# On-column digestion of mAbs for automated middle-level analysis by LC-MS

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Middle-level analysis has become a widely accepted analytical strategy for rapid characterisation of antibodies and related products. The IdeS enzyme (Immunoglobulin G-degrading enzyme of Streptococcus pyogenes) specifically digests IgG just below the hinge, which after reduction of disulphide bonds yields fragments of 23-25kDa that are amenable to high-resolution mass spectrometry. Here we present a rapid, automated solution for antibody subunit generation and analysis using a standard HPLC-MS setup with only minor modifications. FabRICATOR® (IdeS) enzyme was immobilised in an HPLC column format to allow for on-column digestion of IgG-based biologics. The resulting fragments were trapped on the column head of a reverse phase (RP) column connected in-line with the FabRICATOR-HPLC column. After uncoupling the enzyme column from the flow path using a column switching valve, the fragments could be reduced by injection of a reducing agent before being separated by RP-HPLC and analysed by mass spectrometry. This allowed for a fully automated, completely hands-off workflow for analysis of several critical mAb quality attributes.

## Introduction

Monoclonal antibodies (mAbs) and other IgG-based biopharmaceuticals are a fast-growing market [1] and these biologics have an increasing impact on many fields of medicine. In contrast to conventional small-molecule drugs, these large macromolecules are inherently heterogeneous. Many different product quality attributes such as glycosylation, oxidation, deamidation or C-terminal lysine clipping have been shown to affect mAb stability, safety, efficacy, toxicity and/or serum half-life [2-5]. As these biologics are produced by living cells, rather than synthesised chemically, there are many parameters in the production process that can influence different product quality attributes and need to be tightly controlled. This requires analysis and monitoring of critical quality attributes (CQAs) at many different stages of the product development cycle.

The analysis of many mAb CQAs is performed using bottom-up peptide mapping by LC-MS. This entails the digestion of the antibody into small peptides which are then separated by LC and analysed by mass spectrometry. As this is often time and resource intensive in sample preparation, data acquisition and analysis, top- and middle-down approaches are a potential alternative.

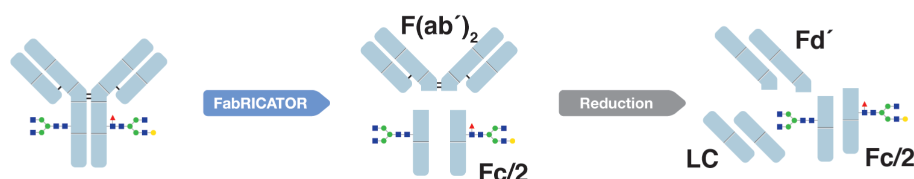


*Figure 1: Middle-level analysis of a mAb using FabRICATOR Digestion of a mAb by FabRICATOR protease leads to the formation of an F(ab´)2 and 2 Fc/2 fragments. Reduction of the disulphide bonds breaks the F(ab´)2 into LC and Fd´ fragments which are or suitable size (23-25 kDa) for direct analysis by LC-MS.*

Recent advances in sensitivity and accuracy of mass spectrometry instrumentation have made it possible to analyse mAbs as intact proteins (top-down) or as large mAb subunits (middle-down). However, analysis of mAb at the intact level is still challenging. Broad charge state envelopes, wide isotope distributions, adduct formation and neutral losses make - in combination with the inherent heterogeneity of the antibody- for very complex mass spectra [6]. This type of analysis therefore requires high-end instrumentation not readily available to many laboratories. Middle-level (middle-up or middle-down) analysis represents a sensible compromise [7]. Antibodies (human IgG1-4) or Fc fusion proteins are digested at one specific site below the hinge region using FabRICATOR [8] protease to yield Fc/2 and F(ab´)$_2$ fragments (Figure 1). This digestion is fast (15-30 min) and complete with no optimisation necessary. Reduction of the disulphide bonds results in the formation of only three mAb subunits requiring analysis (Fc/2, light chain (LC) and Fd´). They are 23-25 kDa in size which makes isotopic resolution achievable on most high-resolution mass spectrometers. This allows for fast and reliable analysis of many mAb CQAs with minimal sample preparation, fast data acquisition and simple data analysis.

