

Highly Sensitive and Robust Workflow for Therapeutic Monoclonal Antibody Analysis from Complex Matrices

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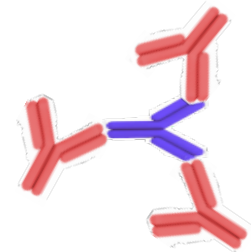
Field Application Specialist, Europe & Emerging
Market Area

Thermo Fisher Scientific

Background: Therapeutic antibody analysis from matrix

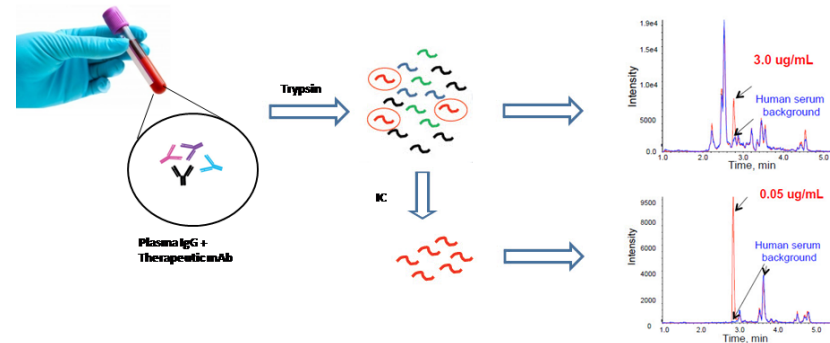
• Ligand Binding Assays Challenges

- Data limited to quantification
- Lack of structural information- Difficult for Drug-Antibody Ratio
- Neutralization events e.g. Anti-Drug Antibodies
- Cross-reactivity & non-specific binding



• LC-MS Challenges

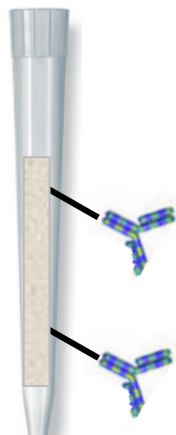
- Lack sensitivity- except with ImmunoCapture
- Data limited to detectable peptides
- Methods are labour intensive
- Automation



- **Goal:** Create a user friendly & reliable approach for affinity purification of therapeutic monoclonal antibodies from plasma for LC-MS analyses

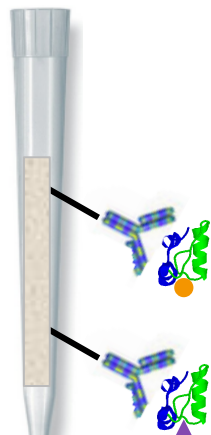
Approach: Mass Spectrometric Immunoassay (MSIA)

