



At-line high throughput site-specific glycan profiling using targeted mass spectrometry

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ABSTRACT

Protein post-translational modification (PTM) plays an important role in many biological processes; of which glycosylation is arguably one of the most complex and diverse modifications and is crucial for the safety and efficacy of biotherapeutic proteins.

Mass spectrometric characterization of protein glycosylation is well established with clear advantages and disadvantages; on one hand it is precise and information-rich, as well as being relative inexpensive in terms of the reagents and consumables despite the instrumentation cost and, depending on the method, can give site specific information; on the other hand it generally suffers from low throughput, restriction to largely purified samples and is less quantitative, especially for sialylated glycan species.

Here, we describe a high throughput, site-specific, targeted mass spectrometric peptide mapping approach to quickly screen/rank candidate production cell lines and culture conditions that give favourable glycosylation profiles directly from conditioned culture media for an Fc-fusion protein. The methodology is fully compatible with automation and combines the speed of 'top-down' mass spectrometry with the site-specific information of 'bottom-up' mass spectrometry. In addition, this strategy can be used for multi-attribute product quality screening/monitoring as an integral part of cell line selection and process development.

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1. Introduction

Variation in glycosylation profiles has been a recognized source of heterogeneity for biologics; it is closely monitored as a critical quality attribute in process development and manufacturing and can have major impacts on drug immunogenicity [1], PKPD profile [2–4], and efficacy [5].

Since the rise of monoclonal antibodies as a modern class of targeted therapy, the industry has moved along to far more complex therapeutic protein formats which may or may not be antibody scaffold related. For instance, Enbrel (etanercept), which consists of part of a TNF α -receptor fused to an Fc-domain and

Epogen, a recombinant erythropoietin, are well-known marketed molecules that have presented difficult analytical challenges [6–12]. One common theme between many of these new formats is that they often contain multiple glycosylation sites [13–15] and more complex glycosylation patterns [16,17] that present challenges from a biopharmaceutical development perspective given the aforementioned importance of glycans on product quality.

Typically, therapeutic glycoproteins are produced from stable recombinant cell lines, such as Chinese hamster ovary (CHO) cells, and it is well known that the inherent differences between individual cell line clones and their culture processes can significantly impact the glycosylation patterns of the resulting product [9,18,19]. During the transition period between late stage lead molecule selection and early stage process development it is not uncommon that multiple lead molecules, cell line clones or bioreactor processes are tracked in parallel and one of the rate limiting factors is often how fast pure sample material can be obtained for analytical profiling and subsequent interpretation. Therefore, in order to support the iterative process of project lead molecule selection, cell line selection and cell culture process development, high throughput analytics, which preferably work directly on crude culture media samples, are key to providing rapid product quality data that can be used to drive decisions on cell line selection and process development.

Abbreviations: CM, conditioned media; TQ-MS, triple quadrupole mass spectrometry; PTM, post-translation modification; 2-AB, 2-aminobenzamide labelled UPLC glycan analysis; ESI, electrospray ionization; QBD, quality by design; DoE, design of experiment; PKPD, pharmacokinetic pharmacodynamic.

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Common approaches to glycan analysis come in a few broad categories [20,21]: (1) release and derivatize approach where the glycans are chemically or enzymatically released, labelled by a fluorophores such as 2-AA and 2-AB and are separated and detected on a U/HPLC or capillary gel electrophoresis system [22,23], (2) 'top- or middle-down' LC-MS-based approach where the intact or IdeS digested glycosylated protein molecule's mass is determined [24–26], or (3) 'bottom-up' LC-MS-based method where the protein is first enzymatically digested and the resulting glycopeptides separated and analysed [27,28]; (4) MALDI-MS with which released glycan [29] or tryptic glycopeptides are analysed either directly or after separation.

Compared to released glycan methods, 'top-down' LC-MS has the advantage of being quick and simple, although the site-specific information is often not explicit. In contrast, 'bottom-up' LC-MS has the advantage of site-specifically characterizing molecules with multiple glycosylation sites and being more information-rich, although it has the drawback of slow and tedious sample preparation and data analysis steps. In addition, regardless of being 'top-down' or 'bottom-up', mass spectrometry has the general disadvantage of the inability to handle crude samples and a lack of sensitivity and precise quantitation power for sialylated glycopeptides [30]. MALDI-MS is a flexible alternative to the top-down and bottom-up methods although it does not circumvent difficulty in quantitating sialylated glycans and the requirement of some levels of purification.

In this work, we address some of current issues of 'bottom-up' LC-MS approaches described above by using targeted peptide mapping on a triple quadrupole mass spectrometer (TQ-MS). Specifically, we focused on: (1) Simplification of the sample preparation procedure; (2) The reduction in the duration of the enzymatic digestion and the resulting glycopeptide mis-cleavage for an unbiased yet faster glycosylation profile readout and (3) The application of this methodology to samples direct from a bioreactor without purification. We have developed a denaturation and tryptic digestion protocol that completely eliminates the necessity of buffer-exchange and is directly applicable to therapeutic protein samples in conditioned media. In addition, the entire process from denaturation to data reporting can be complete within a day for up to 96 samples and generates site-specific data that is suitable for neutral glycan quantitation and sialylated glycan ranking; thereby providing quick, information-rich and potentially multi-attribute data to drive bioprocess development with an integrated, high throughput approach unprecedented in industry.

2. Materials and methods

2.1. Reagents and chemicals

Tris base (cat#T6791) and urea (cat#U0631/U5128) were from Sigma. HPLC gradient acetonitrile (cat#83639.320) was from VWR. LC-MS grade water was from Merck (cat#1.15333.250). TFA (cat#9470.0010) was from J T Baker. FA cat#28905 and DTT cat#20291 was from Thermo Fisher. Sequencing grade modified trypsin cat#V5111 was from Promega.

2.2. Model fusion protein

The model fusion protein contains an antibody domain (with expected Fc glycosylation) and tandem receptor agonist ligand domains (each with a single glycosylation site) joined by flexible linkers and was expressed by a stable CHO clonal cell line. The clarified harvest was purified using MabSelect Sure Protein A resin (GE Healthcare, Amersham), followed by pH viral inactivation, Mustang Q anion exchange (Pall, Portsmouth) and finally diafiltered using a

30 kDa ultrafiltration membrane against formulation buffer and concentrated to 10 mg/mL as measured by UV absorbance at 280 nm.

