Modernizing the Platform Characterization Peptide Map for Accurate Assessment of Deamidation and Isomerization by LC-MS/MS

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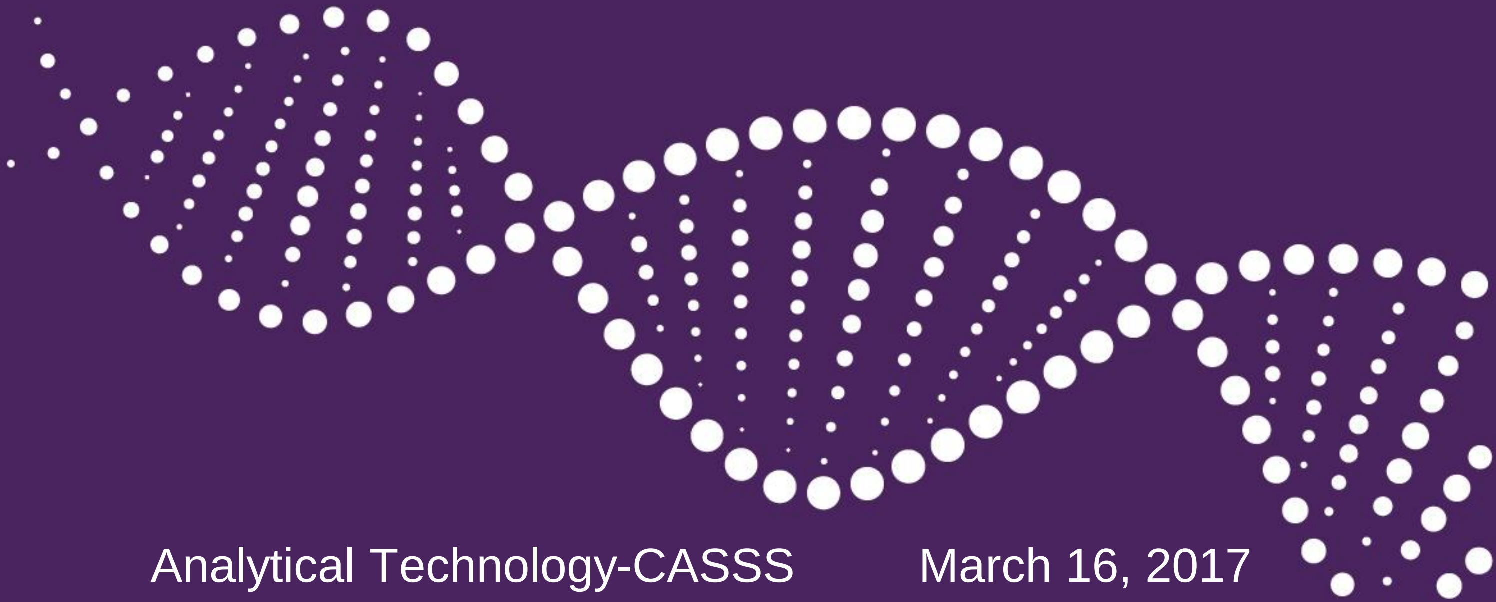
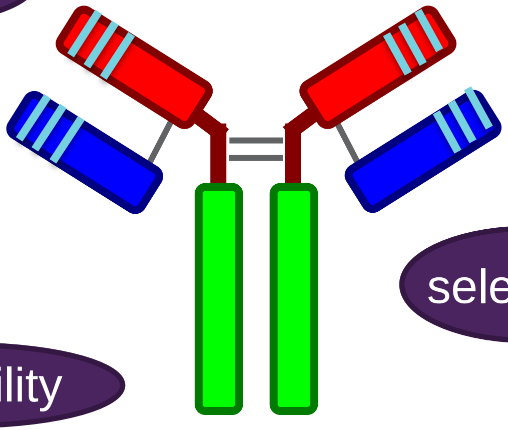


Analytical Technology-CASSS March 16, 2017

Antibody Considerations as a Drug Candidate

Typical parameters in selecting lead antibody:



manufacturability

activity

affinity

poly-reactivity

selectivity

viscosity

stability

aggregation propensity

Antibodies have a propensity to degrade:
manufacturing, storage and in vivo administration

Early Product Development Challenge

Determine Degradation Hotspots

Sequence Liabilities or CQAs?

Engineer to Eliminate Sequence Liability?

- Oxidation: Met & Trp
- Deamidation: Asn and Gln
- Isomerization: Asp
- Glycation: Lys
- Truncations: Asp-Pro

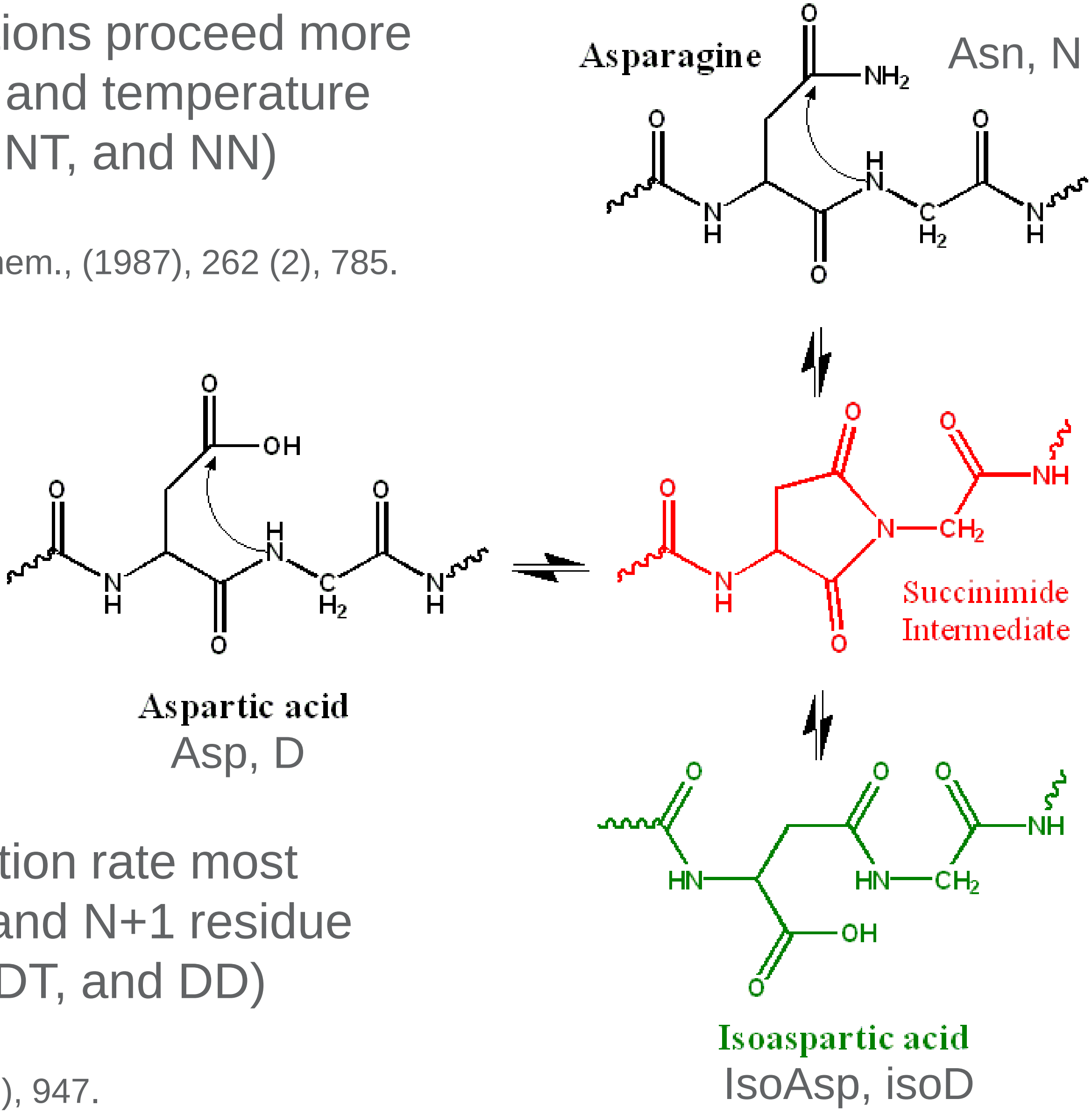
- Molecular modeling
- Peptide mapping

- Does hotspot impact antigen binding?
- Focus on complementarity determining regions (CDRs)
- Constant region hotspots may be potential liabilities

Deamidation/Isomerization Scheme

- Asn deamidation reactions proceed more quickly at elevated pH and temperature
- N+1 residue (NG, NS, NT, and NN)

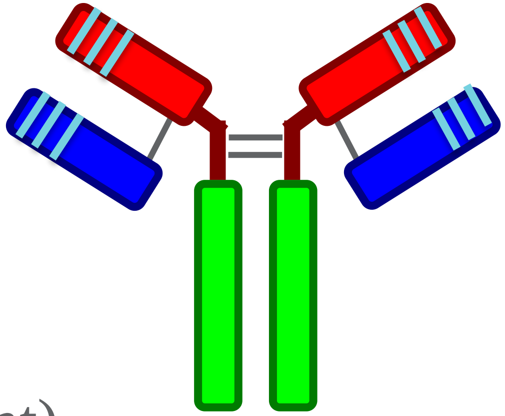
Geiger T. and Clarke S., J of Biol. Chem., (1987), 262 (2), 785.



