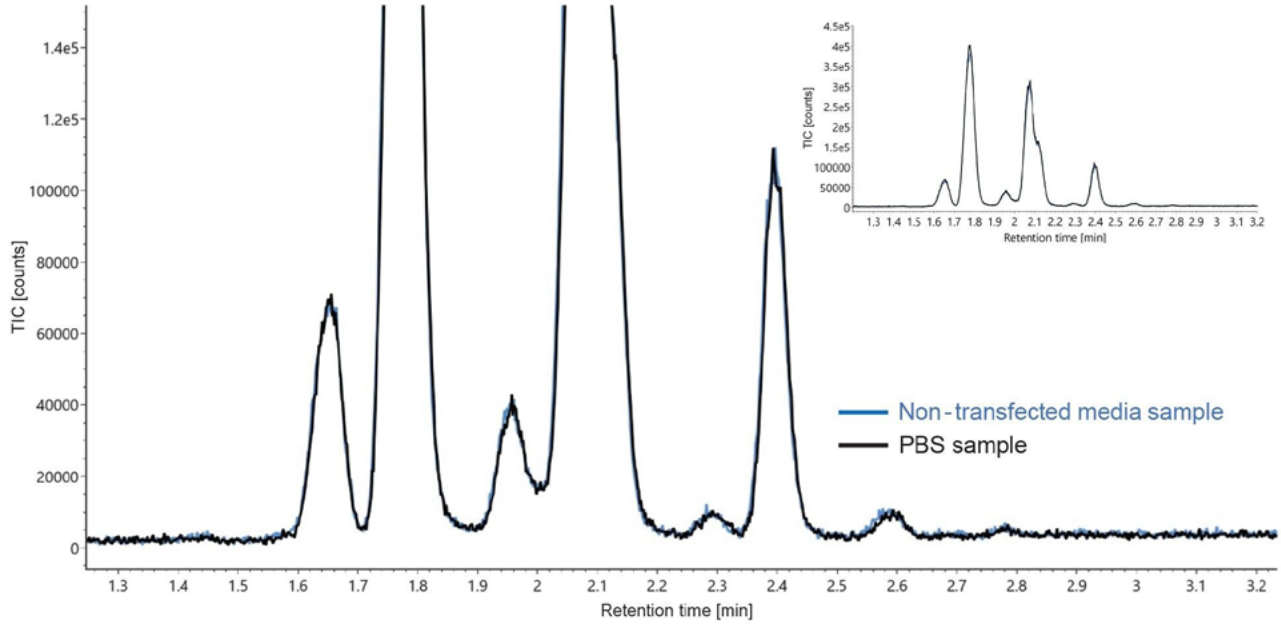

Figure 5. UPLC-MS total ion chromatograms of released and labeled N-glycans from trastuzumab-anns Protein A purified from PBS (black trace) and non-transfected media (blue trace) and prepared using the automated diafiltration protocol. Zoomed chromatograms are y-axis normalized; full-scale chromatograms are absolute and shown in the inset. The total run time is five minutes per sample.

The UPLC-MS data was analyzed in waters_connect using the Accurate Mass Screening workflow; 15 glycans of interest were imported from a Glycan Scientific Library to create a targeted list. This workflow enables facile analysis of highly abundant glycans and potentially immunogenic glycans that may be present in lower quantities (*i.e.* sialylated, high-mannose, or afucosylated glycans). The reproducibility of the protocol's glycan recovery was assessed using the percent relative standard deviation (%RSD) of the FA2 glycoform abundance across the eight analyzed samples. The %RSD was calculated to be 13%, demonstrating suitable repeatability of the diafiltration method.

