AdvanceBio Peptide Mapping

An HPLC Column Technology for Faster Protein Biocharacterizations

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BioColumn Technical Specialist

What Is Peptide Mapping?

- The chemical or enzymatic treatment of a protein to produce peptide fragments
- Separation and identification of these fragments in a reproducible manner
- For bio-therapeutic proteins and peptides peptide mapping is:

  In-depth analysis that can identify minor and even isobaric differences in protein primary structure such as errors in the transcription of complementary DNA, point mutations., and PTMs

Proteins and Monoclonal Antibodies – Complex Molecules, Complex Manufacturing

Complex Molecules

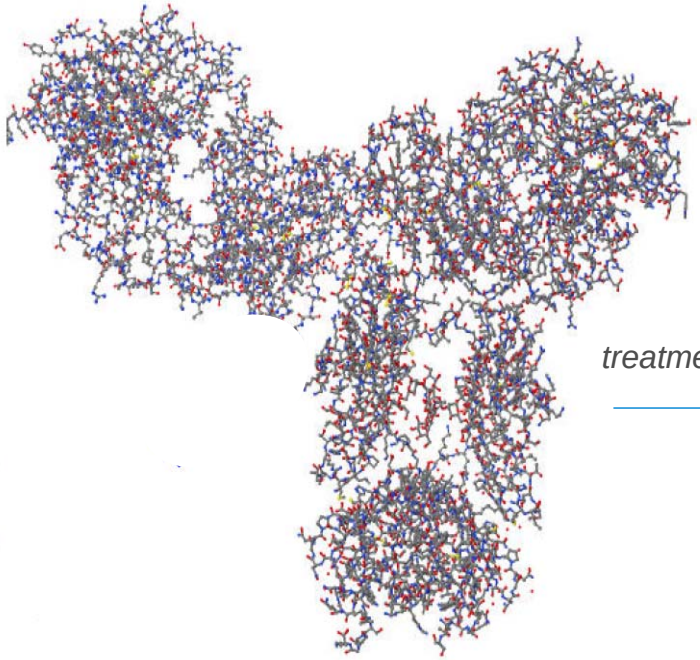
- Proteins have multiple layers of structure – primary, secondary, tertiary.., that all must be characterized
- Monoclonal antibodies/proteins have glycosylations/post-translational modifications that can vary

Complex Manufacturing

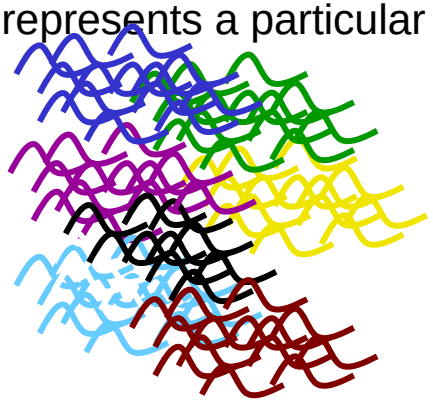
- Manufacturing requires living cells – which can vary, creating variable results
- Variation in process can change structures, post-translational modifications
- These changes need to be characterized

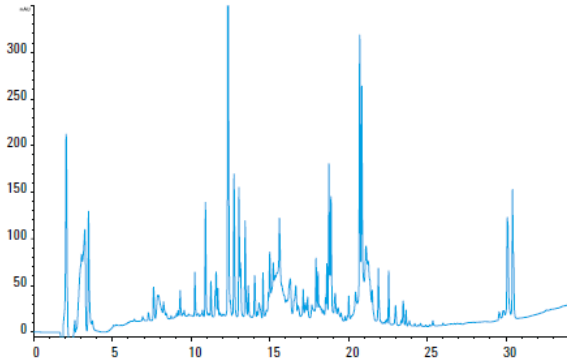
Why peptide mapping?

- Analysis of a single modification on a large protein may not be possible because the change is too small to be seen in the large molecule
- Results in a collection of peptides of varying length & number depending on the protease used. The resulting peptides can be analyzed where each peak represents a particular peptide



treatment with a protease enzyme





Peptide mapping is the single most important technique in analytical characterization…

- Confirm primary structure by comparison of a product to a reference protein (detect point mutations, mis-translations & confirm genetic stability)
- Identify location of disulfide bonds
- Characterize & analyze degradation processes such as deamidation & oxidation
- Isolate digest fragments for sequencing or further identification
- Identify sites of glycosylation
- Drug substance identity test
- Drug substance purity test

NEW: Peptide Mapping ‘How To’ Guide #5991-2348EN

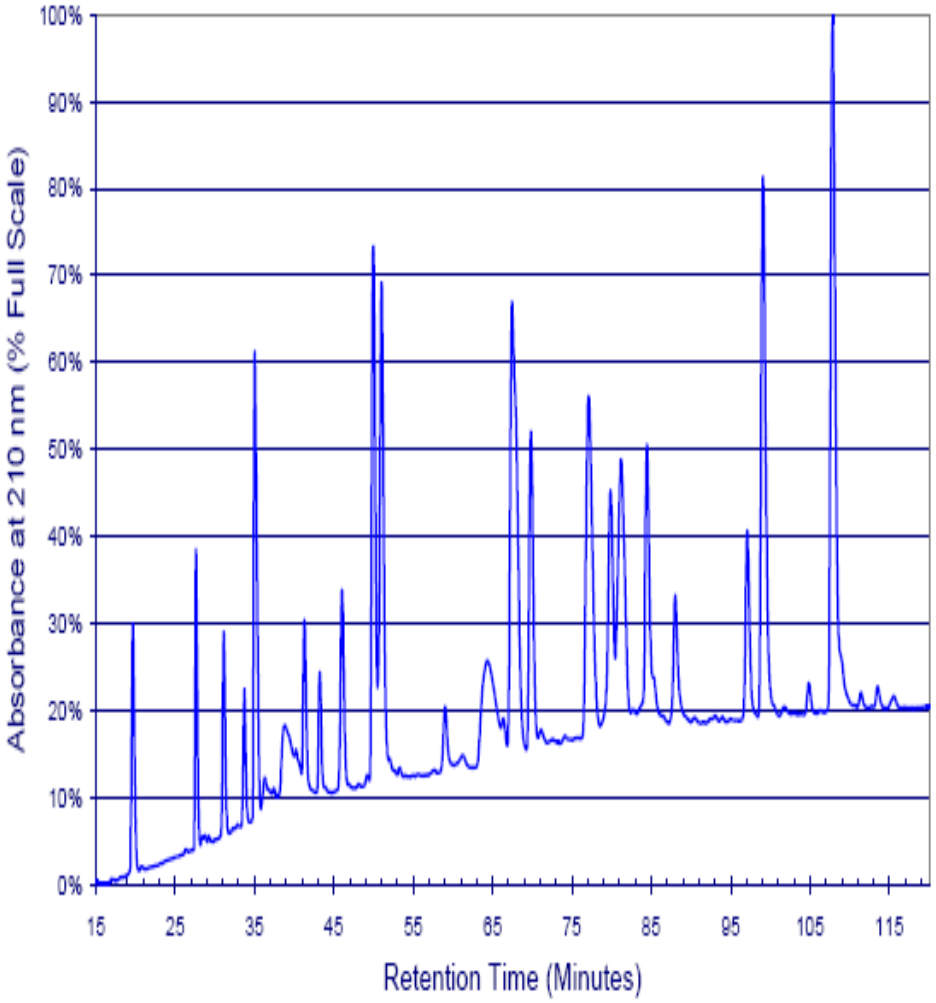
THE COLUMN

This Talk Will Show That The AdvanceBio Peptide Mapping Column Allows You To:

1. Achieve high efficiency separation performance with rapid run times. Faster than traditional UV and LC/MS analysis times
2. Conserve the bio-characterization information content with accelerated run times
3. Achieve superior UV and LC/MS separation performance to competitive products
4. Achieve UHPLC-like speed and peptide mapping performance at HPLC operating conditions, in direct comparison to competitive uHPLC columns

“Traditional” Peptide Map

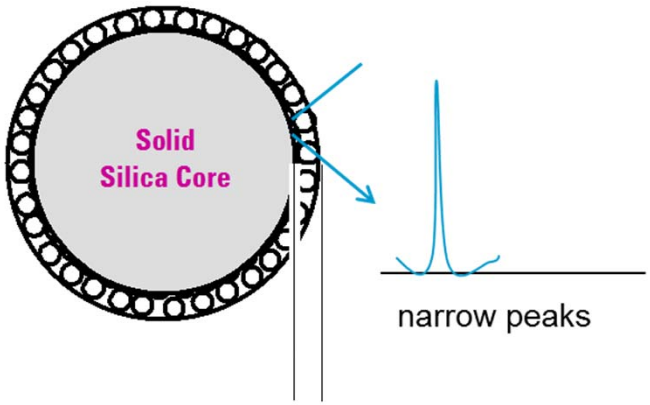
Column: 4.6 X 250 MM
C18 stationary phase
5 micron particle size,
Mobile Phase: A= 0.1%TFA in water, B=0.1%TFA in ACN
Gradient: 10% B to 90 B in 100 minutes
Runtime: 115 minutes
Re-equilibration time: 20 minutes



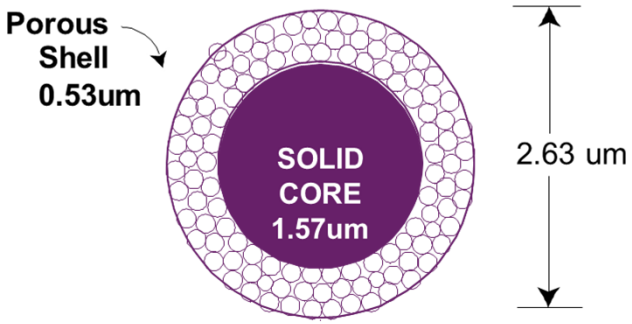
AdvanceBio Peptide Mapping Column Technology

2.7um Superficially porous particle technology delivers UHPLC type column performance but without high column back pressures

Superficially porous (core-shell, poroshell, shell particle, etc)



Actual



120A pore size
C18 phase

Decrease the diffusion time for macromolecules and limit the diffusion path!