Start



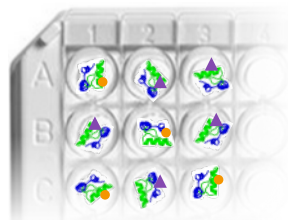
Immobilized
Affinity ligand

Select



Target
selection

Elute



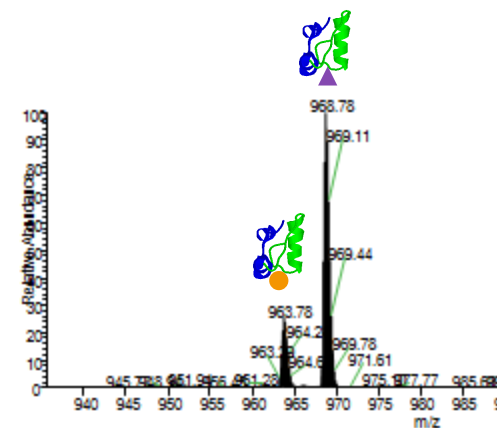
MS-ready
samples

Inject



LC-MS
detection

Analyse



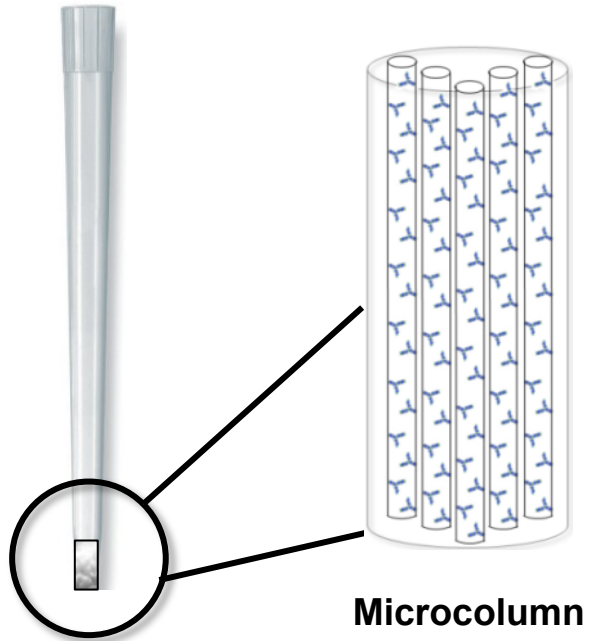
Qualitative and
Quantitative data

Sample Preparation and Liquid Handling

Detection and Analysis

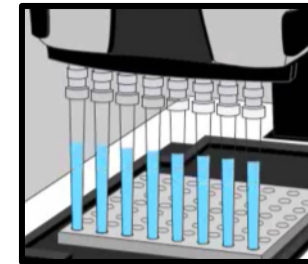
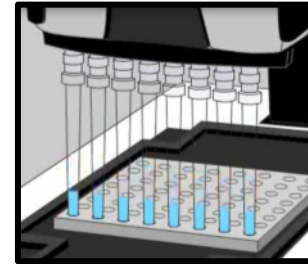
Comprehensive MSIA Workflow

MSIA Microcolumn Technology & Continuous Flow



12 channels: Novus I electronic pipettes

96 channels: Versette automated liquid handler



Microcolumn channels coated with affinity ligands

Continuous aspirating/dispensing cycles to drive reaction kinetics.

Mass Spectrometric Immunoassay (MSIA)
D.A.R.T.'s Pipette Tips

Samples flow up and down through the microcolumn over cycles & target is purified by affinity ligand

- No matrix loss
- Consistent amount of ligand on surface
- Cleaner samples for MS
- Low (1-12) & High Sample (1-96) throughput

Multiple Workflows for Therapeutic Antibody Analysis

MSIA D.A.R.T (tip)	Affinity Ligand	Target Analyte	Comment
Streptavidin	Antigen	Therapeutic Ab or ADC	Antigen specific to therapeutic mAb
Streptavidin	Anti-human Fc antibody	Therapeutic Ab or ADC	Antibody Binds to Fc region of mAb
Streptavidin	Antidrug conjugate antibody	ADC and drug conjugate	Drug conjugate is target
Streptavidin	Idiotypic antibody	Therapeutic Ab/ADC	Idiotypic Ab required
Protein A/G, A or G	Anti-human Fc Ab	Therapeutic Ab/ADC	For pre-clinical models
Protein A/G, A or G	Antidrug conjugate Ab	ADC + drug conjugate	Protein A series orientate antibody
Protein A/G, A or G	Idiotypic antibody	Therapeutic Ab/ADC	Both antibodies will elute off

**Therapeutic Monoclonal Antibody
Analysis from Complex Matrices:**

● **Adalimumab model system**
Antigen as affinity ligand

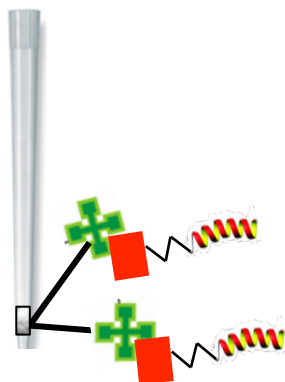
MSIA Workflow: Antigen as Affinity Ligand

Start



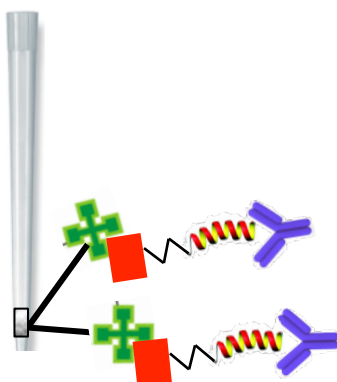
Pre-Rinse
MSIA
D.A.R.T.'S™
Streptavidin

Load



Bind
biotinylated-
TNF α in PBS

Purify



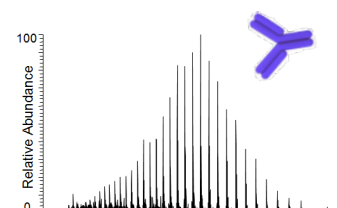
Capture
Adalimumab from
diluted plasma*

Elute



Elute Adalimumab
with 0.4% TFA in
33% AcN

Analyze



LC-MS Data

Processing time: 1 hour

*Sample pre-treatment is possible to counter neutralisation

Bottom up Analyses: LC/MS Conditions

LC Conditions

- Thermo Scientific™ Hypersil GOLD™ Synchronis™ aQ LC Column (2.1 x 100 mm)
- Ultimate 3000 XRS
- Mobile phase A: 0.2% Formic Acid in water
- Mobile phase B: 0.2% Formic Acid in acetonitrile (MeCN)
- Gradient: 2%-35% B @ 150 μ L/min
- Injection volume: 84 μ L

