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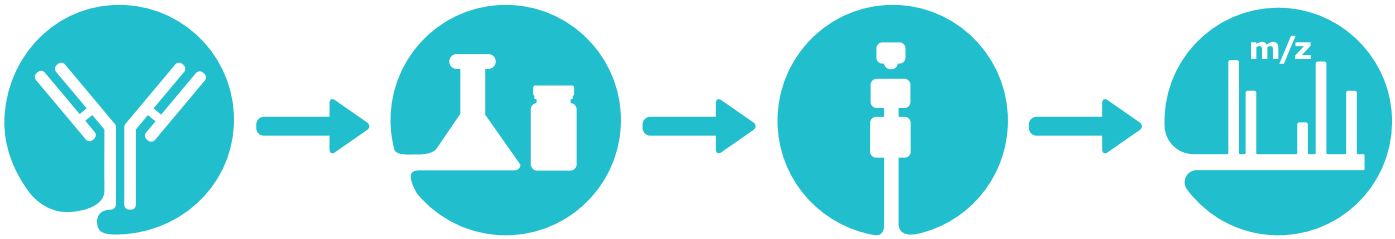
Application Note

Middle-up Mass Analysis of Protease Digested Cetuximab

Protocol for purification, proteolysis, reduction, and UHPLC-MS analysis of unlabeled and labeled monoclonal antibodies

Workflow for Middle-up Mass Analysis of Protease Digested Cetuximab



A complete reversed phase UHPLC-MS workflow has been developed to simplify middle-up mass analysis of an immunoglobulin G antibody. The protocol describes the analysis of proteolyzed and reduced samples. This type of antibody fragment analysis is a much faster assay than peptide mapping for establishing the presence or absence of antibody fragment modifications.

In detail, the workflow includes:

- Antibody purification using immobilized protein A
- Antibody proteolysis utilizing IdeS enzyme
- Antibody reduction procedure
- Mass spectrometer calibration
- UV spectrophotometric mAb quantification method
- System suitability test utilizing an intact protein LC-MS standard
- RP-UHPLC-MS method for separation and analysis of unlabeled and labeled samples

Introduction to Monoclonal Antibody Sample Preparation and Analysis

Monoclonal antibodies (mAbs or immunoglobulins - IgGs) are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/Mol). They are composed of two identical light chains (LC, molecular weight ca. 25 kDa each) and two identical heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, and migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. MAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules. Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits are requirements for both innovator and biosimilar therapeutics.[1,2]

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In many cases, the characterization of an antibody-based drug is performed using a specific chromatographic technique (e.g., size exclusion, reversed phase or hydrophilic interaction liquid chromatography, respectively – SEC, RP or HILIC) [3,4] coupled with mass spectrometry (MS). This combination allows for different types of analyses to be carried out, e.g., accurate mass measurement of the intact mAb and subunits, peptide mapping, and the determination of post-translation modifications such as glycosylation, oxidation, and deamidation.

Several techniques are applied to simplify antibody analysis by either fragmentation or removal of glycans. The latter can be performed by treatment with PNGase F, whereas proteolysis with IdeS[5] or reduction of inter-chain disulfide bonds with reducing agents, such as dithiothreitol, result in the formation of different antibody fragments with masses of 25 – 50 kDa. Various combinations of these techniques can be applied. For analysis of mAbs in cell culture supernatants, these may be combined with a preceding affinity purification step.[6] These approaches are referred to as intact mass and middle-up analysis methods.[7] The former term relates to the measurement of the mass of an intact mAb without controlled dissociation being performed. Such an experiment reveals information about stoichiometry, proteoforms, and modifications. Middle-up experiments include mass measurement after cleaving mAbs into several large fragments/subunits via chemical reduction or proteolytic digestion. An example of this approach is the analysis of mAbs light and heavy chains, providing insight into amino acid and post-translational modifications of the individual chains. **Figure 1** shows an overview of antibody sample preparation and various digestion options prior to intact mass analysis.

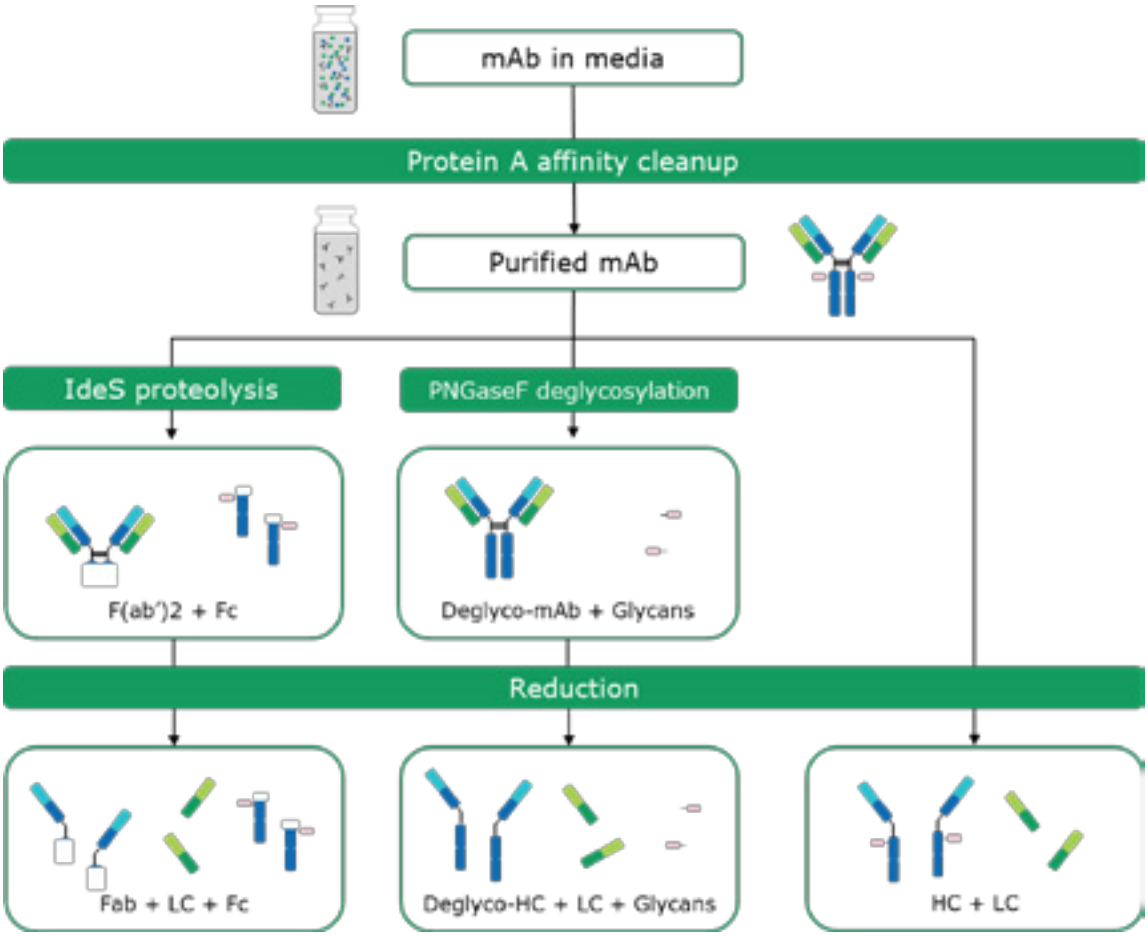


Figure 1. Antibody sample preparation by protein A affinity cleanup and chemical and proteolytic digestion options: Proteolysis with IdeS (formation of Fc and Fab fragments); PNGase F treatment (deglycosylation); chemical reduction (release of heavy and light chains). A combination of proteolysis and chemical reduction is also possible.