This results in more efficient mass transfer at increasing flows = sharper peaks during fast run times.

AdvanceBio Peptide Mapping Column

Primary Benefit – Reduce Peptide Mapping Time without Losing Resolution

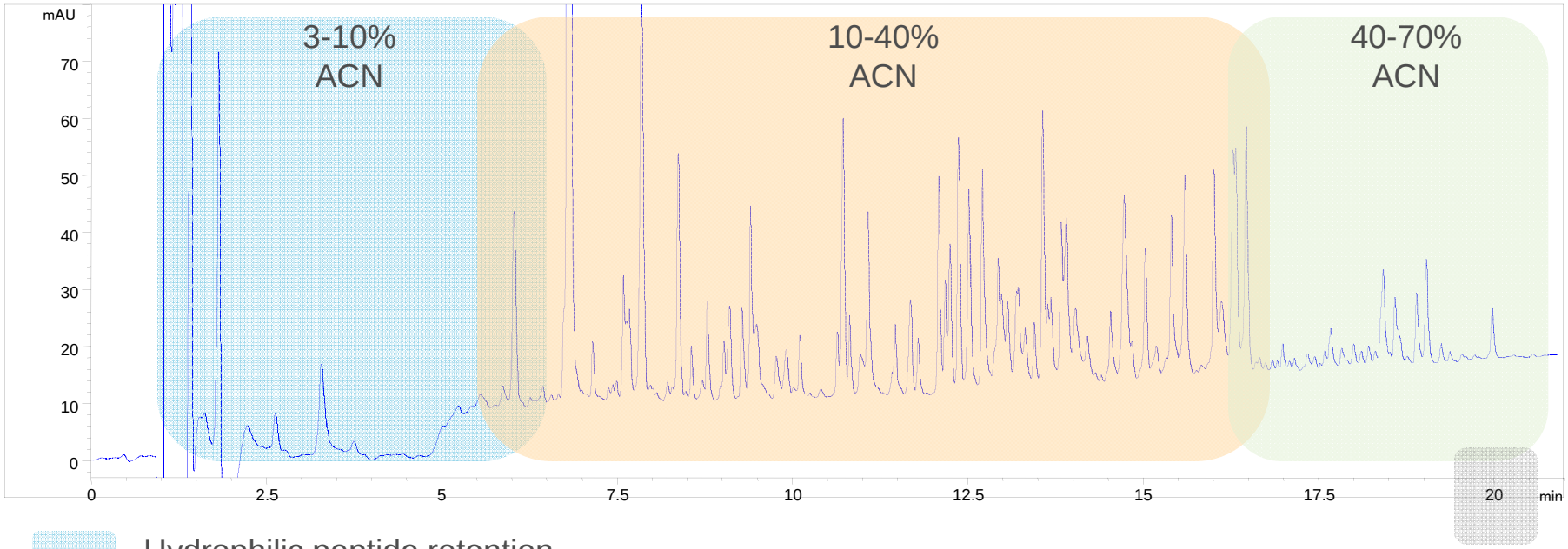
What is It? - A superficially porous column with a 2.7um particle and C18 functionality which enables separation of hydrophilic through hydrophobic peptides to give superior resolution across the gradient range to efficiently resolve peptide fragments.

Major Features

- High Resolution of the Peptide Map
- Fast Analysis Times
- Lower Pressure Drops than sub 2um Columns
- Quality Checked for Peptide Performance

AdvanceBio Peptide Mapping Column Technology

BSA tryptic digest on 2.1 x 150mm AdvanceBio Peptide Mapping Column

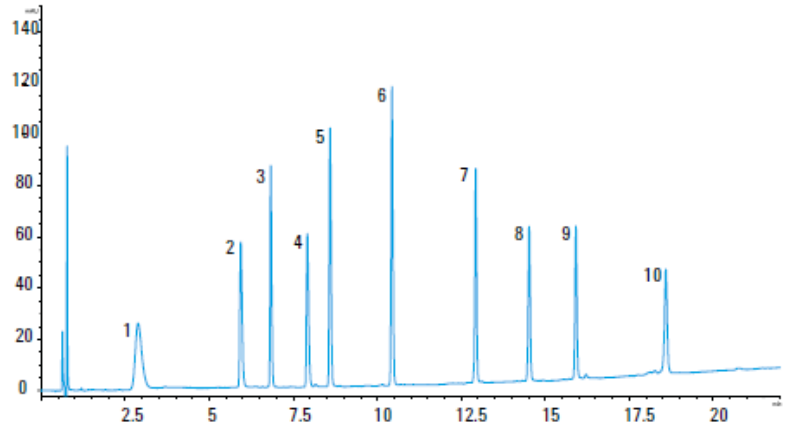


- Hydrophilic peptide retention
- Narrow Peaks w baseline resolution
- Hydrophobic peptide retention
- Reduced and fast analysis time

Critical and desired peptide mapping components to achieve fast, selective and highly efficient peptide separations across a wide dynamic range.

Quality Assurance Testing with Agilent Peptide Mix

Test mix used for every batch of AdvanceBio Peptide Mapping media. The mixture contains 10 hydrophilic, hydrophobic, and basic peptides, ranging in molecular weight from 757 to 2845 Da. Every column is also tested with a small-molecule probe to ensure efficiency.



Pk no.	Peptide
1	Bradykin frag (1-7)
2	Bradykin Acetate
3	Angiotensin II
4	Neurotensin
5	Angiotensin I
6	Renin
7	[Ace-F-3,-2 H-1] Angiotensinogen (
8	Ser/Thr Protein Phosphotase (15-3
9	[F14] Ser/Thr Protein Phosphotase
10	Mellitin (Honey bee venom)

Conditions

Column: AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm, p/n 653750-902

Flow rate: 0.5 mL/min

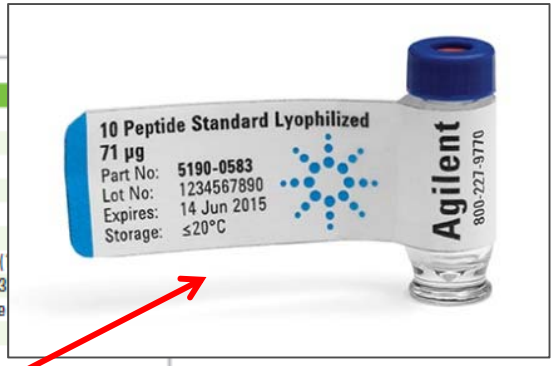
Injection: 3 µL

Temp: 55 °C

Detection: 220 nm

Gradient: A, water (0.1% TFA), B, ACN (0.1% TFA), 0-25 min,15-65% B; 25-26 min, 65-95% B

Sample: Agilent Peptide Mapping Standards Mix (0.5-1.0 µg/µL per peptide) p/n 5190-0583



Peptide Mix Now Commercially Available

Ensures High quality batch-to-batch reproducibility
Confidence in separation performance (peak shape, Rs, selectivity)
Visualization: High performance Peptide Separation Column

Decreasing Run Time

Initial experiment was to decrease run time from traditional 120 min to 75 min

While faster runtime is the goal, we start here to make sure we do not compromise resolution and provide same level of coverage

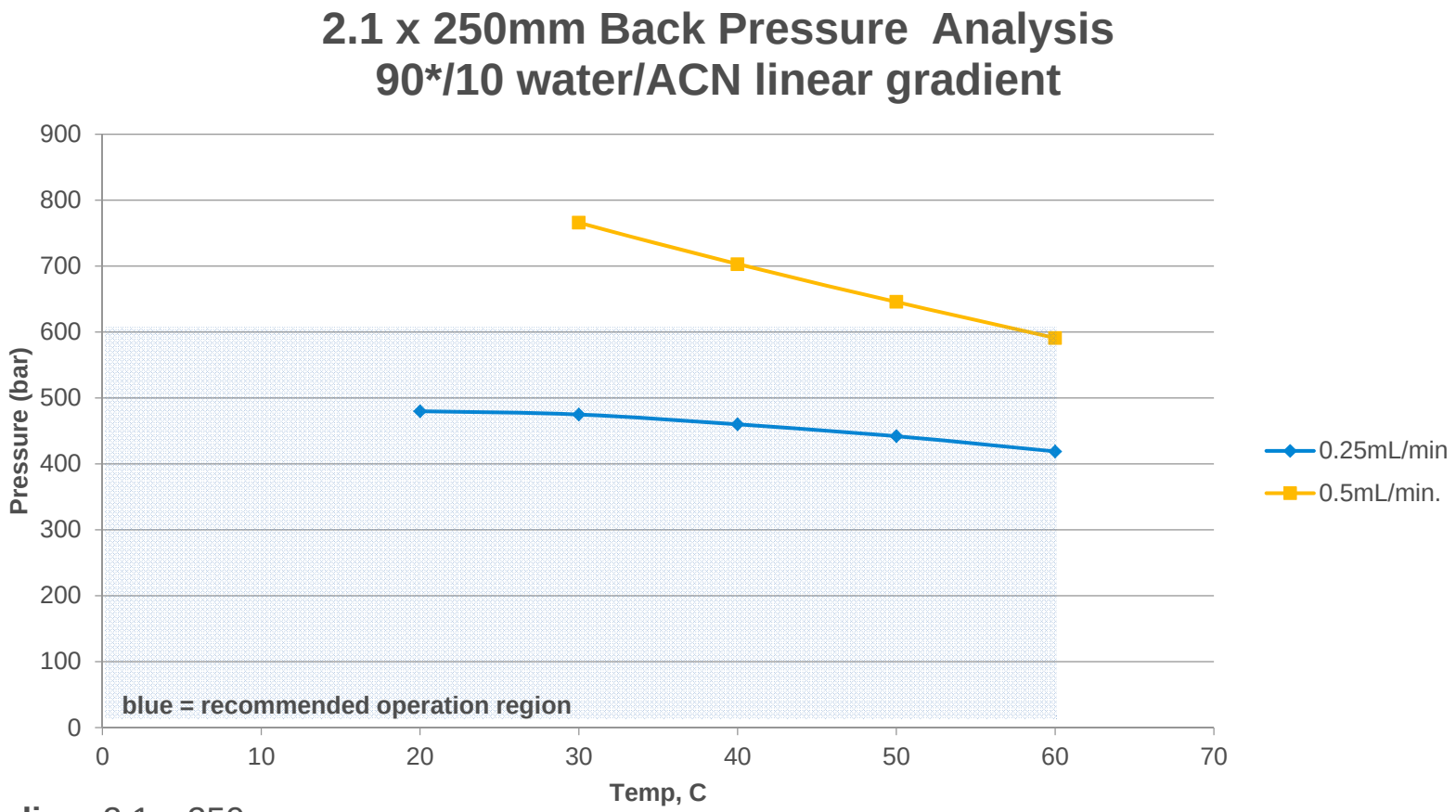
This is followed up with gradient, temperature, and column length optimization

