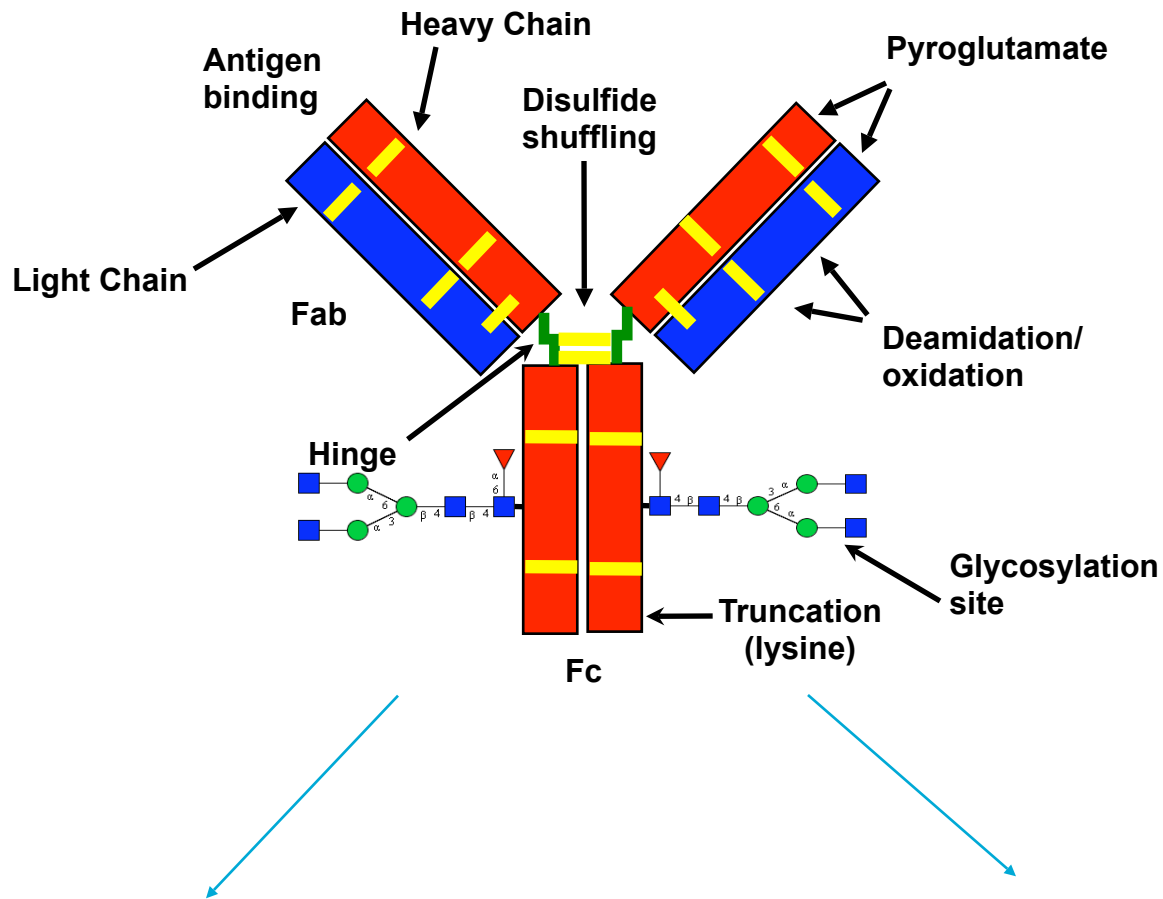


Analysis of Intact Antibodies using HPLC-Chip technology combined with True High Definition QTOF Mass Spectrometry

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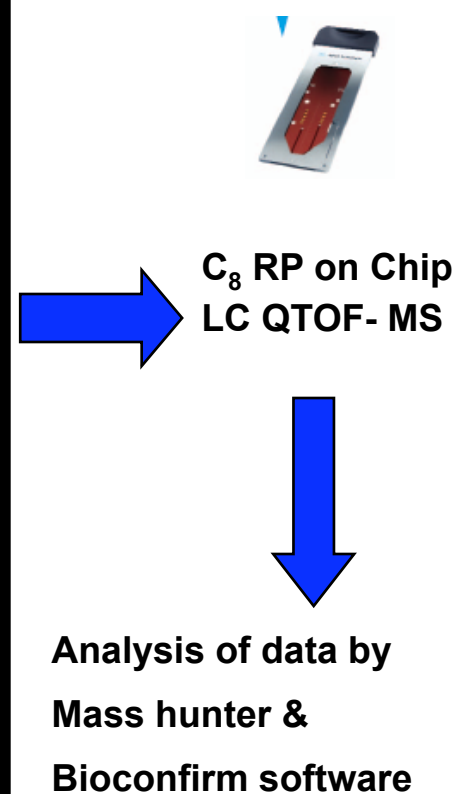
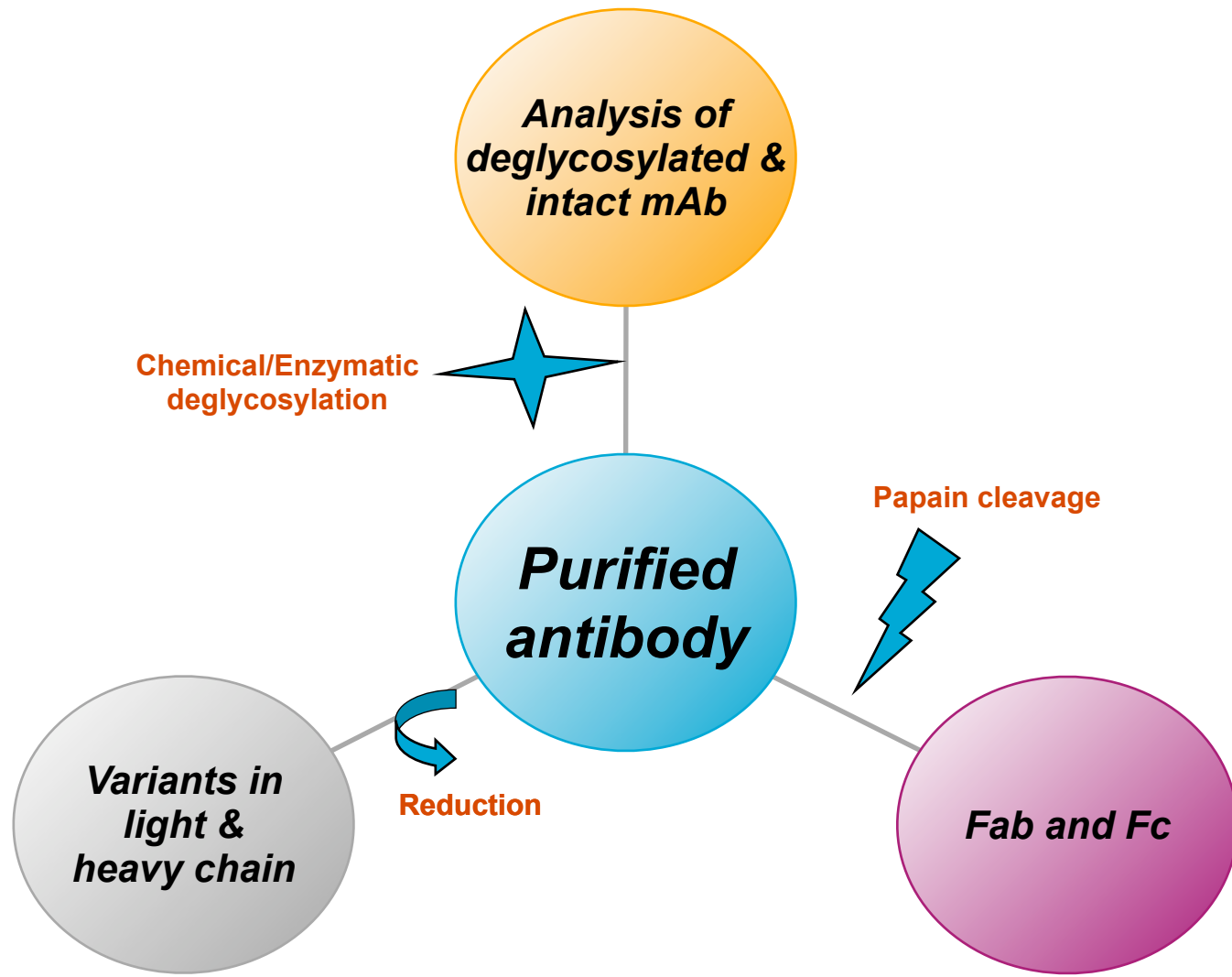
Structure and modifications of Antibody