This report describes a middle-up approach for mass analysis of cetuximab IgG1 antibody samples. After protein A purification, each sample was subjected to IdeS digestion, reduced, and analyzed by reversed phase UHPLC-MS in order to determine the Fc, LC, and Fab masses of unlabelled and labelled antibody samples and reference.

The recombinant human monoclonal antibody SILu™Lite SigmaMAb™ (#MSQC4) in media was utilized as a purification assay control and pre-purified SILu™ Lite SigmaMAb™ reference served as a digestion control. System suitability control was performed using SigmaProt™ Intact Protein LC-MS Standard (#MSRT2 containing a set of nine proteins: ribonuclease B, insulin, lysozyme, transferrin, bovine serum albumin, trypsin inhibitor, β-lactoglobulin A, carbonic anhydrase, lactate dehydrogenase).

General Procedures – Antibody Preparation and System Setup for Reversed Phase LC-MS Analysis

Antibody Purification Procedure

The target antibody purification was performed on cell culture supernatants using immobilized protein A resin in a 96-well format. The suggested minimum working mAb titer is 100 µg/mL.

All procedures were also conducted using a cetuximab and a SigmaMAb™ reference with a molecular mass of ~150 kDa. The assay control sample contains the antibody spiked into or delivered as a mixture with cell culture media or spent media (cell broth including nutrients etc.); it goes through the entire workflow and serves as a control sample within the protein A purification process (and not as an SST for SEC-MS).

In detail, the high-throughput purification of mAbs from cell culture media using protein A resin was performed as follows:

1. System/Workflow Suitability

As part of the workflow suitability, an assay control of SigmaMAb™ in media was purified along with the samples.

a) Reconstitute each vial of MSQC4 in 1.0 mL water to obtain a solution with an antibody concentration of 1 mg/mL.

b) Prepare assay control (spent media sample) by spiking SigmaMAb™ in EX-CELL® CHOZN® Platform Medium, or equivalent, to obtain a final concentration of 100-500 µg/mL.

2. Preparation of equilibration and elution buffers

a) Prepare equilibration buffer (20 mM citrate, 150 mM NaCl, pH 7) by dissolving 5.82 g trisodium citrate dihydrate, 0.04 g citric acid, and 8.77 g sodium chloride in 1 L water. Adjust pH of resulting solution to 7 using 1 M NaOH or HCl as needed; subsequently filter solution using a 0.2 µm filter.

b) Prepare elution buffer (25 mM citrate, pH 3) by dissolving 4.8 g citric acid in 1 L water. Adjust pH of resulting solution to 3 using 1 M NaOH or HCl as needed.

3. Clarify samples

Centrifuge samples in tubes at maximum speed for five minutes and samples in plates at maximum speed for 60 minutes.

4. Protein A loading

a) Add or remove water from top portion of settled protein A slurry to obtain a 50% protein A suspension.

b) Mix slurry by constant pipette action and gentle shaking of reagent reservoir.

c) Use a multichannel pipette to deliver 200 µL of protein A slurry to each well of a 96-well filter plate. Place protein A filter plate on a vacuum manifold. Catch any flow-through from the filter plate by placing the filter plate on top of a used collection plate.

5. Protein A equilibration

a) Add 200 µL of equilibration buffer to each well of protein A and apply vacuum to void the wells of buffer.

b) Repeat both steps twice.

6. mAb binding

a) Remove 750 µL of solution of sample and control without disturbing the pellet and load plate.

b) Cover the plate with film and secure filter and collection plates with a rubber band.

c) Incubate on an orbital shaker at 170 rpm for 30 minutes.

7. Washing bound mAb

a) Place protein A filter plate on vacuum manifold with a waste collection plate inserted and the film cover removed.

b) Apply vacuum to void wells of buffer media and transfer the filter plate onto a waste collection plate.

c) Add 200 µL of equilibration buffer wells and centrifuge plates at 3700 rpm for five minutes (this step helps in clearing the sample film on sides of filter plate wells).

d) Add 200 µL of equilibration buffer to wells and apply vacuum to remove buffer.

e) Repeat once more for a total of three washes.

8. Eluting bound mAb

a) Place protein A filter plate on a new collection plate and secure with a rubber band.

b) Add 100 µL of elution buffer to each well, incubate filter plate an orbital shaker at 170 rpm for five minutes.

c) Centrifuge plates at 3700 rpm for five minutes.

d) Repeat addition of elution buffer, incubation on orbital shaker, and centrifugation for a total of three elution steps (300 µL of total elution volume).

Typical antibody recovery using this procedure is 60%.

Antibody proteolysis

The protein A purified or pre-purified antibody is proteolyzed and reduced into Fab, Fc, and LC components. The immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS, Genovis brand name FabRICATOR®) proteolyzes IgG1 at the heavy chain sequence PAPELLGGP, between adjacent glycines; this gives a F(ab')2 and two Fc fragments. The F(ab')2 fragment, prior to reduction, contains all inter-molecular disulfide bonds of IgG. After reduction, three Fab fragment types with a molecular mass of approximately 25 kDa each are observed.

Additionally, pre-purified SigmaMAb™ reference is used as a digestion control to test if the IdeS workflow/ treatment worked. SigmaProt Intact Prot Protein LC-MS Standard is used as a system suitability control.

The antibody proteolysis utilizing IdeS was performed as follows:

1. pH adjustment buffer: Add 932 µL water to 67.6 µL NH_4OH 28-30% solution to prepare a 1 M ammonium hydroxide solution.
2. Add 5 µL of pH adjustment buffer to 40 µL of the cetuximab sample and SigmaMAb reference (1 mg/mL).

   Note: pH of the sample should be ≥ 7.
3. Add 0.6 µL of IdeS (67 units/µL) to pH adjusted sample.
4. Incubate at 37 °C for 30 minutes.

Antibody Reduction Procedure

Partial disulfide (S-S) bond reduction was performed as follows:

1. Prepare a 1 M dithiothreitol (DTT) solution by dissolving 154.25 mg DTT in 1 mL water.
2. Prepare a 1 M ammonium bicarbonate (ABC) solution by dissolving 79.06 mg ABC in 1 mL water.
3. Combine equal volumes of 1 M ABC and 1 M DTT to prepare the reduction solution.
4. Transfer aliquots of 50 µL of each sample, system suitability reference, and control to autosampler vials.
5. Reduce by addition of 5 µL 0.5 M ABC/0.5 M DTT solution.
6. Incubate for one hour at room temperature or 30 min at 37 °C.