The MS-derived *N*-glycan profiles of the mAb purified from PBS and NTM are shown in Figure 6. The PBS and NTM samples exhibit very similar amounts of the three most abundant glycans (FA2, FA2G1, and FA2G2) and a similar percentage of afucosylated glycans (Figure 6A). Three low-abundance glycans were targeted: high-mannose M5, sialylated FA2G2S1, and bisecting FA2BG1. The bisecting and sialylated glycoforms were present in similar abundances for both PBS and NTM samples. However, the M5 glycoform is more abundant in the NTM

samples than the PBS samples, as shown in Figure 6B and confirmed by the student's t-test (95% CI). The increase in M5 abundance in the NTM samples likely arises from trace amounts of host cell glycoproteins remaining in the samples after Protein A purification. Notably, this UPLC-MS screening method enables comparison of glycan profiles between multiple sample types, even for glycoforms present at very low abundances (<1%).

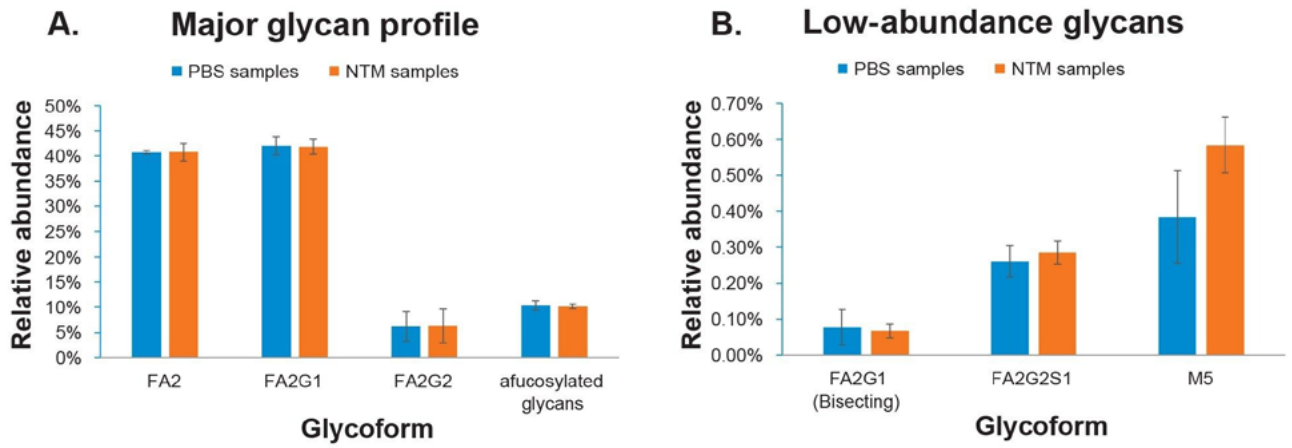

Figure 6. Comparison of UPLC-MS N-glycan profiles of trastuzumab-anns purified from PBS and NTM. Error bars show the 95% confidence interval (n=4). The method enables comparison of N-glycan profiles between multiple sample types for high- and low-abundance glycans.

Conclusion

Glycosylation is closely monitored as a CQA during drug development due to its impact on drug safety and efficacy. Rapid and robust methods for glycan analysis are invaluable in the research, development, and approval of biotherapeutic drugs. Here, we expand the capabilities of Waters' GlycoWorks *RapiFluor*-MS protocol to directly accommodate Protein A-purified mAbs. The automated diafiltration procedure enables facile buffer exchange or sample concentration with high recoveries in a high-throughput, automated format. In addition, the automated diafiltration protocol yields *N*-glycan profiles that are consistent with those obtained using the manual QC/Automation-Friendly GlycoWorks *RapiFluor*-MS protocol. When coupled with a five-minute UPLC-MS method, the protocol can prepare released and labeled *N*-glycans from 48 Protein A-purified samples in

three hours, deliver reproducible UPLC-MS glycan profiles of 48 mAb samples in four hours, and quantify both abundant and trace-level *N*-glycans. Importantly, this method may be adapted for samples prepared at any concentration in any buffer, regardless of RFMS-compatibility, and has potential use for other glycoproteins.