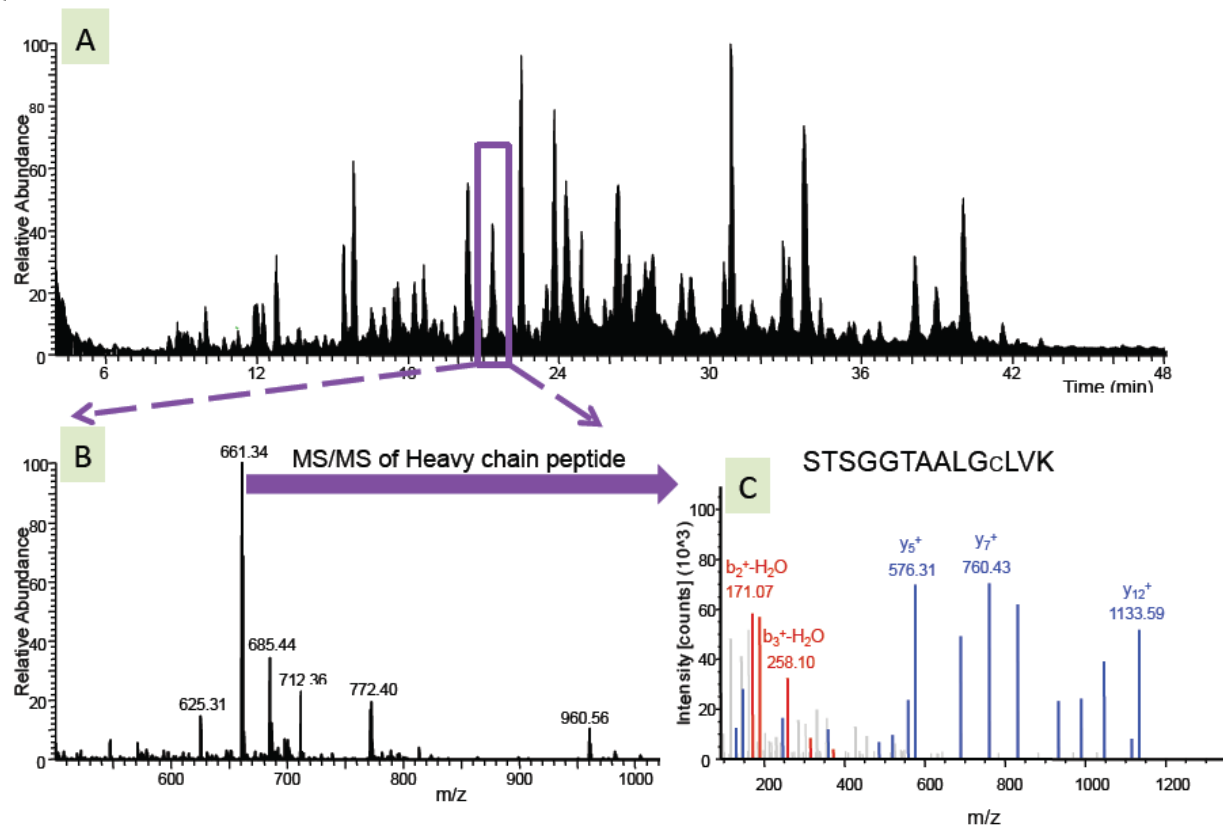
MS Conditions

- Thermo Scientific™ Q Exactive™
- Data-dependent mode, top 10 ions selected for MS/MS.
- Full scans acquired at 70,000 resolution (at m/z 200)
- MS/MS acquired at 17,500 resolution (at m/z 200)