Note: Partial reduction is performed under non-denaturing conditions, where the inter-chain disulfide bonds (which are more susceptible to reduction) will break and produce the light and heavy chains, while the intra-chain disulfide bonds within each individual domain remain intact.

Alternatively, complete reduction of samples can be performed by using this protocol:

1. Prepare a 100 mM solution of tris(2-carboxyethyl) phosphine (TCEP) in 6 M aqueous guanidine hydrochloride by dissolving 2.87 g of TCEP and 57.32 g of guanidine hydrochloride in 100 mL of water (if less solution is needed, scale down accordingly).
2. Combine 30 µL of the resulting solution with 10 µL of sample.
3. Incubate for two hours at 37 °C.

Note: Complete reduction is performed under denaturing conditions, where both the inter-chain and intra-chain disulfide bonds will break.

Calibration

The Waters™ Xevo G2S mass spectrometer was calibrated with a 20 µL/min infusion of 0.4 mg/mL of polyalanine and a lock mass of Glu-Fib was used. Alternatively, calibration can be performed with a 20 µL/min infusion of 0.4 mg/mL of cesium iodide prior to running the samples.

System Suitability

To evaluate performance of the entire workflow, an assay control (SigmaMAb™ in media) was prepared and analyzed along with the samples. SigmaProt™ Intact Prot Protein LC-MS Standard was also tested to ensure system suitability.

UHPLC-MS System Setup and Data Analysis

1. UHPLC-MS System Setup

The essential settings of the UHPLC-PDA chromatography system and the qToF mass spectrometer applied in the analysis of both reduced and non-reduced antibodies are listed in **Tables 1** and **2** below.

Table 1. RP-UHPLC-PDA settings.

Instrument	Waters™ H-Class Acquity UPLC Chromatography System
Software	MassLynx 4.1
Column	BIOshell™ A400 Protein C4 15cm x 1.0mm, 3.4µm
Column temp	Ambient
Autosampler temp	10 °C
Mobile phase	0.1% TFA in water (A), 0.1% TFA in ACN (B)
Gradient	20-46% B in 13.0 min, 0.5 min to 80% B, 2 min at 80% B, 0.5 min to 20% B, 7 min at 20% B
Flow	70 µL/min
Loop volume	20 µL
Injection method	Partial loop or full loop
Injection volume	10 µL
Run time	23 min
Photodiode array	280 nm
Divert valve	0 – 3.5 min

Table 2. qToF-MS settings.

Instrument	Waters™ Xevo G2S Mass Spectrometer
Software	MassLynx 4.1
Capillary (V)	3,500
Sample cone (V)	120
Ion energy (V)	1
Desolvation temp (°C)	300
Source temp (°C)	100
Scan Range (Da)	300 - 5000
Desolvation gas (L/h)	600
Cone gas (L/h)	0
Collision energy (V)	5
Pusher (V)	1900
RF Setting	Automatic

2. MS Data Analysis

Data were processed using Waters™ MassLynx 4.1 software to generate and analyze deconvoluted (zero charged) mass spectra. In general, a summed spectrum was created from the corresponding total ion chromatogram (TIC) of the eluting mAb species. The summed m/z spectrum was then processed by the MaxEnt1 algorithm and converted to a decharged (deconvoluted) mass spectrum. Detailed parameters are listed in **Table 3**.

For glycoform analysis, data were processed using UNIFI software from Waters™ Glycoforms were matched by the software and HC glycoform glycans are listed in the respective section below. Glycoform relative abundance data were tabulated based on peak intensities of the co-eluting glycoform species. A deconvolution filter setting employing a base peak intensity of 2% was used to preclude noise incorporation, and an output resolution setting of 5 Da was used.

Table 3. Deconvolution parameters.

MassLynx deconvolution parameters	
Output mass	MassLynx 4.1
Ranges	20,000 – 30,000
Resolution	1 Da/channel
Damage model	
Uniform Gaussian FWHM	1 Da
Minimum intensity ratios	
Left	33.0%
Right	33.0%
Completion options	
Iterate to convergence	No
Maximum iterations	12
RF Setting	Automatic

Protein Quantification method – Determination of Protein Concentration

Protein quantification of cetuximab samples was performed by analysis of the UV absorbance of all samples at 280 nm (“A280 method”). The system applied was a Varian Cary® UV 50 Bio UV-Vis spectrophotometer. Protein A elution buffer was recorded as a blank prior to sample measurement. To meet system suitability requirements, NIST BSA with a known concentration of 1.04 mg/mL was subjected to analysis. The mAb sample was measured without additional dilution, using 80 µL eluted sample. In detail, the method was run as follows:

1. Sample Preparation

a) Take sample vial out of freezer and leave at room temperature for min. 15 minutes.

b) Centrifuge at 14,000 g for 5 minutes using a bench-top centrifuge (collection of lyophilized product at bottom of vial).

c) Add 500 µL of 0.1% formic acid in water to the vial.

d) Gently invert and mix content in vial, min. 5 times.

e) Leave vial at room temperature for min. 15 minutes.

f) Gently invert and mix content in vial, min. 5 times prior to UV measurement.

2. UV instrument suitability

a) Measure UV absorbance using WAV-7 solid-state reference cell at wavelengths of 270 nm, 280 nm, 300 nm, 320 nm, and 340 nm. Expected absorbance readings are as follows:

Wavelength (nm)	Expected absorbance reading
270	1.0050
280	0.9060
300	0.8000
320	0.5120
340	0.4000

The observed absorbance reading should be within 5% of the expected absorbance reading.

b) Measure UV absorbance using H_2O (blank for BSA protein standard solution).

c) Measure UV absorbance at 280 nm and 320 nm of control, BSA protein standard (sourced from NIST).

- Calculate concentration of control (ε = 0.67 mL/mg), conc. should be within ± 0.1 mg/mL.

3. Sample UV measurement

a) Measure UV absorbance using 0.1% formic acid in water (blank for sample) at 280 nm and 320 nm.

b) Transfer adequate volume of solution (e.g., 80 µL) to UV cell and measure absorbance at 280 nm and 320 nm.

c) If difference between absorbance at 320 nm for blank and sample is < 0.1 skip to step f.

d) If difference between absorbance at 320 nm for blank and sample is > 0.1, centrifuge samples at 14,000 g for 5 minutes using a bench-top centrifuge.

e) Measure UV absorbance at 280 nm and 320 nm again.

f) Calculate corrected UV absorbance at 280 nm by subtracting blank at 280 nm.

g) Calculate recovered mAb amount in µg (ε = 1.4) using this formula:

$[mAb]=AU/\varepsilon$

AU = corrected absorbance reading at 280 nm

ε = 1.4 mL/mg

[mAb] = Antibody concentration (mg/mL)

[mAb]×500 (µL) = content amount (µg)

Results of RP-UHPLC-MS Analysis of Proteolyzed and Reduced Cetuximab

The analysis objective was to perform middle-up mass analysis of all submitted cetuximab samples. After protein A purification, each sample was subjected to IdeS digestion, reduction, and analysis with reversed phase UHPLC-MS for the determination of Fc, LC, and Fab fragment masses.