2.3. Fast trypsin digestion

Model fusion protein stock at 10 mg/mL was diluted into 8 M urea to give a protein concentration of 3 mg/mL. DTT stock at 500 mM was then added to give a final DTT concentration of 10 mM. The sample was then denatured and reduced at 50°C for 10 min. Subsequently, the denatured and reduced sample was diluted 2X using 100 mM Tris buffer, pH 7.2 and trypsin was added at a 1:20 enzyme: substrate ratio. The mixture was incubated at 37°C for up to 4 h with time course samples taken and frozen at 0.5, 1, 2 and 4 h before subsequent analysis. After initial data analysis it was determined 1-h digestion is sufficient for generating unbiased glycan data and all subsequent experiments were performed using 1-h digestion time. Unpurified samples in culture medium were treated exactly as purified samples.

2.4. Targeted LC-MS methods

An aliquot of 7.5 µg of the digest was injected onto a Waters Peptide BEH C18 column (300A, 1.7 µm, 2.1 mm x 150 mm) at 55°C. Mobile phase A was 0.1 % formic acid in water, and mobile phase B was 0.1 % formic acid in acetonitrile. The gradient started at 0 % B for 2 min and was stepped to 7 % where it was then increased gradually to 32 % in 6 min. The column was then washed with 90 % B for 1.6 min followed by equilibration at 0 % B for 0.2 min.

Mass spectrometer data was acquired on a Waters Xevo TQ-S instrument on positive ion unit mass resolution mode with the following settings: electrospray capillary voltage 3 kV, cone voltage 20 V, source temperature 70°C, desolvation temperature 400°C and desolvation gas flow 800 L/h. MS1 was set at monoisotopic masses of the tryptic glycopeptides and MS2 was set at 138 *m/z* (GlcNAc oxonium ion) with collision energy at 60 V and dwell time of 27 ms. Automatic peak integration was performed in TargetLynx and percentage glycan species calculated in Microsoft Excel.

2.5. LysC limited digestion and purification

Model antibody-fusion protein was buffered to neutral pH by addition of a 10 % volume of 1 M Tris pH 8.0. Lyophilized LysC was resuspended in PBS to 0.01 µg/µL before being mixed with the neutralized model antibody-fusion protein at 2000:1 protein to enzyme ratio and left to incubate at 4 °C overnight. The extent of digestion was confirmed by SEC-HPLC method. The digestion mixture was then passed through a small-scale protein A column, where the antibody domain bound and was eluted with 25 mM acetate buffer pH 3.65 and collected separately from the flow through which contained the fusion protein domain. The two components were then neutralized with Tris base and concentrated to about 2 mg/mL before 2-AB and GXII glycan analysis.

2.6. GXII capillary electrophoresis glycan analysis

The N-linked glycans profile for the model antibody-fusion protein was conducted using the ProfilerPro Glycan Screening Assay (Perkin Elmer) according to the manufacturer's instructions. The resulting electropherograms were analysed using LabChip GX software and the glycans released were annotated by comparing its electrophoretic migration time to that of glycan standards.

2.7. 2-aminobenzamide (2AB)-labelled UPLC glycan analysis

N-glycans were released at 5 mg/mL in 50 mM Tris HCl, pH 7.8 with 7.5 units of N-glycosidase F by overnight incubation at 37 °C.

Released glycans were labelled with 2-AB using a Prozyme 2AB labelling kit. Excess 2AB was removed using HILIC cartridges and a clean up 96-well plate (Prozyme) operated via a vacuum manifold. Analysis of the 2AB-labelled N-glycans was performed on a Waters Acquity UPLC BEH Glycan column (2.1 mm × 150 mm, 1.7 μm particle) and an Acquity UPLC with a fluorescence detector (Waters, Milford, MA, U.S.A.). 2AB-labelled dextran ladder standard containing glucose unit (GU) oligomers (Prozyme) was used for glycan retention time normalisation, converting retention times into glucose units.

3. Results

3.1. Developing a streamlined denaturation and digestion protocol

To be able to use mass spectrometry as a screening tool for bioprocesses, one prerequisite is a short turnaround time. Besides data analysis time, which is predominantly determined by the software and is beyond the scope of this article, the total turnaround time for current practice consists of the instrument running time and the sample preparation time. Using TQ-MS means quicker acquisition and better sensitivity can be achieved when multiple transitions are monitored simultaneously in MS/MS mode compare to standard QTOF instruments. In addition, a considerably shorter LC gradient can be coupled to the MS and hence ensures a streamlined run time. However, the current widely employed sample digest preparation procedure for peptide mapping is lengthy and often rate limiting, impacting throughput.

The approach of fast digestion has been attempted before, with Wang et al. [31] reporting an ‘ultrafast’ tryptic digestion protocol for a slightly different application and this was the starting point for this work. The primary aim of Wang et al.’s work was to use a quick digestion protocol to minimize chemical modification artefacts when monitoring critical quality attributes such as deamidation and oxidation in a full peptide mapping (scanning) setting. Mis-cleavage was observed, based on the publication’s supplementary information, but due to the scouting/non-targeted nature of the analysis the proportion of cleaved to mis-cleaved species was not further explored. In contrast to Wang et al., this work focused on a targeted approach and hence the proportion of fully cleaved versus mis-cleaved became much more relevant, because of the need to identify which peptide species to target and whether mis-cleavage is glycan dependent.

In order to elaborate the precise extent of mis-cleavage in relation to the digestion time, a model IgG4 antibody-fusion protein which contains a standard Fc-glycan and an extra glycosylation site with more mature, sialylated glycans on the fusion protein domain was employed in this study. This molecule was chosen as a model because a method developed for the Fc portion can potentially be used generically for all IgG4 molecules whilst method development for the fusion protein partner gives an insight into optimizing sialylated glycopeptide detection. The intention was initially to replicate Wang et al.’s protocol but, unlike for normal antibodies at 50 mg/mL, the 70 °C denaturation step caused the antibody-fusion protein to precipitate (data not shown). An adjustment was therefore made to bring down the protein concentration to 3 mg/mL and lower the denaturation temperature to 50 °C. To compensate for the reduction in initial protein concentration, the subsequent dilution before trypsin addition was also reduced to 2-fold instead of 5-fold. This reduction in initial protein concentration also has direct benefit for at-line bioreactor sampling application, as in the earlier days of a time course experiment or for some difficult-to-express molecules the titre may be low.

3.2. Total Fc-glycan signal intensity and distribution of charge-forms and cleavage state over time

The abundance and charge form distribution between the fully cleaved and singly mis-cleaved Fc glycopeptide over digestion time course is shown in Fig. 1. It demonstrates that in the modified fast tryptic digestion protocol, fully cleaved glycopeptide increased steadily for at least 4 h post trypsin addition. In clear contrast, the signal intensity of singly mis-cleaved glycopeptide peaked around 1–2 h post trypsin addition before decreasing again. Over the entire 4-h time course, the signal intensity of the fully cleaved Fc glycopeptide was less than half the signal from the singly mis-cleaved peptides. Interestingly, the proportion of the most abundant charge forms, 2+ and 3+ ions changed between mis-cleaved and fully cleaved glycopeptides. Specifically, 3+ ions take roughly 99 % of the entire signal for mis-cleaved glycopeptide, while they only account for 75 % for the fully cleaved signal. It is perhaps not surprising as the mis-cleaved glycopeptide is larger and contains at least one extra positively ionisable arginine residue. From this result, it is apparent that for this particular tryptic peptide under this digestion protocol and with the particular trypsin product used, single mis-cleavage is generally dominant, time dependent and shifts the charge distribution profile. Comparison of the streamlined digestion protocol to standard in solution digestion for bottom-up mass spectrometry demonstrated comparability between methods (Data not shown).

