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Simple Techniques for Improving the Isolation of Synthetic Peptides

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Principal Scientist
Waters Corporation

Agenda

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- Background
- Techniques
  - Scaling a separation
  - Focusing the gradient
  - At-column dilution
  - Temperature control
- Practical example
- Summary

Why peptides?

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- Unique role
- Attractive as potential drug candidates
- Sources

Synthetic Peptides

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- Synthetic peptides
  - Amino acids linked using organic chemical reactions
  - Average: 10-30 residues
  - Large: 70-120 residues or more

- Impurities
  - Failure Sequences
  - Deletion Sequences
  - Damaged Sequences
  - Cleavage Adducts

- Purification requirements
  - 75-98% purity of final product

Peptide Isolation Workflow

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Amount?

Crude analysis

Isolation

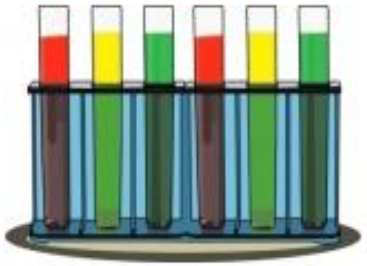
Fraction Analysis

No

Enough pure peptide?

Yes

Pure peptide



Most Common Operating Conditions

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- Purification using reversed-phase chromatography
- C18 columns
- Elution with gradient
- Monitor with UV detection at 214-220nm
- Mass-directed isolation

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Scaling Equations

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Mass

$$M_2 = M_1 \times \frac{L_2}{L_1} \times \frac{d_2^2}{d_1^2} \quad \textit{where:}$$

M_1 is Mass on Column1

M_2 is Mass on Column2

L_1 is Length of Column1

L_2 is Length of Column2

d_1 is Diameter of Column1

d_2 is Diameter of Column2

Gradient

$$t_2 = t_1 \times \frac{L_2}{L_1} \times \frac{d_2^2}{d_1^2} \times \frac{F_1}{F_2} \quad \textit{where:}$$

t_1 is Gradient Duration for Column1

t_2 is Gradient Duration for Column2

L_1 is Length of Column1

L_2 is Length of Column2

d_1 is Diameter of Column1

d_2 is Diameter of Column2

F_1 is Flow rate for column1

F_2 is Flow rate for column2

Flow rate

$$F_2 = F_1 \times \frac{d_2^2}{d_1^2} \quad \textit{where:}$$

F_1 is Flow Rate for Column1

F_2 is Flow Rate Column2

d_1 is Diameter of Column1

d_2 is Diameter of Column2

Scaling a Method

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Analytical Method

Time	Flow	%A	%B
0.00	1.46	95	5
5.00	1.46	50	50
5.50	1.46	5	95
6.50	1.46	5	95
7.00	1.46	95	5
10.00	1.46	95	5

Column: 4.6 x 50 mm
System dwell volume: 0.71 mL
Column volume: 0.698 mL
System dwell volume (in column volumes): 1.02

Preparative Method

Time	Flow	%A	%B
0.00	25	95	5
0.30	25	95	5
5.30	25	50	50
5.80	25	5	95
6.80	25	5	95
7.30	25	95	5
10.30	25	95	5

Column: 19 x 50 mm
System dwell volume: 4.75 mL
Column volume: 11.9 mL
System dwell volume (in column volumes): 0.40
Gradient offset: 0.30

Gradient Scaling

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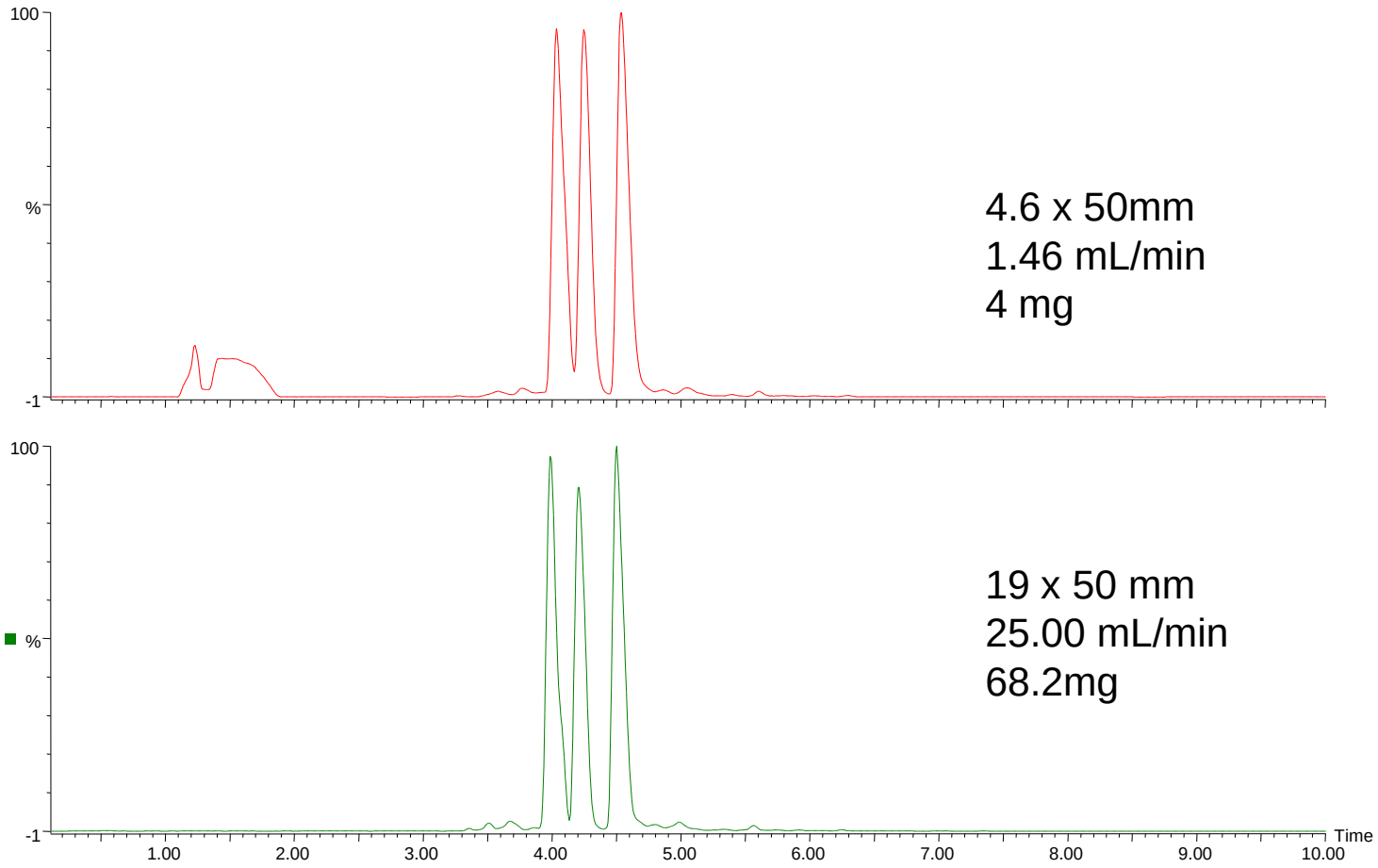

Estimated Peptide Mass Loading Capacity (mg)

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Length (mm)	Diameter (mm)					
	2.1	4.6	10*	19*	30**	50
50	0.04-0.11	0.3-0.6	1.5-3.0	4-9	11-22	31-62
100	0.11-0.22	0.5-1.0	2.5-5.0	9-18	22-45	62-125
150	0.15-0.33	0.8-1.6	4.0-8.0	13-27	34-68	93-186
250	0.26-0.55	1.3-2.6	6.0-12.0	22-45	56-112	155-310

*5 µm and 10 µm OBD Prep Columns; ** 10 µm OBD Prep Columns

* [OBD™], Optimum Bed Density; US Patent # 7,399,410, UK Patent # GB2408469

Estimates of mass load are broad because capacity depends on solubility of the peptide in the mobile phase.