Temperature

- Initial temp of 30-50 C recommended
- Optimal temp will be application dependent, effected by composition and digestion type
- Change in temp can change selectivity and result in RT switching
- Elevated temp produces narrower peak bands and lower pressure

Column Characterization

Linear Flow Analysis (back pressure)



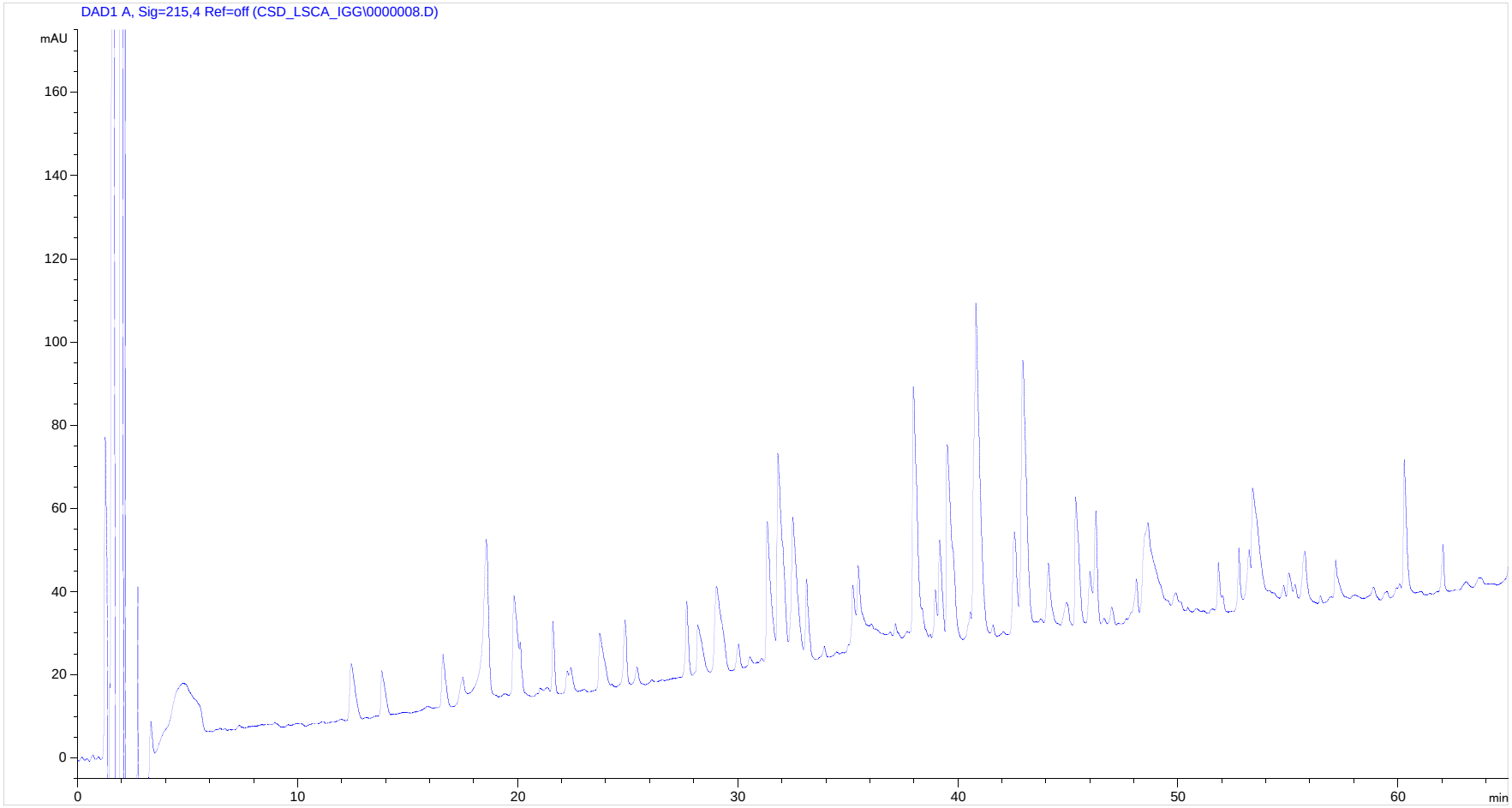
Column dim.: 2.1 x 250mm
Mobile phase: A-water (0.1%TFA), B- ACN (0.08%TFA)
Gradient: 0-15min ,10-90%B
Instrument: Agilent Infinity 1290

* Press reading obsv. (max)

Gradient Optimization

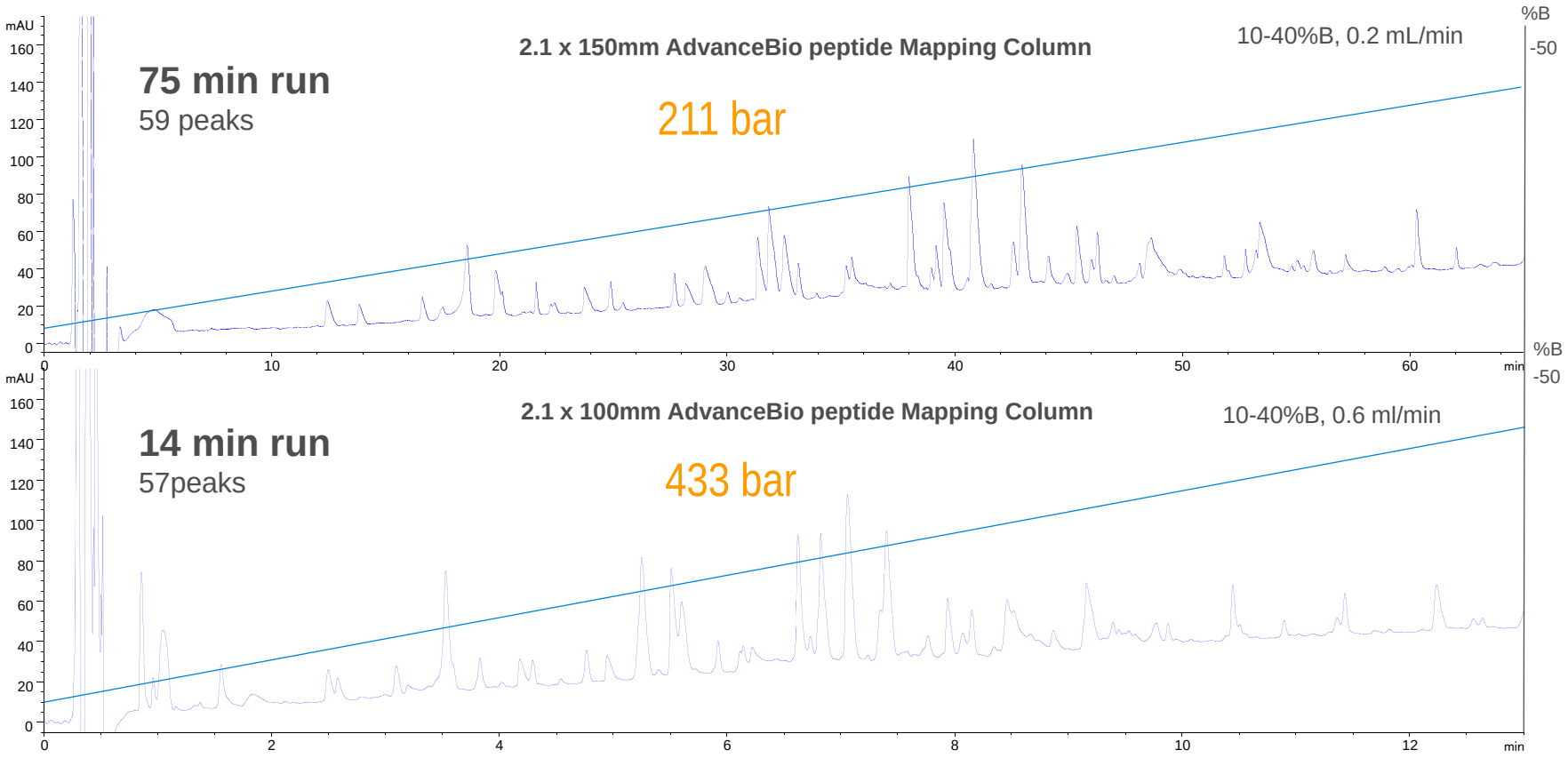
- Changes in gradient steepness can improve band spacing and change selectivity
- Varied by either changing the %B change over the same run time
- Or by keeping the % change in B the same, but changing the run time with flow rate change.