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Featured Products

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

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

ACQUITY RDa Detector <https://www.waters.com/waters/nav.htm?cid=135077027>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

720007854, February 2023

BIOPROCESS WALK-UP SOLUTIONS

A streamlined interface and dedicated workflows with automated sample preparation and LC-MS analytics

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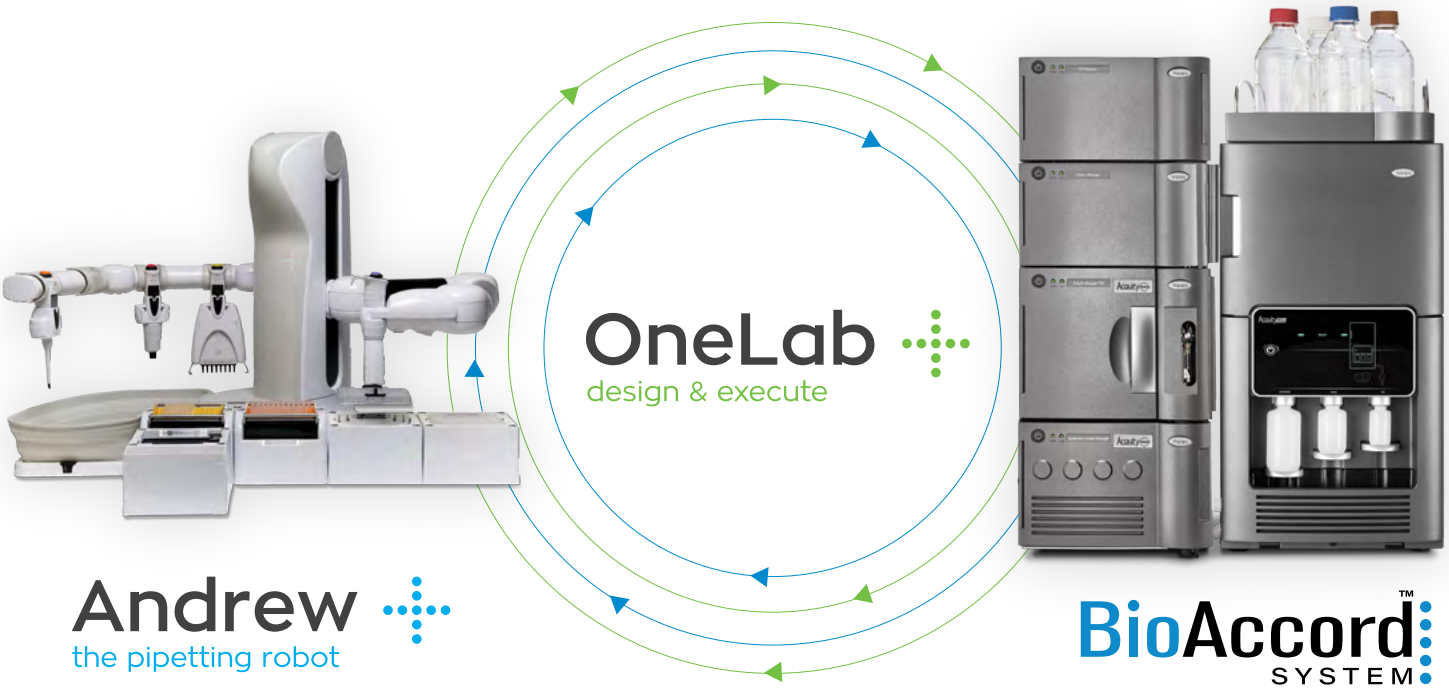
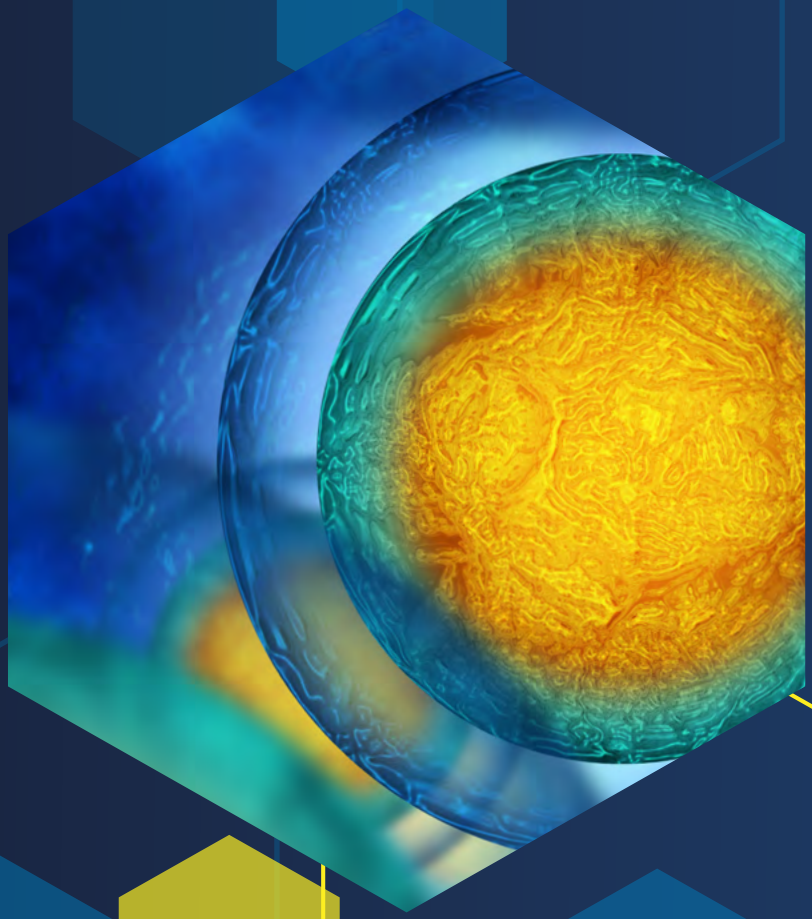