3.3. Overall Fc-glycan profile over time

To understand whether there is any preferential digestion of certain glycoforms that can bias the results from a fast digestion protocol, charge-form sorted Fc-glycan profiles were plotted against time (Fig. 2). It is clear that glycan profiles stay highly similar over the time course of 4 h within each charge and cleavage state. The seemingly higher percentage of G2F52 from 3+ fully cleaved glycopeptide is unlikely to be true due to low signal intensity for this entire sub-dataset and thus the associated low signal to noise ratio. Despite the slight differences, the fact that the Fc-glycan is overwhelmingly occupied by G0F and G1F glycans makes it harder to further analyse the extent of difference in other glycan species linked to a particular charge form and cleavage

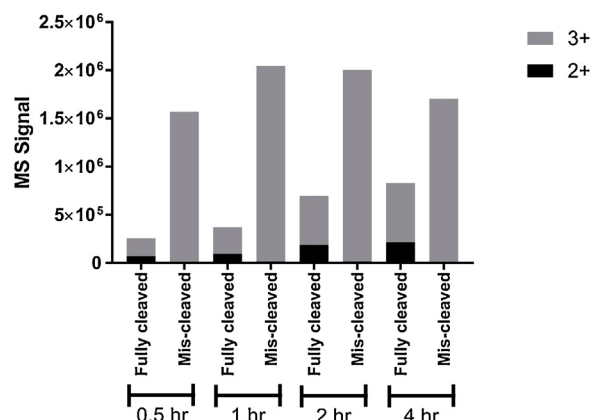


Fig. 1. Mass spectral time course showing shifts in charge form to overwhelmingly 3+ state in singly mis-cleaved compare to fully cleaved Fc-glycopeptides and the general dominance of singly mis-cleaved glycopeptide signal over 4 h digestion time. From left to right: fully cleaved glycopeptide at 0.5 h digestion; singly mis-cleaved glycopeptide at 0.5 h digestion; fully cleaved glycopeptide at 1 h digestion; singly mis-cleaved glycopeptide at 1 h digestion; fully cleaved glycopeptide at 2 h digestion; singly mis-cleaved glycopeptide at 2 h digestion; fully cleaved glycopeptide at 4 h digestion; singly mis-cleaved glycopeptide at 4 h digestion.

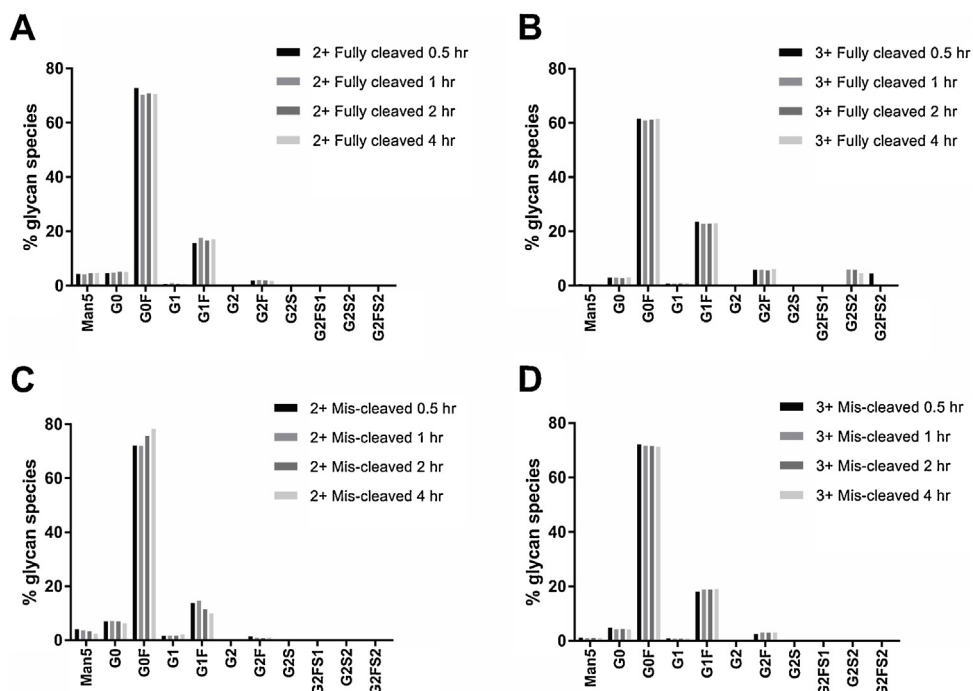


Fig. 2. Cleavage state and charge-form sorted Fc-glycan profiles against trypsin digestion time showing despite changes in total signal intensities over time the overall glycan profile remains the same. (A) 2+, fully cleaved glycopeptides; (B) 2+, singly mis-cleaved glycopeptides; (C) 3+, fully cleaved glycopeptides; (D) 3+, singly mis-cleaved glycopeptides.

state. This was explored in more detail for the fusion protein domain glycan profiling study in the subsequent sections.

Nevertheless, the results indicate there is no preferential tryptic digestion dependent on glycan types, supporting the hypothesis that an accurate representation of the molecule's glycosylation profile can be obtained by this streamlined protocol before the digestion goes to completion. Specifically, denaturation and reduction at 50 °C for 10 min followed by tryptic digestion at pH7.2 for 1 h at 37 °C.

3.4. Electrospray source ionisation (ESI) parameter optimization

A similar protocol was applied to the fusion protein domain on the same molecule that is known to contain significant amounts of more mature and sialylated glycans. Existing experience and knowledge suggest there is likely to be an underestimate of sialylated glycans due to poor ionization [32] and in-source fragmentation [20,30,33]. Therefore, a response surface map (RSM) DoE experiment was performed to optimize the ionization parameters to improve the quantification of sialylated glycans. The parameters optimized included capillary voltage, cone voltage and source temperature and the ranges used in the DoE were defined from an initial scouting experiment (data not shown). The responses chosen were to optimize both the total sialylated glycan signal (combined signal from G2S1, G2FS1, G2S2 and G2FS2 glycopeptides) and the G2FS2:G2FS1 ratio (the two most abundant sialylated glycans of this fusion protein).