All media samples (see **Table 4**) were received and stored at -20 °C prior to protein A purification.

To meet system suitability requirements, NIST BSA with a known concentration of 1.04 mg/mL was subjected to an A280 analysis. Concentration of the NIST BSA was calculated to be 1.13 mg/mL, which is within the +/- 0.1 mg/mL system suitability requirements. A280 value, corrected A280 value, and the calculated concentration of the purified Cetuximab samples are listed below in **Table 4**.

Table 4. Denotations and properties of submitted samples.

Sample	A280 value	Corrected A280 value	Concentration (mg/mL)
Blank	0.3486	0	-
Cetuximab E2 Light	0.6761	0.3275	0.24
Cetuximab E2 Heavy	0.5875	0.2389	0.17

System Suitability Test Results

1. Cetuximab Reference

Cetuximab reference sample (10 µL) was injected on the Waters™ Xevo G2S. **Figure 2** illustrates the TIC (total ion current) and photodiode array (280 nm) traces of the digested and reduced antibody, while **Figure 3** displays the charged and decharged mass spectra of the reference. The observed intact mAb glycoform masses matched the common glycoform masses of cetuximab, as listed in **Table 5** below. They. The measured discrepancies between the observed masses and the theoretical values for four glycoforms are all within 0.004% mass error or less.

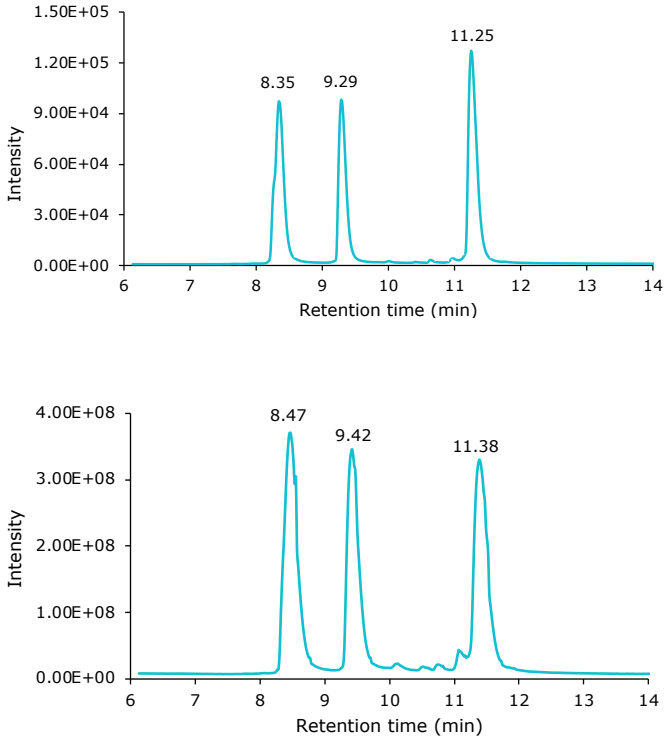


Figure 2. Cetuximab reference after proteolysis and reduction. Top: Photodiode array (280 nm) trace, Bottom: Total ion chromatogram (TIC).

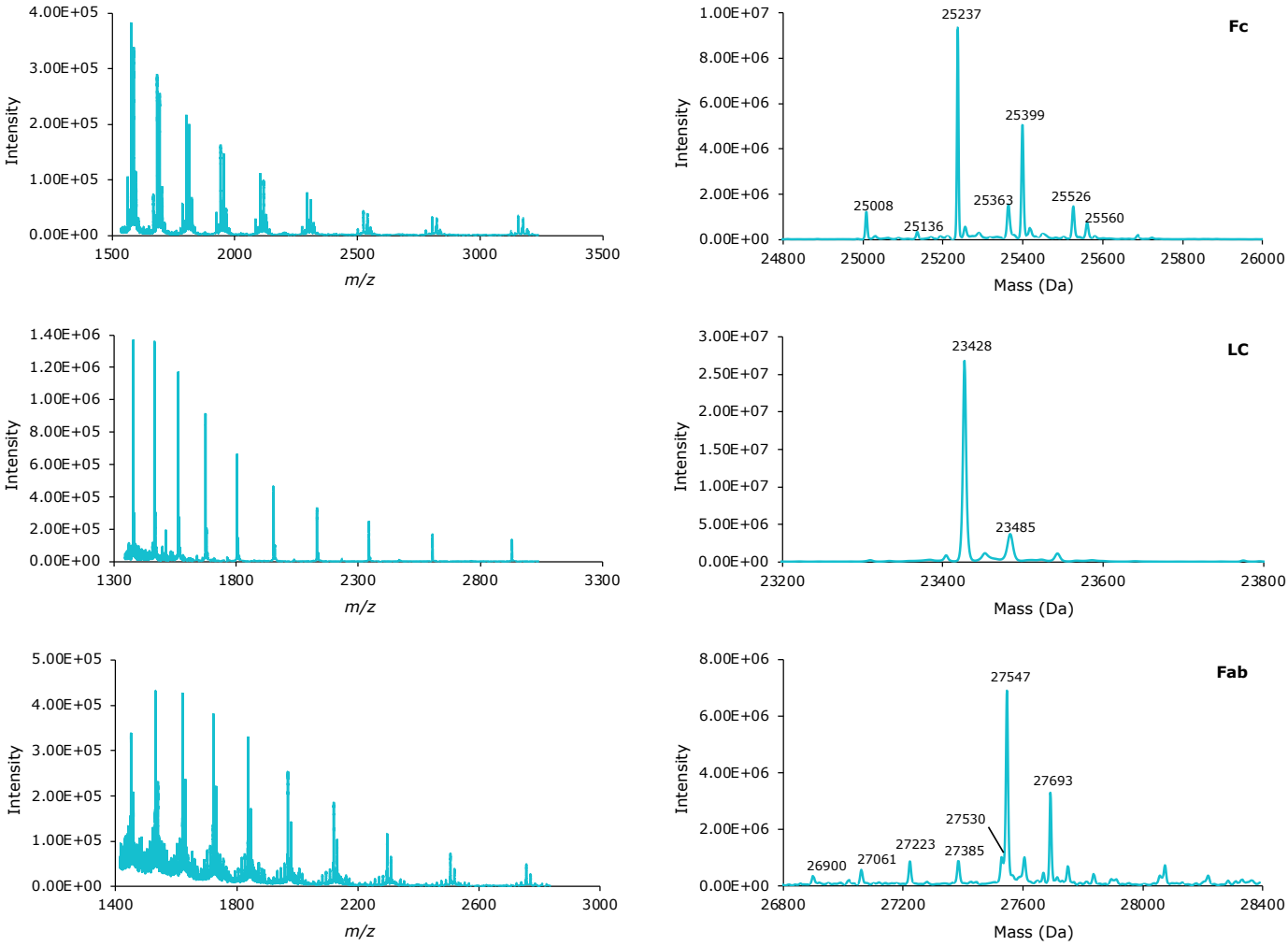


Figure 3. Cetuximab reference after proteolysis and reduction. Summed (left) and deconvoluted (right) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 5. Observed peaks for IdeS-treated and reduced cetuximab reference.