- Asp isomerization reaction rate most impacted by lower pH and N+1 residue
- N+1 residue (DG, DS, DT, and DD)

Li et al, J. Pharm. Sci., (2013), 102 (3), 947.

The Ideal Peptide Map



- Robust and easy to perform
- Complete sequence coverage
- High quality separations (in a MS-friendly solvent)
- High quality UV chromatograms
- Minimal method-induced artifacts (short digestion times, low pH)
- Accurate targeted quantitation (Extracted Ion Chromatograms)

Developed in-house, automated software to highlight potential "hotspots"

Trastuzumab L Chain CDR Hotspots

1	DIQMTQSPSSLSASVGDRVTITCRASQDV**N**	30
31	**T**AVAWYQQKPGKAPKLLIYSASFLYSGVPS	60
61	RFSGSRSGTDFTLTISSLQPEDFATYYCQQ	90
91	HYTTPPTFGQGTKVEIKR	

Trastuzumab H Chain CDR Hotspots

1	EVQLVESGGGLVQPGGSLRLSCAASGFNI**K**	30
31	**DT**YIHWVRQAPGKGLEWVARIYPT**NG**YTRY	60
61	A**DS**V**K**GRFTISADTSKNTAYLQMNSLRAED	90
91	TAVYYCSR**W**GG**DG**FYA**M**DYWGQGTLVTVSS	120
121		

- Automatically underlines CDR1, CDR2 and CDR3
- Highlights potential hotspots in blue

Case Study #1 Using Trastuzumab (Herceptin®) as a Benchmark for Peptide Map Method Development

Trastuzumab L Chain CDR Hotspots			Rel. Abundance
1	DIQMTQSPSSLSASVGDRVTITCRASQDV**N**	30	High level (>5%)
31	**T**AVAWYQQKPGKAPKLLIYSASFLYSGVPS	60	Low level (1-5%)
61	RFSGSRSGTDFTLTISSLQPEDFATYYCQQ	90	Potential (<1%)
91	HYTTPPTFGQGTKVEIKR		

Trastuzumab H Chain CDR Hotspots			Rel. Abundance
1	EVQLVESGGGLVQPGGSLRLSCAASGFNI**K**	30	High level (>5%)
31	**DT**YIHWVRQAPGKGLEWVARIYPT**NG**YTRY	60	Low level (1-5%)
61	A**DS**V**K**GRFTISADTSKNTAYLQMNSLRAED	90	Potential (<1%)
91	TAVYYCSR**W**GG**DG**FYA**M**DYWGQGTLVTVSS	120	

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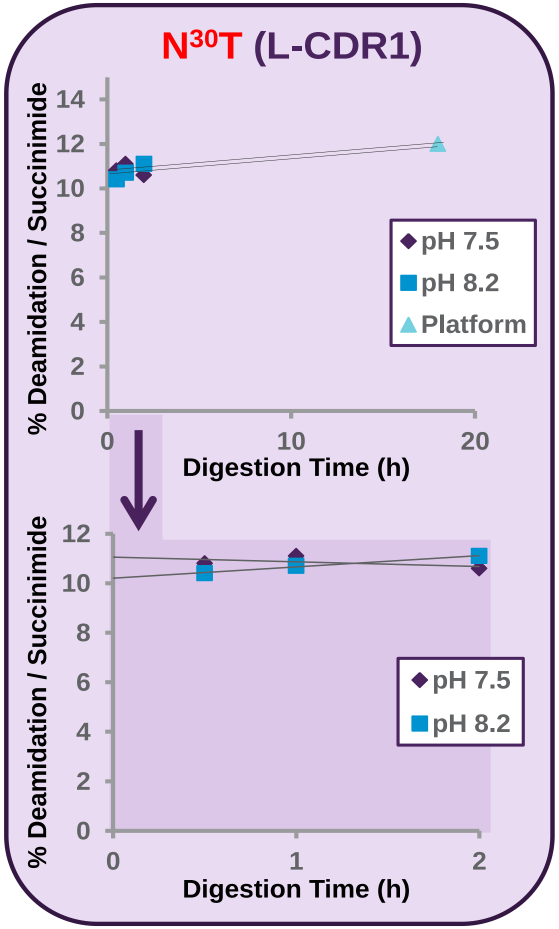
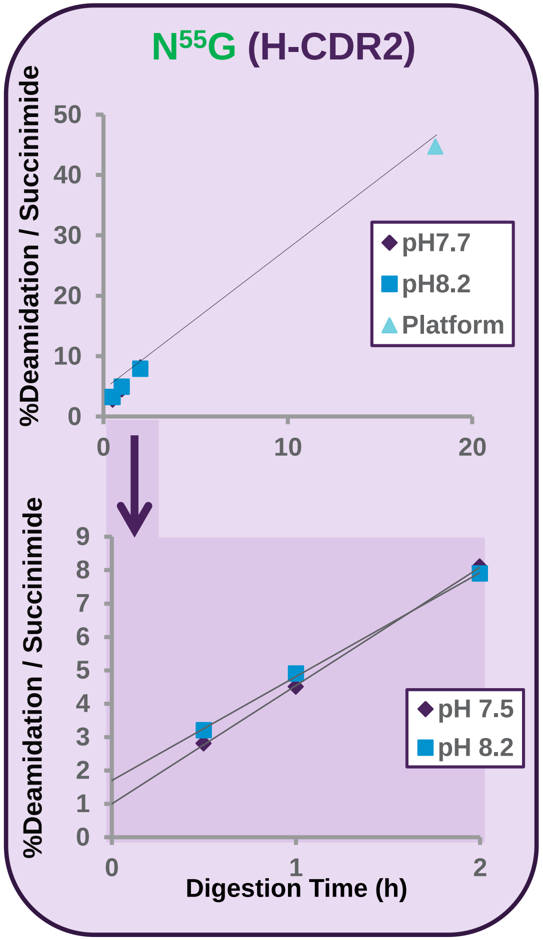
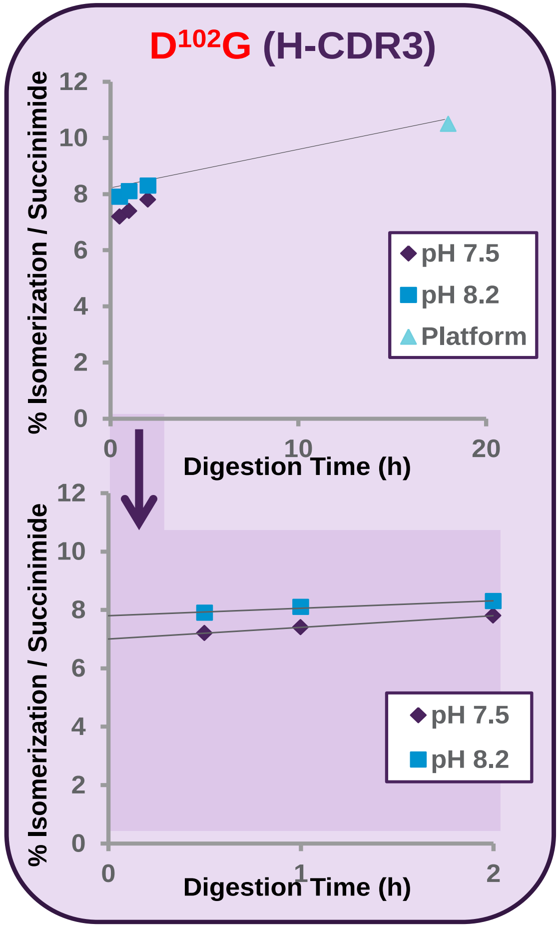
Trastuzumab H Chain CDR Hotspots			Rel. Abundance
1	EVQLVESGGGLVQPGGSLRLSCAASGFNIK	30	High level (>5%)
31	DTYIHWVRQAPGKGLEWVARIYPT**NG**YTRY	60	Low level (1-5%)
61	ADSVKGRFTISADTSKNTAYLQMNSLRAED	90	Potential (<1%)
91	TAVYYCSRWGG**DG**FYAMDYWGQGTLVTVSS	120	