Flow rate (mL/min)
Injection Volume (µL)

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	Diameter (mm)					
	2.1	4.6	10*	19*	30**	50
Flow rate	0.19-0.39	0.9-1.8	4.5-9.0	16-32	40-80	111-222
Inj Vol	4.3	20	100	350	880	2450

*5 µm and 10 µm OBD Prep Columns; ** 10 µm OBD Prep Columns

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Gradient Types

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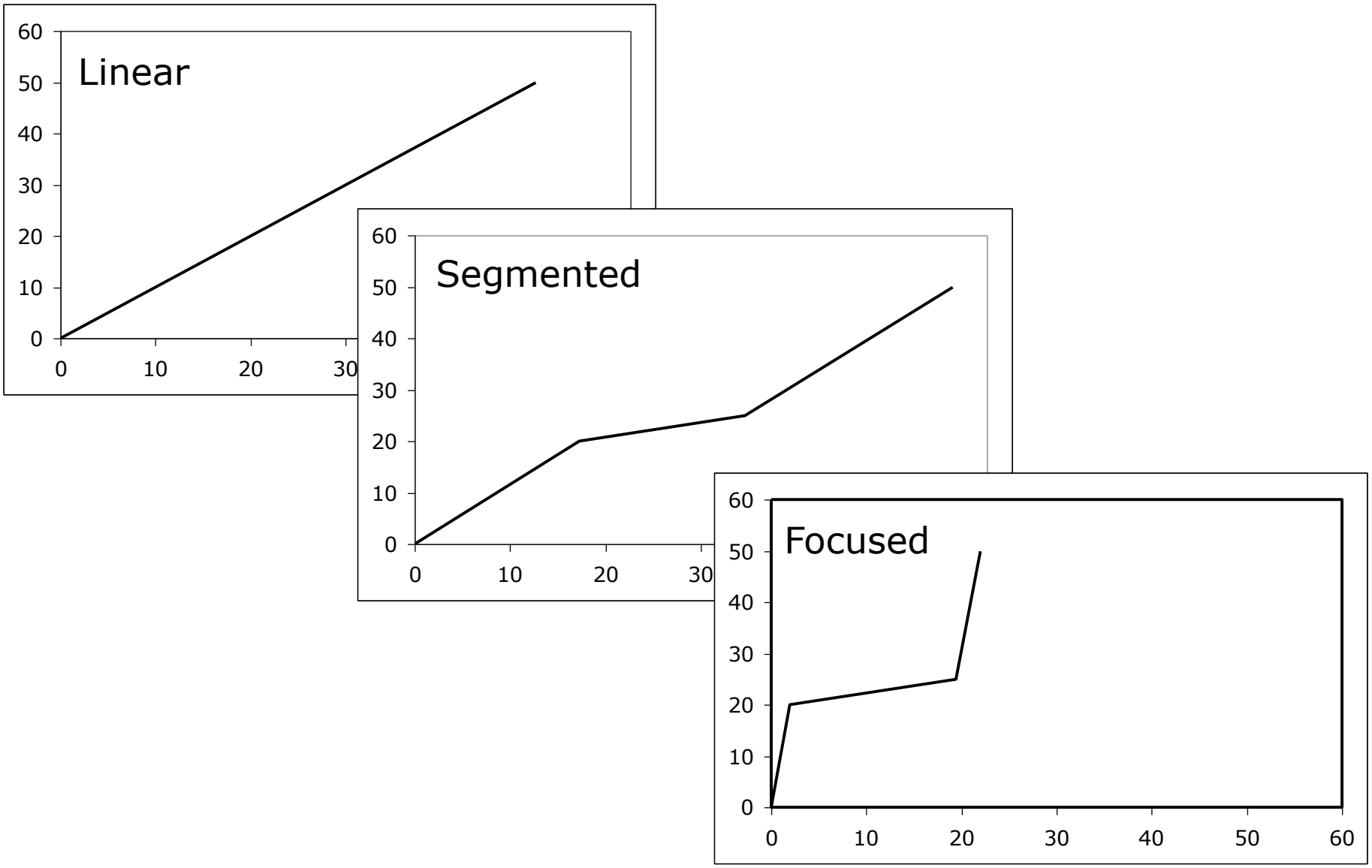

Developing a Focused Gradient

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- Estimate volume of system and column
- Make pilot run of sample
- From elution time, system volume and gradient table, estimate % that elutes the peptide
- From gradient and column volume, estimate gradient slope in pilot run in % change per column volume
- Create shallow gradient segment from 5% below to 3% above estimated elution percentage

- Full Description in Application Note:
  Developing Focused Gradients for Isolation and Purification
  720002955en

Crude Peptide Analysis

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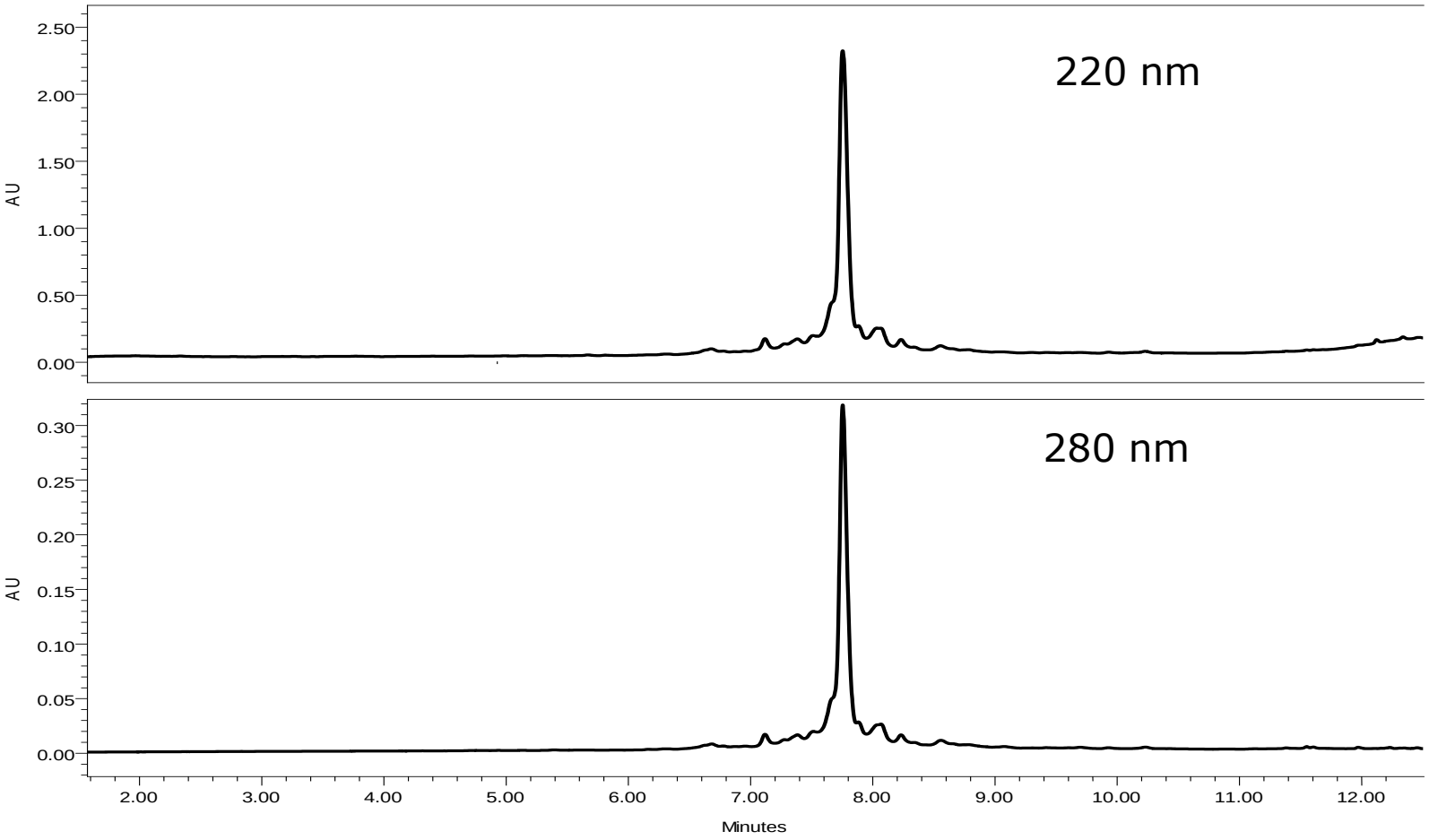

XBridge Peptide BEH C_{18} 5µm Column, 4.6 x 100 mm
Gradient: 5-50%B in 10 minutes, 3.4% change per column volume, 5 µL
Peptide eluted at 33%B.