Primary characterization of the Antibody

Identification of Site specific modification of mABs

Primary characterization of the Antibody



Why use Agilent instrumentation for this?

High Sensitivity QTOF MS results on Antibody proteins

High Mass accuracy QTOF MS results

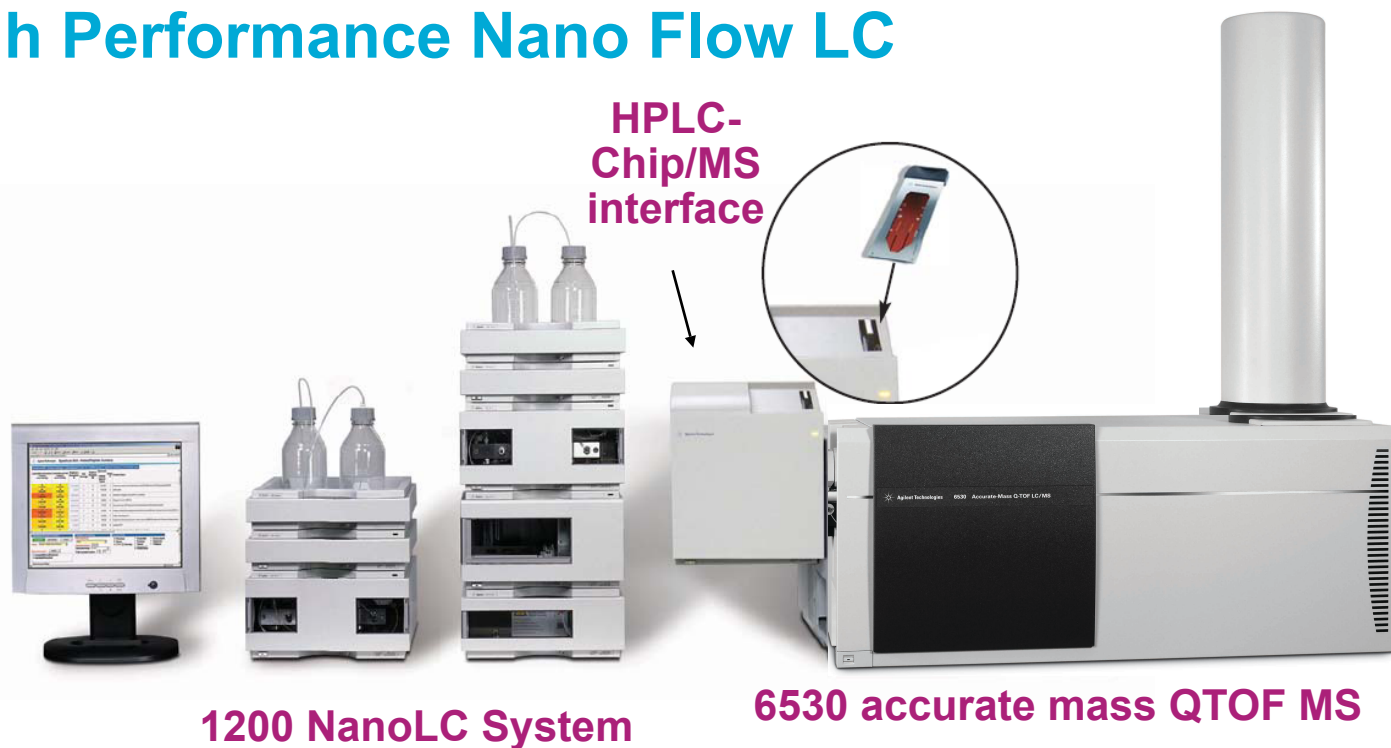
Ability to save on sample by using HPLC-Chip technology to achieve high quality nanoLC introduction to the MS

Agilent nanospray source using HPLC – Chip does not exhibit adduct formation as per other set ups.

Software allows automated high resolution Maximum Entropy based charge deconvolution.

Agilent HPLC-Chip NanoLC coupled to the Agilent 6530 Accurate Mass Q-TOF

- High Def-TOF technology
- High Performance Nano Flow LC



Experimental Parameters

• LC Parameters:

HPLC-Chip : 5 μ m, ZORBAX 300SB-C8 (300A), 40 nl enrichment column and a 75 mm x 43 mm analytical column

Flow rate was 3 μ L/min from capillary pump for the enrichment column and 300 nL/min from nano pump for the analytical column.

0.1% formic acid in water (A) & 90% acetonitrile in water with 0.1% formic acid (B)

Flush volume : 7 μ L

Sample diluted in 3% acetonitrile/water with 0.1% formic acid

Gradient: Starting condition : Nano pump 97%(A) and 3%(B)

Cap Pump 97%(A) and 3%(B)

time	B(%)
1	25
4	60
8	80
12	95
15	5

Stop time: 15 min

• MS Parameters

Spectra were recorded in positive ion mode

Vcap- 1750V and drying gas flow of 5 L/min at 325°C.

