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Nota de aplicación

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

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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.

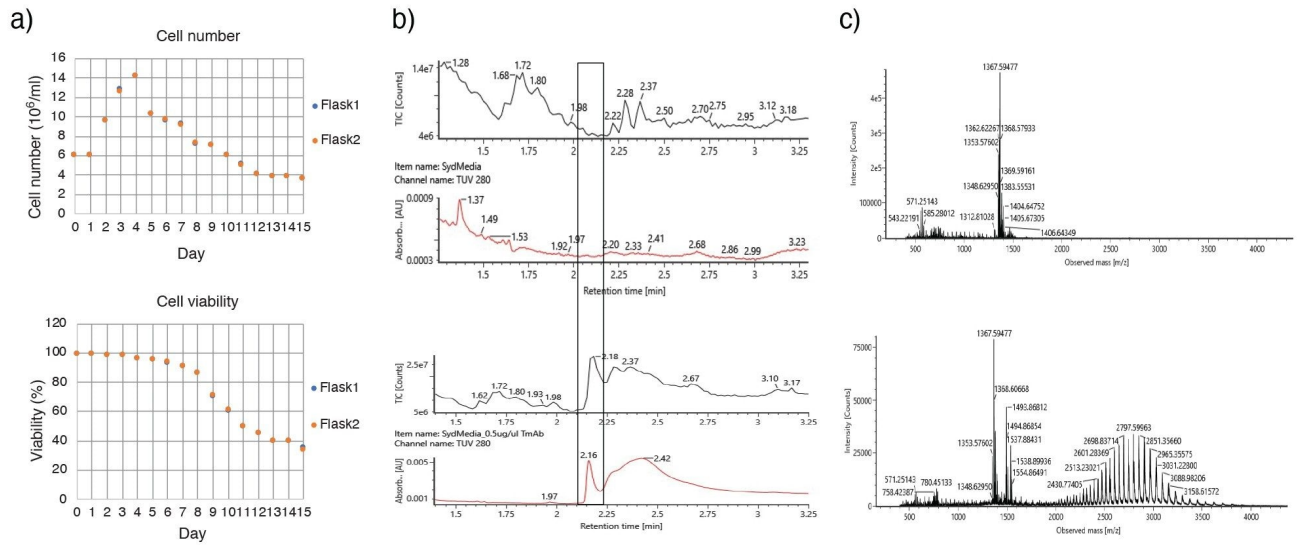


Figure 1. a) Cell count, and viability of non-transfected CHO cells cultured for 15 days. b–c) Direct LC-MS analysis (b: TIC traces, c: MS spectra) of CHO cell culture media (top) and after spiked addition of trastuzumab (bottom).

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.

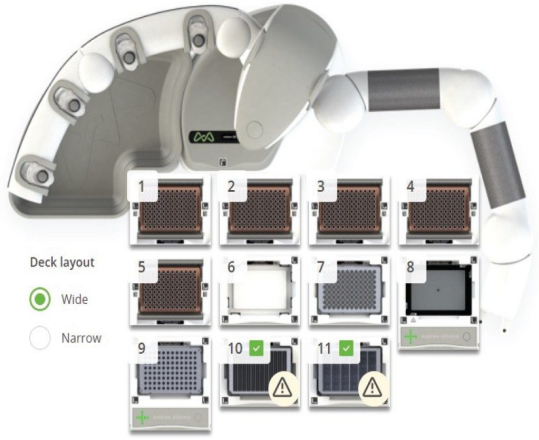
Afterwards, an aliquot of 20 µL purified mAb (with <0.5 µg/µL concentration) were digested and the disulfide bonds reduced to yield three mAb subunits (Fc/2, light chain and Fd') by adding 10 µL FabRICATOR (Ides) at 2 units/µL and 30 µL dithiothreitol (DTT) at 40 mM to each sample and incubating for 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).