Focused Gradients

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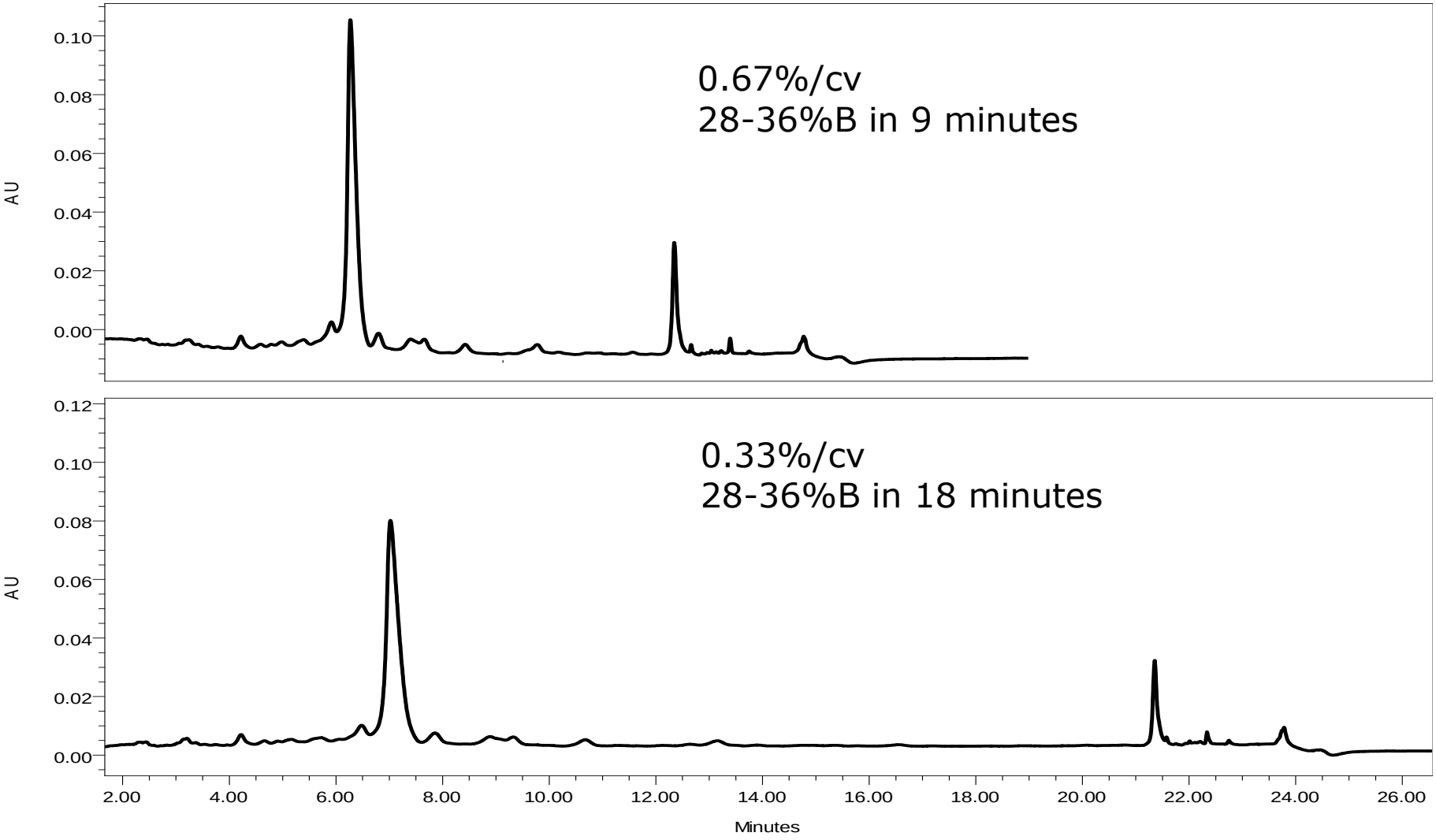

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Dissolving Synthetic Peptides

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- No universal technique or solvent
- Apparent insolubility may include synthesis by-products
- Useful choices
  - Water with 0.1-2.0% TFA
  - Wet with small amount of DMF, dilute with water to ~10% organic
  - 100% DMSO
  - 100% Hexafluoroisopropanol (HFIP)
  - 6-12M Guanidine-HCl (not for use with mass spectrometers)
- Always results in relatively large volume of relatively strong solvent

Standard Gradient System

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Conventional Separations
Simplified Flow Diagram

System Gradient Pump
(Aqueous flow delivers sample plug to column.)

20 ml/min
95% Aqueous
5% Organic

Auto Sampler Valve

C o l u m n

Configurations vary.

Fraction Collector

Detector(s)

Standard Prep Separation With DMSO As Sample Solvent

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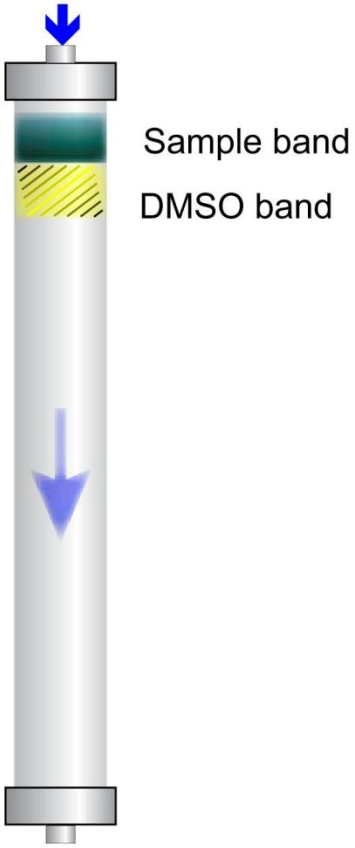

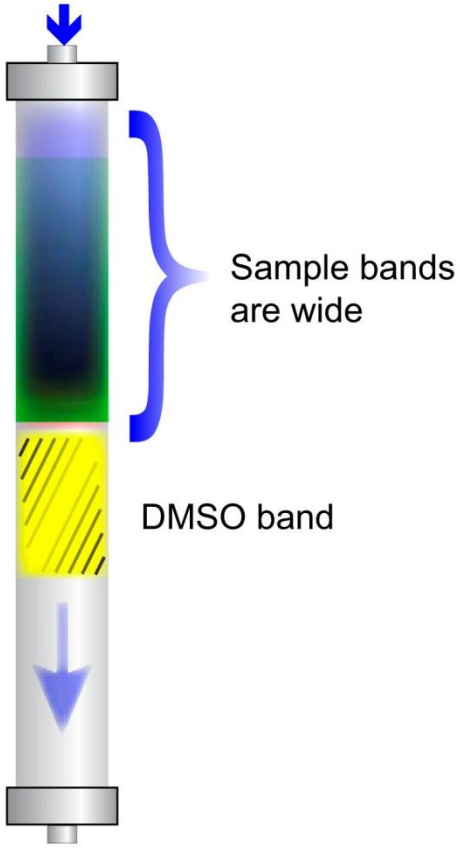

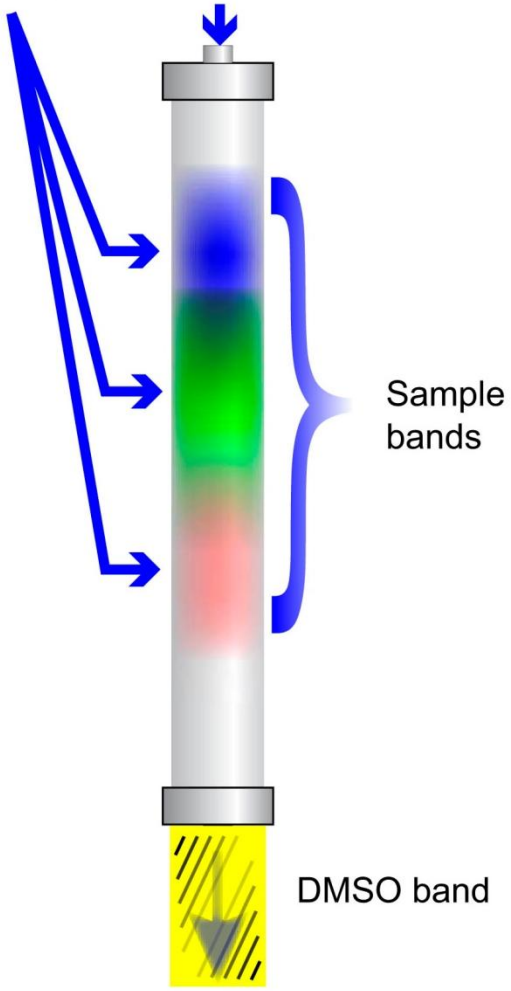

At-Column Dilution

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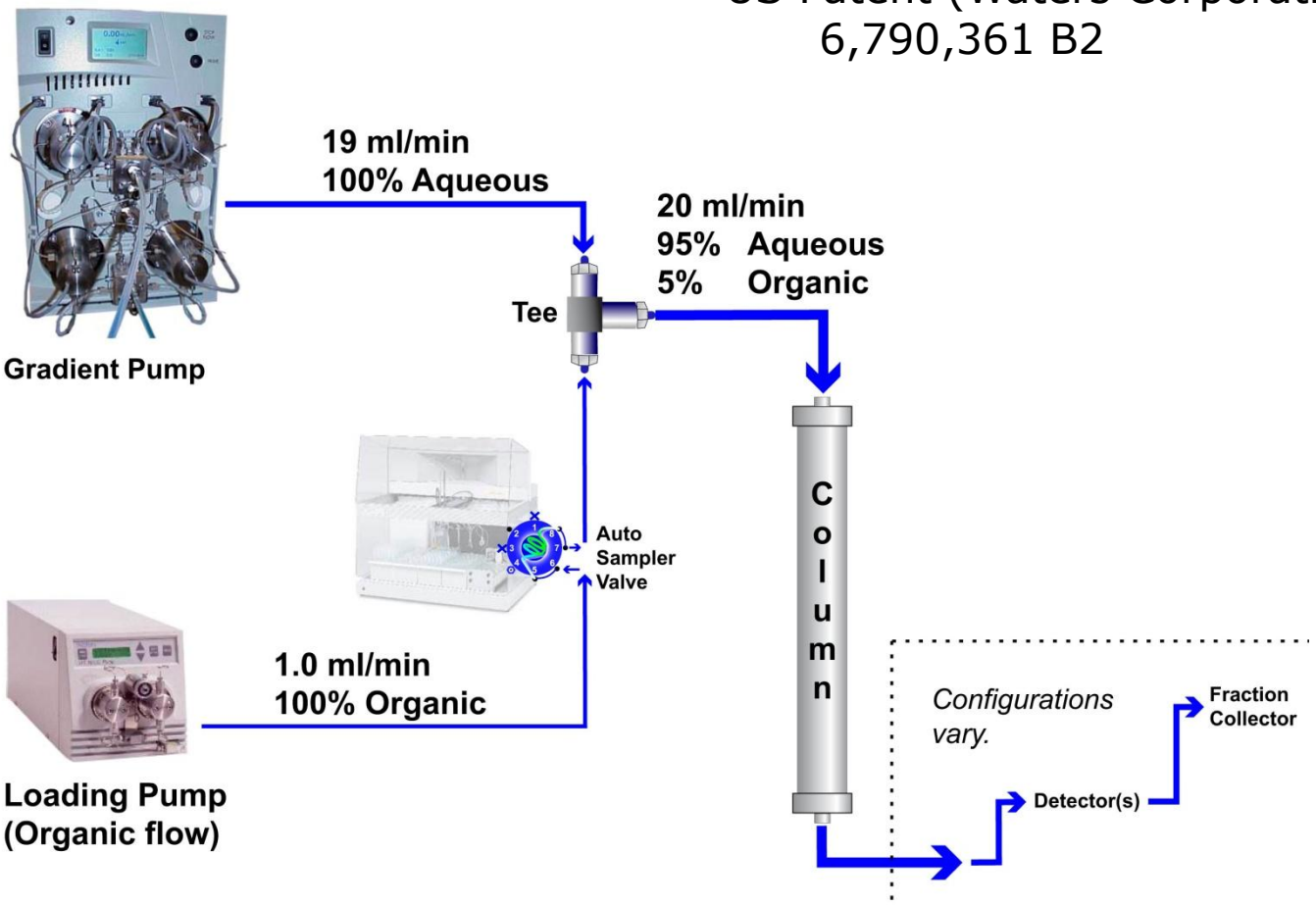
At-Column-Dilution Separations
Simplified Flow Diagram