The results demonstrate that capillary voltage has a large impact on both responses, followed by source temperature and minimal effect from cone voltage (Suppl. Fig. 1). Based on the DoE outcome, an optimized ionization setting of 3 kV capillary voltage, 20 V cone-voltage and 70oC source temperature was chosen. This optimized method was also tested on the non-sialylated Fc glycans of the model molecule and found no significant difference compared to the results obtained from the original method (data not shown).

3.5. Total fusion protein-glycan signal intensity and distribution of charge-forms and cleavage state over time

With DoE optimized ionization settings, the same fast sample preparation protocol was applied to this model molecule and the fusion protein glycan was targeted instead of Fc-glycans described above. A very similar trend of change in signal intensity and charge-distribution was seen for the fusion protein glycopeptide as for the Fc-glycopeptide (Suppl. Fig. 2). Interestingly, in this case the fully cleaved glycopeptide signal never exceeded a third of the singly mis-cleaved form, in clear contrast to what was seen in Fc-glycopeptide. In addition, the fully cleaved form signal increased at a slower rate compared to that seen from Fc-glycopeptide (Fig. 1 and Suppl. Fig. 2). The two observations together imply that the presence of a significant amount of more matured glycan on this glycopeptide likely contributed to the lack of efficiency of tryptic cleavage at this particular site, possibly due to steric hindrance. Not surprisingly, a similar shift towards 3+ ions from roughly 78% to 96% in the singly mis-cleaved population was also observed in this case.

3.6. Overall fusion protein-glycan profile over time

Similar to the analysis of the Fc-glycopeptide, the charge-form and cleavage state for glycopeptides from the fusion protein domain were plotted against time to understand whether there was any preferential digestion of certain glycoforms that biased the results from the fast digestion protocol. As seen in the Fc-glycopeptide, the fusion protein-glycan profiles were very similar over the time course of 4 h for all individual charge and cleavage states (Fig. 3). This together with the above observation for the Fc glycan site (Fig. 2) indicates the glycan profiles obtained from the fast digestion protocol are representative of what would be obtained from longer digestion protocols. In contrast to the Fc-glycopeptide, there was variation in glycan profiles between the two major charge forms with significant skew towards lower and

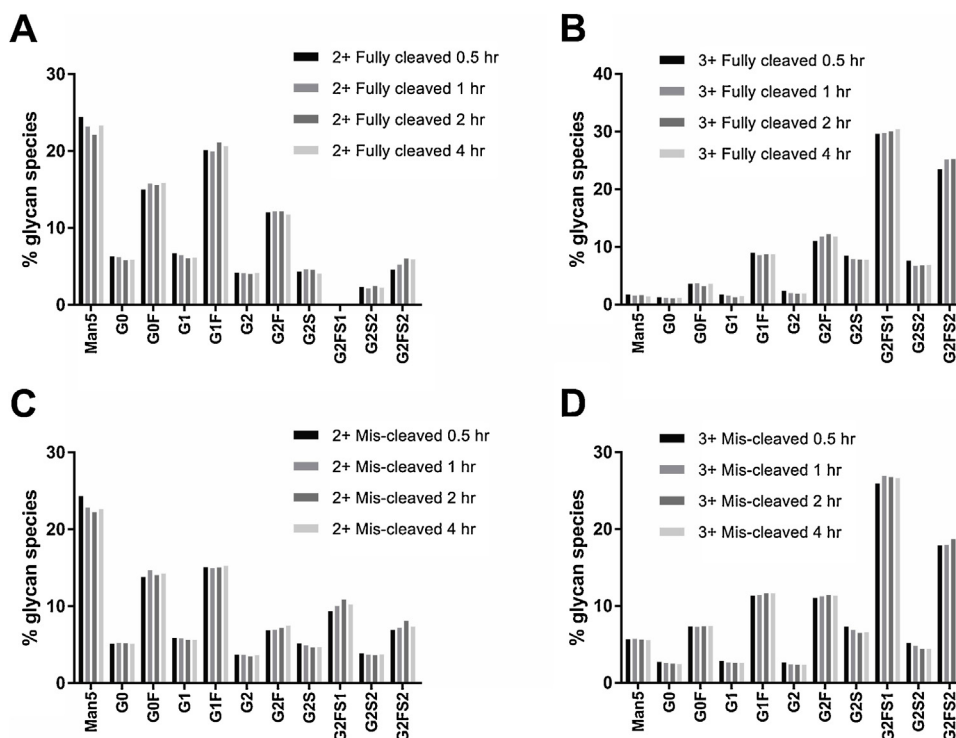


Fig. 3. Cleavage state and charge-form sorted fusion protein-glycan profiles against trypsin digestion time. Despite changes in total signal intensities over time, the overall glycan profile remains the same. In contrast to the Fc-glycopeptide, here glycan profiles between 2+ and 3+ states show a marked difference. (A) 2+, fully cleaved glycopeptides; (B) 2+, singly mis cleaved glycopeptides; (C) 3+, fully cleaved glycopeptides; (D) 3+, singly mis-cleaved glycopeptides.

higher glycans from 2+ and 3+ ions, respectively. However, the observed glycan profile differences between fully and singly mis-cleaved glycopeptide was minor.

3.7. Comparison with 'gold standard' methodologies

As described in the introduction of this article, mass spectrometric glycan profiling has been generally regarded as being less quantitative, especially with sialylated glycan species. One of the aims of this study was therefore to investigate this by comparing our TQ-MS results with those obtained from two widely accepted orthogonal methods; 2-AB and glycan analysis on the LabChip GXII system. Both 2-AB and LabChip represent the 'release and label' category of glycan quantitation methods and the separation is facilitated by a UPLC system and a capillary, respectively. In comparison, TQ-MS offers a distinct opportunity to obtain site-specific glycan quantitation instead of global glycan quantitation; this has significant advantages if site-specific characterization is required, by enabling monitoring of the glycosylation site during reengineering of the molecule or the bioprocess. It is worth mentioning that both categories of methodology involve the use of enzymes and for which the former's efficiency is likely affected by the target protein's tertiary structure while the latter's more on the glycan architecture. Considerations must therefore be given to potential observed differences due to PNGase and trypsin accessibilities of the particular target protein's fold and glycosylation patterns, respectively.

For the validation of this method we wanted to be able to directly compare the site-specific and global methodologies and therefore the antibody-fusion protein was digested (limited LysC digestion) to separate the Fc-glycan domain from the fusion protein glycan domain. These domains were then analysed separately by GXII and 2-AB glycan analysis.