Fragmentor voltage - 420V for intact antibody and 350V for the fragments

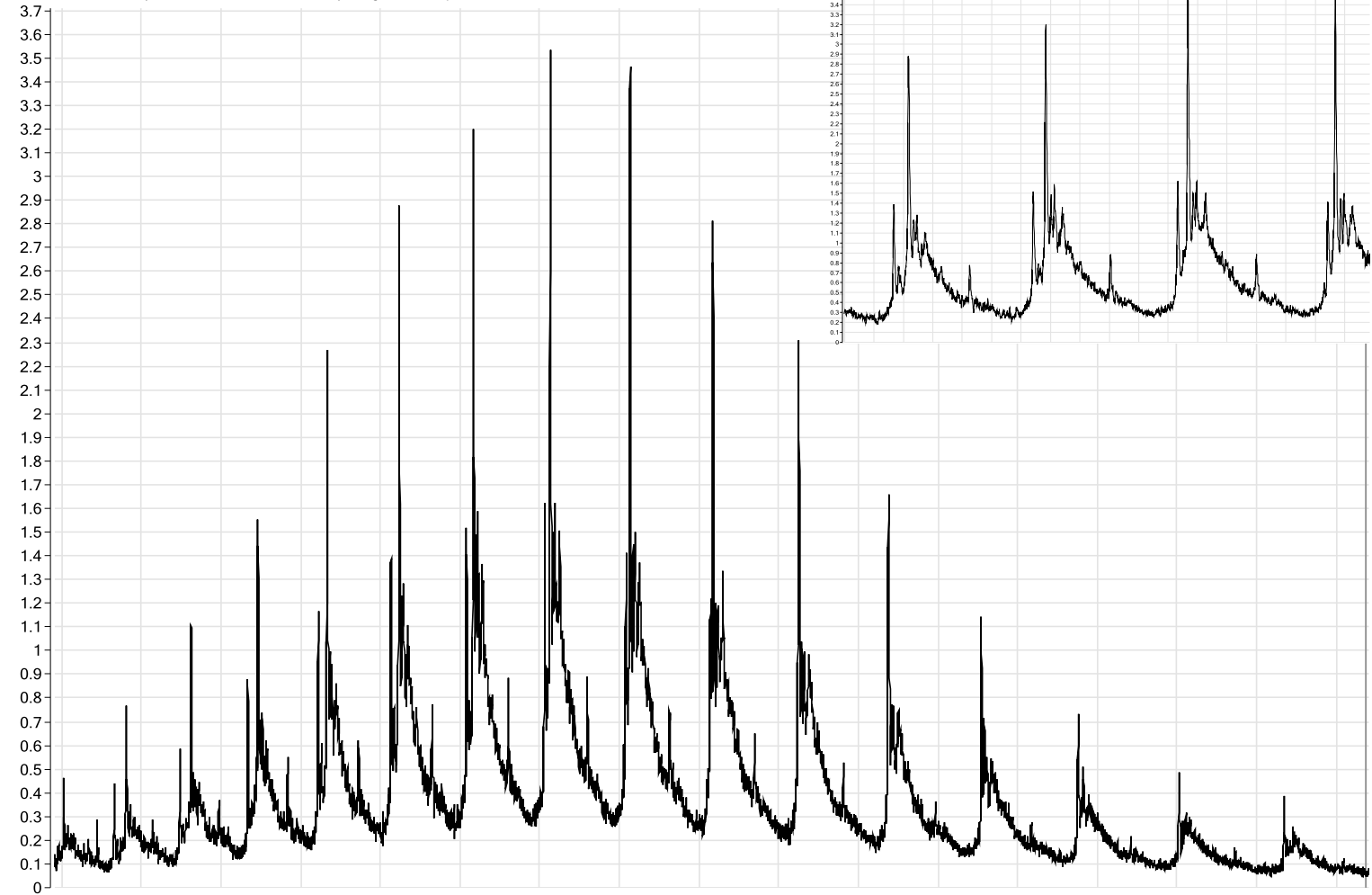
Data were acquired on standard (20000 m/z), 1GHz, MS only mode

range 500-4000m/z (for fragmentsof mAB) ; 1000-4000m/z (for intact mAB)

Intact mAb mass spectrum

x10³ +ESI Scan (6.723-8.473 min, 45 scans) Frag=420.0V promab-r003.d

x10³ +ESI Scan (6.723-8.473 min, 45 scans) Frag=420.0V promab-r003.d



Structures of N-linked sugars commonly found in IgG molecules

Code	Oligosaccharide structure	Average mass
G2F		1769.6
G1F		1607.5
G0F		1445.4
G0		1299.3