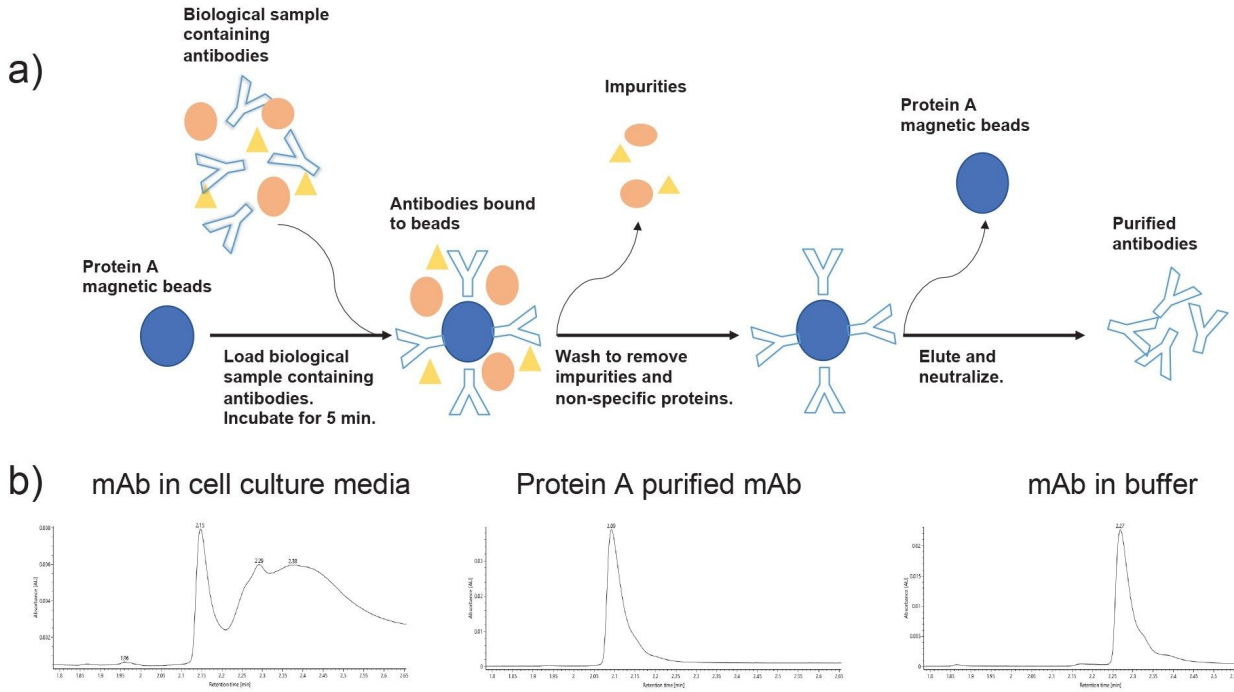


Figure 3. a) Schematic of antibody purification using Protein A-coupled magnetic beads. b) Chromatograms of mAb in cell culture media, after Protein A purification and mAb In buffer.

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).