Asn Deamidation and Asp Isomerization vs. Trypsin Digestion Time (Time Course) for Trastuzumab (Herceptin®)


$N^{30}T$ (L-CDR1)

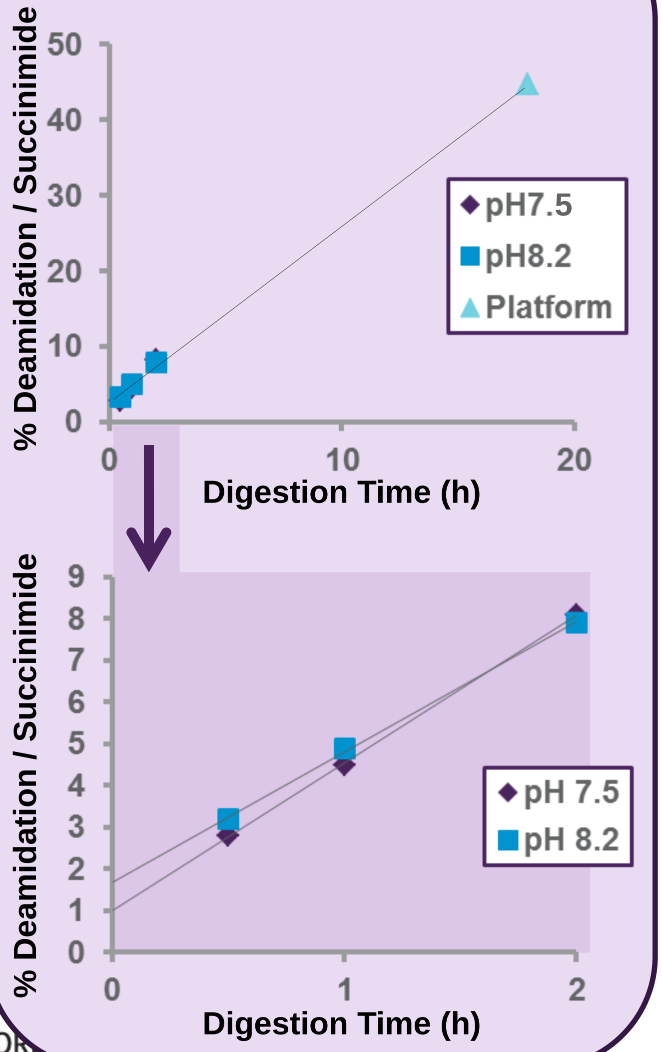

$N^{55}G$ (H-CDR2)


$D^{102}G$ (H-CDR3)

Digestion Time Can Influence Level of Deamidation

Deamidation for Innovator Trastuzumab

Pfizer: N⁵⁵G (H-CDR2)



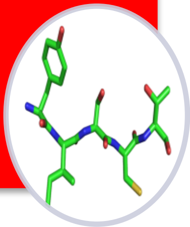
- Time-course digestion facilitates the accurate measurement of deamidation at T=0
  - Digest for 30, 60 and 120 min
  - Report accurate deamidation levels

Literature	% Deamidation Level at $N^{55}G$ (H-CDR2)
Genentech Harris 2001	~1 (CEX-HPLC)
Roche Sydow 2014	4 (pH 6.0 map)
Pfizer Results	
Innovator Trastuzumab	44 to 2

New Trypsin Peptide Map
Shorter Digestion Times and Time-Course

Goal: minimize method-related artifacts

Peptide Mapping



- Time-course digestion facilitates the accurate measurement of deamidation at T=0
  - Digest for 30, 60 and 120 min

- Efficient reduction, alkylation and digestion with Lys-C/trypsin (Promega) (pH 8.2)
- Waters XSelect XP CSH C18 column(formic acid)
- Reproducible UV chromatographic profiles

- Analyzed on a Thermo Orbitrap Fusion™ Tribrid™ Mass Spectrometer
  - Unprecedented depth of analysis
  - High quality MS/MS data (CID, ETD)
  - Use Fusion for PTM discovery

Low-artifact Trypsin Digest Methods

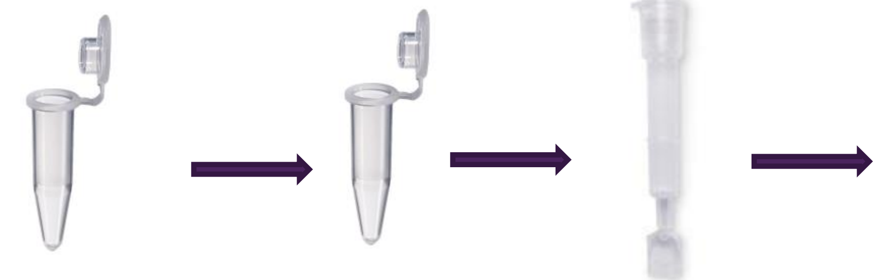
Digest at pH 8.2

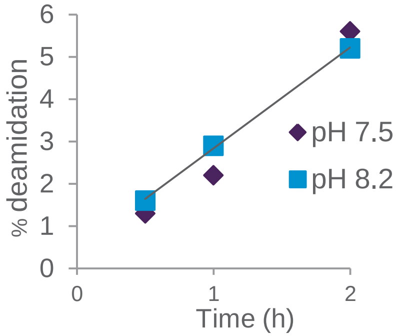
Denaturation / Reduction

Alkylation

Buffer exchange Using Bio-Spin 6

Tryptic Digestion





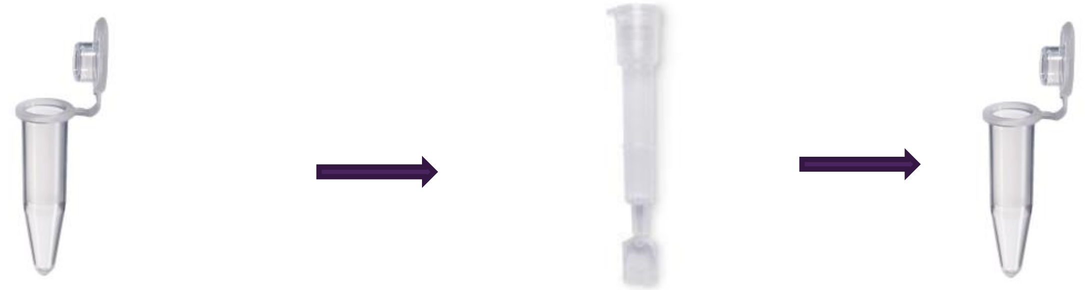
7M GdHCl /
100mM Tris pH 8.2
DTT
30 min at 37 ˚C

IAA
30 min, dark, RT

Buffer exchange
Bio-Spin 6 column
(Bio-Rad)
130 µL load volume

Lys-C/trypsin (Promega)
30, 60, and 120 min
37˚C in 100 mM Tris (pH 8.2),
1:10; enzyme: protein (w:w).
Digestion quenched using 1% TFA

Digest at pH 6.0



7M GdHCl /
20 mM MES (pH 6.0)
TCEP
60 min at 37 ˚C

Buffer exchange
Bio-Spin 6 column
(Bio-Rad)
130 µL load volume

Lys-C/trypsin (Promega)
18 hours at 37˚C in 0.5 mM TCEP
in 20 mM MES (pH 6.0)
1:10 enzyme: protein (w:w).
Digestion quenched using 1% TFA