Bottom up Analyses: LC/MS Data

5 ng/mL adalimumab from 200 μ l plasma diluted with 200 μ l PBS



A: Total Ion Chromatogram of digested adalimumab

B: Mass Spectrum of peptides eluting at 21 mins

C: MS/MS & amino acid sequence of heavy chain peptide at m/z 661.34

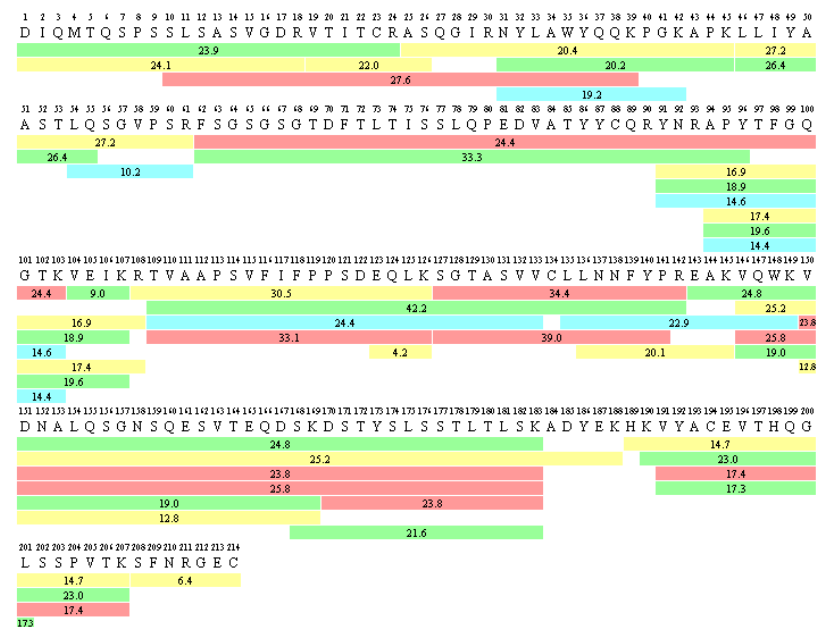
Bottom-up data: Peptide Coverage

• PepFinder Results (5 ng/mL adalimumab from 200µl plasma + 200µl PBS)

HeavyChain



LightChain



Color Code for Signal Intensity

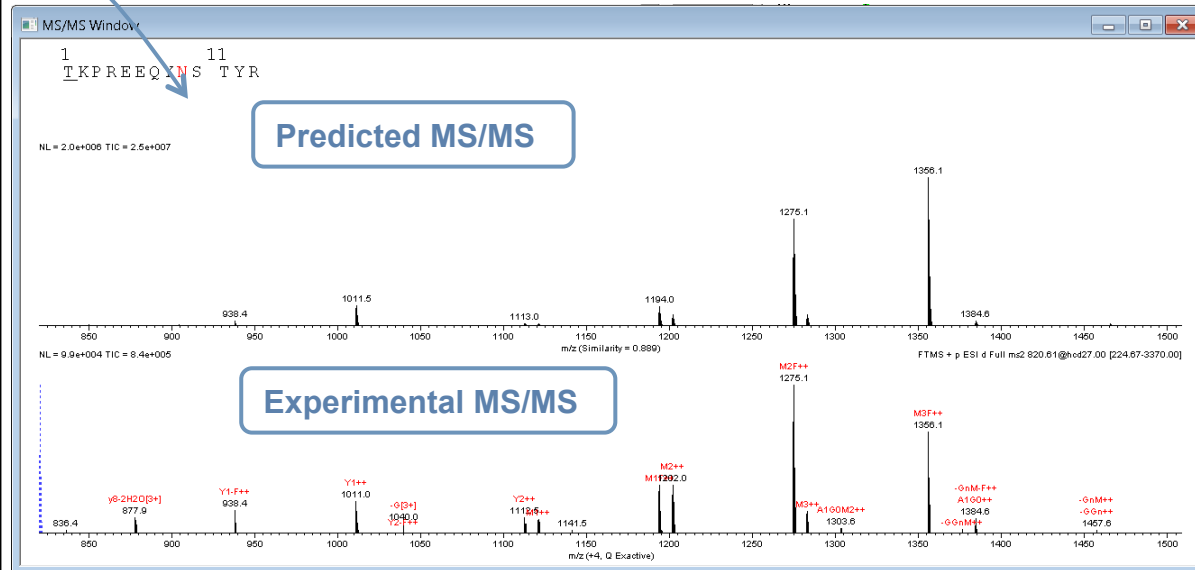
>1.4e+006 >1.5e+005 >1.5e+004 >1.5e+003 >1.5e+002