Waters Application Note :71500078010rA

US Patent (Waters Corporation)
6,790,361 B2



At-Column Dilution Prep Separation

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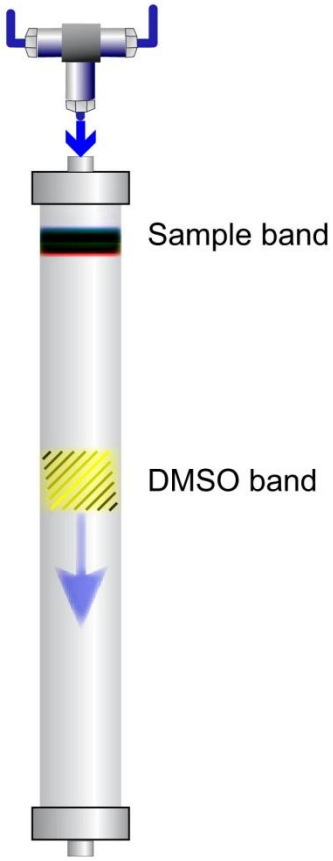

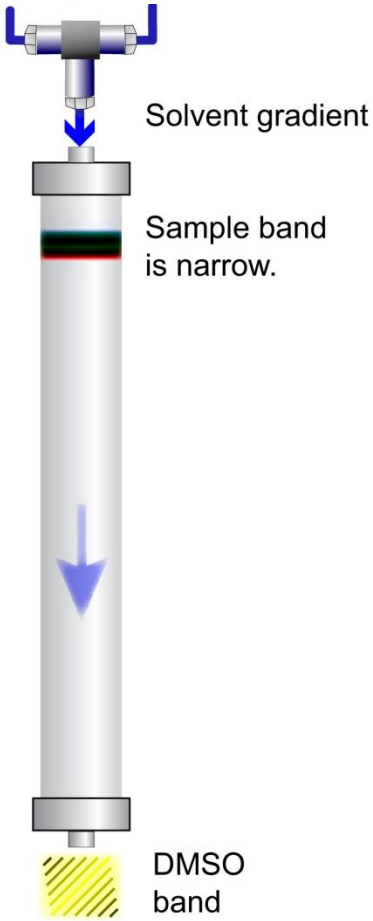

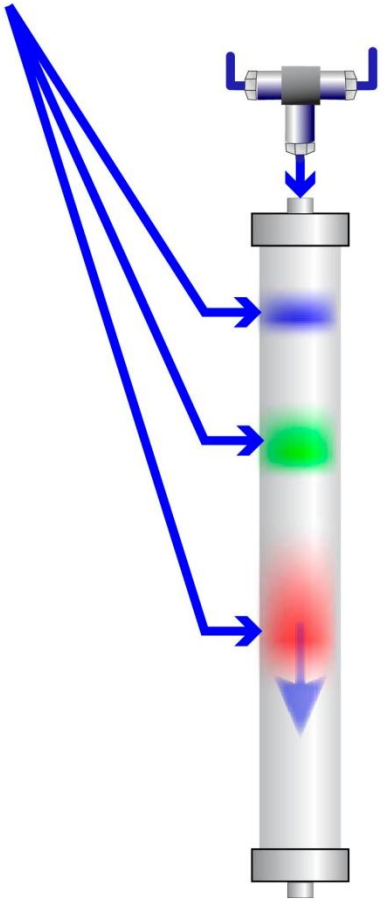

Conventional vs. At-Column Dilution

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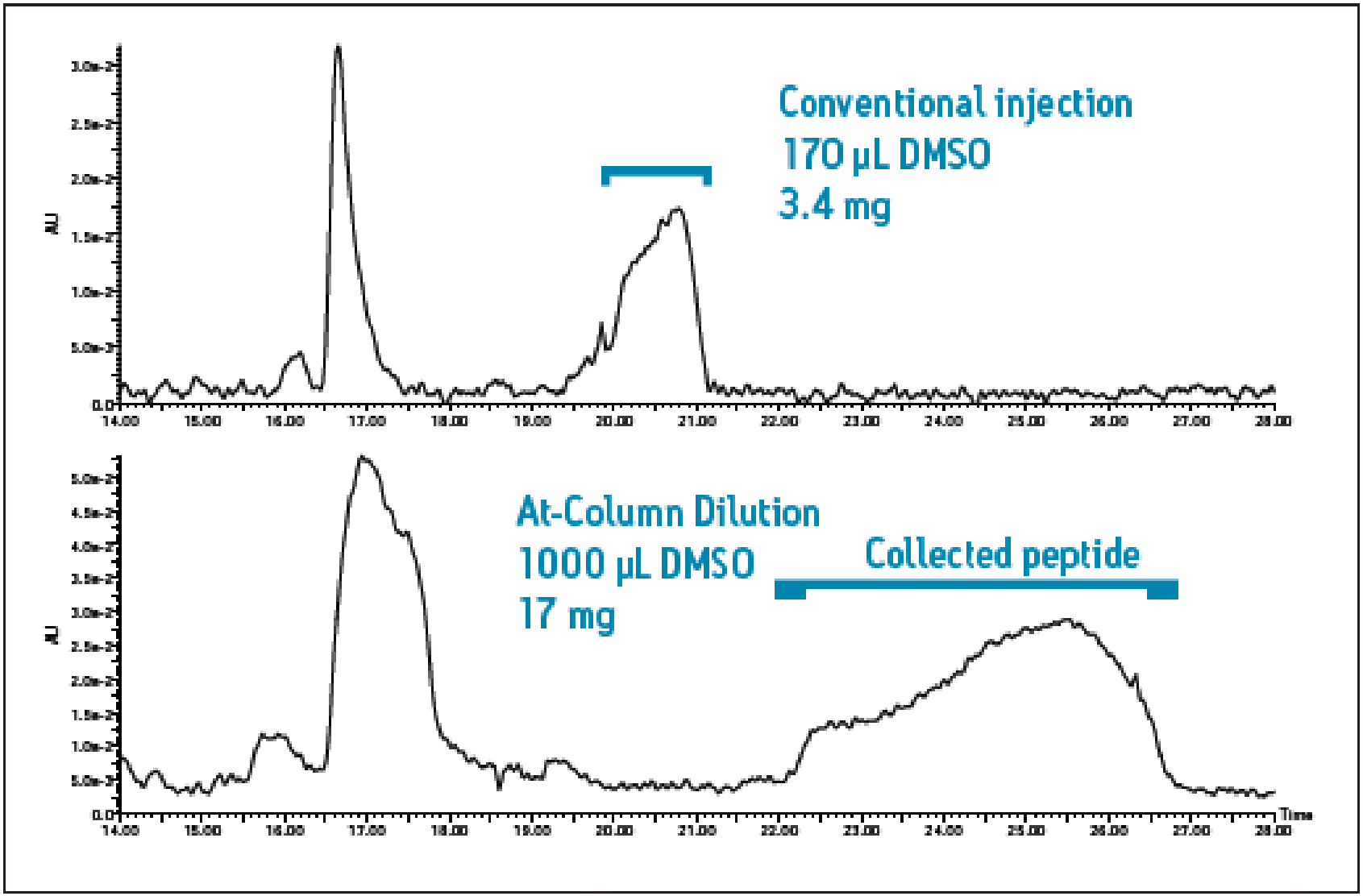

At-Column Dilution

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- Permits injection of large volumes of strong solvents
- Improves both mass capacity and resolution
- Increases system ruggedness
- Extends column life
  - Reduces introduction of particles
  - Reduces pressure shock from viscous solvent

- Waters Application Note :71500078010rA
- US Patent (Waters Corporation)
  - 6,790,361 B2

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Effect of Temperature Crude Synthetic Peptide