Automation

Application Notes



Waters™

Application Note

Simplifying Bioreactor In-Process Monitoring with Waters Bioprocess Walk-Up Solutions

Yun Wang Alelyunas, Elizabeth Embrey, Lindsay Collins, Adrien Pegaz-Blanc, Guillaume Mignard, Mark D. Wrona

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

A walk-up solution that will enable bioprocess engineers to collect attributes easily and rapidly for process related monitoring and optimization is described. The Waters™ bioprocess walk-up solutions afford simple and single point access for automated sample preparation and data acquisition. It manages sample information coming from bioreactors, creates a LC-MS sample run list, executes automated sample preparation and, initiates analysis and intact protein data processing. Automated sample preparation and data acquisition for both intact protein and cell culture media is achieved back-to-back on a single system, using a predefined workflow, to maximize ease of use and minimize human intervention.

Benefits

- Walk-up system for single access to conduct automated sample preparation and LC-MS data acquisition
- Ease-of-use in obtaining intact protein and cell culture media sample data using well-established methodologies
- Automated and user-friendly sample preparation and LC-MS platform to obtain high quality data in process and product related monitoring including intact protein analysis, cell culture media analysis and many other product analytics when desired

Introduction

Cell culture or microbial based inoculation for protein production is a time-consuming process, typically lasting about two weeks. It is increasingly desirable to routinely monitor critical process and product attributes such as changes in nutrient profiles and high level glycoform information for the drug substance. Monitoring feed and metabolite components will aid in designing or optimizing feed strategies for nutrient replenishment; detect and quantify the formation of toxic metabolites; and can be used to elucidate what reactor conditions are conducive to optimal cell growth and bioproduction. Higher monitoring frequency of the protein formation in the process enables determination of most favorable cell culture duration; yield optimization; and most importantly ensure that the drug substance quality is within the specification criteria. Moving these assays and technologies in the hands of bioprocessing groups facilitates faster access to more in-depth information for the process engineers. However, to enable non-MS experts to perform routine applications of this technology, we need to ensure the systems are easy to use and quickly provide informative results and reporting outputs. In previous Waters application notes, we have published automated sample preparation and LC-MS analytical methods for both protein and culture media analysis.[1] In this technology brief, the bioprocess walk-up solutions are described as a step forward towards automated LC-MS analyses in bioprocess laboratories. A single user interface, based on OneLab™ software, provides a seamless connection between sample input, automated sample preparation and LC-MS data acquisition and analysis (Figure 1). In addition, users of Sartorius Ambr® 15 and 250 High Throughput bioreactors can utilize the data interface to automatically upload all sample information and return their results back to the Ambr software for further evaluation. The interface, combined with predeveloped analytical workflows for critical product and process quality attributes, are designed to allow bioprocess engineers to perform assays generating high quality data with minimal training or knowledge in LC and MS technologies.