Elaine Stephens

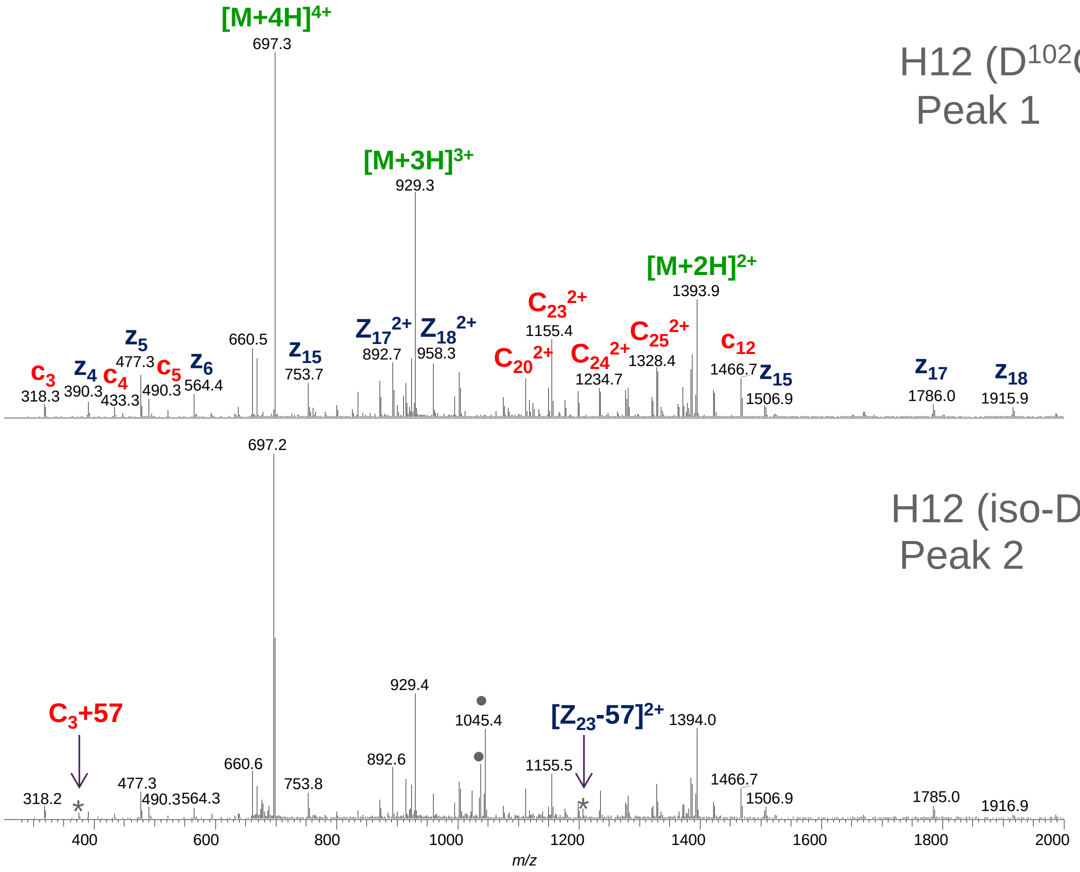
Asn Deamidation and Asp Isomerization in Trastuzumab (Herceptin®) Using Various Methods

Site	Modification	Method	% Modification
N^{30}T (L-CDR1)	**Deamidation**	CEX-HPLC (UV)[1]	~15
		Trypsin pH 6.0 (overnight) map [2]	11
		PFE-Trypsin pH 7.5 (overnight) map	12
		PFE-Lys-C/Trypsin pH 8.2 (T=0) map	10
N^{55}G (H-CDR2)	**Deamidation**	CEX-HPLC (UV)[1]	~1
		Trypsin pH 6.0 (overnight) map [2]	4
		PFE-Trypsin pH 7.5 (overnight) map	44 ★
		PFE-Lys-C/Trypsin pH 8.2 (T=0) map	2
D^{102}G (H-CDR3)	**Isomerization**	CEX-HPLC (UV)[1]	~9
		Trypsin pH 6.0 (overnight) map [2]	7
		PFE-Trypsin pH 7.5 (overnight) map	Not detected ★
		PFE-Lys-C/Trypsin pH 8.2 (T=0) map	8

1. R.J. Harris, B. Kabakoff, F.D. et al, J of Chromat. B, Biomedical sciences and applications, 752, 233-245 (**2001**).
2. J.F. Sydow, F. Lipsmeier, V. et al, PLoS One, 9, e100736 (**2014**).

Efficient Separation and ETD Spectra Confirm ID of H12 ($D^{102}G$) and H12 (iso-$D^{102}G$)

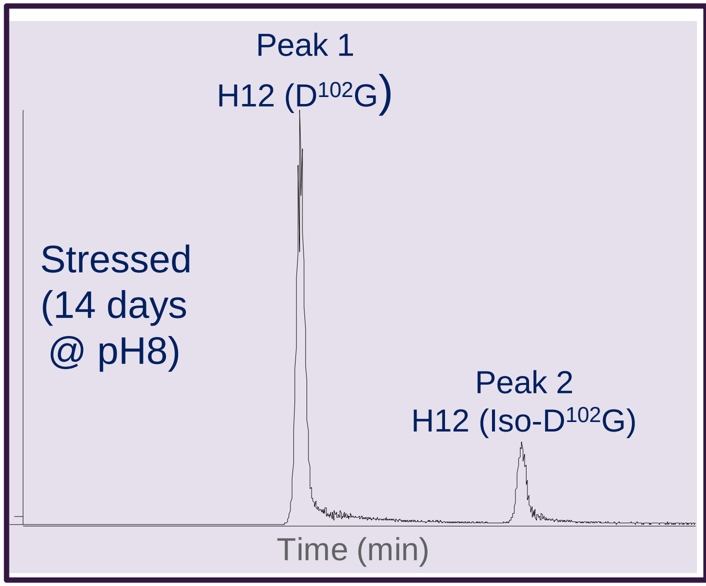
H12 peptide (WGG**$D^{102}G$**FYAMDYWGQGTLVTVSSASTK)



H12 ($D^{102}G$)
Peak 1

H12 (iso-$D^{102}G$)
Peak 2

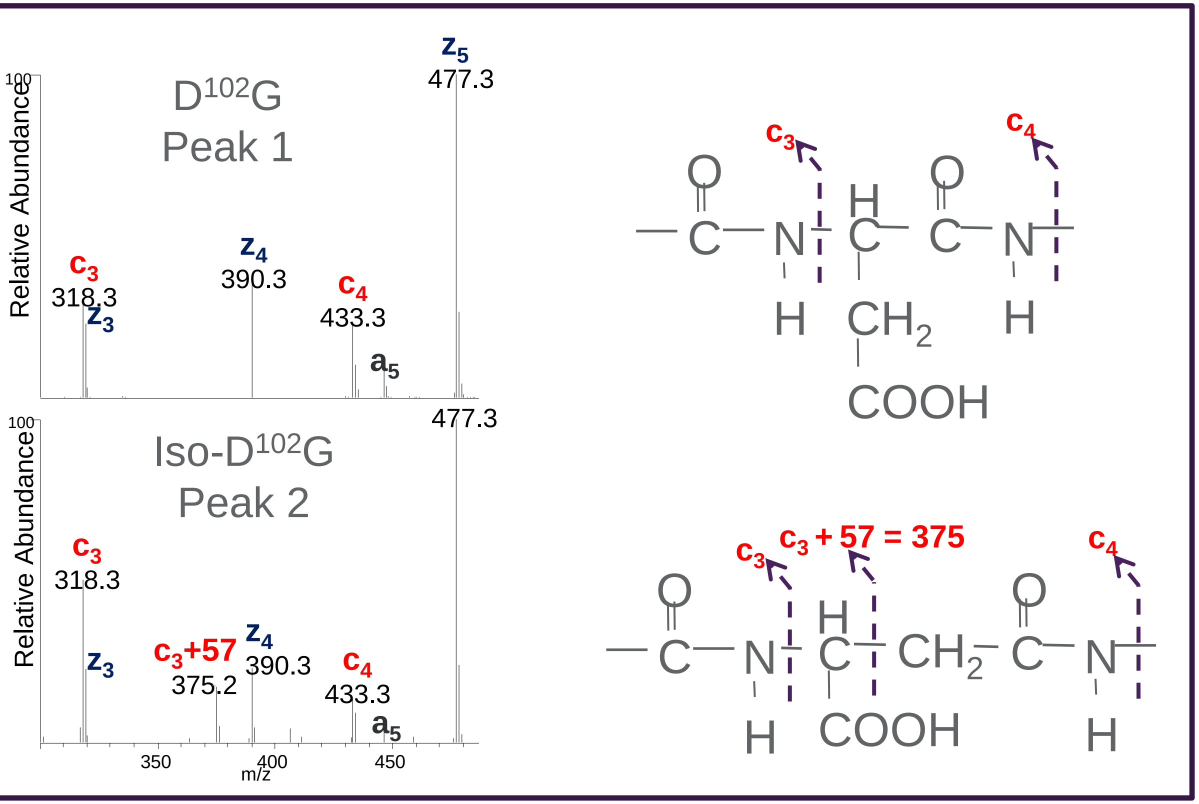
Extracted Ion Chromatogram (EIC) for H12