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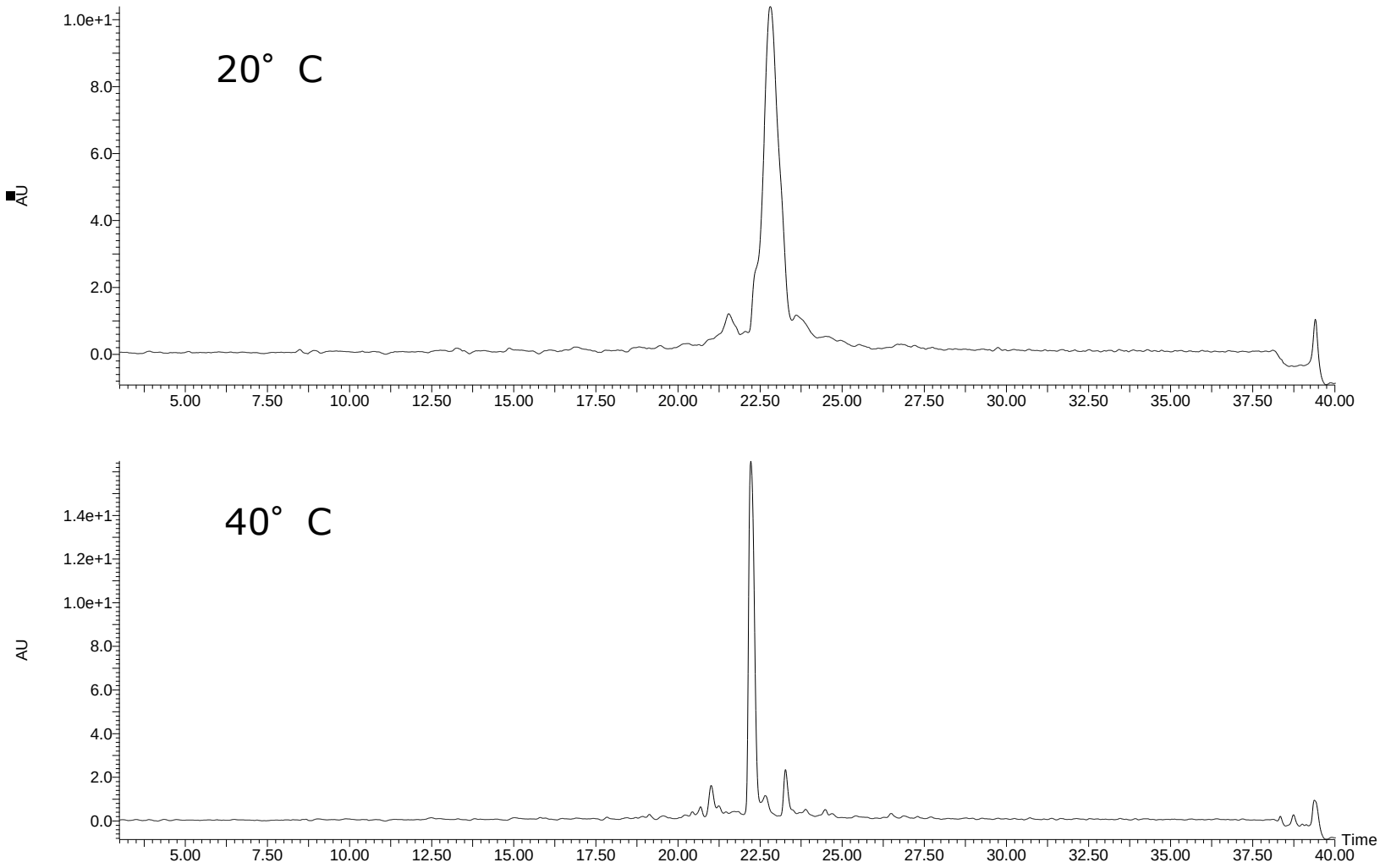

Effect of Temperature Peptide Separation

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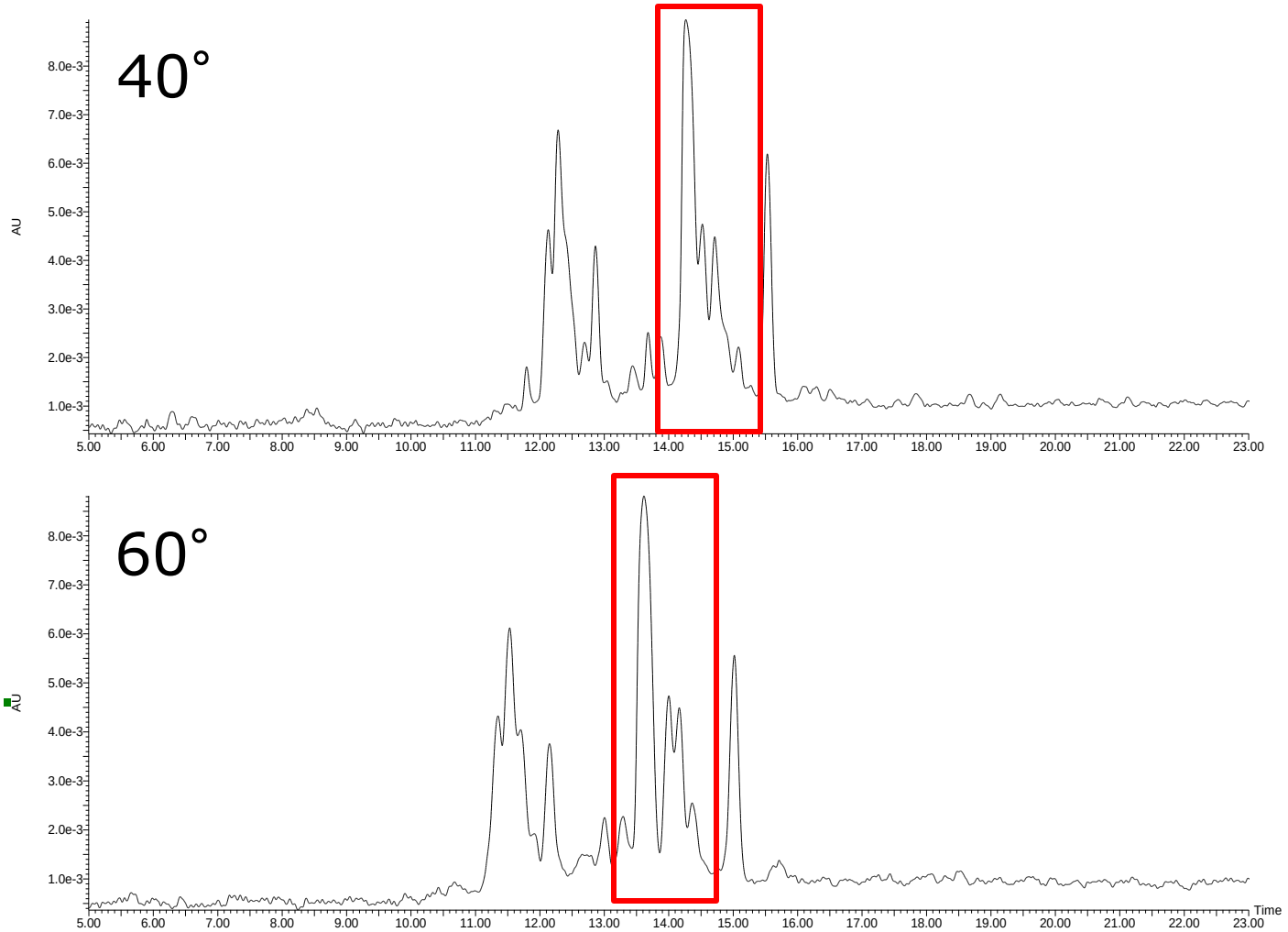