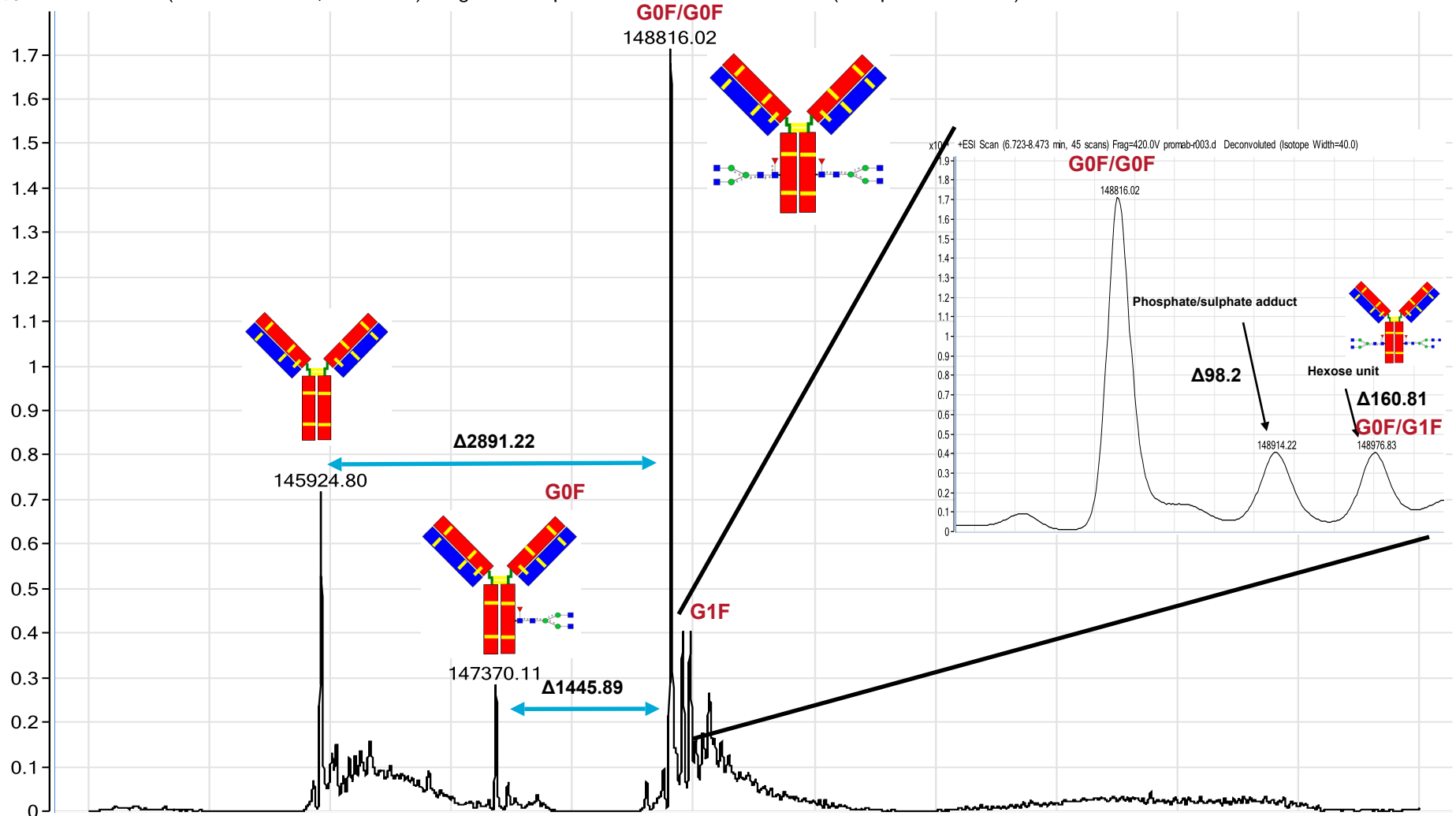
small amt found in this mAb

Major form found in this mAb

- Glucose
- N-acetylglucosamine
- ▲ Fucose
- Mannose

Deconvoluted spectrum of intact mAb

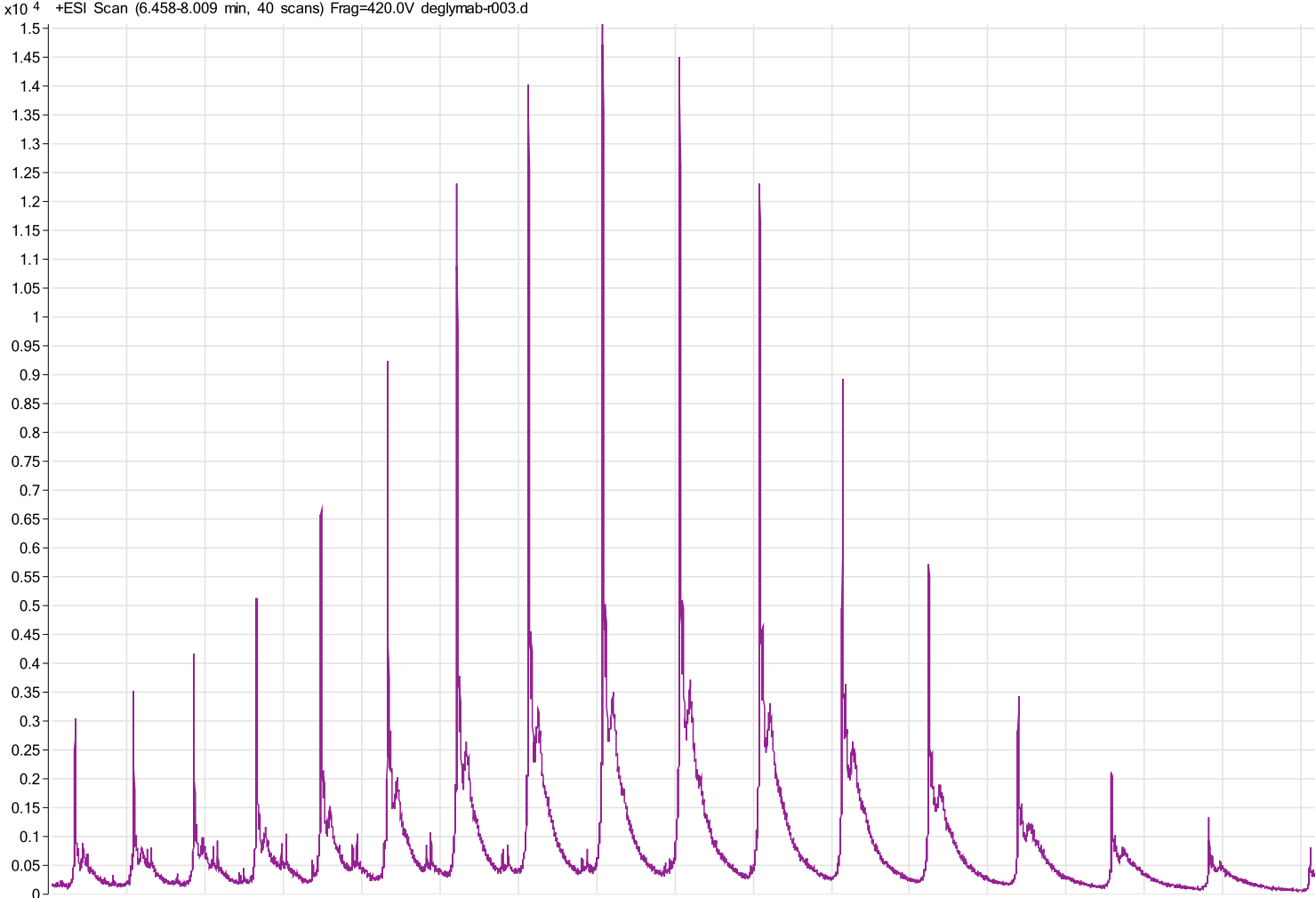
x10⁴ +ESI Scan (6.723-8.473 min, 45 scans) Frag=420.0V promab-r003.d Deconvoluted (Isotope Width=40.0)



Deglycosylated mAb TIC

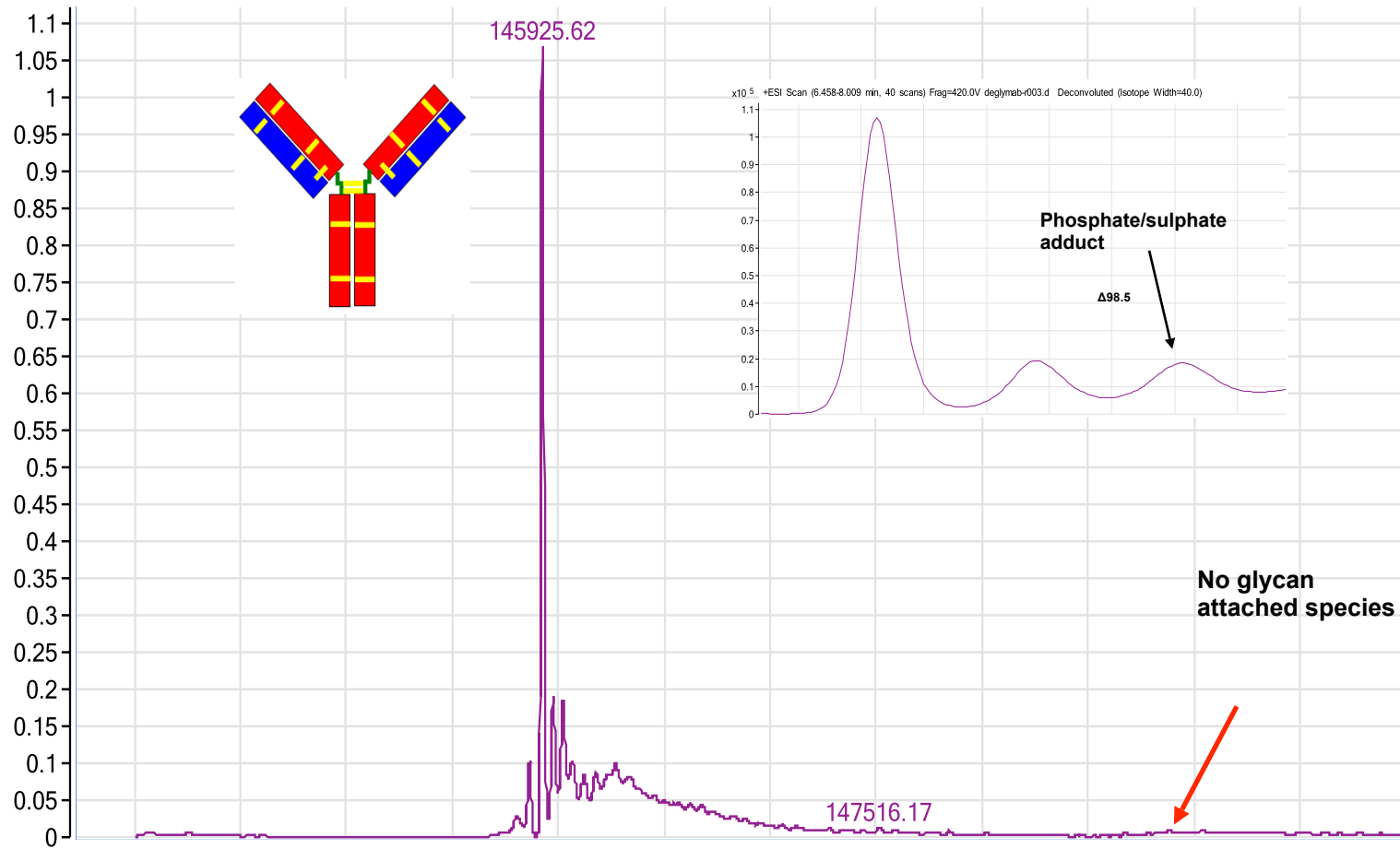
- Deglycosylation of antibody was done using PNGase from Prozyme in 20 mM Tris-HCl buffer pH 8.2.
- 3 μ g of antibody in 20 mM Tris-HCl buffer pH 8.2 and was added 0.3 μ l of 100U/ml solution of PNGase and kept for 24hr incubation at 37°C