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	Man5	$C_{1112}H_{1724}N_{284}O_{357}S_7$	25008	25008	0.000
	Man5 + Lys	$C_{1118}H_{1736}N_{286}O_{358}S_7$	25136	25136	0.000
	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25236	25237	0.004
	G0F + Lys	$C_{1128}H_{1752}N_{288}O_{362}S_7$	25364	25363	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25398	25399	0.004
	G1F + Lys	$C_{1134}H_{1762}N_{288}O_{367}S_7$	25526	25526	0.000
	G2F	$C_{1134}H_{1760}N_{286}O_{371}S_7$	25560	25560	0.000
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23427	23428	0.004
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23484	23485	0.004
Peak 3 (Fab)	G0F	$C_{1192}H_{1839}N_{301}O_{391}S_8$	26899	26900	0.004
	G1F	$C_{1198}H_{1849}N_{301}O_{396}S_8$	27061	27061	0.000
	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27223	27223	0.000
	G3F	$C_{1210}H_{1869}N_{301}O_{406}S_8$	27385	27385	0.000
	G2FS’	$C_{1215}H_{1876}N_{302}O_{410}S_8$	27530	27530	0.000
	G4F	$C_{1216}H_{1879}N_{301}O_{411}S_8$	27547	27547	0.000
	G4F2	$C_{1222}H_{1889}N_{301}O_{415}S_8$	27693	27693	0.000

*The +57 Da mass shift correlates with one glycine

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G3F: Gal3GlcNAc2Man3GlcNAc2Fuc

G2FS’: NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G4F: Gal4GlcNAc2Man3GlcNAc2Fuc

G4F2: Gal4GlcNAc2Man3GlcNAc2Fuc2

2. SigmaMAb™ Reference

SigmaMAb™ reference sample (10 µL) was injected on the Waters™ Xevo G2S. **Figure 4** illustrates the photodiode array (280 nm) and TIC traces of the digested and reduced antibody, while **Figure 5** displays the charged and decharged mass spectra of the reference. The observed masses are listed in **Table 6** below.

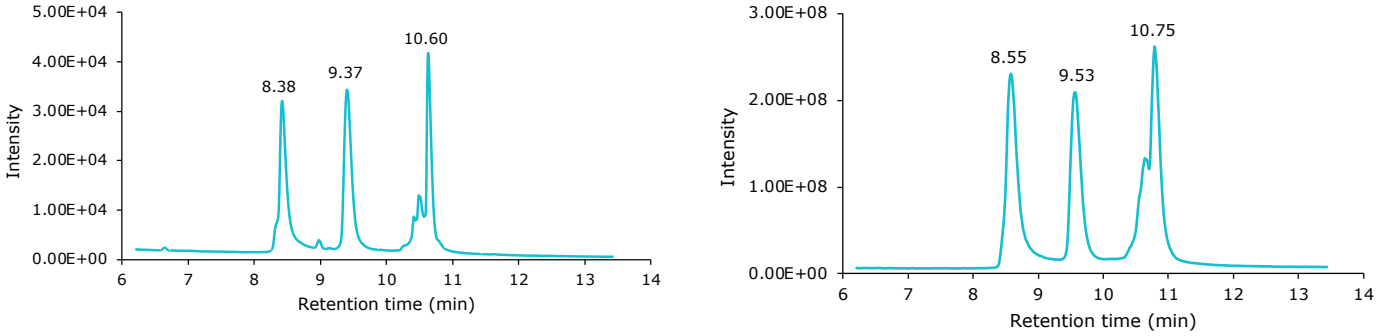


Figure 4. Photodiode array (280 nm, left) and TIC traces (right) of proteolyzed and reduced SigmaMAb™.

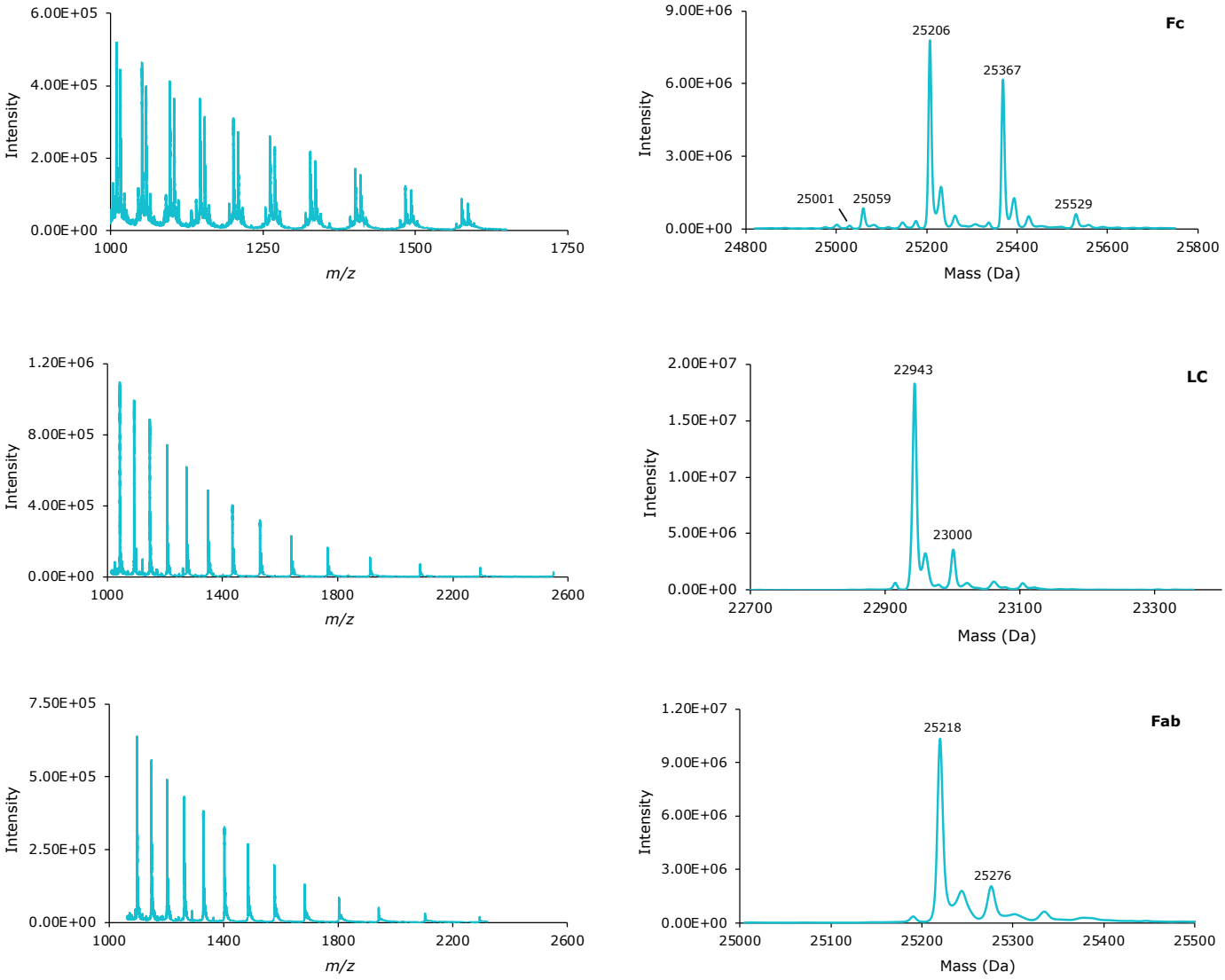


Figure 5. Proteolyzed and reduced SigmaMAb™. Summed (left) and deconvoluted (right) spectra for Fc, LC, and Fab (top to bottom) regions. Peaks 1, 2, and 3 correspond to Fc, LC, and Fab fragments.