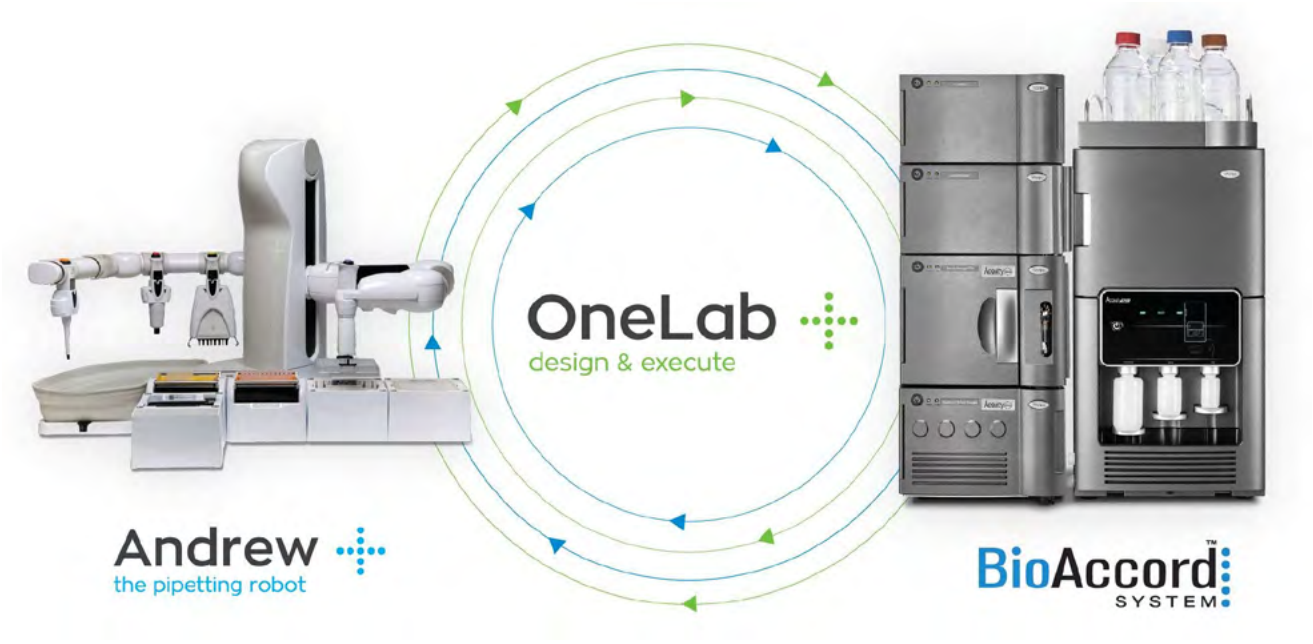


Figure 1. The Waters bioprocess walk-up solutions provide a single access OneLab software platform for automated sample preparation and LC-MS data acquisition and reporting.