75 Minute Run IgG Tryptic Digest
0.2ml/min, 0-40% B



IgG Tryptic Digest Peptide Mapping

Decreasing Analysis Times Without Sacrificing Separation Performance or Reaching Back Pressure Constraints

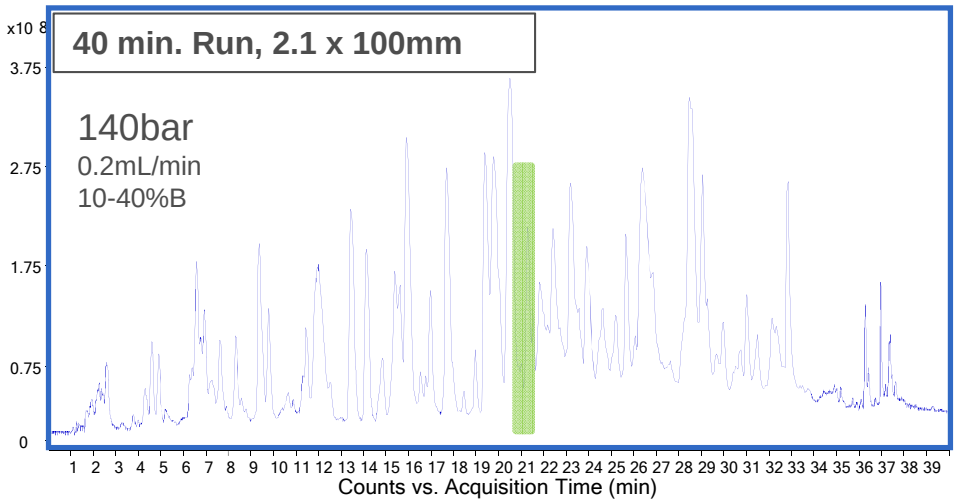


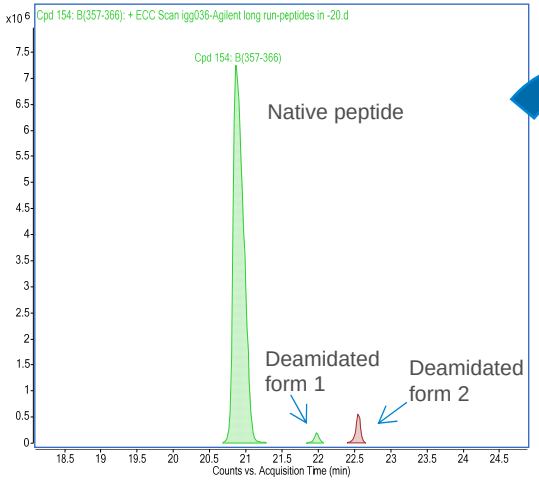
Keeping gradient change/column volume the same ensures selectivity remains constant

DO WE LOSE ANY CRITICAL DATA WHEN WE SPEED THINGS UP?

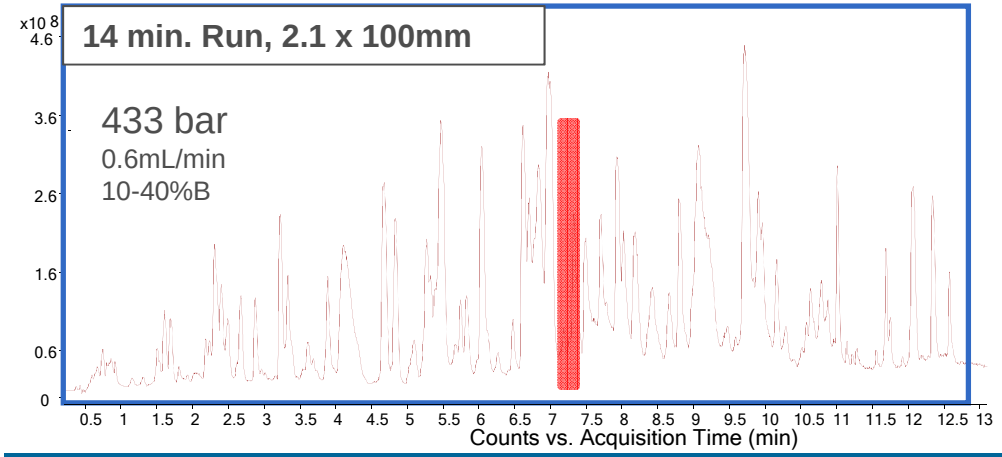
Rapid LC/MS IgG Tryptic Digest Peptide Mapping

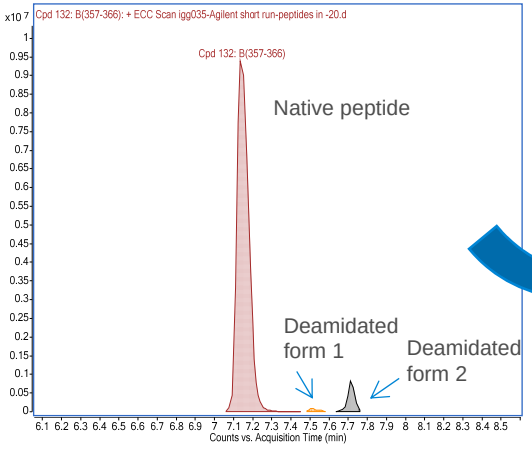
Critical Post Translational Modification (PTM) Conserved during Reduced Run Times





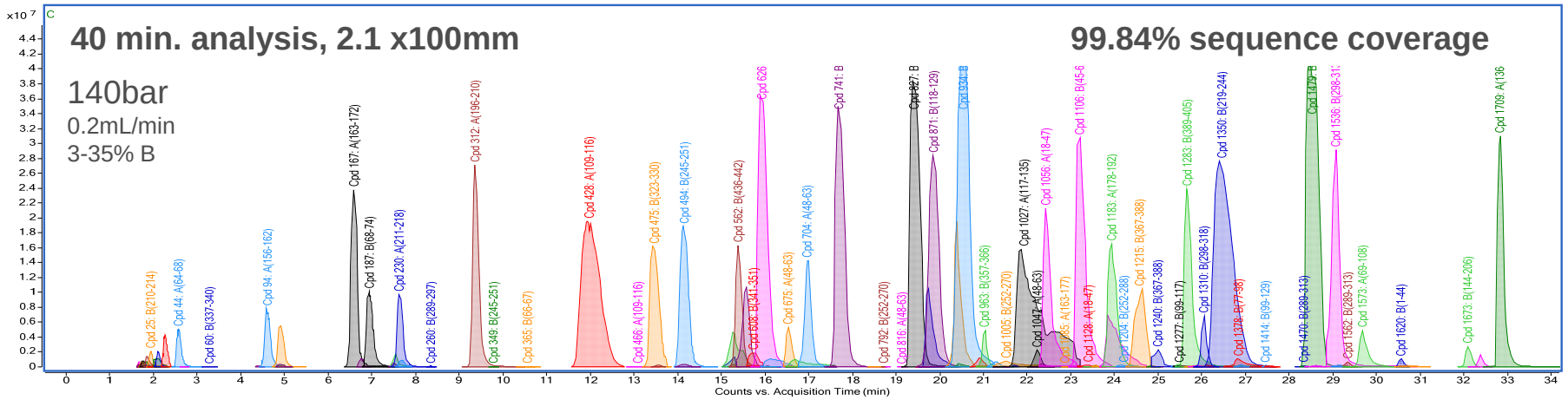
Heavy Chain Peptide 357-366 and its two deamidated forms conserved

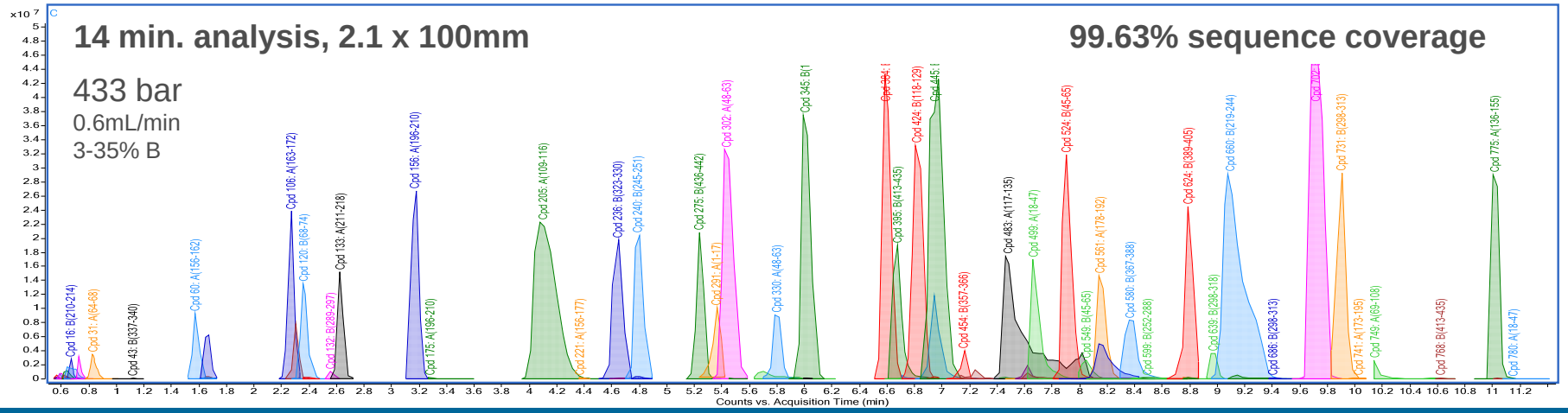




Rapid LC/MS IgG Tryptic Digest Peptide Mapping

Sequence Coverage Remains Unchanged When Gradients are Shortened to increase Analysis Times