Table 6. Observed peaks for IdeS-treated and reduced SigmaMAb™.

Peak	Fragment glycoform	Theoretical mass (Da)
Peak 1 (Fc)	Fc unmodified	23761
	G0F-N	25001
	G0	25059
	G0F*	25206
	G1F**	25367
	G2F	25529
Peak 2 (LC)	LC	22943
	LC + glycine	23000
Peak 3 (Fab)	Fab unmodified	25218
	Fab + glycine	25276

G0F-N: GlcNAc2Man3GlcNAcFuc
G0: GlcNAc2Man3GlcNAc2
G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*, **: G0F and G1F species are expected to be the most abundant

Cetuximab Sample Results

The submitted cetuximab E2 Light (unlabelled) and cetuximab E2 Heavy (labelled) samples were analyzed in a proteolyzed and reduced form using RP-UHPLC-MS.

1. Cetuximab E2 Light (unlabelled)

The TIC, photodiode array (280 nm) trace, and summed and deconvoluted MS spectra for the unlabeled sample cetuximab E2 Light are shown in individual **Figures 6** and **7**. Corresponding observed masses are displayed in **Table 7**. Minor amounts of oxidized species were observed and are not listed.

The IdeS-treated and reduced cetuximab E2 Light unlabeled fractions Fc, LC, and Fab match with the theoretical masses within an error of 0.004% or less. Comparison of the submitted samples to the reference revealed a difference in the glycosylation profile.

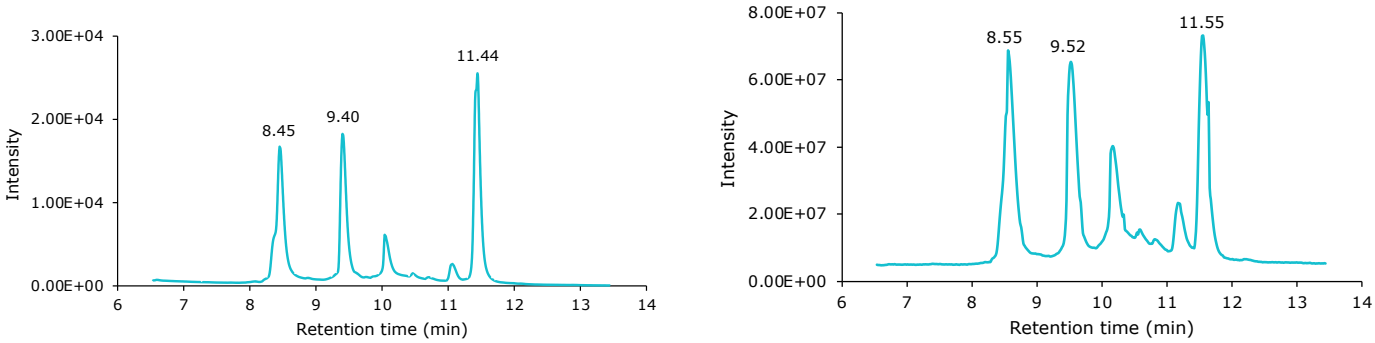
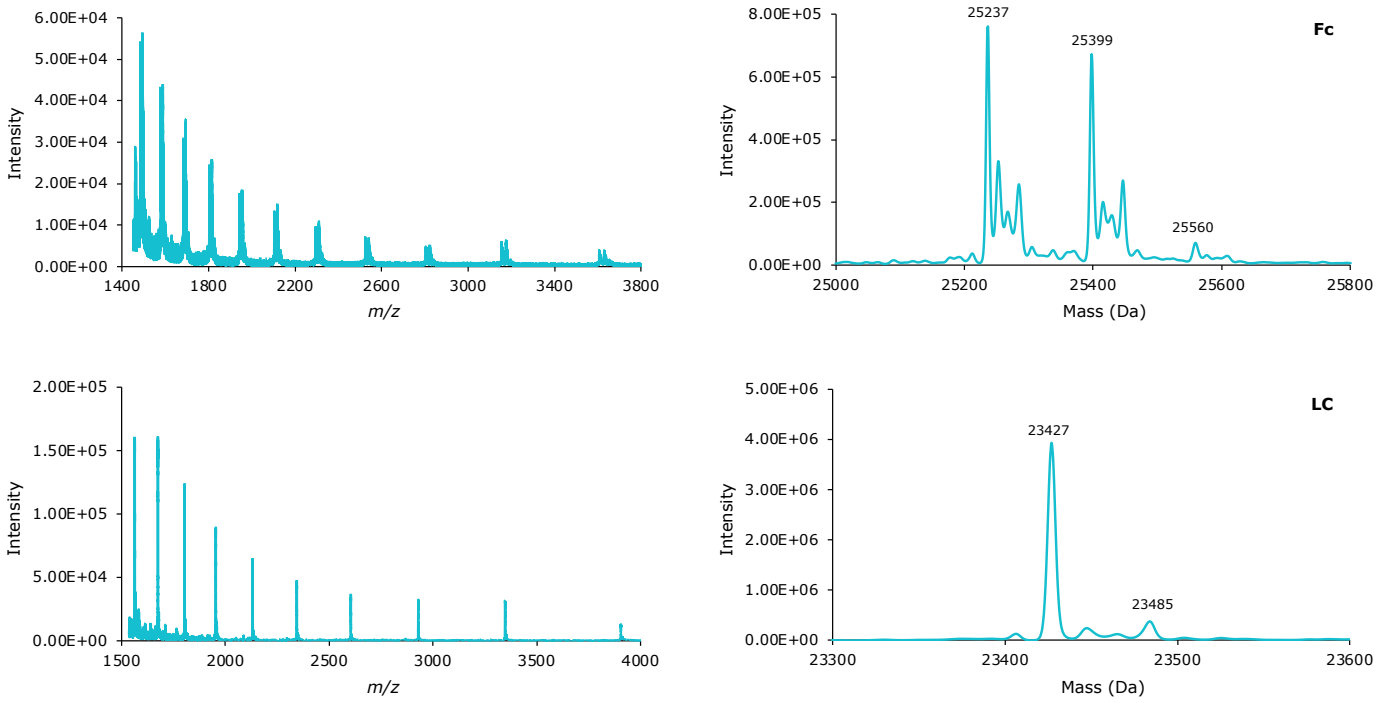


Figure 6. Cetuximab E2 Light unlabeled sample after proteolysis and reduction. Photodiode array (280 nm, left) and TIC traces (right).