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:LightChain	202	7.5%	100.0%	45.03%
2:HeavyChain	486	13.0%	98.2%	54.97%

Bottom-up data: Glycopeptides

- PepFinder Modifications (5 ng/mL adalimumab from 200µl plasma + 200µl PBS)

Protein	Modification	Recovery	Abundance
HeavyChain	N290+Deamidation	Good	0.7090%
HeavyChain	~N290+NH3 loss	Good	0.0000%
HeavyChain	N301+A1G1F	Fair	1.4305%
HeavyChain	N301+A2G0F	Fair	47.8488%
HeavyChain	N301+A2G1	Fair	1.0735%
HeavyChain	N301+A2G1F	Fair	19.7984%
HeavyChain	N301+A2G2	Fair	0.8692%
HeavyChain	N301+A2G2F	Fair	19.2940%
HeavyChain	N301+A2S1G1F	Fair	8.0900%
HeavyChain	N301+A3G0	Fair	0.4442%
HeavyChain	N301+A3G0F	Fair	4.1488%
HeavyChain	N301+A3G1F	Fair	4.9133%
HeavyChain	N301+A3G2F	Fair	1.1204%
HeavyChain	N301+A4S1G0F	Fair	2.8024%
HeavyChain	N301+A4S2G0F	Good	18.6272%
HeavyChain	N301+A4Sg2G2	Fair	2.3738%
HeavyChain	N301+A4Sg2G2F	Fair	1.0490%



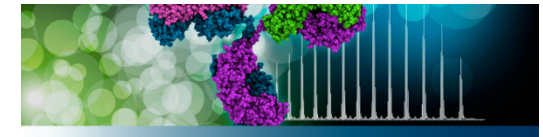
Reduced Heavy/Light Chain LC-MS conditions

LC Conditions

- Thermo Scientific™ **Proswift™** **RP-4H LC column (500 μ m x 10 cm)**
- Ultimate 3000 XRS
- Mobile phase A: 0.2% Formic Acid in water
- Mobile phase B: 0.2% Formic Acid in acetonitrile (MeCN)
- Gradient: 15 - 35% B @ 200 μ L/min
- Injection volume: 84 μ L

MS Conditions

- Thermo Scientific™ Q Exactive™
- Full scans acquired from 900-4500 m/z at 17,500 resolution
- Deconvolved with Protein Deconvolution Software (ReSpect)



Protein Deconvolution 3.0

Confident Analysis of Intact Protein Data

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ReSpect™ deconvolution algorithm. © Copyright 2012 by Positive
Probability Ltd. This program is protected by copyright law and
international treaties as described in help>About.

Thermo
SCIENTIFIC



Heavy/Light Chain data

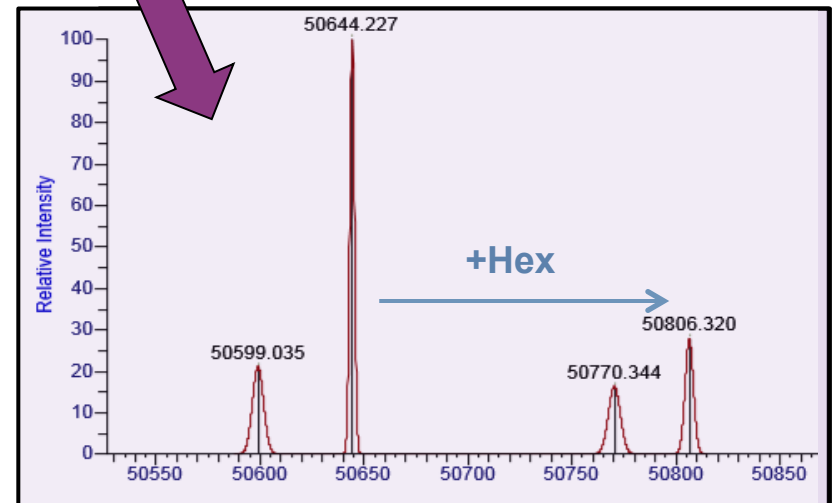
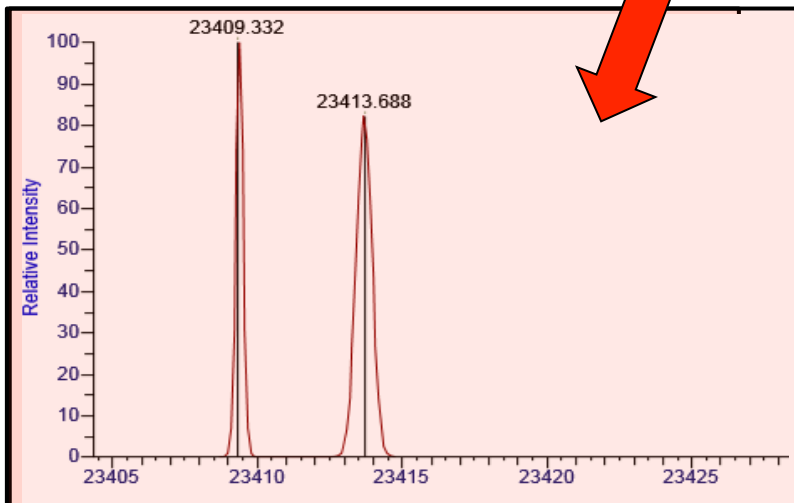
Light Chain