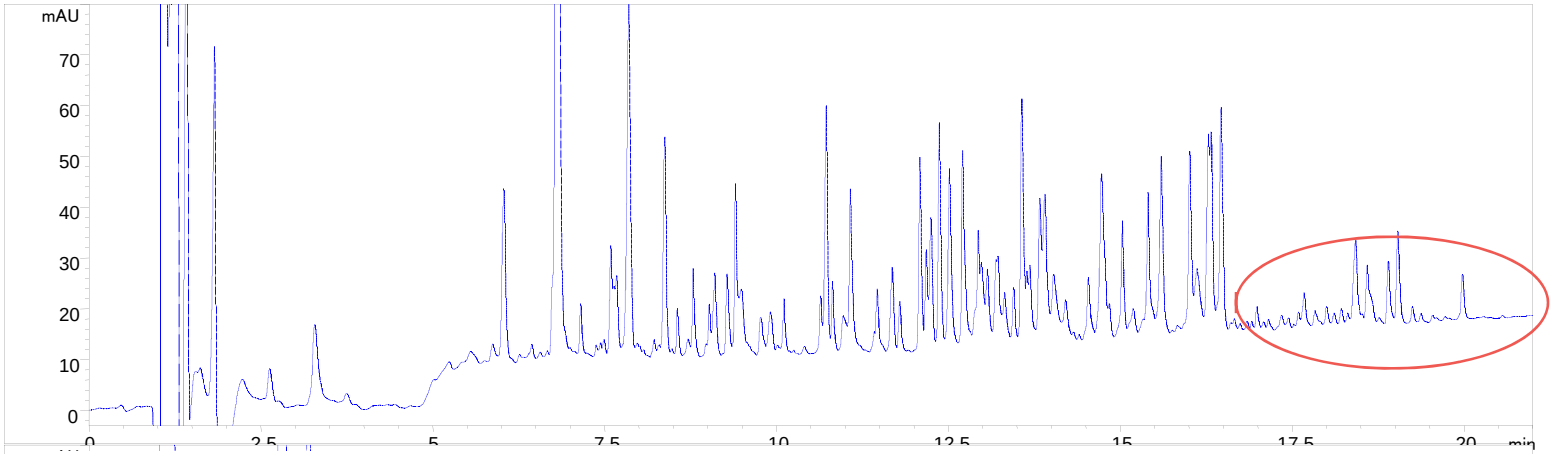


How Does AdvanceBio Peptide Mapping compare to other options?

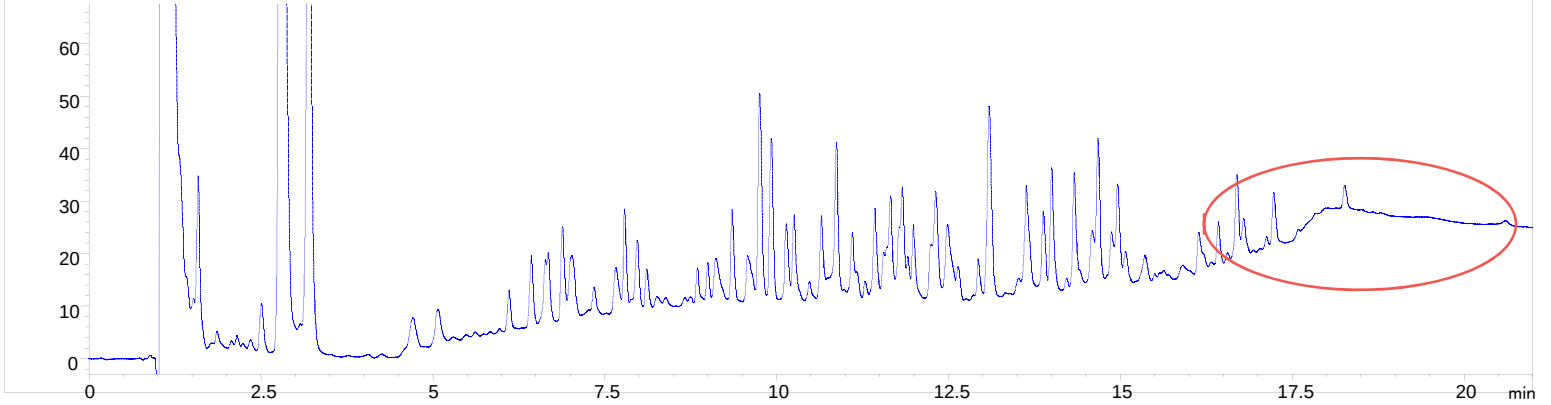
Competitive Superficially Porous: Peptide Column

Higher Peak Capacity Achieved by AdvanceBio Peptide Column during 20 min. Rapid Run

2.1 x 150mm AdvanceBio Peptide Mapping Column, BSA tryptic digest



2.1 x 150mm 3.6um Superficially Porous Peptide Column, BSA tryptic digest



HPLC (UV) Results

104 peaks
319 bar

76 peaks
166 bar

Competitor Superficially Porous Peptide Column Comparison

Higher Sequence Coverage Achieved by AdvanceBio Peptide Column during 14min. Rapid Run

Mobile phase A:
water (0.1%FA)
Mobile phase B:
90% ACN (0.1%FA)

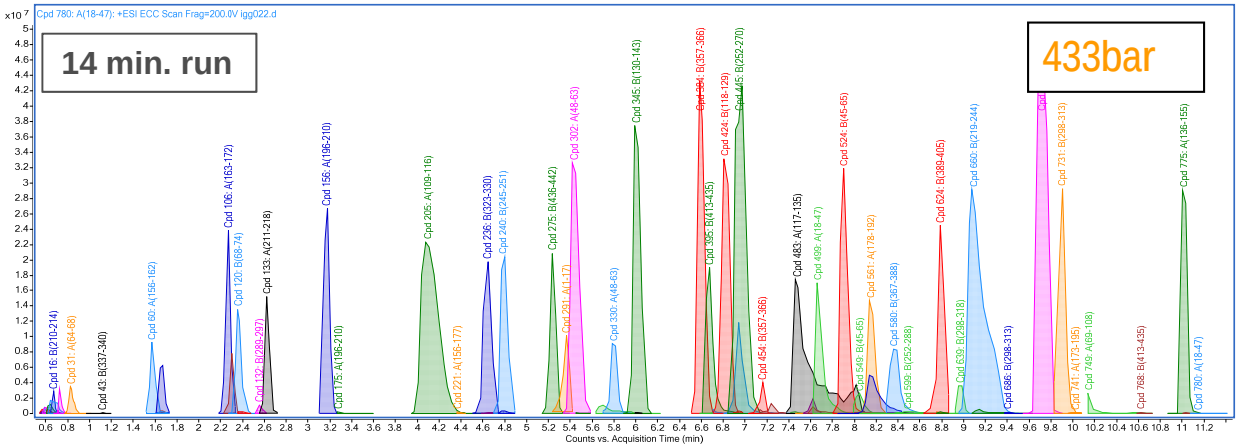
Inj.: 15uL
Flow: 0.6 ml/min
Temp.: 40 °C

Gradient (14min.)