Results and Discussion

I. A General Description of Waters Bioprocess Walk-Up Solutions

An integrated walk-up system intended for bioprocess engineers to automatically carry out sample preparation and LC-MS data acquisition has been developed. The Waters bioprocess walk-up solutions are based on OneLab software that enable bioprocess engineers to initiate automated sample preparation using the Andrew+™ Pipetting Robot and subsequent LC-MS data acquisition and processing. This walk-up solution is designed to ease the burden of routine process monitoring that is typically carried out by core analytical labs and allow bioprocess engineers, who are not experienced LC-MS users, to automatically retrieve this data at point of need in their lab. A top-level flow chart of the solution is shown in Figure 2. Specifically, the OneLab LC-MS interface will perform the following:

1. Select test protocol, ***e.g.,*** intact mass and/or cell culture media analysis. The protocol is a collection of methods used for sample preparation using Andrew+ Pipetting Robot and LC-MS analysis/reporting using the BioAccord™

2. Enter sample information from the bioreactors. For users running Ambr 15 or 250 HT systems, a OneLab file watcher will automatically import sample information files
3. Enter analysis name and associated information, by clicking “continue”, it will initiate sample preparation using the Andrew+ Pipetting Robot (Figure 2C)
4. When the sample preparation is completed, it instructs the user to place the sample in the BioAccord LC-MS System. Upon clicking “continue”, it will automatically start BioAccord data acquisition (Figure 2D)
5. Access from OneLab for users to activate viewing of intact mass results and the status of their cell culture media data acquisition

In the background, the OneLab interface interacts with waters_connect™ software for BioAccord instrument control and data acquisition. The current OneLab interface will perform intact mass and culture media analyses back-to-back in a single run through column switching using a two-column compartment column manager. A description of Andrew+ sample preparation and LC-MS method using the BioAccord LC-MS System have been described in a separate application note.[1] A summary list of consumables needed for sample preparation is shown in the Appendix.