With an increasing number of new mAb-based therapeutics in the pipelines [9] with more and more biosimilars being developed, the analytical challenges have risen considerably. In the biopharmaceutical industry, there is a pronounced need for faster and automatable tools for the analysis of these biologics in order to cope with the demand for throughput and robustness while minimising the effects of human error. FabRICATOR-HPLC (Genovis) is a biocompatible PEEK column packed with POROS resin where active FabRICATOR enzyme has been immobilised. This allows for on-column digestion of mAbs and mAb-derived biologics into subunits directly in the LC-MS system, completely automating the sample preparation step for a middle-level analysis. In this article we describe

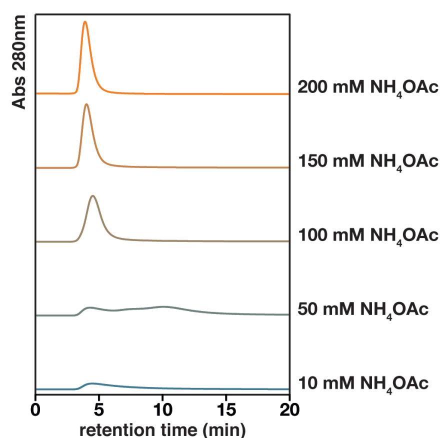


*Figure 2: Elution from the FabRICATOR-HPLC column at different salt concentrations. 10 µg trastuzumab was injected onto the FabRICATOR-HPLC column at a flow rate of 50 µl/min and elution was monitored for 20 min. In low ionic strength mobile phases, the mAb elutes as low, broad peaks with the Fc/2 eluting first and the F(ab')2 being retained longer.*

| Table 1: HPLC setup | |
|---|---|
| Pump | Agilent 1260 Infinity quaternary pump |
| Columns | Genovis FabRICATOR-HPLC (2.1 x 50mm, 37°C)<br>Thermo Scientific MAbPac RP (2.1 x 100 mm, 70°C) |
| Valves | 2 x 2pos/6port, divert valve (DV) in the MS source |
| Mobile phases | A: 0.1% FA in water, B: 0.1% FA in ACN, C: 150 mM $NH_4OAc$, pH 7 |
| Detector | Variable wavelength detector (280 nm) |

| Table 2: MS setup | |
|---|---|
| Instrument | Bruker Impact II ESI-Q-TOF |
| Mass range | 300-3000 m/z |
| ESI source voltage | 4.5 kV |
| Nebuliser gas pressure | 1.8 bar |
| Drying gas flow rate | 8 l/min |
| Drying gas temperature | 220°C |

a workflow for automated, completely hands-off middle-level analysis of a mAb, based on a 2D-HPLC setup where the mAb is digested in the first dimension and the resulting fragments separated and analysed in the second dimension. The workflow can be implemented on any standard HPLC-MS setup with only minor modifications. It is suitable for routine analysis where throughput and minimal sample handling is key and might even be employed to automatically monitor CQAs directly from culture supernatant taken from a bioreactor during an ongoing mAb production.

# Experimental

## Sample Preparation

Trastuzumab and adalimumab (both human IgG1) were obtained in manufacturer's formulation buffer. Before analysis the buffer was exchanged to 150 mM ammonium acetate ($NH_4OAc$), pH 7 using a ZebaSpin desalting column (0.5 ml, 40kDa MWCO, Thermo Scientific) and was diluted to 1 mg/ml. For in-solution digestion with FabRICATOR (Genovis), 50 µg buffer exchanged antibody was treated with 50 units FabRICATOR in 15 µl PBS for 30 min at 37°C. The digested antibodies were reduced and denatured by adding 25 µl 8 M Guanidine hydrochloride (GdHCl) and 10 µl 500 mM dithiothreitol (DTT) (final concentration 1 mg/ml mAb in 4 M GdHCl, 100 mM DTT) and incubated for 30 min at 37°C before analysis.

## FabRICATOR-HPLC Column Activation

FabRICATOR is a cysteine protease [8] and the column therefore needs to be activated with a reducing agent before the first use. This was achieved by making 3 x 20 µl injections of 20 mM DTT in MQ water into the FabRICATOR-HPLC column at a flow rate of 50 µl/min with 150 mM $NH_4OAc$, pH 7 serving as mobile phase. This activation was followed by a column wash of 150 mM $NH_4OAc$, pH 7 for 2 hours.