Deglycosylated mAb mass spectrum

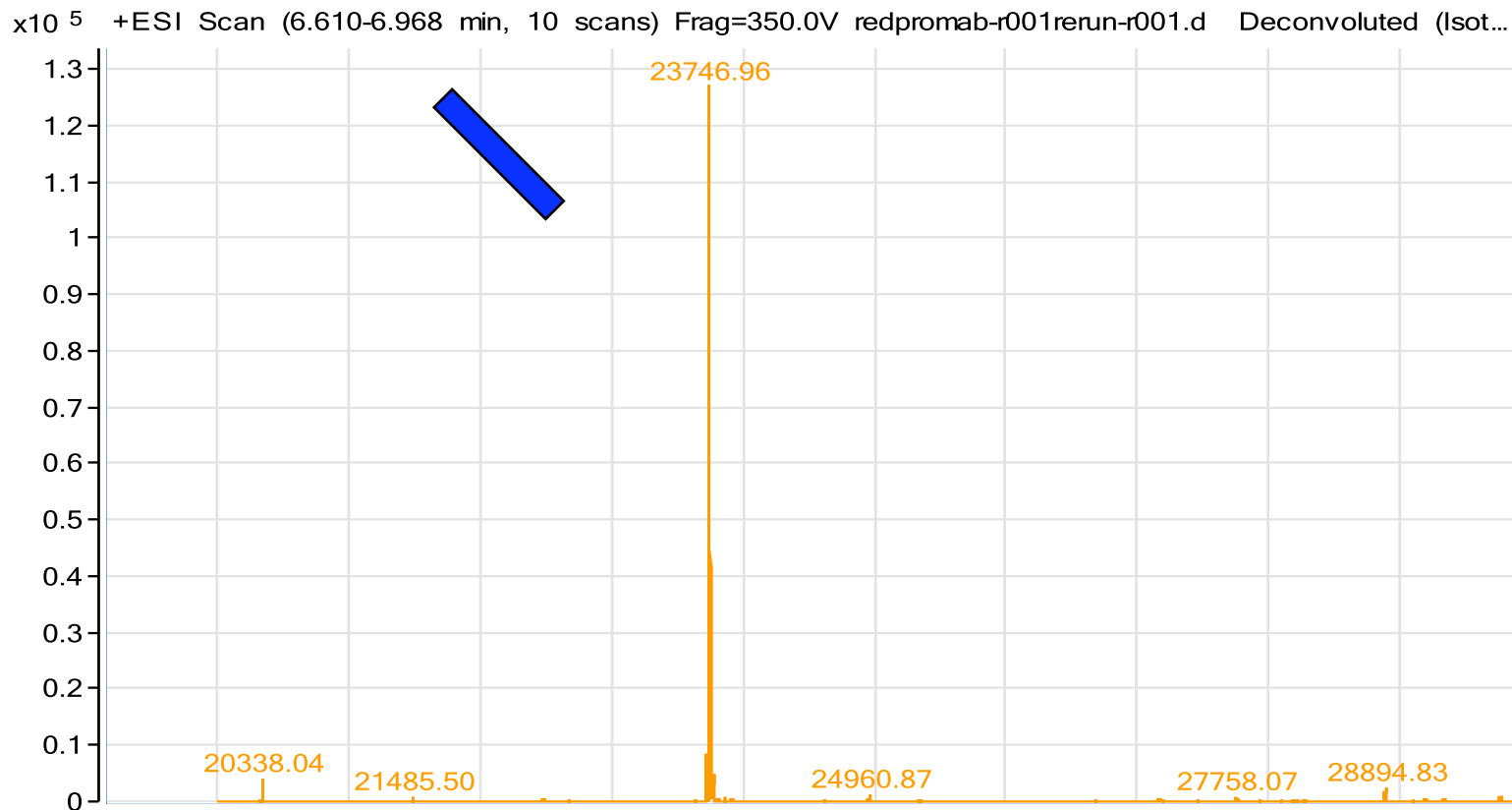


Deconvoluted spectrum of Deglycosylated mAb

x10⁵ +ESI Scan (6.458-8.009 min, 40 scans) Frag=420.0V deglymab-r003.d Deconvoluted (Isotope Width=40.0)