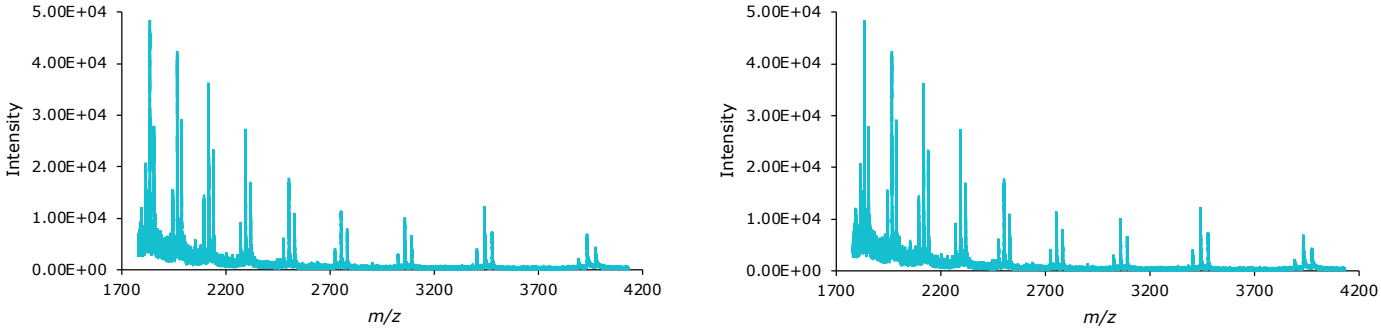


Figure 7. Cetuximab E2 Light unlabeled sample after proteolysis and reduction. Charged (left column) and decharged (right column) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 7. Observed peaks for IdeS-treated and reduced Cetuximab E2 Light unlabeled sample..

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25236	25237	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25398	25399	0.004
	G2F	$C_{1134}H_{1760}N_{286}O_{371}S_7$	25560	25560	0.000
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23427	23427	0.000
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23484	23485	0.004
Peak 3 (Fab)	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27223	27223	0.000
	G2FS	$C_{1215}H_{1876}N_{302}O_{409}S_8$	27514	27514	0.000
	G2FS2	$C_{1226}H_{1893}N_{303}O_{417}S_8$	27806	27805	0.004

*The +57 Da mass shift correlates with one glycine

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G2FS: NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G2FS2: NeuAc2Gal2GlcNAc2Man3GlcNAc2Fuc

2. Cetuximab E2 Heavy (labelled)

The TIC, photodiode array (280 nm) trace, and summed and deconvoluted MS spectra for the labelled sample cetuximab E2 Heavy are shown in individual **Figures 8** and **9**. Corresponding observed masses are displayed in **Table 8**. This sample shows oxidized species that are also listed and that may have occurred during sample preparation.

The IdeS-treated and reduced cetuximab E2 Heavy labelled fractions Fc, LC, and Fab match with the theoretical masses within an error of approx. 0.01%. Comparison of the submitted samples to the reference revealed a different glycosylation profile.

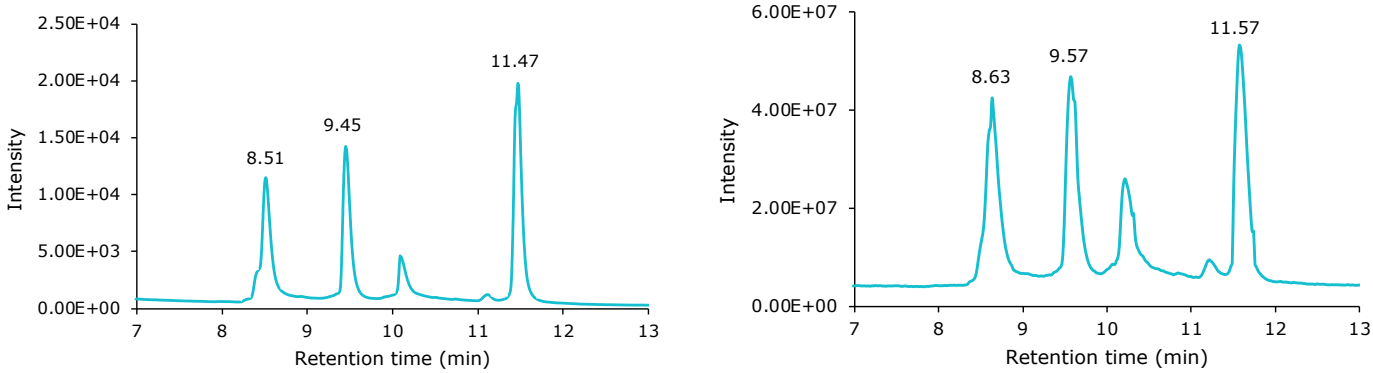


Figure 8. Cetuximab E2 Heavy labelled sample after proteolysis and reduction. Photodiode array (280 nm, left) and TIC traces (right).