Effect of Temperature Peptide Separation

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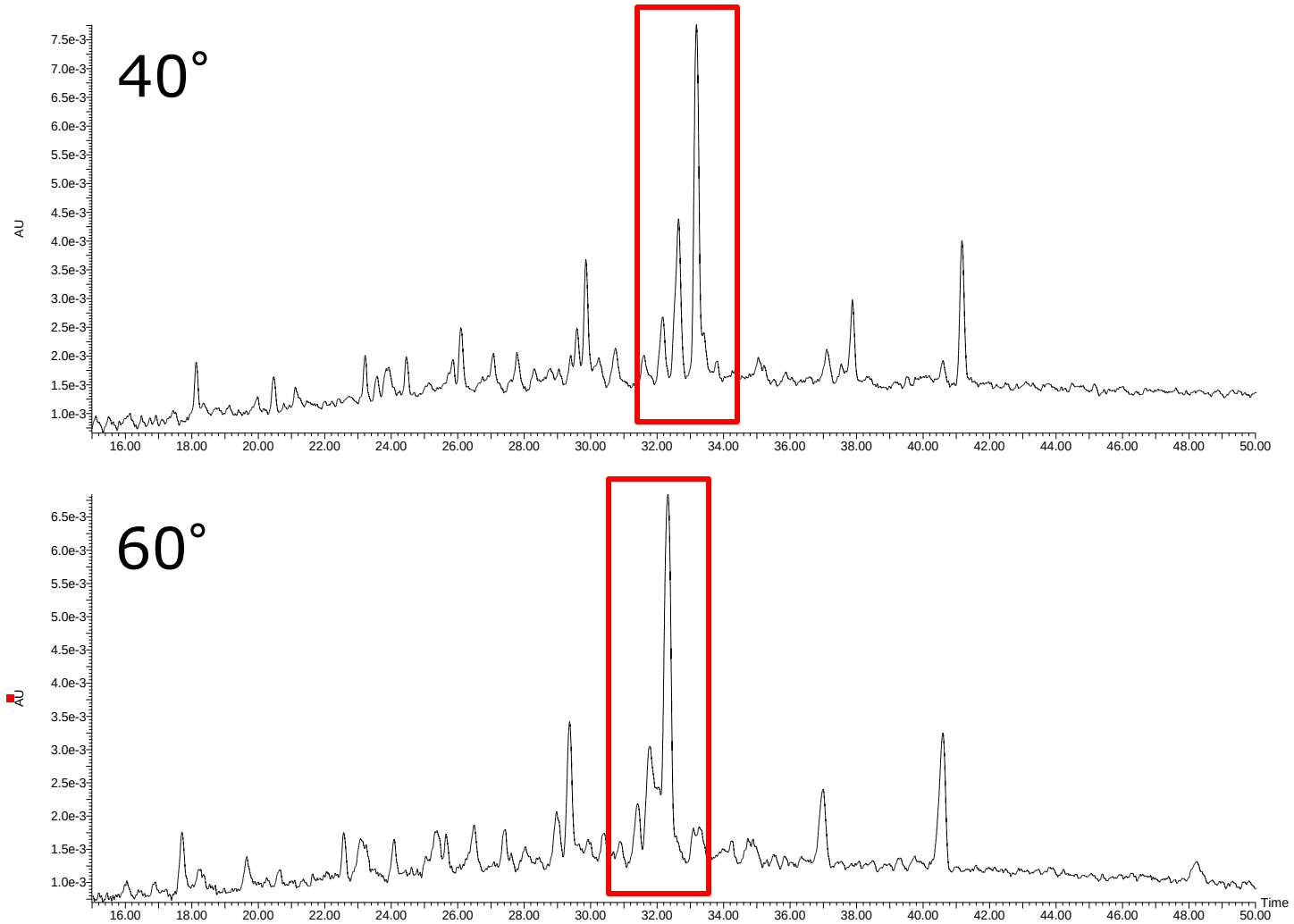

Controlling Temperature in Purification
Practical Considerations

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- Large diameter columns cannot be effectively heated from the outside
- High flow rate separations occur at the temperature of the incoming solvent
- Temperature gradient within column
- Add a coil of stainless steel tubing to the inlet end of the column for pre-heating (e.g. 5-mL sample loop)
- Immerse column and pre-heating coil into a water bath

Column and Pre-heater Loop Immersed in Water Bath at 60°

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At-Column-Dilution: Sample Loading with Temperature Control

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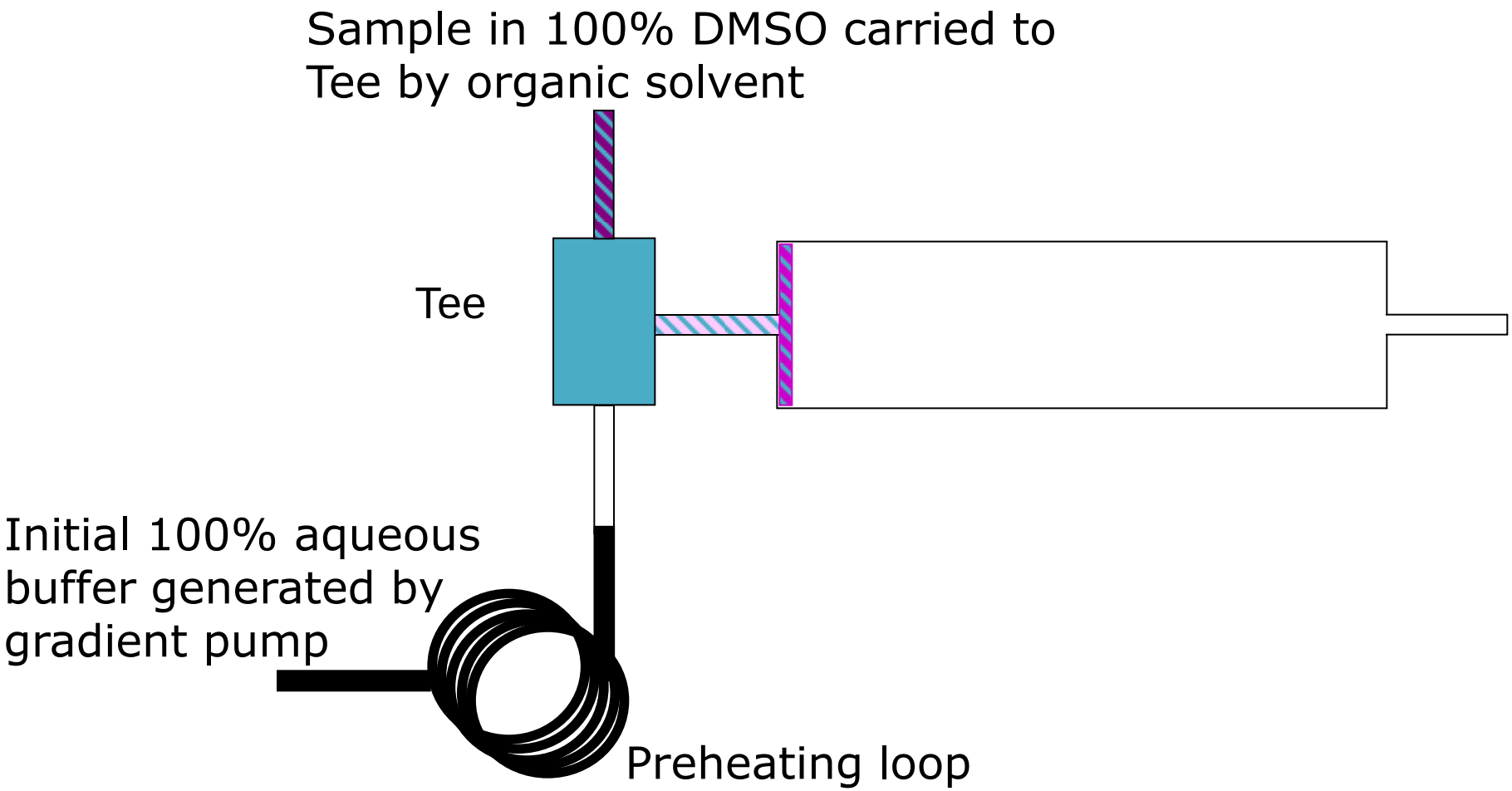

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Temperature Evaluation
Crude Peptide

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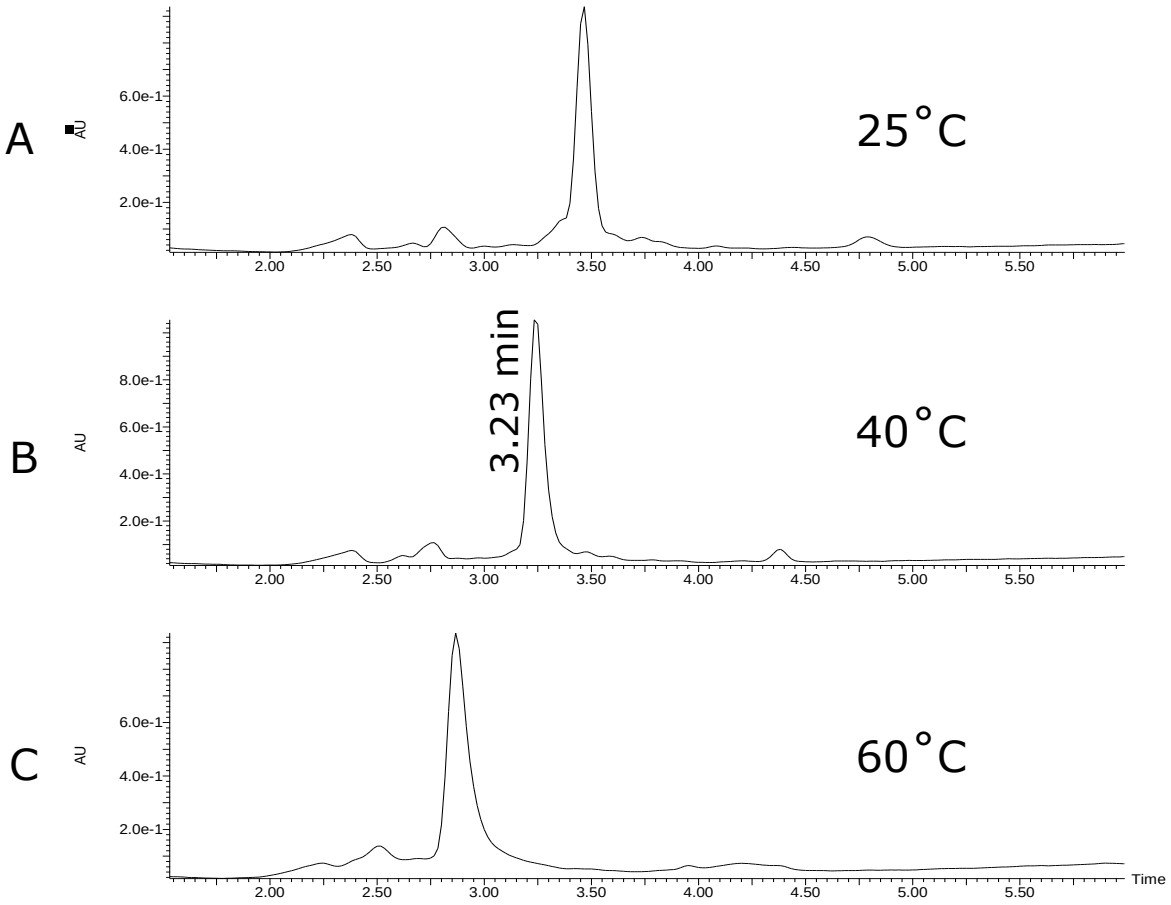