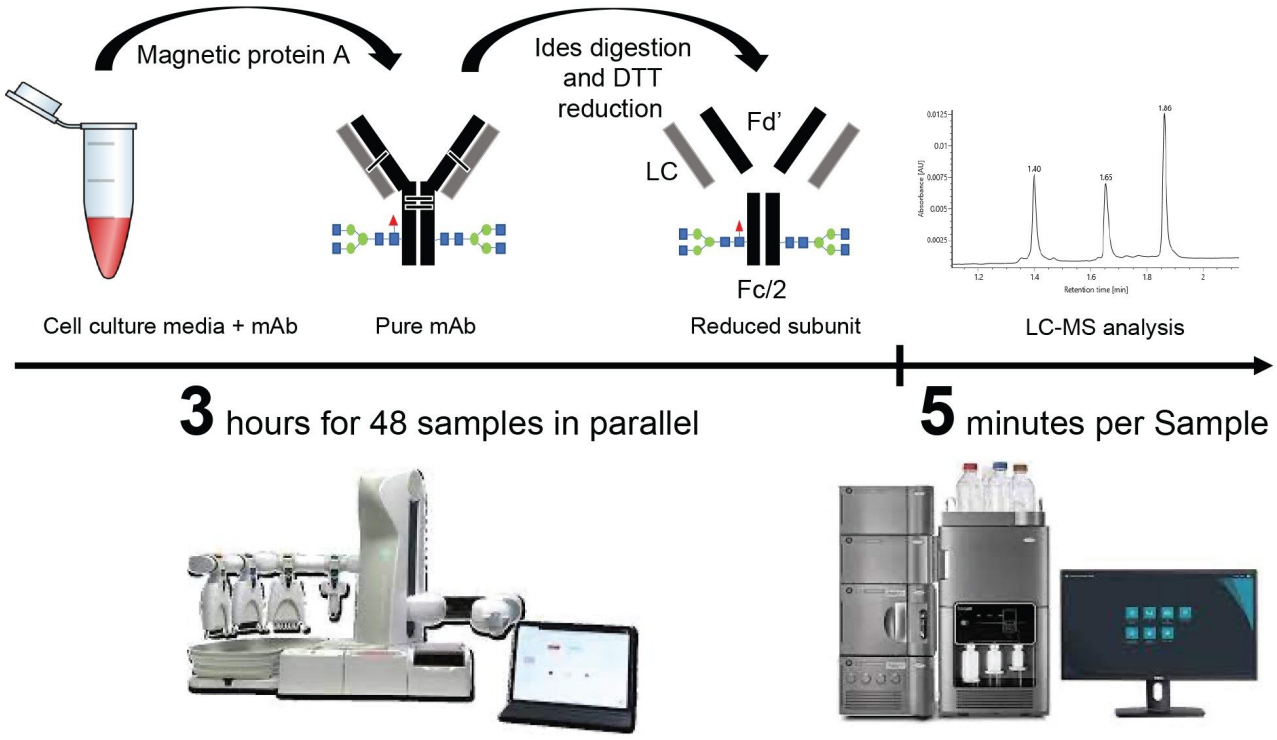


Figure 4. Workflow of automated antibody purification and digestion with Andrew+ and rapid LC-MS analysis using Waters BioAccord System.

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.