Time(min.)	%B
0	3
10	35
12	90
14	90

LC/MS:
Agilent 1290 coupled to 6530 QTOF

2.1 x 100mm AdvanceBio Peptide Mapping Column, IgG1 tryptic digest

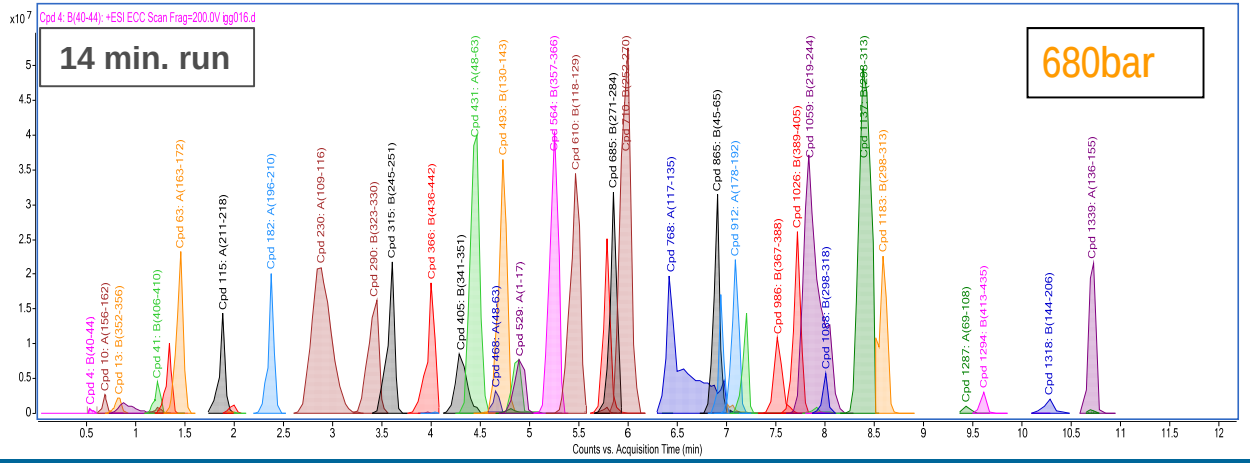


Mapping Results

Tryptic peptides i.d:
76 peptides

Sequence coverage.:
99.63%

2.1 x 100mm 1.7um Superficially Porous Peptide Column, IgG1 tryptic digest



Mapping Results

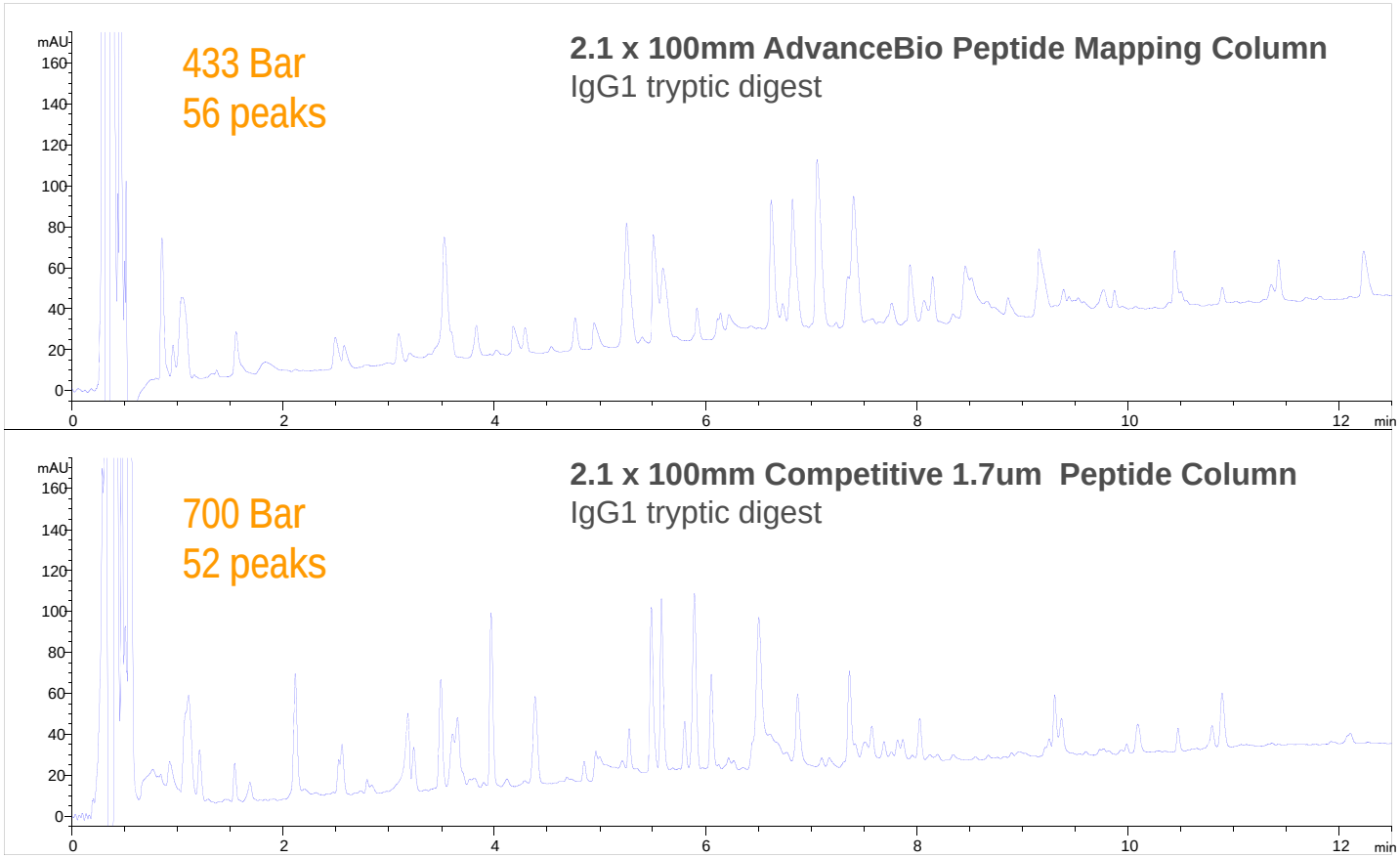
Tryptic peptides i.d:
65 peptides

Sequence coverage.:
89.54%

Achieving UHPLC-like Speed and Peptide Mapping Performance at HPLC Operating Conditions

Competitive UHPLC Peptide Column Comparison

Delivering UHPLC-type Speed and Separation Performance under HPLC Operation



Mobile phase A:
water (0.1%FA)
Mobile phase B:
90% ACN (0.1%FA)

Inj.: 15uL
Flow: 0.6 ml/min
Temp.: 40 °C

Gradient (14min.)

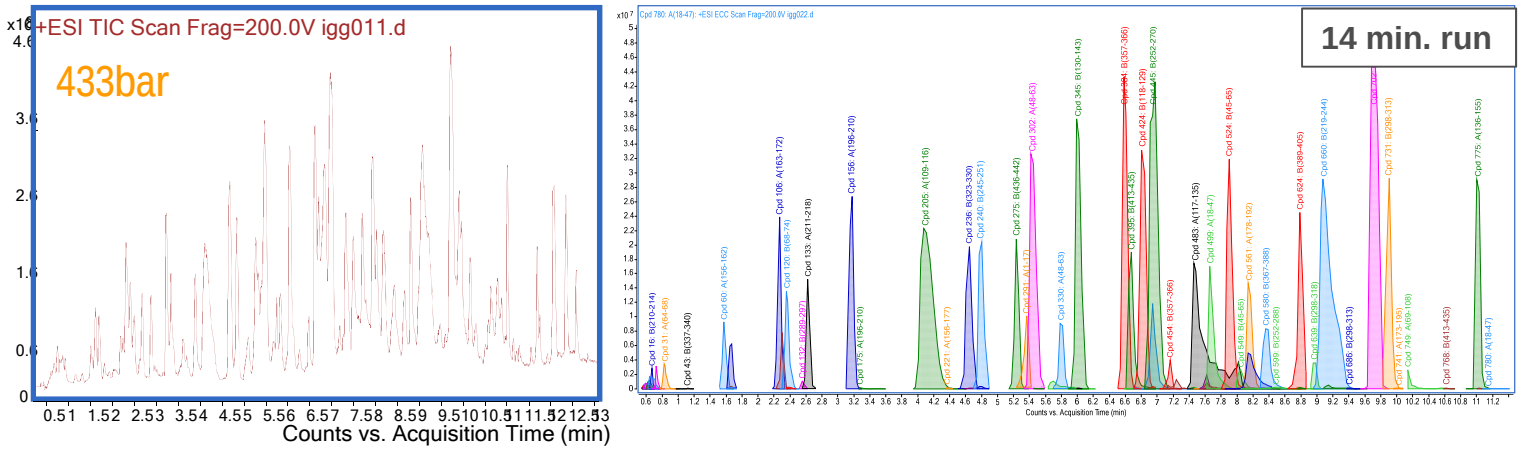
Time(min.)	%B
0	3
10	35
12	90
14	90

LC/MS:
Agilent 1290 coupled to 6530 QTOF

TOP Competitor UHPLC Peptide Column Comparison

Mass Spec BioCharacterization Performance: UHPLC vs HPLC

2.1 x 100mm AdvanceBio Peptide Mapping Column, IgG1 tryptic digest



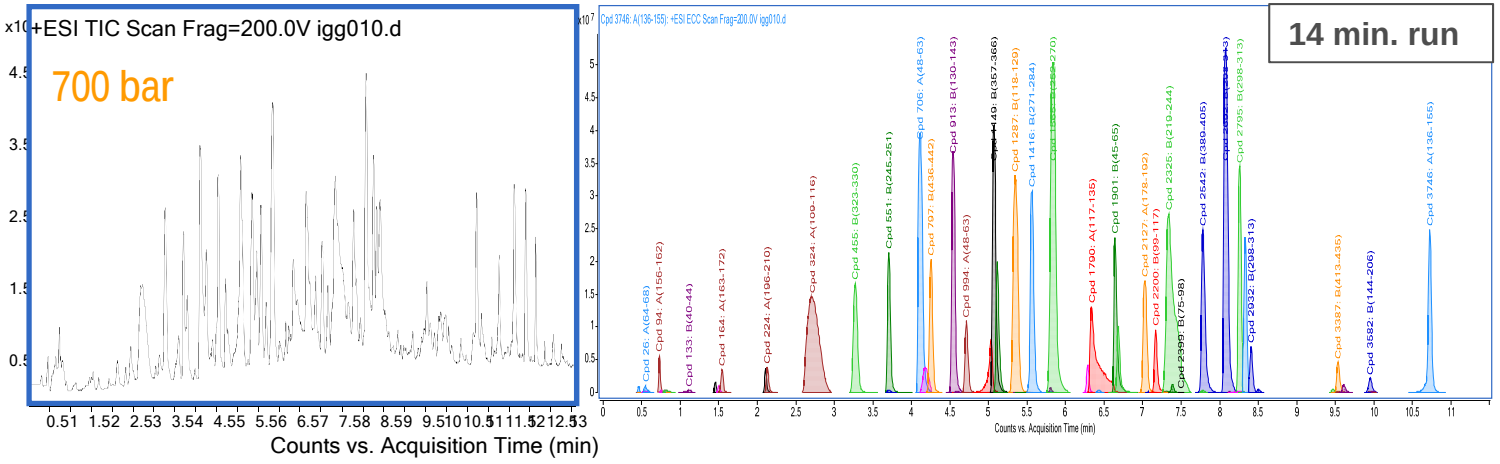
HPLC Results

Tryptic peptides i.d:
76 peptides

Sequence coverage.:
99.63%

LC Pressure:
433 bar

2.1 x 100mm Waters 1.7um CSH Peptide Column, IgG1 tryptic digest



UHPLC Results

Tryptic peptides i.d:
76 peptides

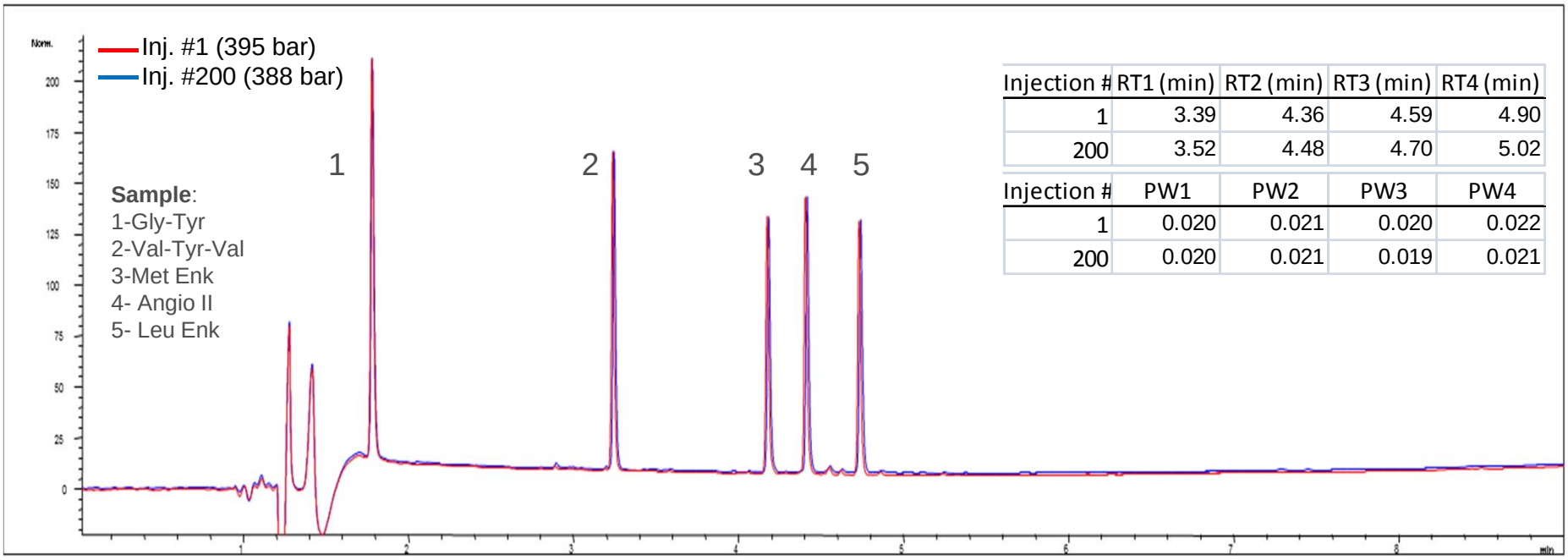
Sequence coverage.:
99.00%

LC Pressure:
700 bar

ROBUSTNESS

pH stability (phase)