## Determination of the Digestion Buffer's Optimal Ionic Strength

The FabRICATOR-HPLC column was coupled directly to the UV detector of the LC system and run at a flow rate of 50 µl/min. Different buffer ionic strengths were achieved by mixing 10 mM $NH_4OAc$, pH 7 and 500 mM $NH_4OAc$, pH 7 at appropriate ratios using the mixing capabilities of the HPLC system's quaternary pump. 10 µg trastuzumab was injected and monitored under isocratic conditions for 20 minutes to determine the ionic strength delivering maximum UV absorption at 280 nm wavelength (Figure 2).

## Automated mAb Subunit Analysis

The configuration of the LC-MS system is shown in Figure 3 and instrument parameters are shown in Tables 1 and 2. The automated middle-level LC-MS analysis was performed as described previously [10]. From the resulting chromatograms the digestion efficiency was determined by quantifying the peaks corresponding to Fc/2 Fd´ and intact heavy chain (HC). From the deconvoluted mass spectra of the Fc/2 the Fc glycosylation profile could be determined by comparing peak intensities of the different glycoforms.

# Results and Discussion

## Mobile phase considerations

Non-volatile buffer salts in the sample or the mobile phase of a LC-MS analysis can have severe negative effects on data quality. Even low millimolar concentrations are sufficient to cause considerable ion suppression and adduct formation. The standard PBS buffer, usually recommended for FabRICATOR digestion in solution, is therefore less suitable as a mobile phase for FabRICATOR-HPLC in an LC-MS setup. The FabRICATOR enzyme tolerates many different buffer compounds without loss of activity if the pH is kept between 6 and 8. We therefore tested FabRICATOR-HPLC's digestion performance in ammonium acetate ($NH_4OAc$) at pH7, an MS-friendly buffer that is often used as a mobile phase for native mass spectrometry [11]. Digestion performance was unaffected by the new buffer, but it became obvious that the ionic strength of the mobile phase greatly affected the retention of mAb subunits on the FabRICATOR-HPLC column (Figure 2). At low ionic strength, the mAb fragments are retained longer on the FabRICATOR column leading to a low, broad elution peak or no elution at all (Figure 3). Especially the F(ab´)2 fragment is very sensitive to the ionic strength of the buffer. At least 150 mM $NH_4OAc$ was necessary to have both fragments elute in one single, narrow peak. This was true for all IgG1-based mAbs we tested. Conditions might differ when digesting other IgG subclasses or Fc fusion proteins and might require some optimisation of mobile phase composition.