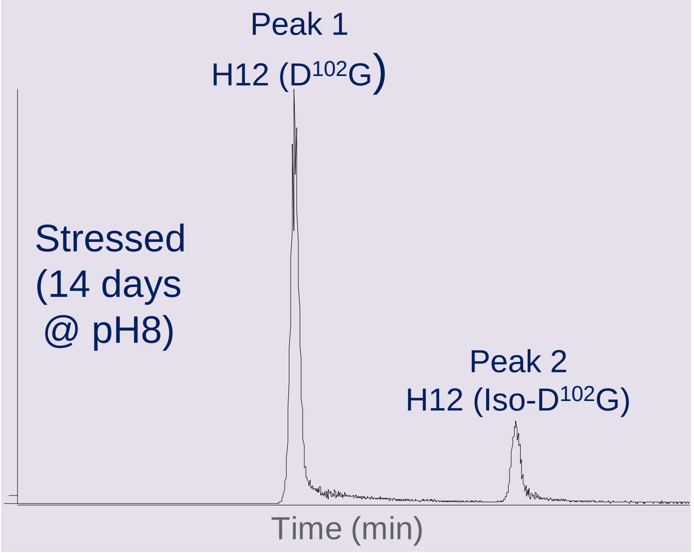
- Waters XSelect XP CSH C18 column with formic acid can resolve isomerized peak

Isomerization of Asp-102 led to lower potency (Harris et al, 2001)

Diagnostic ion (C_3 + 57) Confirms Presence of isoAsp in Trastuzumab



Extracted Ion Chromatogram (EIC) for H12



- Waters XSelect XP CSH C18 column can resolve isomerized peak

J.J. Cournoyer, J.L. Pittman, V.B. Ivleva, E. Fallows, L. Waskell, C.E. Costello, P.B. O'Connor, Prot. Sci., 14, 452-463 (2005).

Case Study #1
Peptide Map of Trastuzumab (Herceptin®)

- Trastuzumab (Herceptin®) is useful as a benchmark for peptide map method development

- Artifactual deamidation is a real possibility
  - Some motifs are sensitive to digestion time and pH (ex. $N^{55}G$ in H-CDR2)

- Resolution of Asp isomerized peptides ($D^{102}G$ vs iso$D^{102}G$) is challenging
  - Peptides have the same mass
  - Can only quantitate Asp/isoAsp if separated

Case Study #2: Deamidation in an IgG4 mAb

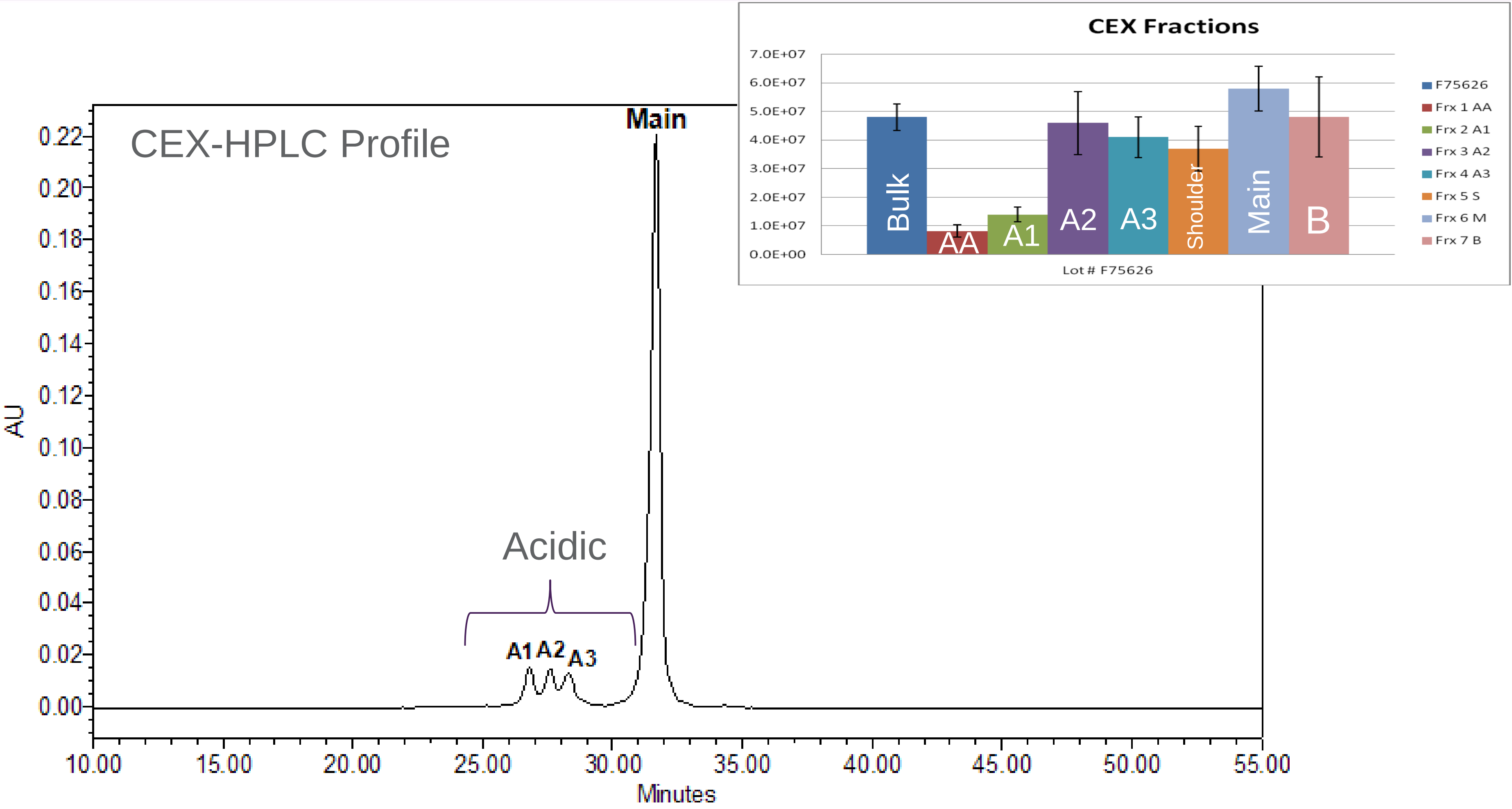
L Chain CDR Hotspots

1	XXXXXXXXXXXXXXXXXXXXXXXRSSQSLA	30
31	**NS**YG**NT**FLSXXXXXXXXXXXXXXXGISNRF	60
61	SXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	90
91	XXXLQGTHQPYTXXXXXXXX	

H Chain CDR Hotspots

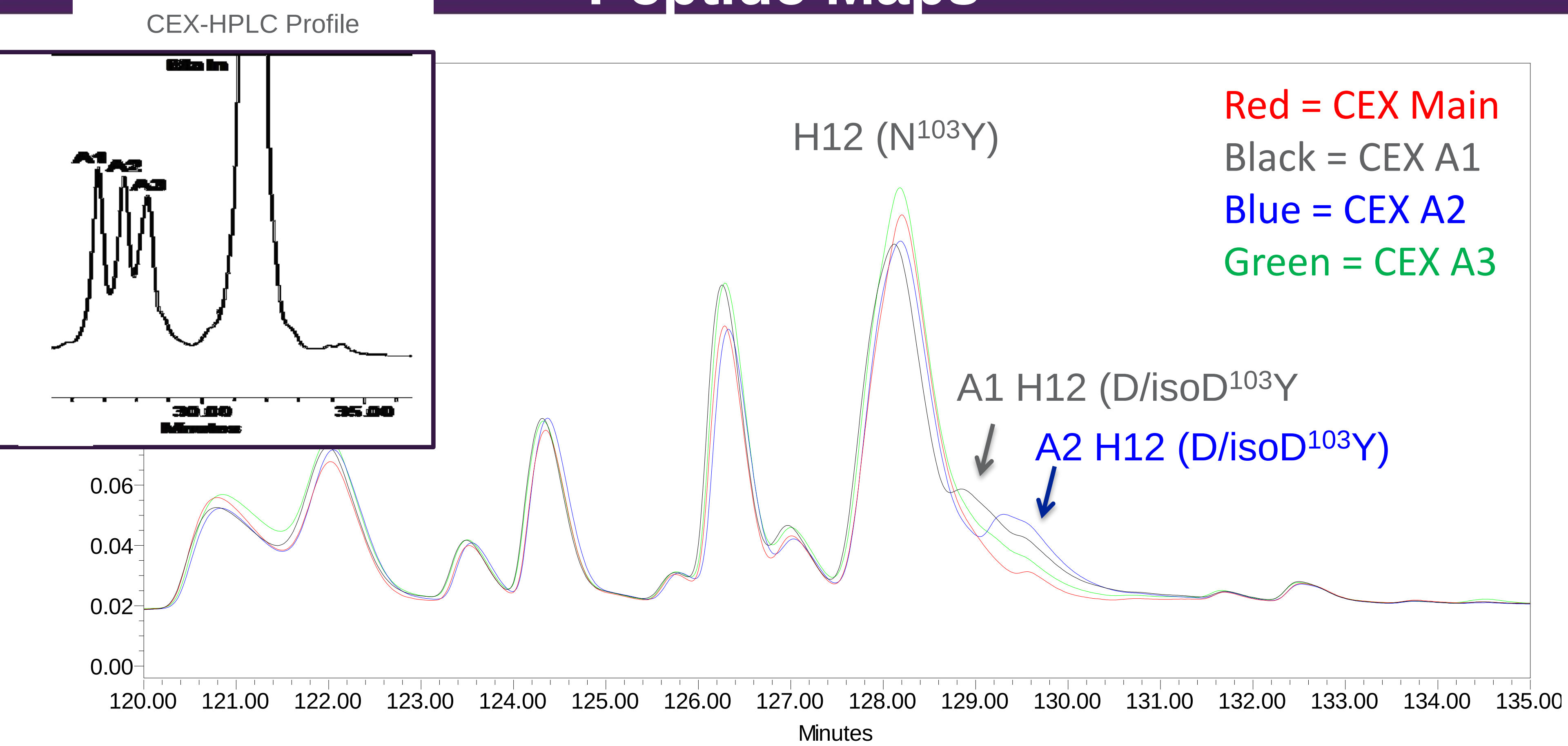
1	XXXXXXXXXXXXXXXXXXXXXXXXXGYRFT	30
31	NYWIHXXXXXXXXXXXXXXGINPG**NN**YATY	60
61	RRKFQGXXXXXXXXXXXXXXXXXXXXXXXX	90
91	XXXXXXXXEGYG**NY**GAWFAYXXXXXXXXXX	120
121		