Site-specific glycan profiles obtained by mass spectrometry were generated using either all or subsets of charge and cleavage forms, and compared with data obtained from GXII and 2-AB. Specifically, these are glycan profiles generated using data from (1) all charge states and cleavage forms (Fig. 4A), (2) singly mis-cleaved 2+ and 3+ glycopeptides only (Fig. 4B) and (3) 3+ singly mis-cleaved glycopeptide signals only (Fig. 4C). The rationale being that firstly the 2+ signal only accounts for a very small percentage of the total signal intensity and secondly the glycan profile of 3+ fully and singly mis-cleaved glycopeptide are highly similar and hence using only the 3+ singly mis-cleaved signal as a surrogate would facilitate simpler data acquisition and analysis throughput without compromising quantitative accuracy significantly. This hypothesis is indeed confirmed as the mass spectrometric glycan distribution profiles obtained from Fig. 4A–C are highly similar, suggesting targeted data acquisition and analysis to the singly mis-cleaved 3+ only species is enough to give a good quantitation of the site-specific glycan profile.

Upon comparing the results from the three orthogonal glycan analysis methodologies, the Fc-glycan profiles were found to be highly similar between the three (Suppl. Table 1). However, not surprisingly, the fusion protein glycan profiles showed some differences between each technique especially in sialylated glycan species. Mass spectrometry appears to underestimate the amount of G2FS2 by about 1.5-fold compared to 2-AB, although the percentage difference of all other glycans seem to be similar. This possibly indicates the presence of some in-source fragmentation, where higher sialylated glycans degrade into lower ones despite the ionization optimization effort. In contrast, the distribution of sialylated glycans from the LabChip GXII appeared less consistent with the other two methodologies. This is to some extent not surprising as sialylated glycans elute from the capillary earlier than the lower marker and hence the kit is not officially recommended for sialylated glycan quantification by its vendor. Nevertheless, it

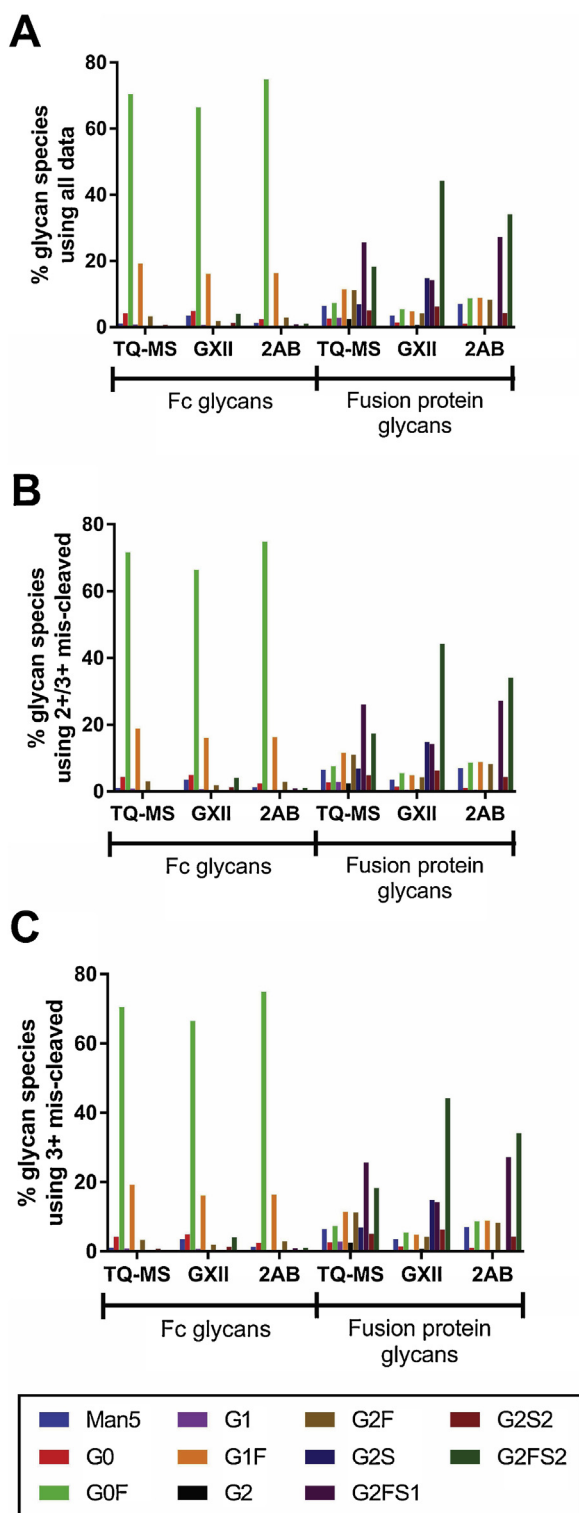


Fig. 4. Site-specific glycan profiles showing that targeting the subset of 3+ charge state of the singly mis-cleaved glycopeptides in TQ-MS is sufficient to generate results comparable to those obtained by 2-AB methodology despite intrinsic underestimation of the sialylated glycan species. Results obtained by GXII are in contrast, less consistent with the former techniques. Comparison of (A) glycan profiles generated using data from all charge states and cleavage forms, (B) singly mis-cleaved 2+ and 3+ glycopeptides only and (C) 3+ singly mis-cleaved glycopeptide signals only. From left to right: TQ-MS fusion-protein glycan profile; TQ-MS Fc-glycan profile; GXII fusion-protein glycan profile; GXII Fc-glycan profile; 2-AB fusion-protein glycan profile; 2-AB Fc-glycan profile.

has been demonstrated that under limited experimental circumstances the technique can be pragmatically exploited to provide rapid estimates of the sialylation level, although the exact quantitation ability is not as reliable compared to other techniques as has been observed in this particular instance (data not shown).

The data demonstrate that the method has been streamlined by targeting only the singly mis-cleaved 3+ glycopeptides for both the Fc (non-sialylated) and the fusion-protein (sialylated) glycans. The data obtained by TQ-MS is quantitative and highly comparable to 'gold standard' methods for non-sialylated glycans; although this quantitation power drops when analysing sialylated glycans. Whether this suppression in sialylated glycopeptide signal is consistent between different molecules is subject to further study.

3.8. Overall Fc- and fusion protein glycan profiles: purified versus crude culture supernatant

In order to achieve the potential of this method for at-line analysis of bioprocesses, the ability to work with crude culture supernatant samples taken directly from bioreactors is crucial. To verify the applicability of the fast digestion protocol for samples in conditioned media (CM), purified model protein was spiked into 'null' CM. This CM was generated from a 14-day culture of a 'null' CHO cell line transfected with a vector that contains a selectable marker, but no model fusion protein. Exactly the same digestion protocols and targeted mass spectrometry method were applied to these samples as previously described. Despite the fact that one might expect some lost in signal intensity due to elevated sodiated and other adducted species in crude sample preparation, the same analyses were applied to CM spiked samples and the data clearly shows that both glycosylation sites gave profiles comparable to purified samples in all charge states and cleavage forms (Fig. 5).