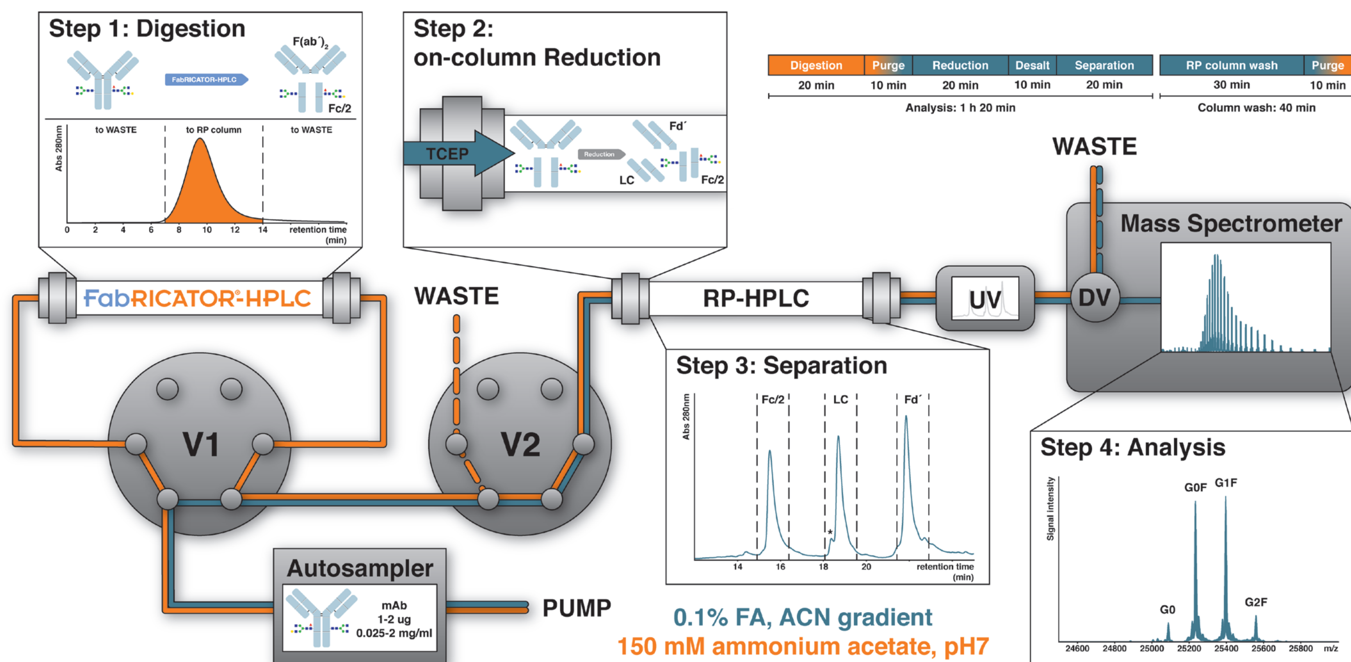


*Figure 3: An automated workflow for middle-level analysis of mAbs*

*Step 1: The mAbs are injected onto the FabRICATOR-HPLC column in 150 mM NH4OAc, pH 7. They are digested on-column and the elution peak is transferred to a RP-HPLC column where the antibody fragments are trapped.*

*Step 2: The FabRICATOR-HPLC column is uncoupled from the flow path using valve V1 and the mobile phases are changed to 0.1% FA, ACN suitable for RP-HPLC. The antibody fragments are reduced and denatured by injection of 50mM TCEP in 20% ACN and desalted while trapped on the column head.*

*Step 3: The reduced antibody fragments are separated on the RP-HPLC column (Thermo Scientific MAbPac RP, 2.1 x100mm) using an ACN gradient (25-35%).*

*Step 4: The fragments eluting from the RP-HPLC are analysed by ESI-Q-TOF mass spectrometry (Bruker Impact II) and the resulting spectra are deconvoluted using the MaxEnt algorithm.*

## Automated middle-level analysis of trastuzumab

FabRICATOR-HPLC allows for the complete automation of the entire sample preparation phase of a middle-level analysis of a mAb or mAb-derived biopharmaceutical by LC-MS. A possible configuration for the LC-MS system is shown in Figure 3. The FabRICATOR-HPLC column is equilibrated in 150 mM $NH_4OAc$, pH 7 and a flow rate of 25 µl/min and a mAb sample is injected. 1-2 µg antibody has been shown to be enough to achieve high quality mass spectra and a broad range of concentrations (0.025 - 2 mg/ml) is tolerated without affecting digestion performance. The antibody is digested into Fc/2 and F(ab')2 while it passes through the column at a flow rate of 25 µl/min (Figure 3, Step 1). Under these conditions, residence time on the column is approximately 7 mins which is sufficient to achieve >95% digestion. Higher flow rates might compromise digestion efficiency. The resulting antibody fragments are transferred to a reverse phase column by coupling both columns in-line with the help of valve V2 (Figure 3). Due to the low eluotropic strength of the digestion buffer, the fragments are efficiently trapped on the head of the RP column. The FabRICATOR-HPLC column is then flushed to waste to avoid carry-over and disconnected from the flow path using a switching valve (Figure 3, valve V1). At this point the mobile phase is changed to FA/ACN suitable for reverse phase chromatography. The trapped mAb fragments are reduced by injection of a tris(2-carboxyethyl)phosphine( TCEP) solution into the RP column (Figure 3, Step 2) whereby the high column temperature (70°C) and the organic solvent (20% ACN) help to fully denature the protein in order to achieve a complete reduction of all inter- and intramolecular disulphide bonds of the mAb. If only the Fc/2 fragment is of interest (e.g. for analysis of Fc glycosylation), the reduction step can be omitted. Upon reduction, the mAb subunits are desalted, separated by RP-HPLC (Figure 3, Step 3) and finally analysed by mass spectrometry (Figure 3, Step 4). A divert valve between the LC system and the mass spectrometer avoids non-volatile compounds from the sample, the FabRICATOR-HPLC digestion buffer and the reducing agent from reaching the MS source during the digestion, reduction and desalting steps. Only when the RP-HPLC gradient starts is