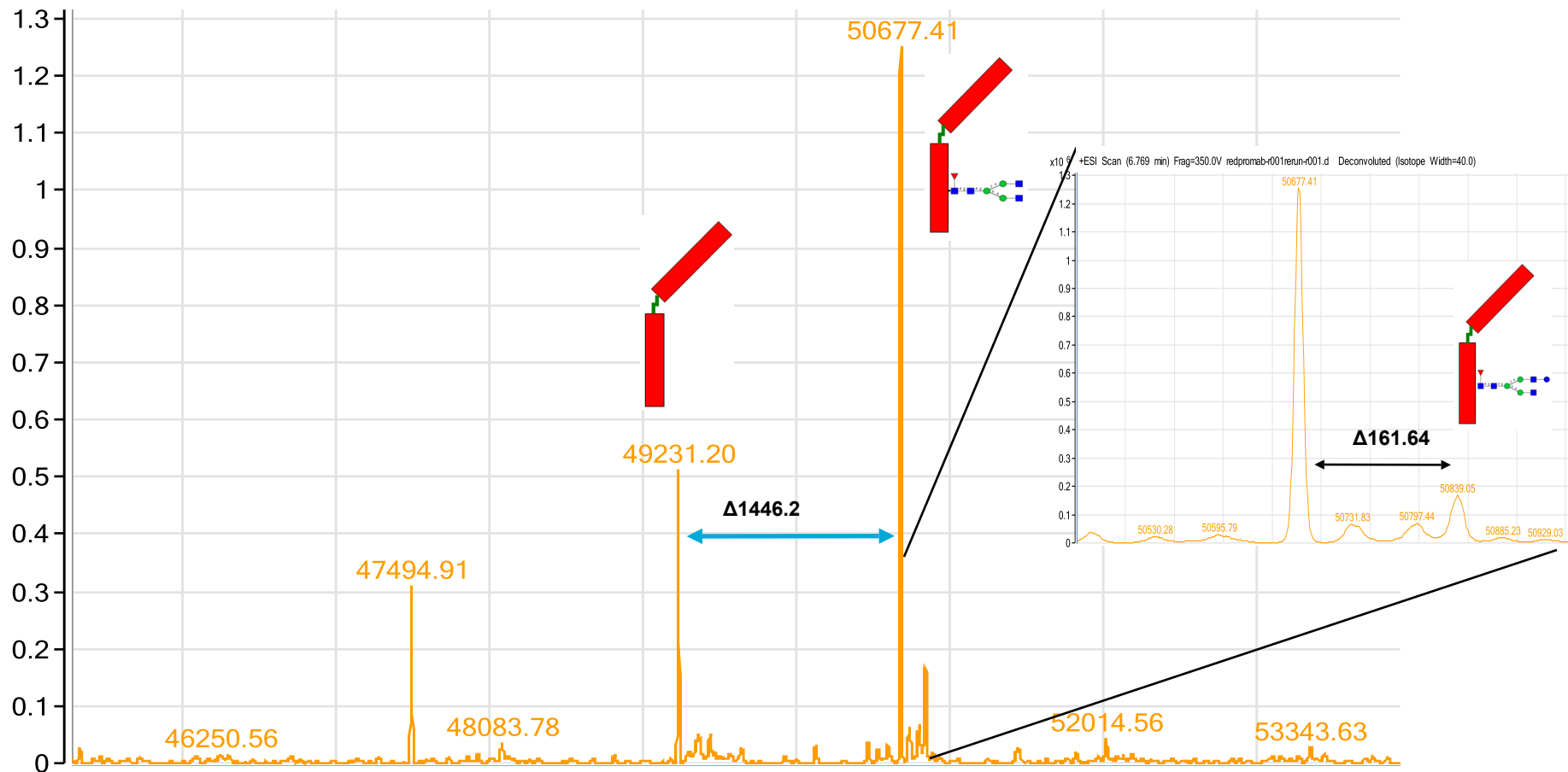
Reduced mAb results: Deconvoluted spectrum of Light Chain of mAb



The antibody(1.5 μ g) was reduced with 25mM DTT at pH 8.6 for 1hr at 80°C.

Deconvoluted spectrum of Heavy Chain of mAb

x10⁶ +ESI Scan (6.769 min) Frag=350.0V redpromab-r001rerun-r001.d Deconvoluted (Isotope Width=40.0)



Lets do some math!----Counting number of Disulfides

From literature :Intact antibody has two light and two heavy chains with **16** disulfides

Light Chain (1)	23746.96Da
Heavy Chain(1)	50677.41Da (with G0F/G0F glycan)
Total	<u>74424.37Da</u>

Total mass of two light and two heavy chains:

$$74424.37 \times 2 = 148848.74\text{Da}$$

Intact mass of Promab: 148816.02Da(with G0F/G0F glycan)

Difference : 32.72Da

For each disulfide reduced you add 2Da, Therefore this antibody has $32.72/2 = 16.36$ or 16 disulfides

Papain digestion

Papain is a cysteine protease from *Carica papaya* latex. It has a broad peptide bond cleavage specificity . It is used to generate Fab and Fc fragments from antibody for various biotechnological/therapeutic applications

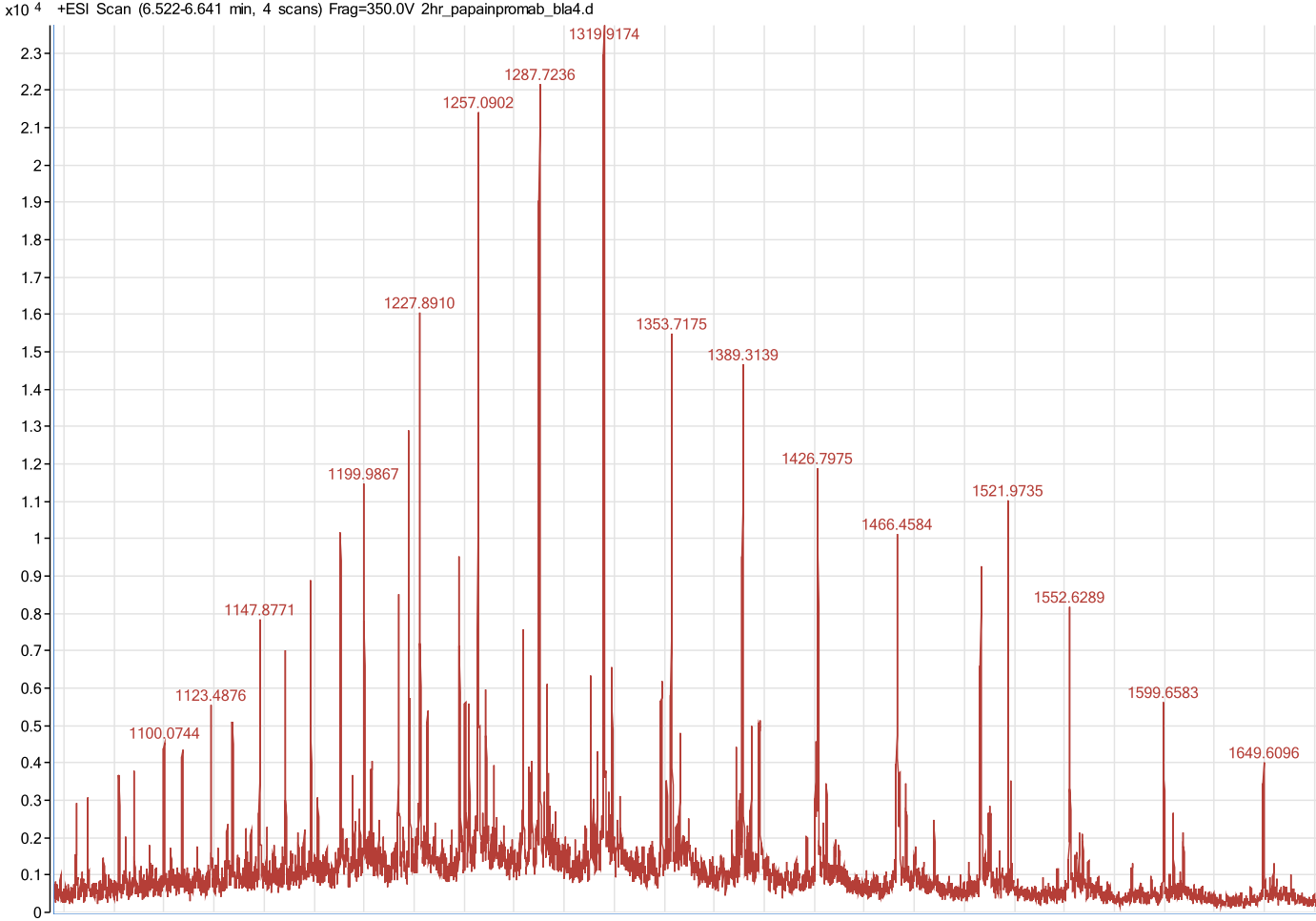
Papain digestion Conditions:

Papain activation/digestion buffer: 1mM EDTA, 50mM sodium phosphate buffer and 10mM cysteine, pH=7.0

The papain was activated using freshly prepared activation buffer for 15min at 37°C.