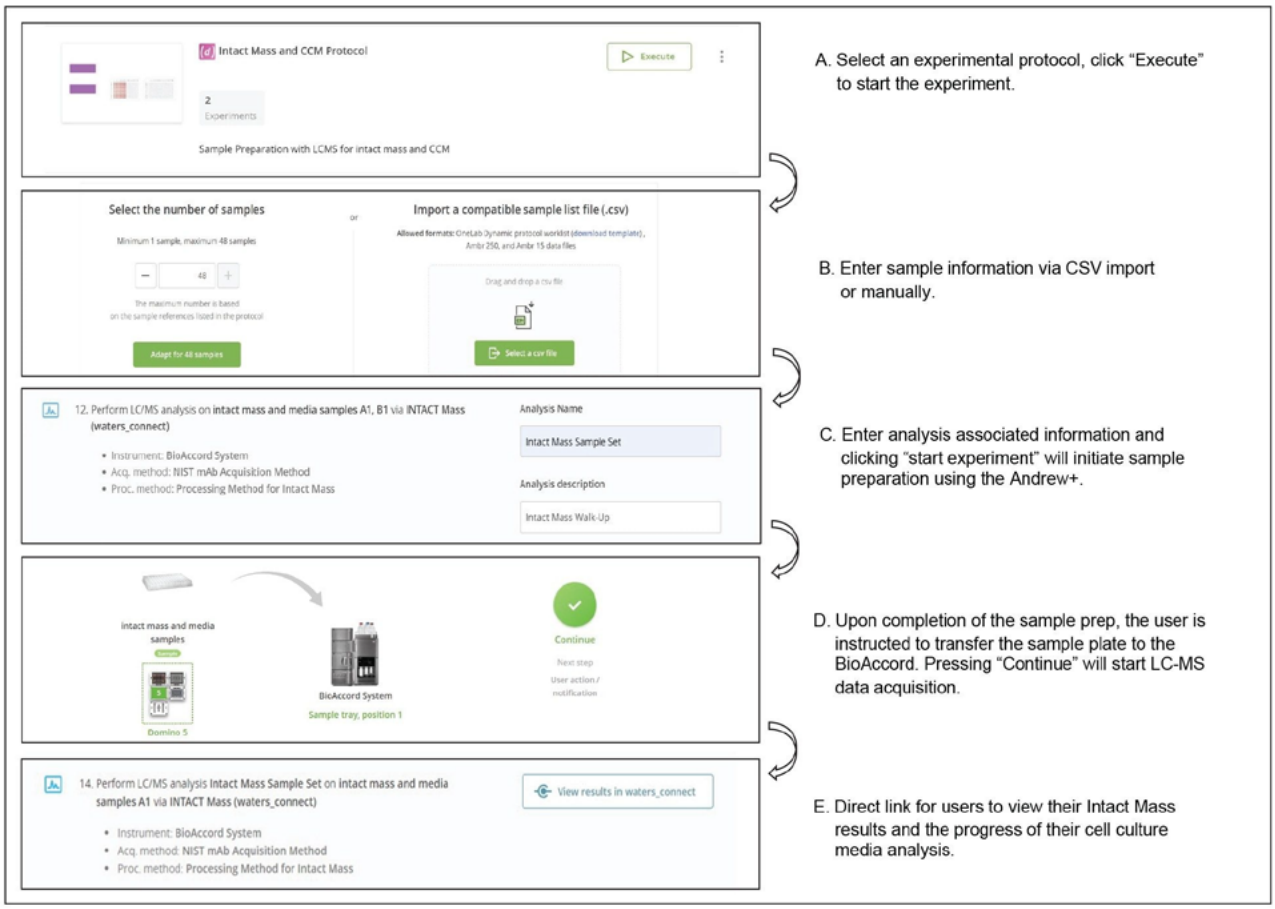


Figure 2. Top level flow chart for the bioprocess walk-up solution using single access in the OneLab software platform.

II. Intact Protein Analysis Results

For intact protein analysis, the acquired data is automatically processed using the Intact Mass™ App. The Intact Mass App is an easy-to-use dedicated data processing application that will automatically process raw spectral data (MS deconvolution) and then perform a determination of protein modifications (major glycoforms) and molecular weight identification (pass/fail) of monoclonal antibody (mAb) biotherapeutics. Detailed description of the Intact Mass App can be found in a previously published Waters application note.[2] Figure 3 is the resulting display for an analyzed mAb sample. In the top-level dashboard display, all samples are visualized with a color-coded system with green showing passing status which indicates that the sample identification meets criteria set

in the analysis method. By clicking on each sample, sample level information is displayed such as LC-MS chromatogram, protein modification result table, observed mass spectrum, and deconvoluted spectrum (Figure 3 B-D). In data export, the results are exported in a format that can be readily read into Ambr software or other third-party software for further data integration, viewing, and analysis. An example of Ambr data display showing an overlay of %modifications as a function of incubation time and bioreactors can be found in a previously published application note.[1]

Figure 3. Display of Intact Mass App in the waters_connect™ platform. (A) Dashboard view showing injections and status of mAb detection, green color indicates the sample has passed detection criteria set in the method. (B) Summary table of modifications observed for the mAb produced including r.t., MS response, %modifications and other info. (C) Observed TUV chromatogram, which is used for peak detection. The first peak shown is light chain (LC) of the mAb, second peak is intact mAb. (D) Observed spectrum of the intact mAb peak, showing charge state distribution. (E) Deconvoluted spectrum of intact mAb, exhibiting the expected four major glycan modifications.

III. Cell Culture Media Analysis

For cell culture media nutrient and metabolites analysis, the acquired data is currently processed using the UNIFI™ screening application and workflow in the waters_connect software. The screening workflow performs

small molecule quantitative and qualitative analysis. In this example, for process monitoring, choline and its metabolite, choline phosphate, are monitored for each sampled media solution during the study (Figure 4). A detailed description of cell culture media analysis using UNIFI can be found in a previously published application note.[3] The analysis can be easily expanded to additional cell culture components and identified metabolites. Additional tools to mine the data (previously described)[3] to enable a deep understanding of compounds present in the media solution and relationships to metabolic and cellular processes through multivariate data analysis (MVDA) approaches.