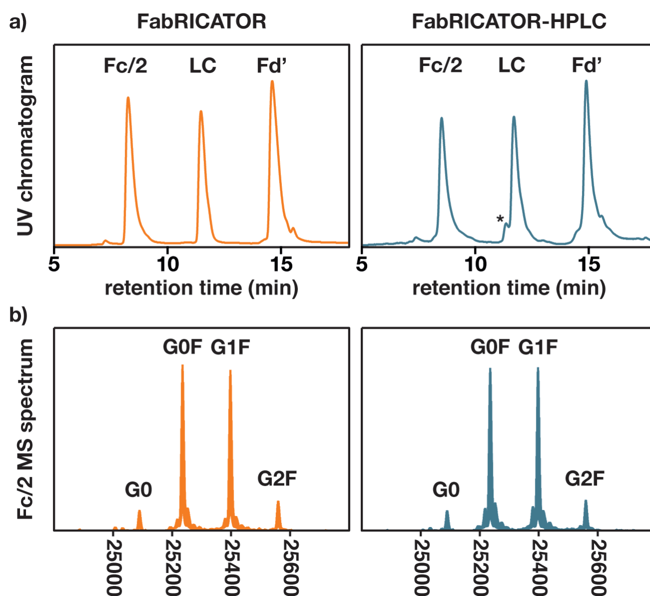


*Figure 4: Comparison between digestion on FAbRICATOR-HPLC and FabRICATOR in solution*

*a) UV chromatogram of the trastuzumab subunits generated by the in-solution FabRICATOR protocol (left, orange) and the automated FabRICATOR-HPLC workflow (right, teal). The asterisk marks LC fragments that are not completely reduced with one intramolecular disulphide bridge intact.*

*b) Deconvoluted MS spectra of the Fc/2 fragment generated by in-solution FabRICATOR digestion (left, orange) or the automated FabRICATOR-HPLC workflow (right, teal).*

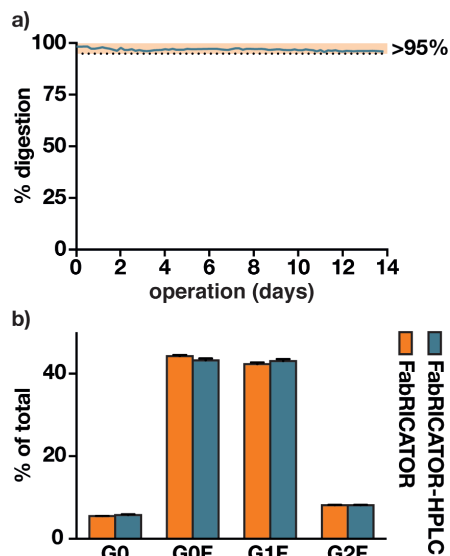


*Figure 5: FabRICATOR-HPLC performance during a 14-day period of continuous operation*

*a) Digestion efficiency of FabRICATOR-HPLC was followed for 14 days with 6 trastuzumab samples (2ug each) being analysed automatically per day. The digestion percentage was determined from the resulting UV chromatograms.*

*b) Comparison of the obtained Fc glycosylation profiles from middle-level LC-MS analysis using the standard in-solution FabRICATOR protocol (n=10, orange) or the automated FabRICATOR-HPLC workflow (n=28, 2 samples per day, teal). The bars represent the mean, the error bars the standard deviation.*

the divert valve switched to the MS source and mass spectra are acquired. This workflow yielded both chromatograms and MS spectra virtually indistinguishable from the ones obtained using a well-established in-solution FabRICATOR digestion protocol [12] (Figure 4).