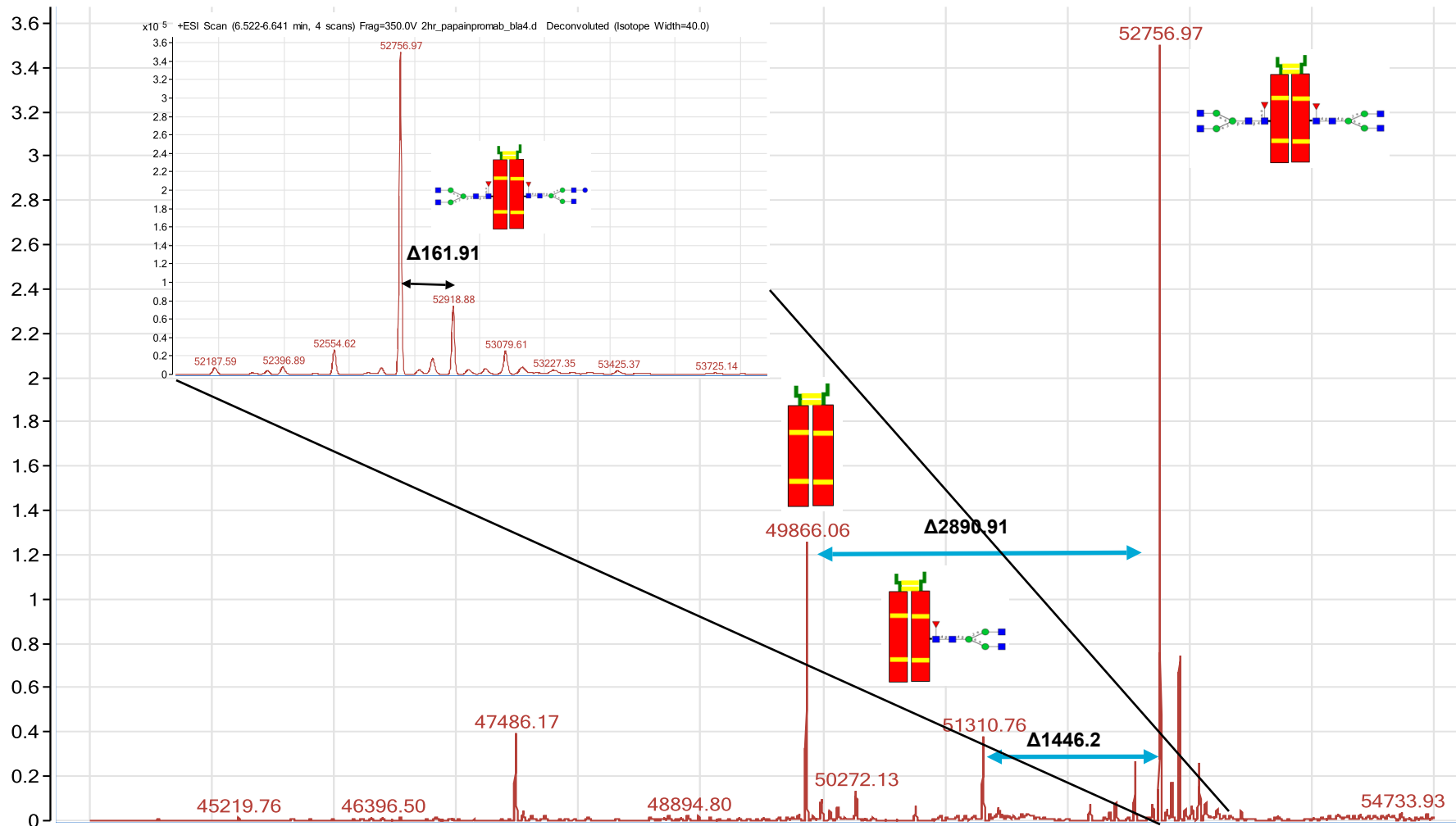
Activated papain solution was added to antibody in digestion buffer. The antibody to protease ratio was 60:1(w/w). The reaction was kept for 2hr digestion at 37°C before mass spectrometry analysis.

Fc fragment of mAb mass spectrum



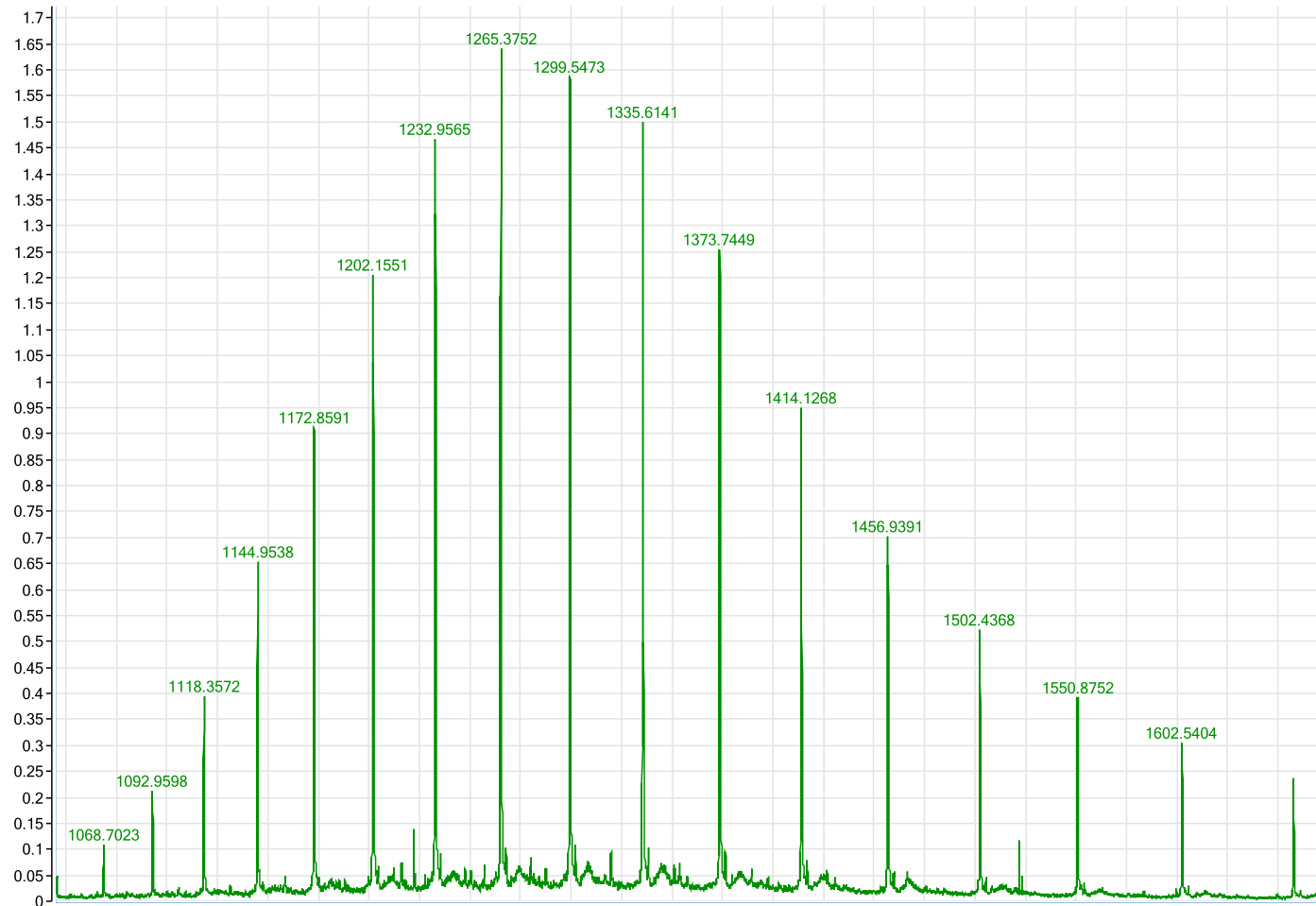
Deconvoluted Fc fragment of mAb

x10⁵ +ESI Scan (6.522-6.641 min, 4 scans) Frag=350.0V 2hr_papainpromab_bla4.d Deconvoluted (Isotope Width=40.0)