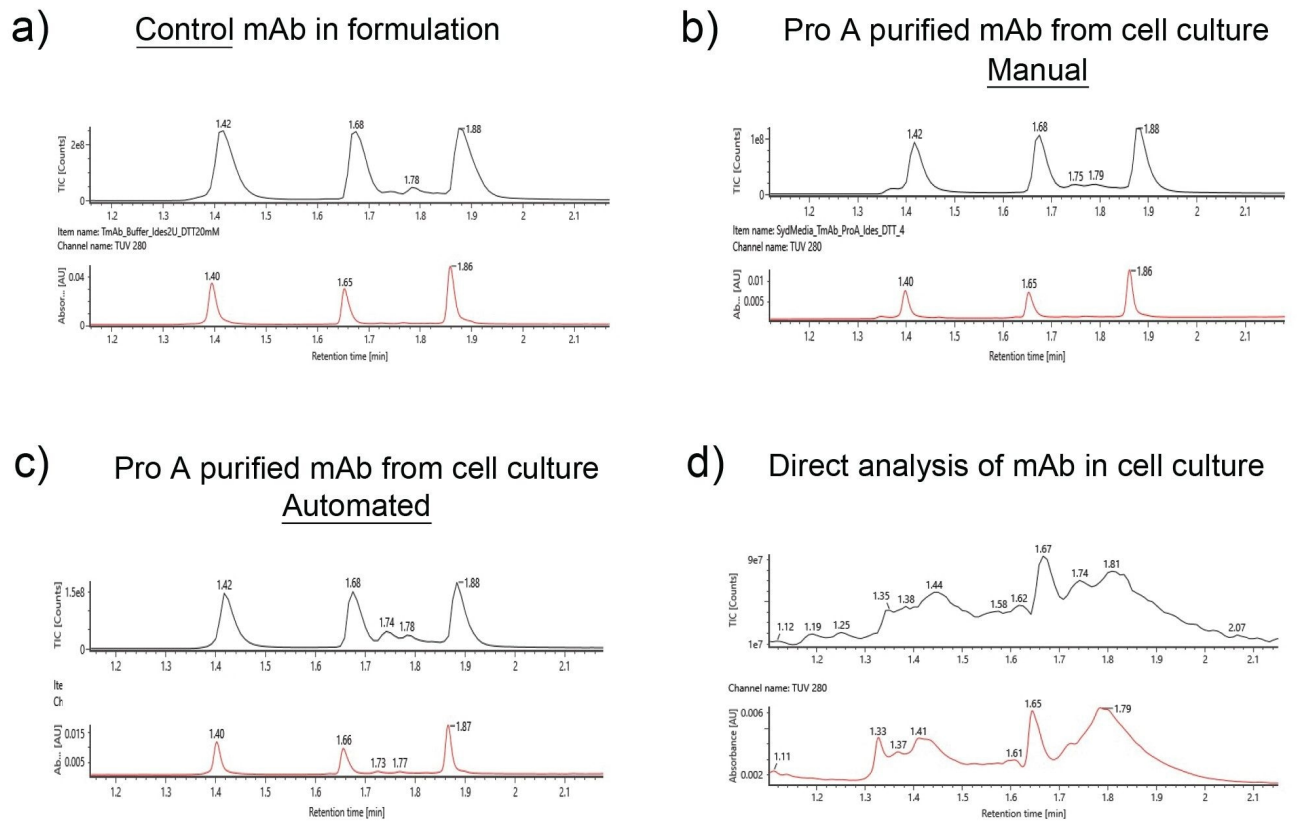


Figure 5. a-c) LC-MS analysis of subunits from purified and digested mAbs. d) unpurified and digested mAbs.

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.

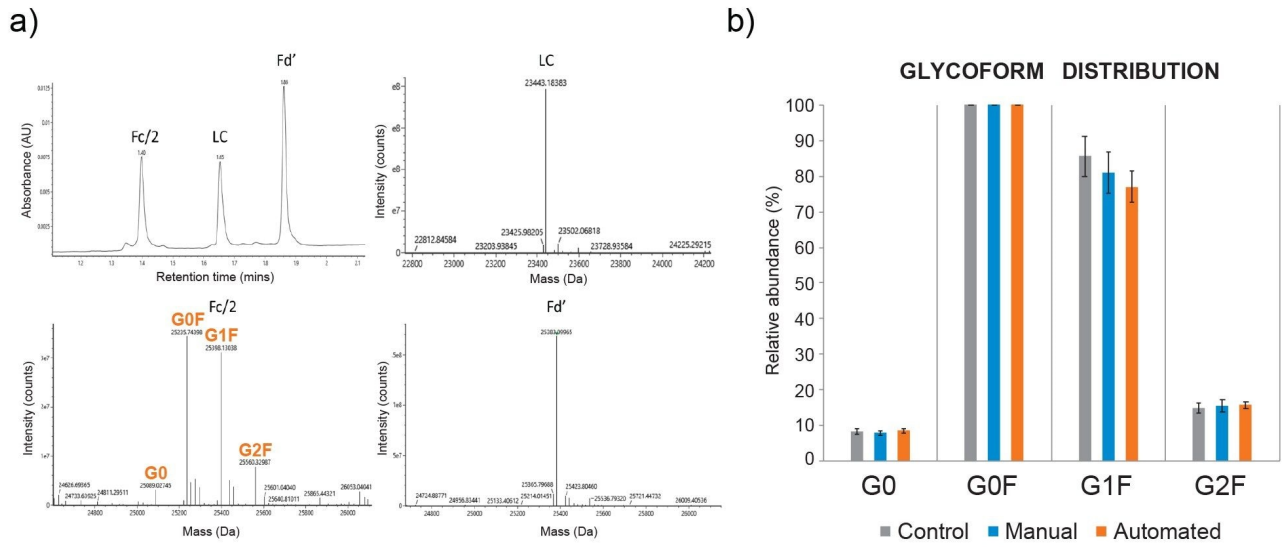


Figure 6. a) Subunit level analysis interpreted results for LC, Fd', and Fc/2 b) Fc N-glycosylation profile from deconvoluted mass spectrum of the Fc/2 fragment generated by manual (blue) and Andrew+ assisted (orange), compared to control (grey). N=8 for each condition.

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.

References

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