200 repeated injections at pH 2.2 at 55C – continuous HPLC operation for 2.5 days

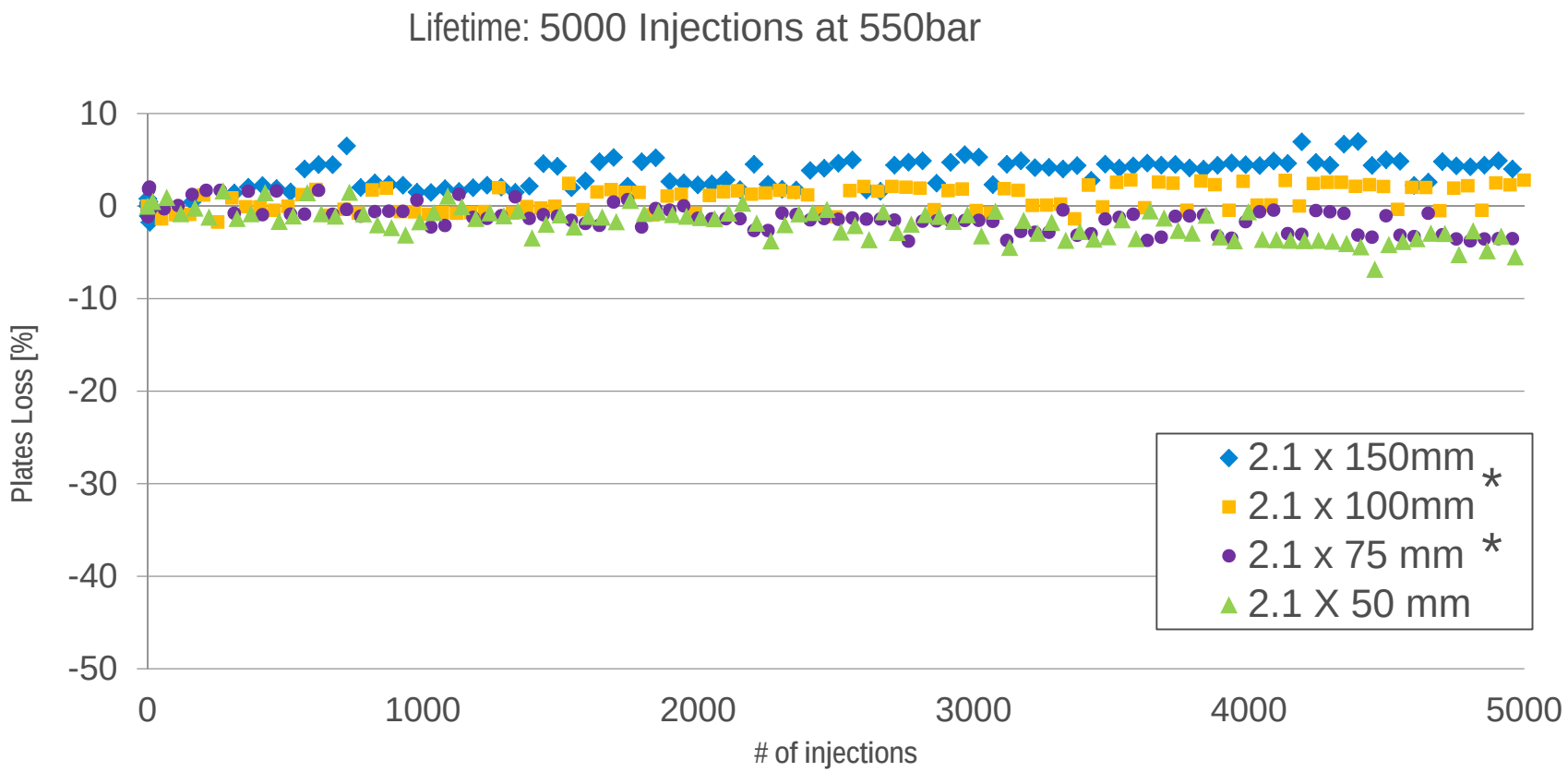


Injection #	RT1 (min)	RT2 (min)	RT3 (min)	RT4 (min)
1	3.39	4.36	4.59	4.90
200	3.52	4.48	4.70	5.02

Injection #	PW1	PW2	PW3	PW4
1	0.020	0.021	0.020	0.022
200	0.020	0.021	0.019	0.021

Column dim.: 2.1 x 250mm
Conditions: flow: 0.50ml/min., inj: 1uL, Temp: 55C, det:220nm,
Mobile phase: A-water (0.1%TFA), B- ACN (0.08%TFA)
Gradient: 0-8min ,10-60%B; 8.1-9min, hold 95%B, re-equilibration time: 8 mins
Instrument: Agilent 1260 Bio-Inert

Column Lifetime Efficiency (packed bed stability)

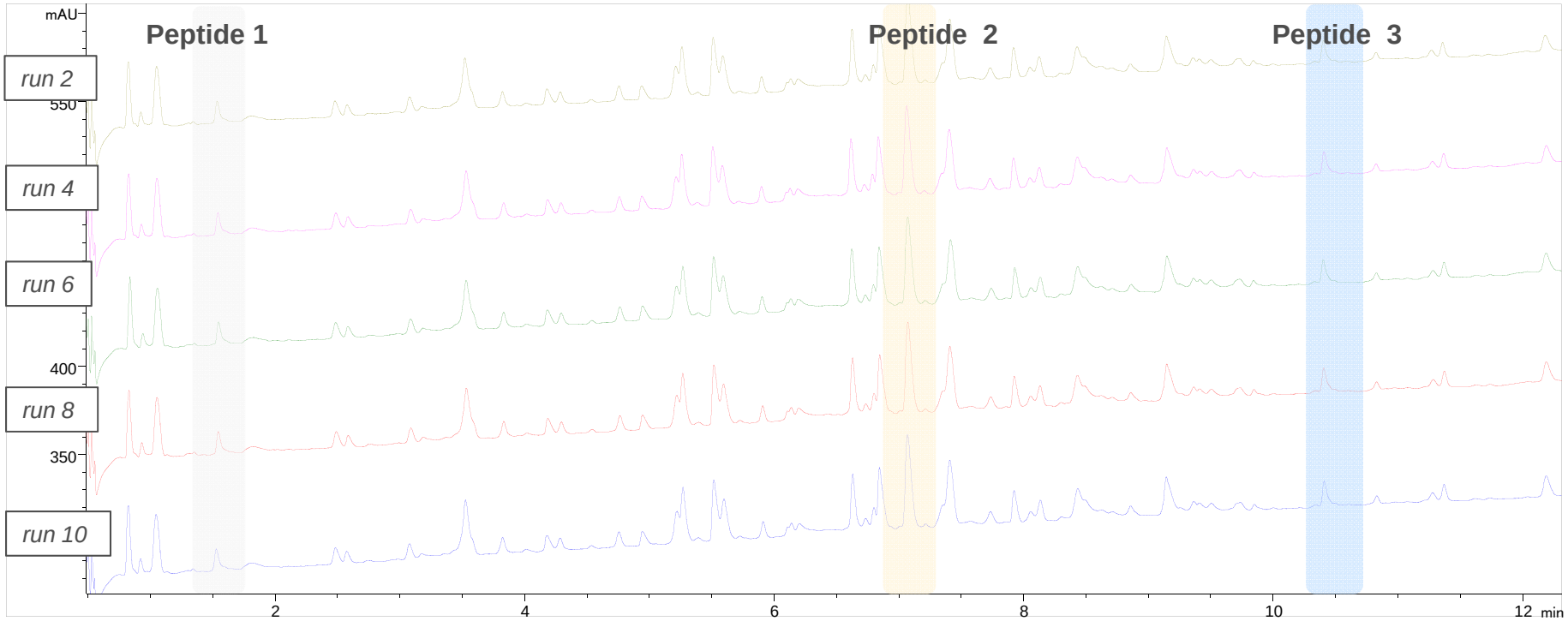


Conditions: flow: varied for column length., inj: 1uL, Temp: 25C, det:280nm, isocratic run
Mobile phase: 70%-water, 30%- MeOH, **Sample probe**: Napthalene
Instrument: Agilent 1200