- 4 Da separation- incomplete reduction of 2 disulphide linkages of LC
- 23413.72 mass is 1 Da of the theoretical LC average mass

Heavy Chain

- 50644.22 mass- loss of C-terminal lysine & addition of G0F N-linked glycan
- 50806.32 mass- addition of a hexose group

Deconvolved average mass of LC and HC.



Intact mAb LC-MS conditions

LC Conditions

- Thermo Scientific™ Proswift™ RP-4H LC column (500 μm x 10 cm)
- Ultimate 3000 XRS
- Mobile phase A: 0.2% Formic Acid in water
- Mobile phase B: 0.2% Formic Acid in acetonitrile (MeCN)
- Gradient: 15% - 35% B @ 200 $\mu\text{L}/\text{min}$
- Injection volume: 100 μL

MS Conditions

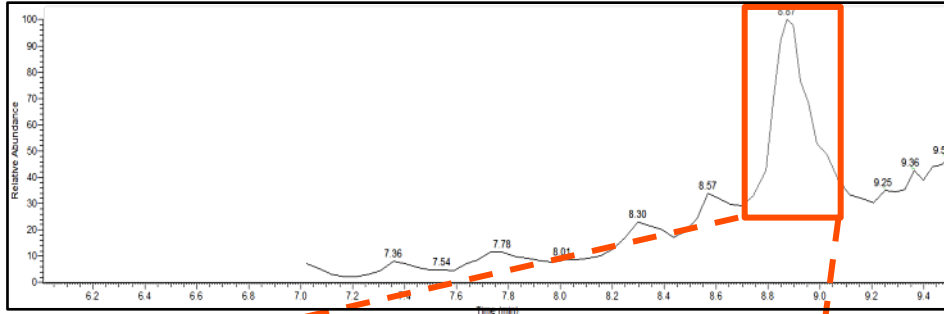
- Thermo Scientific™ Q Exactive™
- Full scans acquired from 1000-4500 m/z at 17,500 resolution
- Deconvolved with Protein Deconvolution Software (ReSpect)



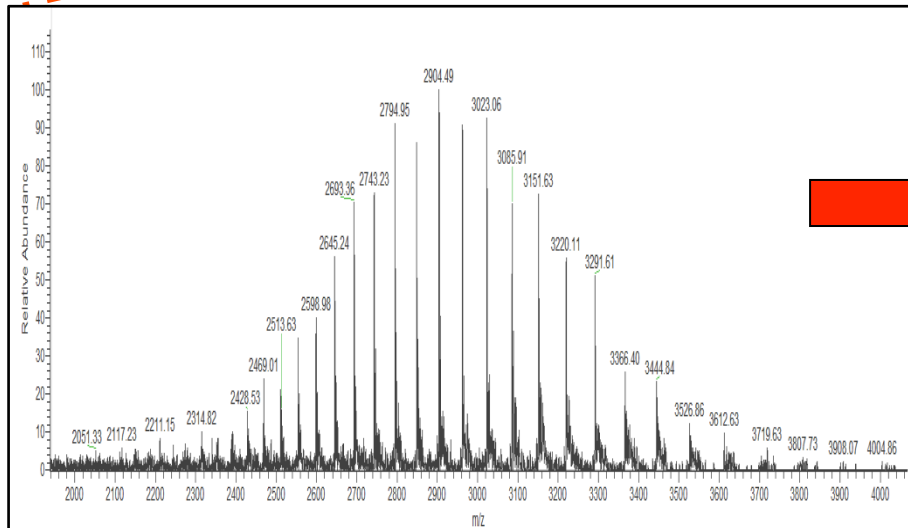
MSIA Workflow: Intact mAb data (ongoing work)