Case Study #2: Deamidation in an IgG4 mAb



- Three distinct acidic isoforms (~15%)
- Acidic fractions collected and characterized for structure/function

Case Study #2: UV Chromatograms of Trypsin Peptide Maps



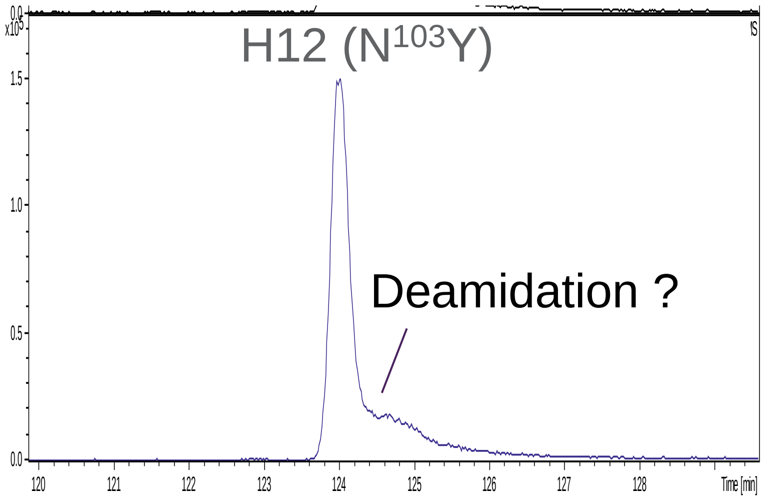
- CEX-HPLC Fraction A1 and A2 show an increase in H12 deamidation
  - Antigen binding impacted A1 charge variant

Case Study #2: New Peptide Map Column for Enhanced Chromatographic Separation

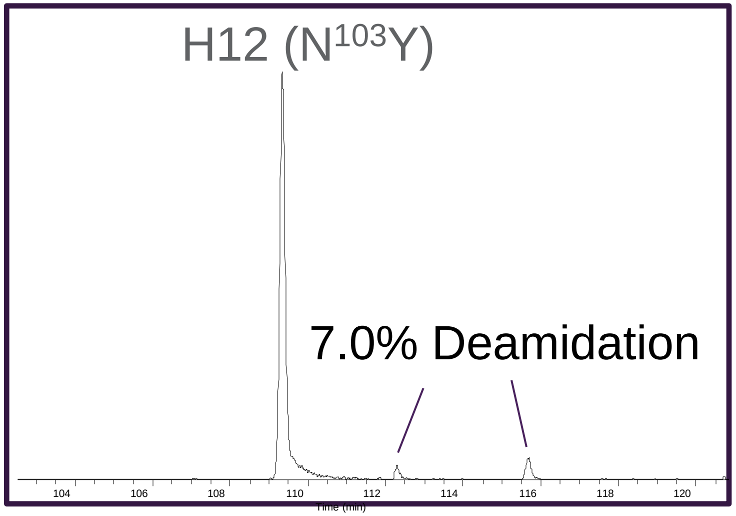
- IgG4 mAb: H chain deamidation in CDR3
- Localized deamidation site to: EGYG**N^{103}Y**GAWFAYWGQGTLVTVSSASTK

- NY site not expected to deamidate (NG, NS, NT, NN)
- Causes loss of target binding

Previous Trypsin Map
Waters BEH C18, TFA



New Trypsin Map
Waters XSelect XP CSH C18, FA



Case Study #2: Deamidation of an IgG4 mAb

Site	Modification	Modification (total Asp and iso-Asp, %)	CEX Fraction
N^{33}S (L-CDR1)	Deamidation	3.2	A3
N^{55}N (H-CDR2)	Deamidation	0.1	- - -
N^{103}Y (H-CDR3)	Deamidation	7.0	A1 and A2

- New HPLC column allows for better separation and accurate quantitation of deamidation

- Detected unexpected N^{103}Y deamidation hot spot (beyond NG, NS, NT, and NN)
  - In process of updating software reflect new findings

Case Study #3
Forced Deamidation Study of IgG1 mAb

Stress Study

- Incubation at 25 ºC and 37 ºC in 100 mM sodium phosphate, pH 8.0
- T=0, 2 and 4 weeks

Peptide Map Method

- New Lys-C/trypsin map, pH 8.2, 30, 60 and 120 min
- Waters XSelect XP CSH C18 column, FA

MS Analysis

- LC-MS/MS and EICs
- Collision induced dissociation (CID)
- Targeted electron transfer dissociation (ETD)

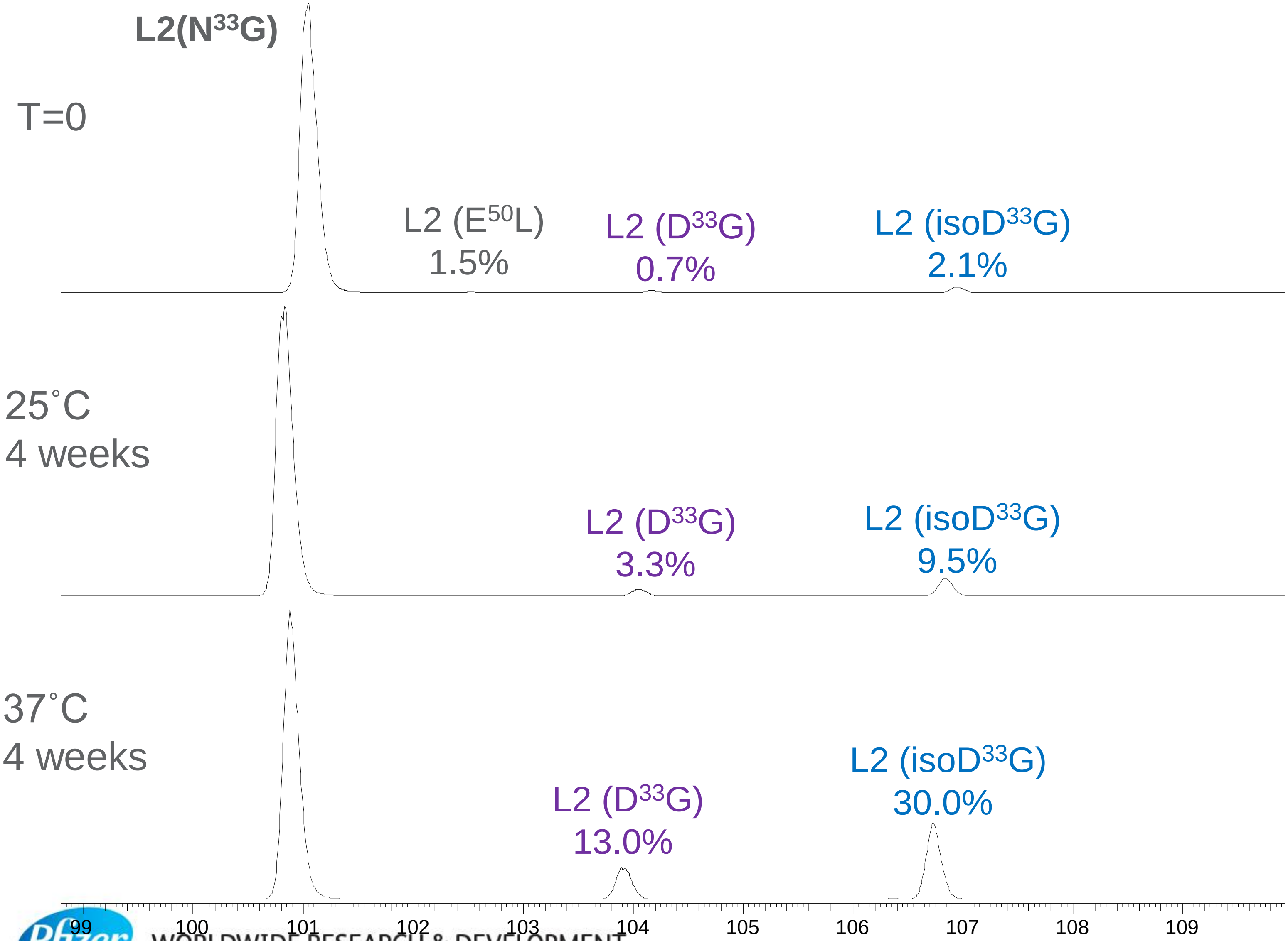
Case Study #3: Forced Deamidation Study of IgG1 mAb