* stocked part #

Rapid LC/MS IgG Tryptic Digest Peptide Mapping Reproducibility

10 Consecutive IgG injections on a 2.1 x 150mm AdvanceBio Peptide Mapping Column

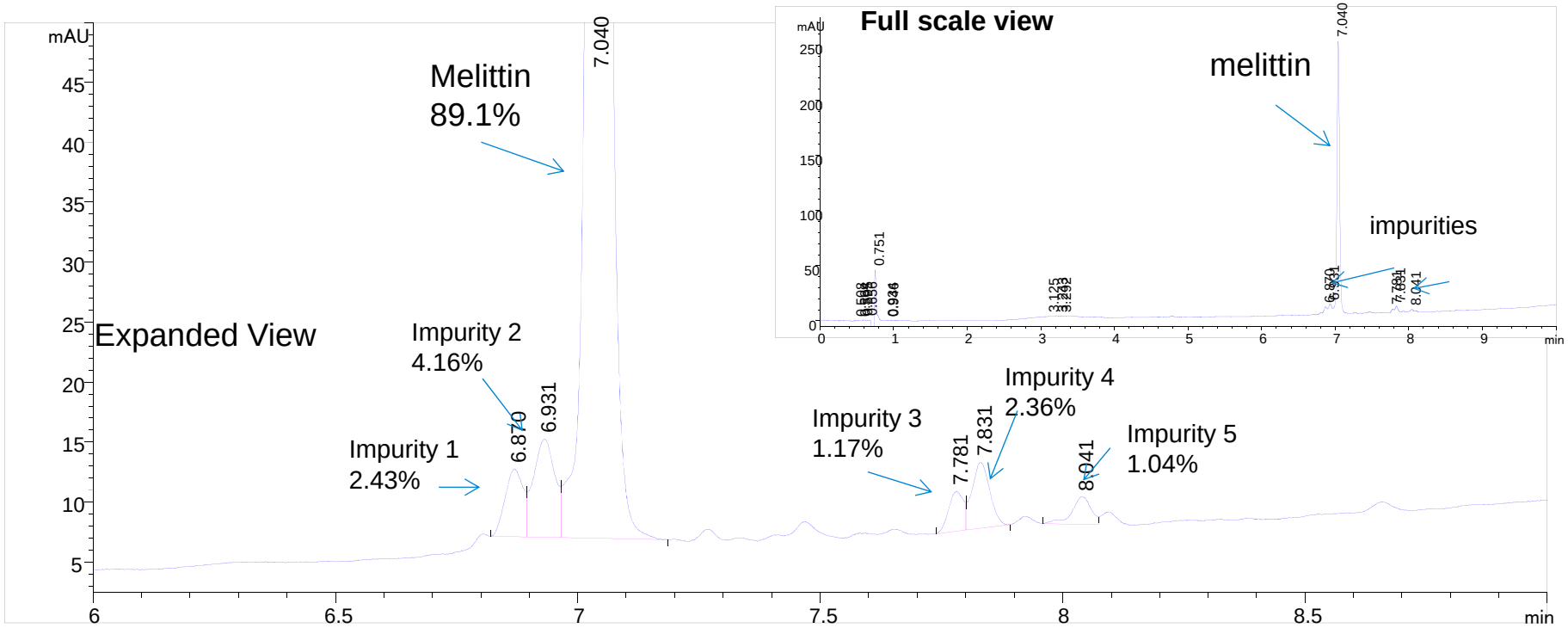


Retention Time 1 hydrophilic		Peptide 1 Peak Area		Retention Time 2 Mid hydrophobic		Peptide 2 Peak Area		Retention Time 3 High hydrophobic		Peptide 3 Peak Area	
Mean (min)	% RSD	Mean (mAU/s)	% RSD	Mean (min)	% RSD	Mean (mAU/s)	% RSD	Mean (min)	% RSD	Mean (mAU/s)	% RSD
1.54	0.51	38.46	3.77	7.06	0.06	172.74	2.62	10.41	0.04	38.460	8.65

CAN THIS COLUMN BE USED FOR OTHER PEPTIDE APPLICATIONS?

Quantitative Peptide Analysis

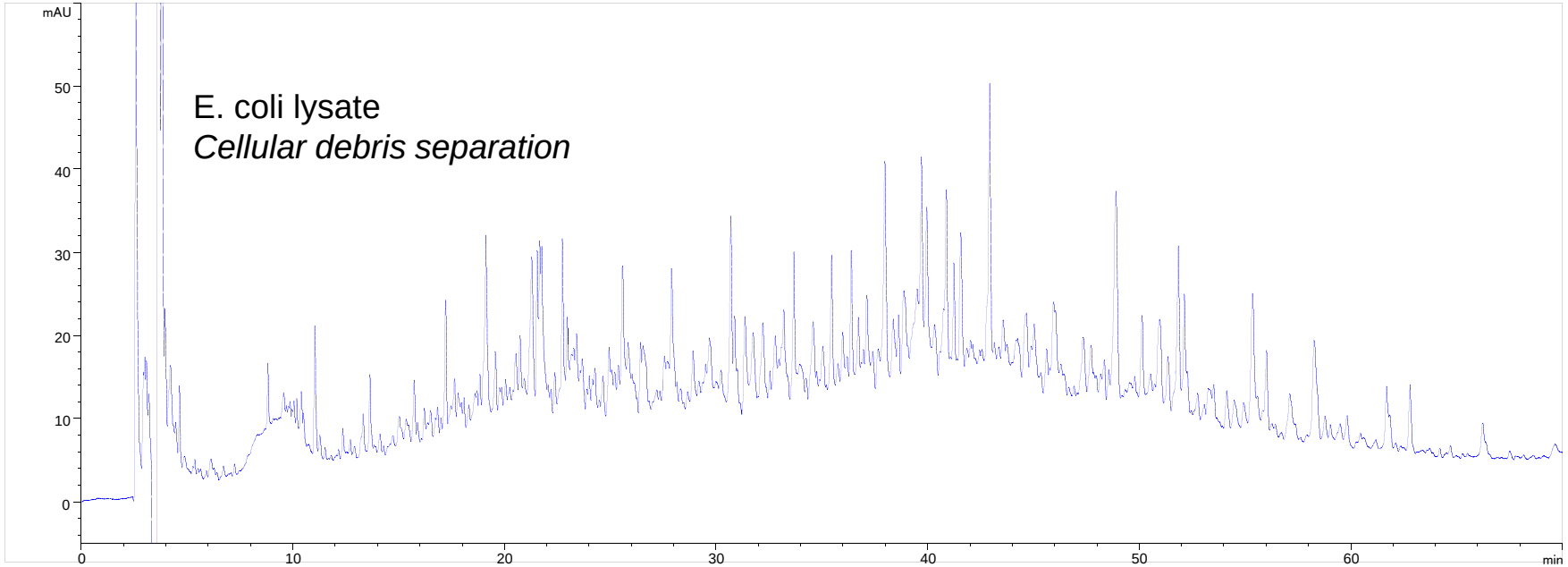
Melittin Analysis



Melittin from bee honey (sigma) separated on a 2.1 x 150mm AdvanceBio Peptide Mapping Column
Mobile phase: A-water (0.1%TFA), B- ACN (0.08%TFA), temp: 55C, Flow: 0.50mL/min

- Delivers excellent resolving power, sensitivity and selectivity for peptide impurity profiling
- Increased flow rate capability maintains resolution and increase analysis times while column back pressures remain below 500 bar .

Complex Peptide Mixtures and Small Proteins



E. Coli lysate separated on a 2.1 x 250mm AdvanceBio Ppetide Mapping Column
Mobile phase: A-water (0.1%TFA), B- ACN (0.08%TFA), temp: 65C, Flow: 0.25mL/min

- Provides compatibility with TFA and Formic acid mobile phases for LC/MS analyses
- Delivers a wide range of separation capability to selectively and efficiently resolve peptides and small proteins with high resolution.

AdvanceBio Peptide Mapping Column

Summary:

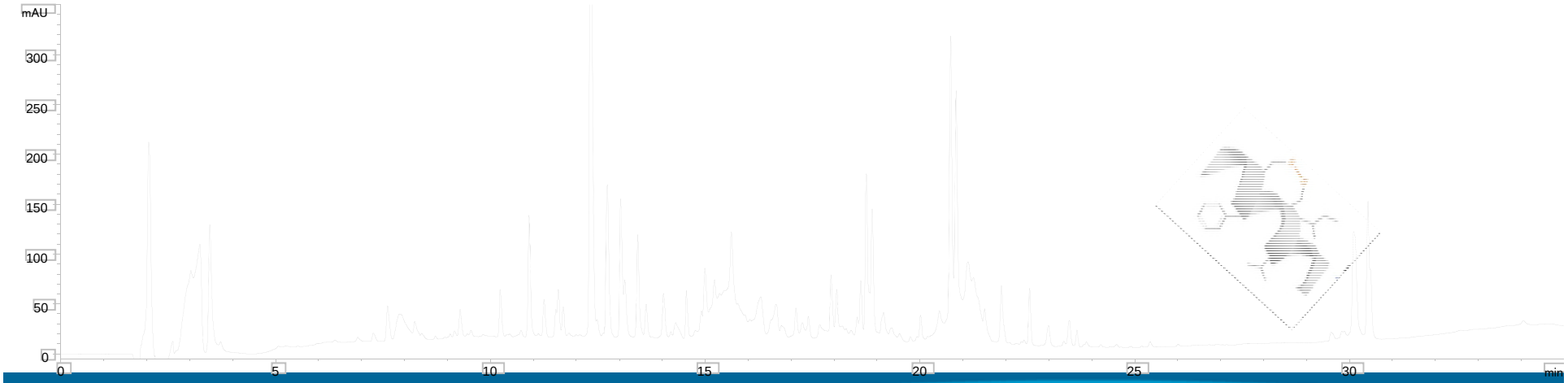
Peptide Mapping Separation Performance does not deteriorate when gradients are shortened and run times decreased

Critical PTM information is conserved with reduced LC/MS analysis times

Higher Sequence Coverage during rapid analysis in comparison to competitive Peptide column

UHPLC like speed and performance was achieved at traditional HPLC pressures and in comparison to competitive uHPLC Peptide Column.

High run to run reproducibility achieved on a complex biological



AdvanceBio Peptide Mapping Column
Literature & Reference Materials

Agilent Peptide Mapping "How-To" Guide

Features AdvanceBio Peptide Mapping Column and How-to techniques/methods. ***pub# 5991-2348EN***

Agilent Biocolumns Selection Guide:

AdvanceBio Peptide Mapping Column configurations, part #s, etc

4 Application Notes (Martosella et. al.)

Glycoprotein EPO mapping

(1) ***pub# 5991-2085EN***
(2) ***pub# 5991-1813EN***

IgG1 mapping

(1) ***pub# 5991-3585EN***
(2) ***in-print***

Thank you!

QUESTIONS?