4. Discussion

Although numerous high-throughput mass spectrometry methods have been developed for a range of applications [34–38], the technique is somehow still being regarded as an offline, relatively low throughput characterization tool that requires highly skilled operators. The aim of the current study was to develop a method that can be used as a high throughput approach by cell culture scientists or non-mass spectrometry experts to facilitate at-line screening of crude bioreactor samples for product glycosylation and potentially other PTMs. Depending on the titre, sialylation states and peptide specific considerations, the method has demonstrated ability to provide comprehensive information ranging from fully quantitative data for Fc-like neutral glycans to semi-quantitative data for sialylated species.

To be able to achieve 'at-line' screening capability, the speed of sample preparation and run time is crucially important. It has been demonstrated in the current study that it is possible to prepare the sample straight from conditioned media with no buffer exchange and reduced digestion time compared to standard in solution peptide mapping procedures. The decisive changes employed in this fast digestion protocol specifically include denaturation in a less potent denaturant urea in contrast to commonly used guanidine to eliminate subsequent buffer exchange before digestion. The denaturation temperature is raised slightly but is kept short to compensate for potential chemical degradation. In addition, the relationship between digestion time and mis-cleavage was investigated to ensure the most abundant glycopeptide is targeted and the resulting glycosylation profiles are unbiased. Although commercially available immobilized digestion such as Thermo Scientific SMART digest trypsin kit achieved comparable digestion speed, the method presented in the current study offered a similar solution without additional cost of goods

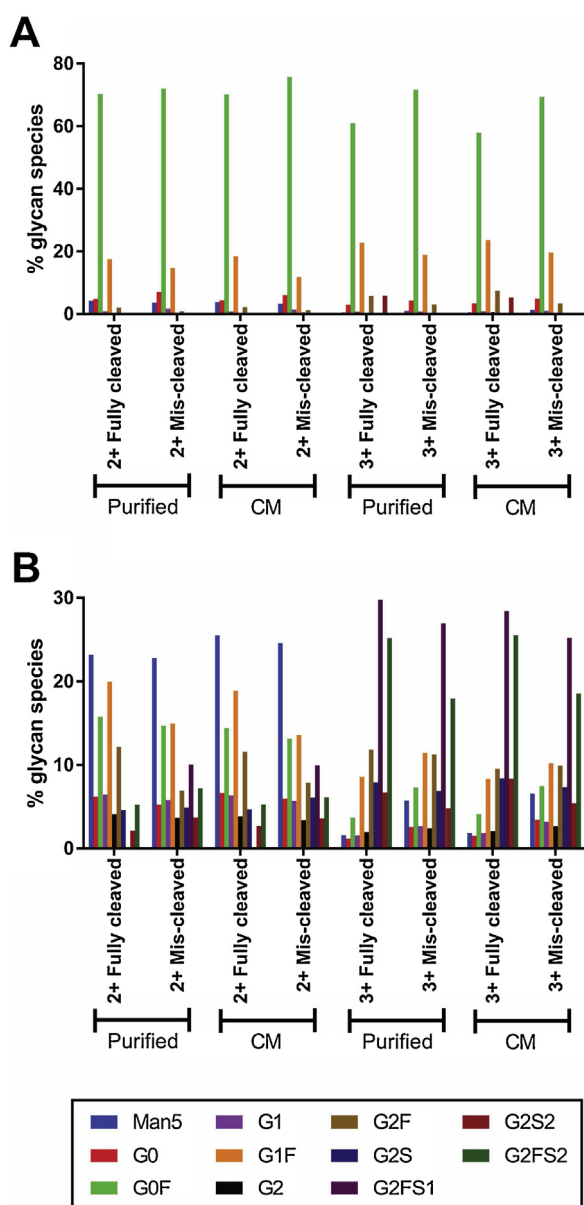


Fig. 5. Comparison of purified and CM-spiked sample glycan profiles at 1 h post trypsin digestion. The results are highly similar, confirming the applicability of the analytical approach on crude samples for both (A) Fc-glycans and (B) fusion-protein glycans. From left to right: 2+, fully cleaved glycopeptide signal from purified sample; 2+, fully cleaved glycopeptide signal from CM-spiked sample; 3+, fully cleaved glycopeptide signal from purified sample; 3+, fully cleaved glycopeptide signal from CM-spiked sample; 2+, singly mis-cleaved glycopeptide signal from purified sample; 2+, singly mis-cleaved glycopeptide signal from CM-spiked sample; 3+, singly mis-cleaved glycopeptide signal from purified sample; 3+, singly mis-cleaved glycopeptide signal from CM-spiked sample.

and avoided extra sample exposure at 70 °C, which can be a source of concern in certain cases due to potential chemical modification. In the current study it has been demonstrated reducing the digestion time increases the amount of at least singly mis-cleaved peptides, but it has been demonstrated that there is no impact on the overall distribution of each glycan species. It is also worth mentioning there are now a range of specifically engineered trypsin variants in the market each with different auto-cleave susceptibility and tend to produce different amount of mis-cleaved target sequences. The specific effects of different trypsin products on the protocol presented here is beyond the scope of this study but would nevertheless be an interesting topic on its own. In

addition to the reduced sample preparation time, the utilization of reduced LC separation from the 90+ minute of typical peptide mapping to 10 min per sample also greatly reduced the run time of this application. The choice of TQ-MS ensured sensitivity and better quantitation power; furthermore, the robustness and low maintenance of the instrument ensured minimal expertise is required from the analyst once the method is setup.

Due to the high throughput nature of this protocol, the technique is well suited for a range of screening activities. This technique has now been routinely used in the authors' lab for lead molecule and cell line selection as well as bioprocess development (data not shown). The quantitation accuracy and digestion efficiency appear robust and reproducible for a wide range of IgG1 and IgG4 Fc-glycans, although the general applicability of this digestion protocol to other protein folds will likely have to be verified individually. This method has generated comparable data to orthogonal methods such as 2-AB and LabChip GXII as seen in this study. The peptide containing the Fc-glycan site is conserved for all Fc-containing molecules of the same isotype and hence both the digestion protocol and the mass spectrometry data acquisition method developed here are universal across multiple projects. For glycosylation sites outside the Fc domain, this type of high throughput method development will have to be performed on a case-by-case basis in combination with traditional peptide mapping. In addition, the resolution and selectivity of the mass spectrometry makes it an ideal multiplexing solution to simultaneously screen for differences in various covalent post-translational modifications in biopharmaceuticals, such as deamidation, oxidation and many more that can be too subtle for classical high throughput methods such as UPLC or capillary electrophoresis alone. This multiplexing potential is particularly useful during the research-development transition period of a drug project where manufacturing and quality control needs to be phased-in from a quality by design (QBD) perspective. Furthermore, this early development phase represents the best opportunity to actively screen out potential problematic molecules or clones when sequence-based liabilities are identified, although their actual impact on the candidate molecule's efficacy maybe unknown at this stage. A high throughput, multiplexing screening will enable molecule leads or clones to be selected on a risk-based approach and ensure smooth transition to subsequent development activities.