IgG1 L Chain CDR Hotspots			Rel. Abundance
1	DIVMTQTPLSLSVTPGQPASISCRSSQSLV	30	High level (>5%)
31	HS**NG**NTFLYWYLQKPGQSPQLLIYRVSNRF	60	Low level (1-5%)
61	SGVPDRFSGSGSGTDFTLKISRVEAEDVGV	90	Potential (<1%)
91	YYCFQATHVPWTFGGGTKVEIKR		

IgG1 H Chain CDR Hotspots			Rel. Abundance
1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	30	High level (>5%)
31	GYYIHWVRQAPGQGLEWMGWIYPG**NF**NTKY	60	Low level (1-5%)
61	NERFKGRVTMTTDTSTSTAYMELRSLRSDD	90	Potential (<1%)
91	TAVYYCARE**DG**SPYYAMDYWGQGTSVTVSS	120	

Deamidation of Light Chain (L2 peptide with CDR1)

^{25}SSQSLVHSN^{33}GNTFLYWYLQKPGQSPQ^{50}LLIYR^{55}

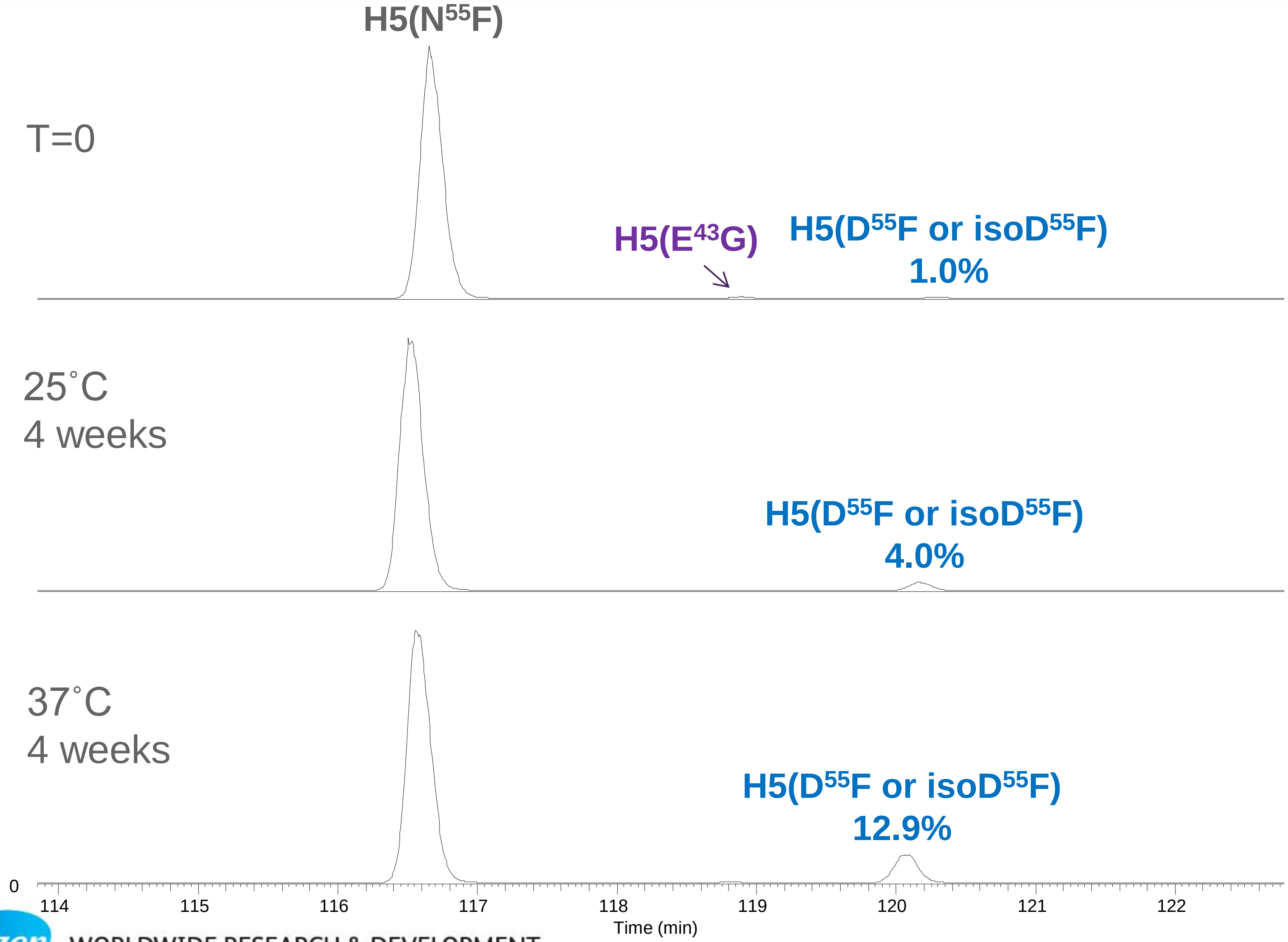


WORLDWIDE RESEARCH & DEVELOPMENT
BioTherapeutics Pharmaceutical Sciences

120 min EICs shown

Deamidation of Heavy Chain (H5 with CDR2)