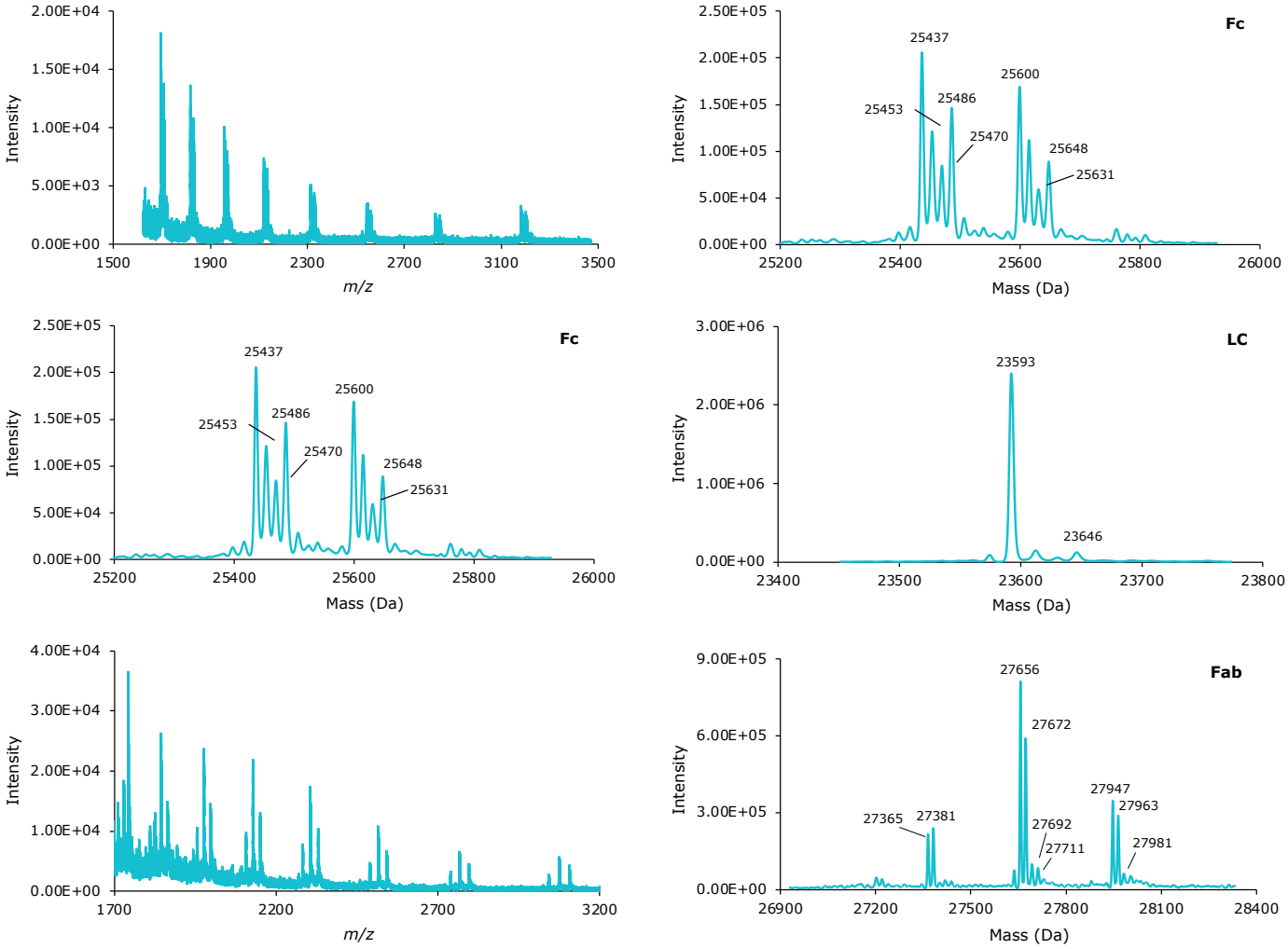


Figure 9. Cetuximab E2 Heavy labelled sample after proteolysis and reduction. Charged (left column) and decharged (right column) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 8. Observed peaks for IdeS-treated and reduced Cetuximab E2 Heavy labelled sample.

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25438	25437	0.004
	G0F+O	$C_{1122}H_{1740}N_{286}O_{362}S_7$	25450	25453	0.004
	G0F+2O	$C_{1122}H_{1740}N_{286}O_{363}S_7$	25469	25470	0.004
	G0F+3O	$C_{1122}H_{1740}N_{286}O_{364}S_7$	25485	25486	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25600	25600	0.000
	G1F+O	$C_{1128}H_{1750}N_{286}O_{367}S_7$	25616	25616	0.000
	G1F+2O	$C_{1128}H_{1750}N_{286}O_{368}S_7$	25632	25631	0.004
	G1F+3O	$C_{1128}H_{1750}N_{286}O_{369}S_7$	25647	25648	0.004
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23594	23593	0.004
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23651	23646	0.020**
Peak 3 (Fab)	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27366	27365	0.004
	G2F+O	$C_{1204}H_{1859}N_{301}O_{402}S_8$	27382	27381	0.004
	G2FS	$C_{1215}H_{1876}N_{302}O_{409}S_8$	27657	27656	0.004
	G2FS+O	$C_{1215}H_{1876}N_{302}O_{410}S_8$	27673	27672	0.004
	G2FS+2O	$C_{1215}H_{1876}N_{302}O_{411}S_8$	27689	27692	0.010
	G2FS+3O	$C_{1215}H_{1876}N_{302}O_{412}S_8$	27705	27711	0.020**
	G2FS2	$C_{1226}H_{1893}N_{303}O_{417}S_8$	27948	27947	0.004
	G2FS2+O	$C_{1226}H_{1893}N_{303}O_{418}S_8$	27964	27963	0.004
	G2FS2+2O	$C_{1226}H_{1893}N_{303}O_{419}S_8$	27980	27981	0.004

*The +57 Da mass shift agrees with one glycine

**The % error was slightly higher than 0.01%

E2 Heavy sample Peak 1 Fc G0F: 25236+ (((18K x 8.01) + (6R x 10.01)) x 99% = 25438

E2 Heavy sample Peak 2 LC: 23427+ (((11K x 8.01) + (8R x 10.01)) x 99% = 23594

E2 Heavy sample Peak 3 Fab G2F: 23423+ (((13KX8.01) + (4R X 10.01)) X 99% = 27366

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G2FS: NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G2FS2: NeuAc2Gal2GlcNAc2Man3GlcNAc2Fuc

G4F2: Gal4GlcNAc2Man3GlcNAc2Fuc2

Conclusion of RP-UHPLC-MS Middle-up Mass Analysis of Proteolyzed and Reduced Monoclonal Antibodies

A workflow for the RP-UHPLC-MS middle-up mass analysis of proteolyzed and reduced monoclonal immunoglobulin G antibodies was developed, using unlabeled and labelled cetuximab as a model mAb and SILu™Lite SigmaMAb™ Universal Antibody Standard human as a reference and assay control sample for purification and digestion control, respectively. The workflow was comprised of an antibody purification process using immobilized protein A, a proteolysis step utilizing IdeS, a mAb reduction procedure, a mass spectrometer calibration method, and a system suitability test applying a recombinant human monoclonal antibody reference. In addition, a generic reversed phase UHPLC-MS method suitable for sample separation and analysis of proteolyzed and reduced mAbs was established. System suitability control was performed using an LC-MS standard comprised of nine proteins. Compared to peptide mapping, the advantage of this type of antibody fragment analysis is its much higher speed for establishing the presence or absence of antibody fragment modifications.

The experimental data demonstrated that the workflow can be used for middle-up mass analysis on cetuximab samples. Deconvoluted masses for unlabeled and labelled cetuximab and SigmaMAb™ fragments Fc, LC, and Fab were generated, and all demonstrate a strong correlation with the theoretical masses within an error of approximately 0.01% or less. A comparison of submitted cetuximab samples to the reference mAb revealed a difference in the glycosylation profile.

References

1. K. Sandra, I. Vandenheede, P. Sandra, J. Chrom. A 1335, 81 – 103 (2014).
2. S. A. Berkowitz, J. R. Engen, J. R. Mazzeo, G. B. Jones, Nat. Rev. Drug. Discov. 11, 527 – 540 (2012).
3. J. H. Knox, High Performance Liquid Chromatography, Edinburgh University Press, 1978.
4. V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Anal. Chem. 89, 2086 – 2092 (2017).
5. J. Sjogren, F. Olsson, A. Beck, Analyst 141, 3114 – 3125 (2016).
6. A. Beck, E. Wagner-Rousset, D. Ayoub, A. Van Dorsselaer, S. Sanglier-Cianferani, Anal. Chem. 85, 715 – 736 (2013).
7. F. Lermyte, Y. O. Tsybin, P.B. O'Connor, J.A. Loo, J. Am. Soc. Mass Spectrom. 30, 1149–1157 (2019).

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EZ-Pierce™ films	**Z721581**

Appendix

Sequence

FC

GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

LC

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSIN
SVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWK
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Fab

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVF
FKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKT HTCPPCPAPELLG

N terminus modification: pyro-Glu

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