The approach is not without limitations though. The inherent difficulty in quantitating sialylated glycopeptides by mass spectrometry has been shown in this case to cause a 1.5-fold reduction in G2FS2 species compared to the 'gold standard' 2-AB method. Nevertheless, for each specific molecule the suppression in signal is consistent (data not shown) and hence ranking between clones and culture conditions is still practical. The other shortcoming associated with the presented targeted glycopeptide method is that full fragment information relating sugar linkage positions are not captured and hence isobaric forms are difficult to distinguish. In addition, targeted mass spectrometry requires prior knowledge of what types of modification are likely to be present in a given sample; to take the example of glycan analysis, this means the operator needs to search for the particular types of glycan likely to be present in order to quantitate accordingly. To this end, it is recommended for novel molecules that this high throughput approach is developed in conjunction with traditional scanning type of mass spectrometry or labelling methods such as 2-AB glycan analysis. Despite the caveats of the technique, with a reasonable understanding of the host cell line's glycosylation pattern and as the knowledge of molecule-specific glycosylation profile improves when a drug project advances, the technique can be employed routinely.

This fast, targeted mass spectrometry method has demonstrated improved throughput compared to traditional full range peptide mapping mass spectrometric analysis; it has also offered definitive mass information whilst maintaining quantitation power, as well as site-specific glycosylation information, compared to widely used high throughput 2AB or capillary gel electrophoresis techniques. Furthermore, the methodology applies equally well to both crude and purified samples, which forms the basis of its 'at-line' capability. This approach has general applicability for glycoprotein development and will greatly benefit the middle ground between the two ends of the current analytical spectrum in terms of speed and resolution. The method developed here can be universal for Fc-containing molecules and requires minimal mass spectrometry expertise to perform once in place. This together with its multiplexing ability to simultaneously monitor other covalent modifications renders it a powerful early stage screening tool for scientists from a range of development disciplines. With the advent of perfusion cell culture and continuous, automated upstream and downstream biopharmaceutical production, this high throughput technique is well suited to provide flexible at-line product quality analysis and to steer the path of early biopharmaceutical development.

CRedit authorship contribution statement

Bertie Chi: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft. **Christel Veysier:** Investigation, Resources. **Toyin Kasali:** Investigation, Resources. **Faisal Uddin:** Investigation, Resources. **Christopher A. Sellick:** Conceptualization, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00424>.