XBridge Peptide BEH C_{18} 4.6 x 50 mm
Gradient: 0-30% isopropanol in 5 minutes @ 40°C; 5.13 mg/mL, 7 µL

Focused Gradient

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Screening Gradient

Time	Flow	%A	%B
0.00	1.46	100	0
0.50	1.46	100	0
5.28	1.46	70	30
6.00	1.46	10	90
7.00	1.46	10	90
7.50	1.46	100	0
10.50	1.46	100	0

System volume: 0.77 mL
Column volume: 0.698 mL
Calculated peptide elution %: 10.85%
Screening gradient slope: 3.0%/column volume
Focused gradient segment range: 5-13%B
Calculated focused gradient slope: 0.6%/column volume
Calculated time for the focused gradient segment: 6.37 min

Focused Gradient

Time	Flow	%A	%B
0.00	1.46	100	0
0.50	1.46	95	5
6.87	1.46	87	13
7.00	1.46	10	90
8.00	1.46	10	90
8.10	1.46	100	0
11.10	1.46	100	0

Gradient segment range: 5-13%B
Gradient slope: 0.6%/column volume
Time for the focused gradient segment: 6.37 min

Loading Study
Focused Gradient

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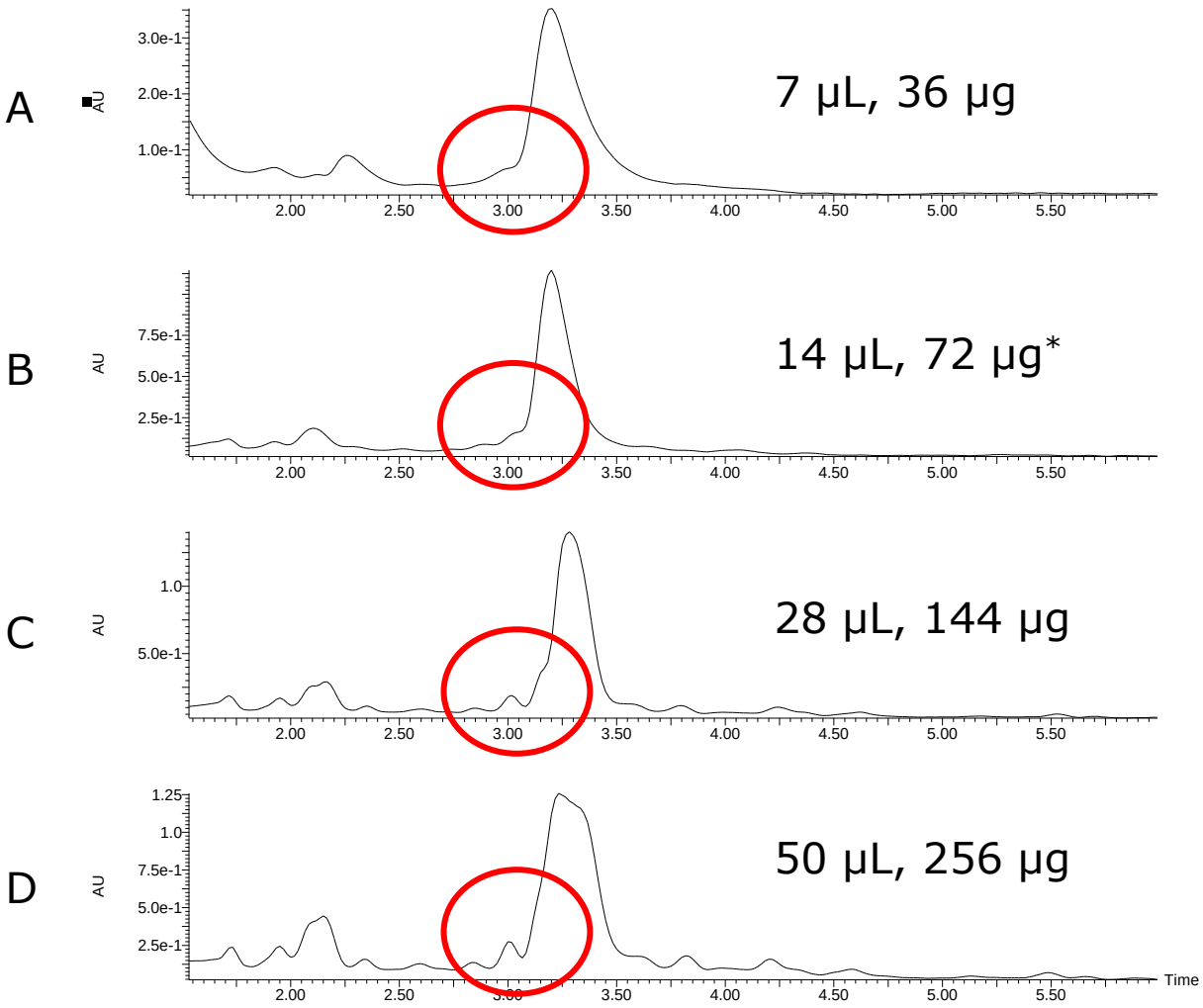

5-13% isopropanol with 0.1% TFA @ 40°C, 214 nm

Scaling

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Analytical Method

Time	Flow	%A	%B
0.00	1.46	100	0
0.50	1.46	95	5
6.87	1.46	87	13
7.00	1.46	10	90
8.00	1.46	10	90
8.10	1.46	100	0
11.10	1.46	100	0

Column: 4.6 x 50 mm
System dwell volume: 0.77 mL
Column volume: 0.698 mL
System dwell volume (in column volumes): 1.10

Preparative Method

Time	Flow	%A	%B
0.00	25	100	0
0.66	25	100	0
1.66	25	95	5
14.40	25	87	13
14.66	25	10	90
16.66	25	10	90
16.86	25	100	0
22.86	25	100	0

Column: 19 x 100 mm
System dwell volume: 9.75 mL
Column volume: 23.804 mL
System dwell volume (in column volumes): 0.41
Gradient offset: 0.66

Preparative Chromatography Conventional Injection

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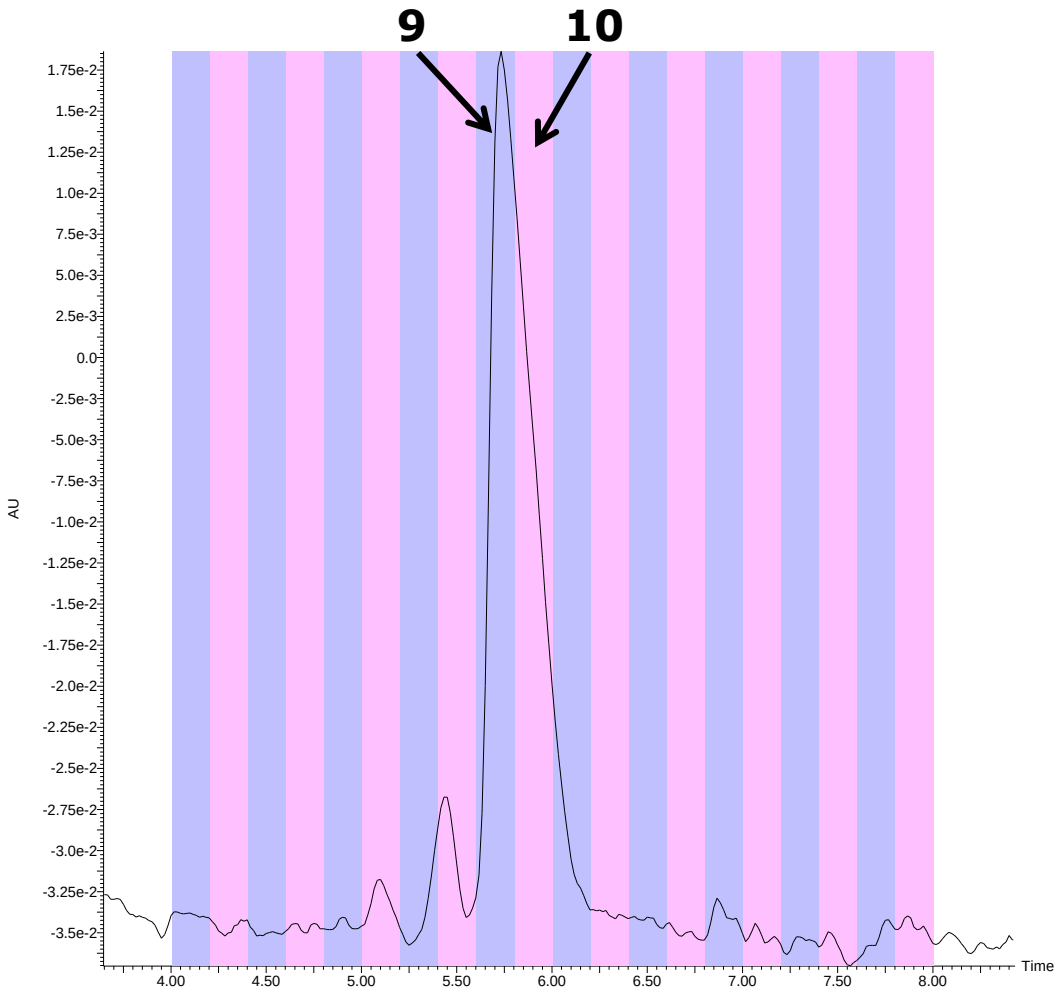