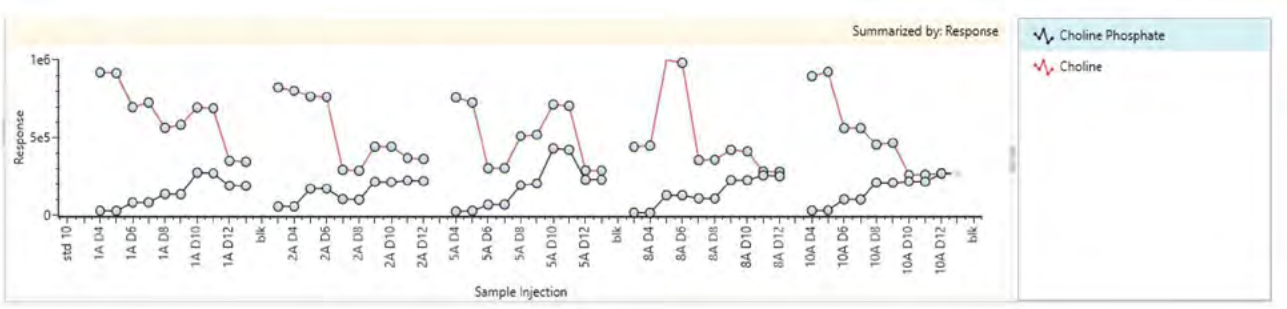


Figure 4. Overlaid trending plots of choline and choline phosphate. X-axis is bioreactor name and sampling date, y-axis is LC-MS response. Red line is choline, black line is choline phosphate.

Conclusion

Waters bioprocess walk-up solutions provide a simple and powerful interface for automated sample preparation using the Andrew+ Pipetting Robot and initiating LC-MS data acquisition. The combination of the Andrew+ Pipetting Robot and the BioAccord LC-MS System provides the capability to rapidly process samples from bioreactor systems and easily provide high quality results with minimal user interaction. Highlights and capabilities include:

- A single access and easy to use interface in OneLab, with predeveloped workflows, that will initiate sample preparation and subsequent data acquisition in a walk-up manner
- Automated sample preparation and sample information transfer to maximize productivity and minimize human error
- Acquisition of both intact mass of protein and cell culture media data back-to-back based on column switching using a two-column compartment column manager

- Seamless collection of protein glycoprofiling and cell culture media composition to aid process monitoring and understanding
- Compact and user friendly BioAccord LC-MS System producing excellent data quality to support the development of more robust processes

In conclusion, Waters bioprocess walk-up solutions will enable process engineers to obtain high quality data easily and routinely on their own to aid in process monitoring and optimization.

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Appendix

Parts description	Part no.	Comments
Dominos and lab ware		
Tip insertion system domino (2)	186009612	Holding 10 µL to 1200 µL optifit non-filtered tips
Microplate domino (2)	186009600	For 350 µL 96 well plate, one each for source and destination plate
8-channel pipette reservoir domino	186009613	Holding two integra 10 mL multichannel reservoir
2 mL HPLC vial rack domino	186010091	Holding 48-well HPLC vial rack, used for standard preparation
Solvent reservoir	Integra Biosciences P/N 4332	10 mL multichannel reagent reservoirs
350 µL 96-well sample collection plate	186002643	Round well, polypropylene, 100/pk
Polypropylene cap mat round well for 96-well plate, 50/pk	186002483	
Polypropylene 12 × 32 mm screw neck vial, 700 µL (no cap)	186005219	
48-well vial rack	700011047	
Pipette and tips		
8-channel Andrew Alliance Pipette, 0.2–10 µL	186009768	
8-channel Andrew Alliance Pipette, 10–300 µL	186009607	
Single-channel Andrew Alliance Pipette, 0.2–10 µL	186009769	
Single-channel Andrew Alliance Pipette, 10–300 µL	186009606	
Pipette adaptor single channel	186009590	
Pipette adaptor multi channel	186009591	
BH Tip 0.1–10 µL, refill (10 × 96) Pk10	700013293	
BH Tip 350 µL, refill (10 × 96) Pk10	700013297	

Summary of consumables used by Andrew Alliance system for intact and media sample and standard preparations.

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

ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>

UNIFI Scientific Information System <https://www.waters.com/134801648>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

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