- 1.6 $\mu\text{g/mL}$ adalimumab spiked into 500 μl plasma diluted with 250 μl PBS

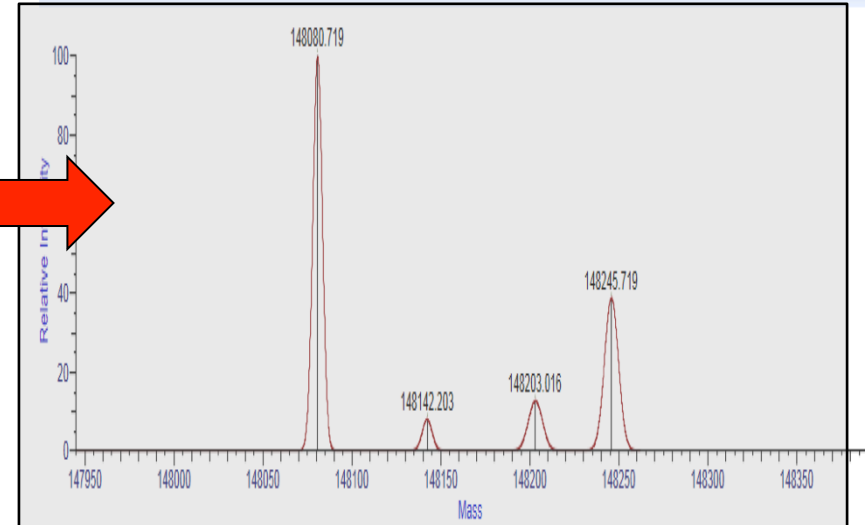
Base peak chromatogram



Raw Mass Spectrum



Deconvolved Mass Spectrum



MSIA Workflow Analytical options: Key Features

- **Bottom Up**
- Most sensitive, can be optimised by slower flow rates (μ LC, nLC).
- Most sequence information
 - Increased ID of PTM sites
- Applicable for quantitative MS
- Amenable to triple-quadrupole MS

- **Heavy/Light chain**
- Sequence coverage depends on number of different fragmentation modes
- Able to observe/identify a variety of PTMs
- Determination of Drug Antibody Ratio (DAR)
- More simplistic methodology (reduces inefficiency in proteolytic digestion)

- **Intact**
- Able to observe/identify a variety of PTMs
- Determination of Drug Antibody Ratio (DAR)
- Less prone to modifications generated during post elution handling steps

Therapeutic Monoclonal Antibody Analysis from Complex Matrices:

Adalimumab model system

Anti-Fc antibody as affinity ligand

Preliminary data

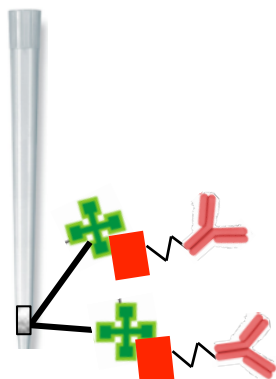
MSIA Workflow: Anti-Fc antibody as Affinity Ligand

Start



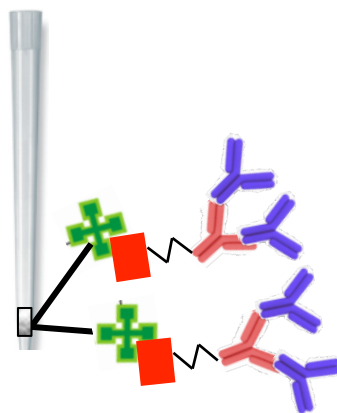
Pre-Rinse
MSIA
D.A.R.T.'S™
Streptavidin

Load



Bind
biotinylated-
anti-Fc antibody

Purify



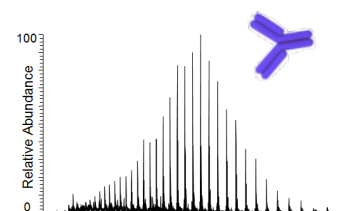
Capture Adalimumab
from diluted plasma:
binds to mAb's FC
region