References

- [1] C.H. Chung, B. Mirakhur, E. Chan, Berlin J. Le QT, M. Morse, B.A. Murphy, S.M. Satinover, J. Hosen, D. Mauro, et al., Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose, *N. Engl. J. Med.* 358 (11) (2008) 1109–1117.
- [2] L. Liu, S. Gomathinayagam, L. Hamuro, T. Prueksaritanont, W. Wang, T.A. Stadheim, S.R. Hamilton, The impact of glycosylation on the pharmacokinetics of a TNFR2:Fc fusion protein expressed in Glycoengineered *Pichia Pastoris*, *Pharm. Res.* 30 (3) (2013) 803–812.
- [3] A. Wright, S.L. Morrison, Effect of altered CH2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1, *J. Exp. Med.* 180 (3) (1994) 1087–1096.
- [4] A. Wright, Y. Sato, T. Okada, K. Chang, T. Endo, S. Morrison, In vivo trafficking and catabolism of IgG1 antibodies with Fc associated carbohydrates of differing structure, *Glycobiology* 10 (12) (2000) 1347–1355.
- [5] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, et al., The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity, *J. Biol. Chem.* 278 (5) (2003) 3466–3473.
- [6] D. Falck, M. Habeger, R. Plomp, M. Hook, P. Bulau, M. Wuhler, D. Reusch, Affinity purification of erythropoietin from cell culture supernatant combined with MALDI-TOF-MS analysis of erythropoietin N-glycosylation, *Sci. Rep.* 7 (1) (2017) 5324.
- [7] U. Kim, M.J. Oh, Y. Seo, Y. Jeon, J.H. Eom, H.J. An, Sensitive and comprehensive analysis of O-glycosylation in biopharmaceuticals: a case study of novel erythropoiesis stimulating protein, *Bioanalysis* 9 (18) (2017) 1373–1383.
- [8] E. Lary, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel, Orthogonal liquid chromatography-mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level, *J. Chromatography A* 1498 (2017) 128–146.
- [9] P.M. O'Callaghan, M.E. Berthelot, R.J. Young, J.W. Graham, A.J. Racher, D. Aldana, Diversity in host clone performance within a Chinese hamster ovary cell line, *Biotechnol. Prog.* 31 (5) (2015) 1187–1200.
- [10] J. Srikanth, R. Agalyadevi, P. Babu, Targeted, Site-specific quantitation of N- and O-glycopeptides using 18O-labeling and product ion based mass spectrometry, *Glycoconj. J.* 34 (1) (2017) 95–105.
- [11] Q. Tan, Q. Guo, C. Fang, C. Wang, B. Li, H. Wang, J. Li, Y. Guo, Characterization and comparison of commercially available TNF receptor 2-Fc fusion protein products, *MAbs* 4 (6) (2012) 761–774.
- [12] R.I. Thomson, R.A. Gardner, K. Strohfeldt, D.L. Fernandes, G.P. Stafford, D.I.R. Spencer, H.M.I. Osborn, Analysis of three epoetin alpha products by LC and LC-MS indicates differences in glycosylation critical quality attributes, including sialic acid content, *Anal. Chem.* 89 (12) (2017) 6455–6462.
- [13] A. Beck, S. Sanglier-Cianferani, A. Van Dorsselaer, Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry, *Anal. Chem.* 84 (11) (2012) 4637–4646.
- [14] A. Lim, A. Reed-Bogan, B.J. Harmon, Glycosylation profiling of a therapeutic recombinant monoclonal antibody with two N-linked glycosylation sites using liquid chromatography coupled to a hybrid quadrupole time-of-flight mass spectrometer, *Anal. Biochem.* 375 (2) (2008) 163–172.
- [15] S. Liu, W. Gao, Y. Wang, Z. He, X. Feng, B.F. Liu, X. Liu, Comprehensive N-glycan profiling of cetuximab biosimilar candidate by NP-HPLC and MALDI-MS, *PLoS One* 12 (1) (2017) e0170013.
- [16] S. Houel, M. Hilliard, Y.Q. Yu, N. McLoughlin, S.M. Martin, P.M. Rudd, J.P. Williams, W. Chen, N- and O-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-flight mass spectrometry equipped with electron-transfer dissociation functionality, *Anal. Chem.* 86 (1) (2014) 576–584.
- [17] L. Zhu, Q. Guo, H. Guo, T. Liu, Y. Zheng, P. Gu, X. Chen, H. Wang, S. Hou, Y. Guo, Versatile characterization of glycosylation modification in CTLA4-Ig fusion proteins by liquid chromatography-mass spectrometry, *MAbs* 6 (6) (2014) 1474–1485.
- [18] C. Burger, M.J. Carrondo, H. Cruz, M. Cuffe, E. Dias, J.B. Griffiths, K. Hayes, H. Hauser, D. Looby, C. Mielke, et al., An integrated strategy for the process development of a recombinant antibody-cytokine fusion protein expressed in BHK cells, *Appl. Microbiol. Biotechnol.* 52 (3) (1999) 345–353.
- [19] P.H. van Berkel, J. Gerritsen, G. Perdok, J. Valbjorn, T. Vink, J.G. van de Winkel, P. W. Parren, N-linked glycosylation is an important parameter for optimal selection of cell lines producing biopharmaceutical human IgG, *Biotechnol. Prog.* 25 (1) (2009) 244–251.
- [20] D. Reusch, M. Habeger, D. Falck, B. Peter, B. Maier, J. Gassner, M. Hook, K. Wagner, L. Bonnington, P. Bulau, et al., Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: mass spectrometric methods, *MAbs* 7 (4) (2015) 732–742.
- [21] E. Wagner-Roussel, A. Bednarczyk, M.C. Bussat, O. Colas, N. Corvaia, C. Schaeffer, A. Van Dorsselaer, A. Beck, The way forward, enhanced characterization of therapeutic antibody glycosylation: comparison of three level mass spectrometry-based strategies, *J. Chromatogr. B* 872 (1–2) (2008) 23–37.
- [22] Y. An, Y. Zhang, H.M. Mueller, M. Shameem, X. Chen, A new tool for monoclonal antibody analysis: application of IdeS proteolysis in IgG domain-specific characterization, *MAbs* 6 (4) (2014) 879–893.
- [23] G. Zauner, M.H. Selman, A. Bondt, Y. Rombouts, D. Blank, A.M. Deelder, M. Wuhler, Glycoproteomic analysis of antibodies, *Mol. Cell Proteomics* 12 (4) (2013) 856–865.
- [24] S. Nicolardi, A.M. Deelder, M. Palmblad, Y.E. van der Burgt, Structural analysis of an intact monoclonal antibody by online electrochemical reduction of disulfide bonds and Fourier transform ion cyclotron resonance mass spectrometry, *Anal. Chem.* 86 (11) (2014) 5376–5382.
- [25] Y.O. Tsybin, L. Fornelli, C. Stoermer, M. Luebeck, J. Parra, S. Nallet, F.M. Wurm, R. Hartmer, Structural analysis of intact monoclonal antibodies by electron transfer dissociation mass spectrometry, *Anal. Chem.* 83 (23) (2011) 8919–8927.
- [26] B. Wang, A.C. Gucinski, D.A. Keire, L.F. Buhse, M.T. Boyne, Structural comparison of two anti-CD20 monoclonal antibody drug products using middle-down mass spectrometry, *Analyst* 138 (10) (2013) 3058–3065.
- [27] D. Reusch, M. Habeger, M.H. Selman, P. Bulau, A.M. Deelder, M. Wuhler, N. Engler, High-throughput work flow for IgG Fc-glycosylation analysis of biotechnological samples, *Anal. Biochem.* 432 (2) (2013) 82–89.
- [28] B. Shah, X.G. Jiang, L. Chen, Z. Zhang, LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins, *J. Am. Soc. Mass Spectrom.* 25 (6) (2014) 999–1011.
- [29] R. Hansen, A.J. Dickson, R. Goodacre, G.M. Stephens, C.A. Sellick, Rapid characterization of N-linked glycans from secreted and gel-purified monoclonal antibodies using MALDI-ToF mass spectrometry, *Biotechnol. Bioeng.* 107 (5) (2010) 902–908.
- [30] K.R. Reiding, D. Blank, D.M. Kuijper, A.M. Deelder, M. Wuhler, High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification, *Anal. Chem.* 86 (12) (2014) 5784–5793.

- [31] Y. Wang, X. Li, Y.H. Liu, D. Richardson, H. Li, M. Shameem, X. Yang, Simultaneous monitoring of oxidation, deamidation, isomerization, and glycosylation of monoclonal antibodies by liquid chromatography-mass spectrometry method with ultrafast tryptic digestion, *MAbs* 8 (8) (2016) 1477–1486.
- [32] S. Zhang, D. Chelius, Characterization of protein glycosylation using chip-based infusion nanoelectrospray linear ion trap tandem mass spectrometry, *J. Biomol. Tech.* 15 (2) (2004) 120–133.
- [33] Q. Hong, L.R. Ruhaak, S.M. Totten, J.T. Smilowitz, J.B. German, C.B. Lebrilla, Label-free absolute quantitation of oligosaccharides using multiple reaction monitoring, *Anal. Chem.* 86 (5) (2014) 2640–2647.
- [34] M.R. Bladergroen, Y.E. van der Burgt, Solid-phase extraction strategies to surmount body fluid sample complexity in high-throughput mass spectrometry-based proteomics, *J. Anal. Methods Chem.* (2015)250131.
- [35] D. Chelius, G. Xiao, A.C. Nichols, A. Vizel, B. He, T.M. Dillon, D.S. Rehder, G.D. Pipes, E. Kraft, A. Oroska, et al., Automated tryptic digestion procedure for HPLC/MS/MS peptide mapping of immunoglobulin gamma antibodies in pharmaceuticals, *J. Pharm. Biomed. Anal.* 47 (2) (2008) 285–294.
- [36] V. Schwammle, M. Vaudel, Computational and statistical methods for high-throughput mass spectrometry-based PTM analysis, *Methods Mol. Biol.* 1558 (2017) 437–458.
- [37] S.W. Taylor, N.J. Clarke, Z. Chen, M.J. McPhaul, A high-throughput mass spectrometry assay to simultaneously measure intact insulin and C-peptide, *Clin. Chim. Acta* 455 (2016) 202–208.
- [38] Z. Yang, J. Ke, M. Hayes, M. Bryant, F.L. Tse, A sensitive and high-throughput LC-MS/MS method for the quantification of pegylated-interferon-alpha2a in human serum using monolithic C18 solid phase extraction for enrichment, *J. Chromatogr. B* 877 (18–19) (2009) 1737–1742.