## Robust performance

The performance of FabRICATOR-HPLC was tested during a 14-day period of continuous operation at 37°C. Every 4 hours a new trastuzumab sample (2 μg) was injected and analysed using the automated middle-level LC-MS approach described above. Digestion efficiency was assessed by quantifying the amount of intact HC in comparison to the Fc/2 and Fd' fragments from the UV chromatogram. During the entire two weeks, FabRICATOR-HPLC was able to digest >95% of the injected mAb (Figure 5). We also compared the results of the middle-level Fc glycan analysis over the entire period of continuous operation. The results were reproducible and fell within a very narrow margin of error (standard deviation <0.5%) with no discernable trends over time, highlighting the robust performance of FabRICATOR-HPLC during prolonged periods of continuous operation. The results also corresponded well with published data on trastuzumab glycosylation [13] and the results achieved using in-solution FabRICATOR digestion [12].

## Minimal carry-over

In contrast to single use consumables such as soluble FabRICATOR enzyme or FabRICATOR immobilised on a spin column (FragIT™, Genovis), the FabRICATOR-HPLC column needs to tolerate repeated injections without previous samples affecting the results of the analysis of the samples that follow. We assessed carry-over between two consecutive samples by alternating injections of trastuzumab with either blank injections or injections of another mAb (adalimumab). The resulting chromatograms (Figure 6a) and mass spectra (Figure 6b) showed only minimal carry over. UV-HPLC analysis estimates the carry over to <1% which did not affect the outcome of the resulting MS analysis of mAb Fc glycosylation (Figure 6b). The carry-over can be further reduced by slightly decreasing throughput in favour of a more stringent column cleaning method.

# Conclusion

An LC-MS workflow for automated middle-level analysis of mAbs and mAb-based biologics has been demonstrated. FabRICATOR enzyme immobilised onto column hardware, suitable for HPLC, facilitates on-line sample preparation for middle-level LC-MS. On-column digestion of mAbs followed by on-column reduction and analysis by RP-HPLC and MS yields results indistinguishable from the ones produced using an off-line sample preparation protocol. With minimal carry-over, low sample requirements and a tolerance for a wide concentration range, this method is very versatile. It is well suited for both routine analysis as well as more advanced applications such as automatic monitoring of mAb CQAs during production in a bioreactor. A simple and generic solution was shown that should be implementable on any standard HPLC-MS setup with only minor modifications such as extra column switching valves. More complex solutions including additional pumps and parallel flow paths might help to further streamline the workflow and increase throughput even further.

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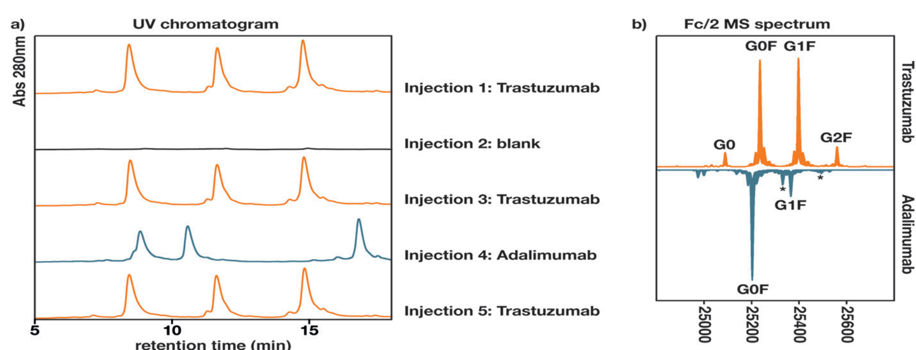


*Figure 6: Evaluation of sample carry-over on FabRICATOR-HPLC*

*a) Quantification of carry-over of trastuzumab subunits from previous injections to a blank and an adalimumab sample respectively. The UV chromatograms show only very minor signal corresponding to the antibody fragments of the previous injection.*

*b) Comparison between deconvoluted Fc/2 MS spectra of trastuzumab (top, orange) and adalimumab (bottom, teal). Note that none of the trastuzumab glycoforms are detectable in the adalimumab sample and vice versa. The asterisk marks antibody fragments that have not undergone C-terminal lysine clipping.*