Elute



Elute
Adalimumab with
0.4% TFA in 33%
AcN

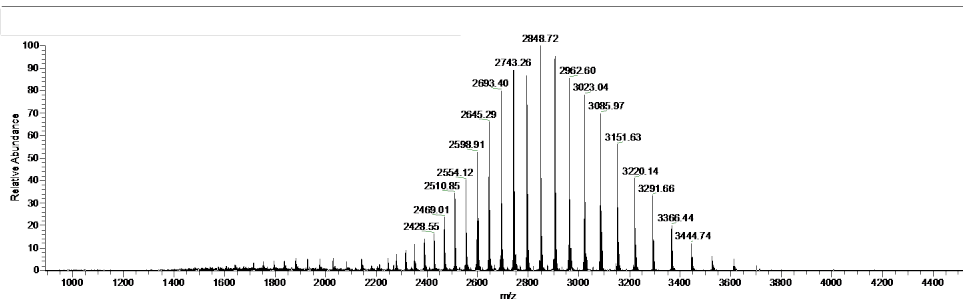
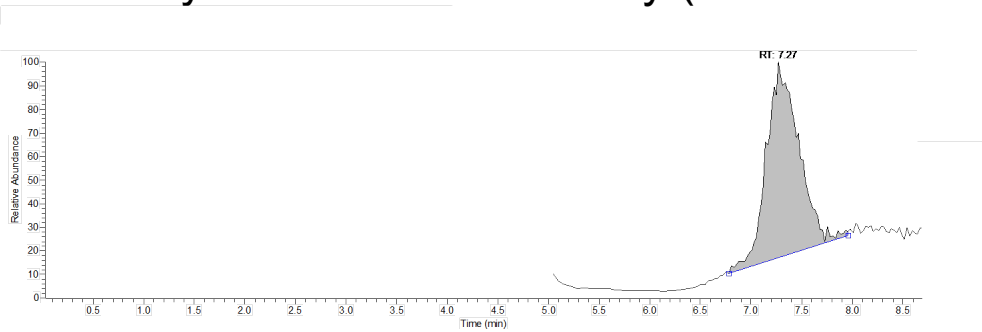
Analyze



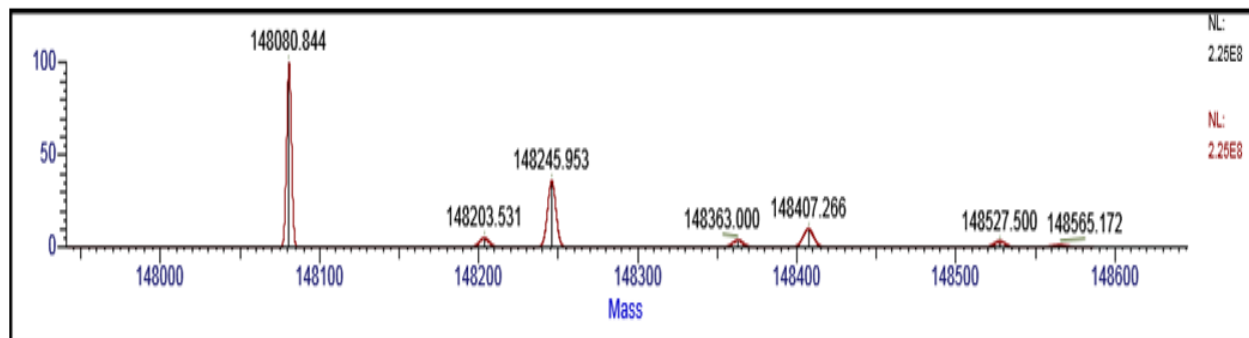
LC-MS Data

MSIA Workflow: Anti-Fc antibody Preliminary data

- 1 μ g Adalimumab spiked into 250 μ l plasma diluted with 250 μ l PBS
- Biotinylated Anti-Fc antibody (Southern Biotech) on Streptavidin MSIA D.A.R.Ts



Deconvolution



Conclusions

- MSIA Streptavidin D.A.R.T.'s were functionalised with biotinylated TNF α & used to purify Adalimumab from plasma.
- Three LC/MS methodologies were presented using HR/AM detection:
 - Bottom up
 - Reduced – Heavy/Light Chains
 - Intact
- MSIA Workflow provides quantitative, qualitative & functional data for Adalimumab.
- MSIA workflow can be applied to other therapeutic antibodies.
 - E.g. there is data on Infliximab
- Initial data- MSIA Streptavidin D.A.R.T.'s can be used to purify adalimumab from plasma using a biotinylated anti-FC antibody



Thank you for listening