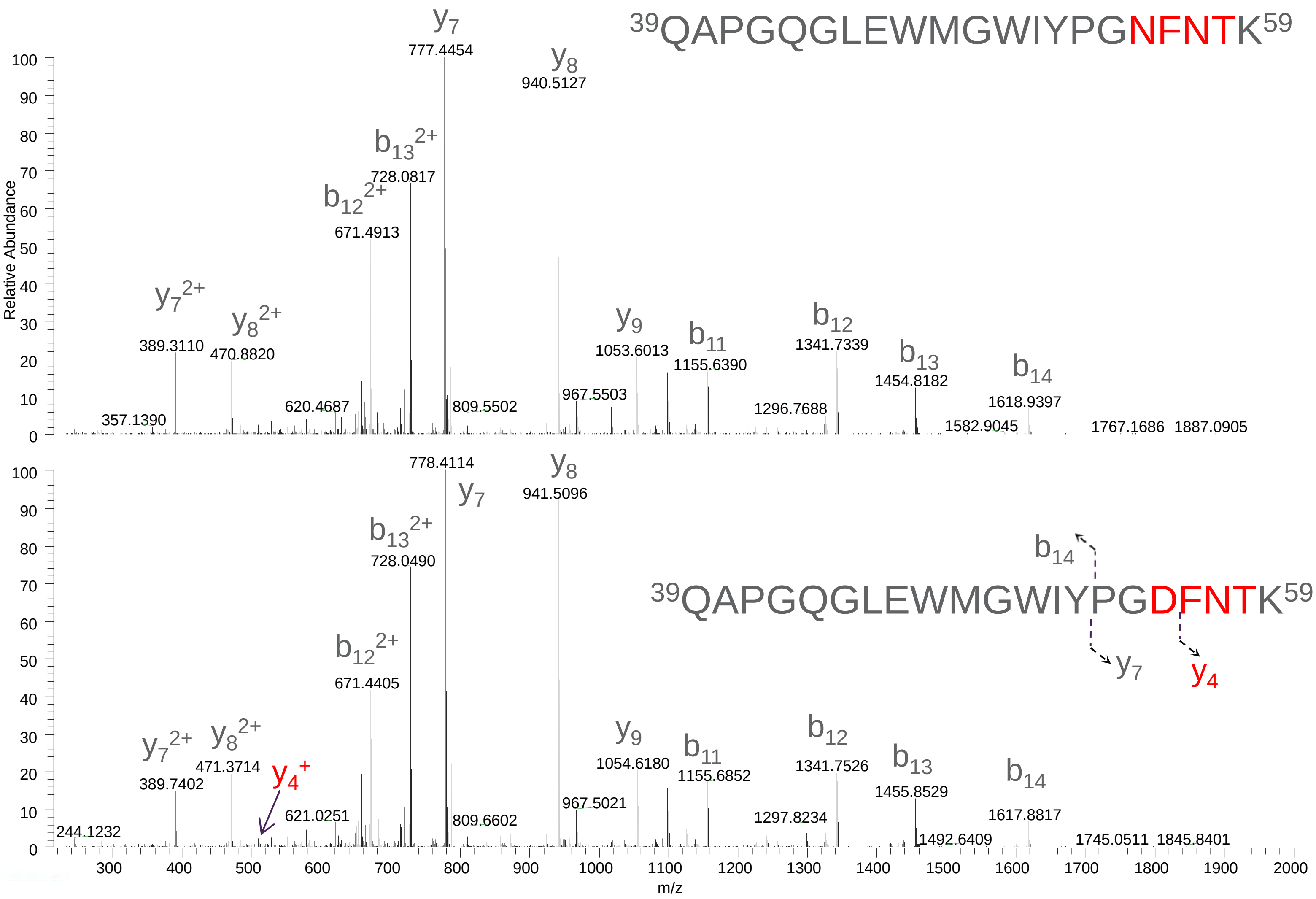
^{39}QAPGQ^{43}GLEWMGWIYPGN^{55}FNTK^{59}



120 min EICs shown

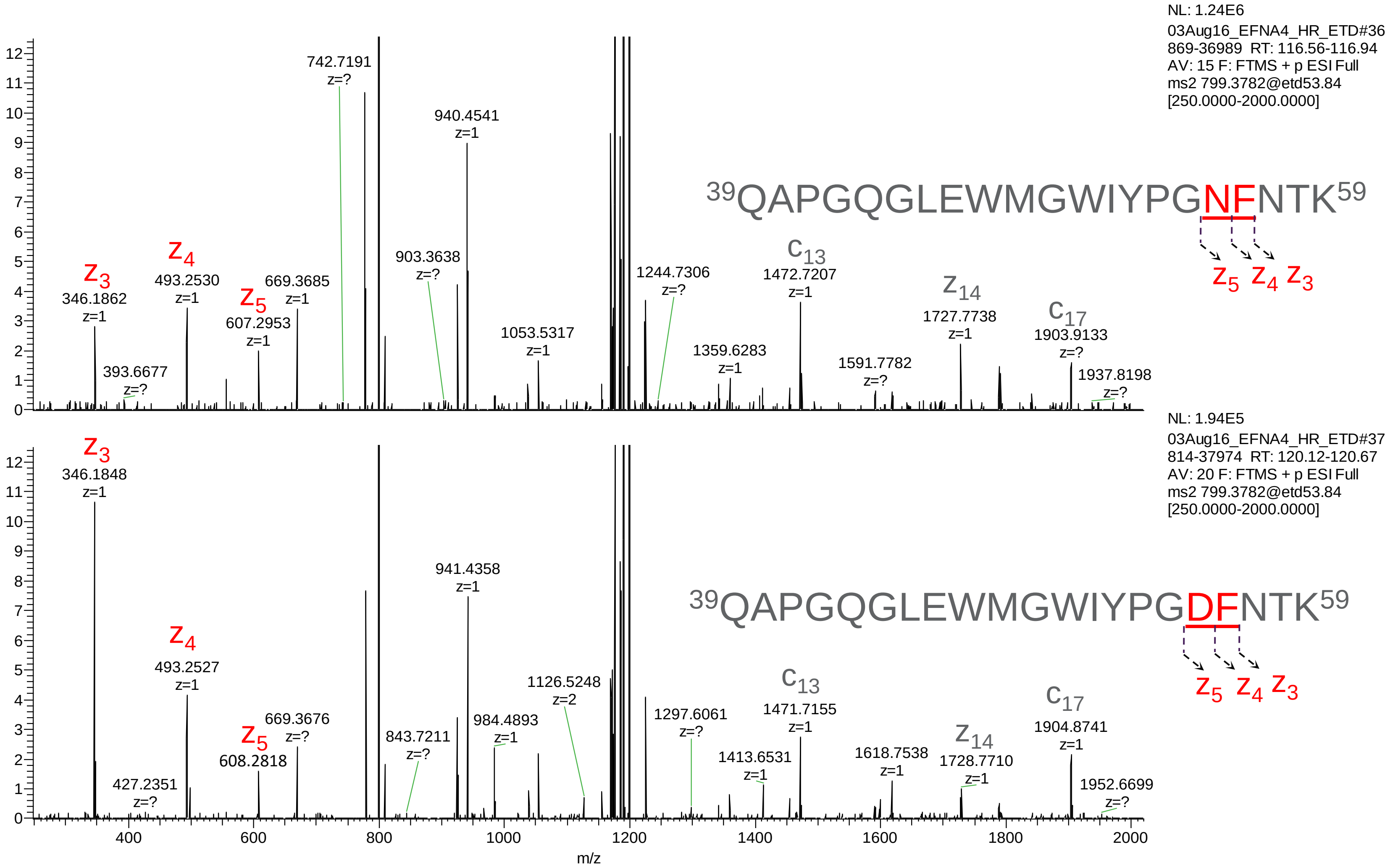
CID Spectra Showing Deamidation at Asn-55

^{39}QAPGQGLEWMGWIYPGN^{55}FNTK^{59}



CID did not provide conclusive evidence for deamidation site

ETD Fragmentation Confirms Deamidation Site at Asn-55 (N^{55}F)



Asn Deamidation and Asp Isomerization Results for IgG1 mAb using New Low Artifact Trypsin Map

Site	Modification	Modification (total Asp and iso-Asp, %)		
		Unstressed	Stressed 4 weeks at 25 °C	Stressed 4 weeks at 37 °C
$N^{33}G$ (L-CDR1)	Deamidation	2.8	12.8	43.0
$N^{55}F$ (H-CDR2)	Deamidation	1.0	4.0	12.9
$D^{100}G$ (H-CDR3)	Isomerization	0.7	1.0	1.7

- Important to analyze stressed material for hotspots (2.8 to 43.0%)
- Detected unexpected $N^{55}F$ deamidation hot spot
- $D^{100}G$ not a hotspot despite motif and location in H-CDR3

Summary

- **New trypsin peptide map-shorter digestion times, efficient enzyme, time-course elements and updated C18 column with formic acid**
  - Detects method-induced artifacts
  - Improved resolution of deamidated and isomerized peptides
  - Accurate quantitation of deamidation/isomerization via time course
  - Fragmentation (CID and ETD) determine sites of modification
    - ETD also distinguishes Asp vs iso-Asp

- **Deamidation and isomerization hotspots are sequence and location dependent and less predictable in proteins**
  - **Detected unexpected deamidation hot spots (beyond NG, NS, NT, and NN)**
    - N^{103}Y in heavy chain CDR3
    - N^{55}F in heavy chain CDR2
    - Low and constant level of Gln deamidation (Q^{50}L, Q^{40}G)

Summary of Hotspots

Case Study 1 Trastuzumab Originator	Case Study 2 IgG4 mAb Ref Material	Case Study 3 IgG1 mAb-stressed Ref Material - Stressed	Rel. Abundance
N^{30}T (L-CDR1)	N^{33}S (L-CDR1)	N^{33}G (L-CDR1) N^{33}G (L-CDR1)	High level (>5%)
N^{55}G (H-CDR2)	N^{55}N (H-CDR2)	N^{55}F (H-CDR2) N^{55}F (H-CDR2)	Low level (1-5%)
D^{102}G (H-CDR3)	N^{103}Y (H-CDR3)	D^{100}G (H-CDR3) D^{100}G (H-CDR3)	Potential (<1%)

Forced degradation studies help identify potential sequence liabilities

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