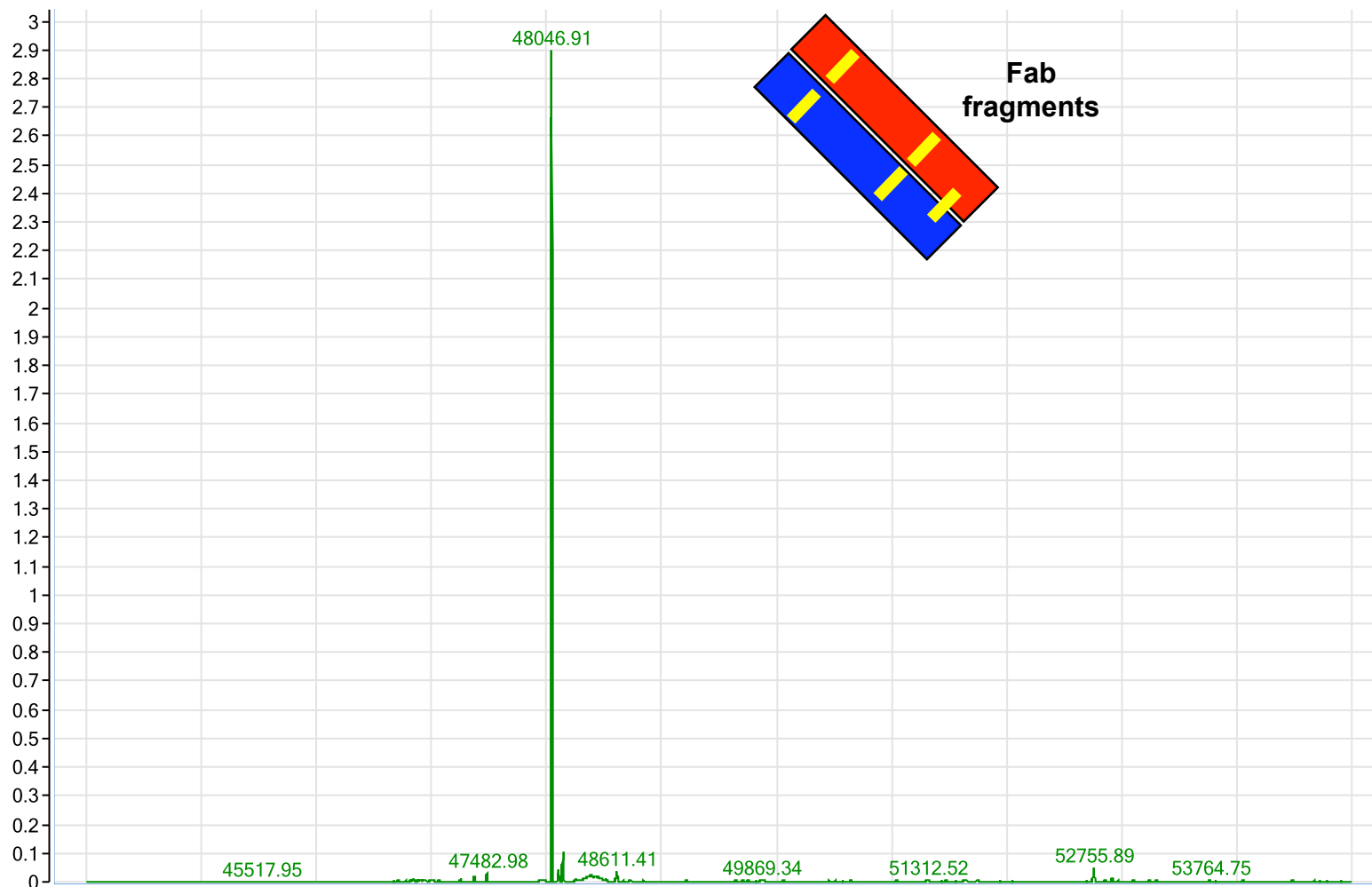
Fab fragment of mAb mass spectrum

x10⁵ +ESI Scan (6.999-7.158 min, 5 scans) Frag=350.0V 2hr_papainpromab_bla4.d



Deconvoluted Fab fragment of mAb

x10⁶ +ESI Scan (6.999-7.158 min, 5 scans) Frag=350.0V 2hr_papainpromab_bla4.d Deconvoluted (Isotope Width=40.0)



Lets do some math!----Counting number of hydrolytic cleavage sites

From literature :Papain digestion generates two Fab and one Fc fragment.
It cuts at two sites (hydrolytic reaction) to generate these fragments.

Fab	$48046.91\text{Da} \times 2 = 96093.82\text{Da}$
Fc	<u>52756.97Da (with G0F/G0F glycan)</u>
Total	148850.79Da
Intact mass of Promab:	<u>148816.02Da (with G0F/G0F glycan)</u>
Difference :	34.77 Da

For each hydrolytic reaction you add 18Da, therefore mass of 34.77Da suggests addition of 2 water molecules(36Da). Hence two cleavage sites as expected from papain digestion.

Sample consumption on different columns

Column	Amt of sample(mg) injected onto column	Reference
HPLC-Chip-LC(43×0.075mm)	5-10ng	This work and [*]
Cap LC,C8 (50 × 1 mm)	~ 4μg	[#]
Poroshell C8(75×2.1mm)	~200ng	[*]

* Dayin Lin and Hongbin Liu *Protein Society* 2008 Poster 236, HPLC-Chip QToF Analysis of MAb

Gadgil HS, Bondarenko PV, Pipes GD, Dillon TM, Banks D, Abel J, Kleemann GR, Treuheit MJ. *Anal Biochem.* 2006 Aug 15; 355(2):165-74.

Conclusion

- **Measurement precision – as confirmed by contrasting Intact antibody with Fab and Fc fragments and with light and heavy chains has been shown to be sub 10ppm or 1 part in 100,000.**
- **High Performance QTOF data can be enhanced with automated charge deconvolution software such that minor phospho/sulfo changes can be observed.**
- **High sensitivity of HPLC-Chip based LC separation with online desalting ensures low sample consumption (a factor of 40 times lower than some techniques), which is an advantage in analysis of expensive and valuable biopharmaceuticals.**
- **This method can be used as an alternative method for routine analysis of biopharmaceuticals with minimal sample preparation.**
- **The chip based LCMS method provides a new tool for the evaluation of product comparability and its feasibility for its use in lot/ product release assays.**