XBridge Peptide BEH C_{18} 5 µm OBD Prep, 19 x 100 mm, 2.4 mg

Fraction Analysis

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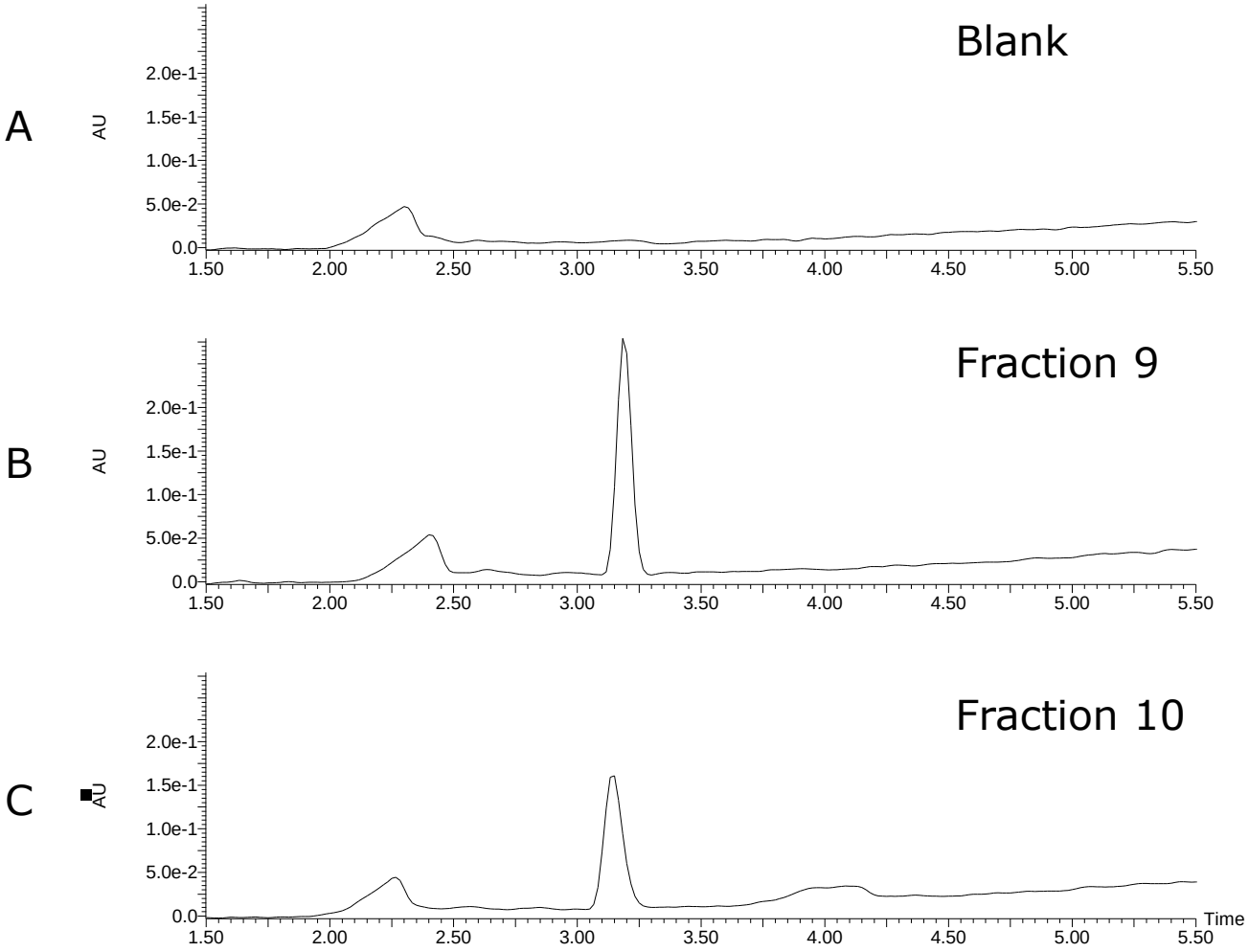

Fraction Analysis

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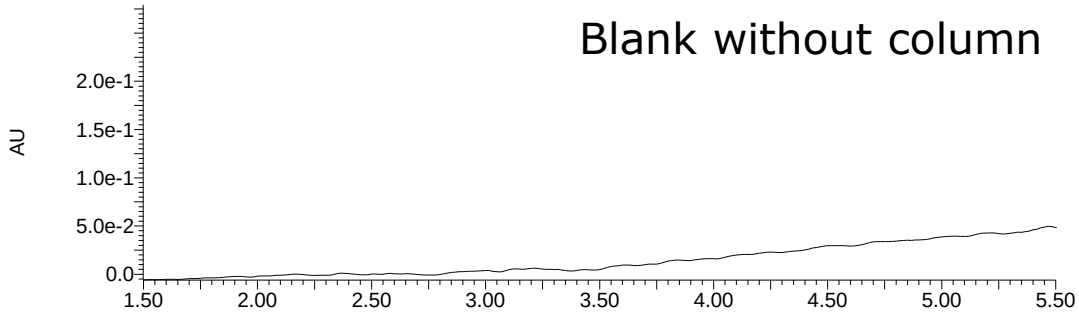
A



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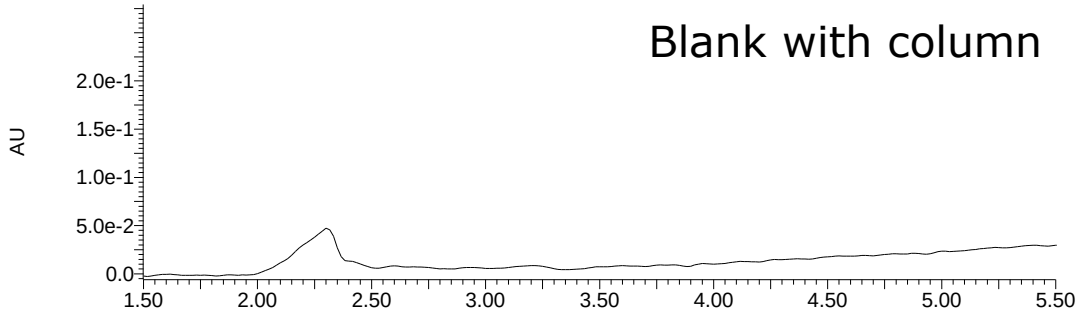

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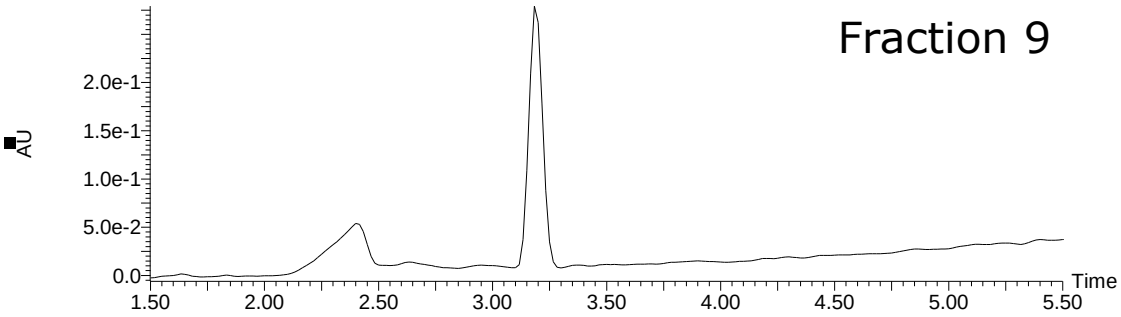

Comparison of Prep Gradient Methods

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Conventional Injection

Time	Flow	%A	%B
0.00	25	100	0
0.66	25	100	0
1.66	25	95	5
14.40	25	87	13
14.66	25	10	90
16.66	25	100	90
16.86	25	100	0
22.86	25	100	0

Gradient time: 14.40 - 1.66 = 12.74 min
Gradient volume: 12.74 min x 25 mL/min = 318.5 mL
Column volumes: 318.5 mL x 1 cv/23.804 mL = 13.38 cv
Gradient slope: 8%/13.38 cv = 0.6%/col vol

At-Column Dilution Injection

Time	Flow	%A	%B
0.00	23.75	100	0
2.26	23.75	100	0
3.26	23.75	100	0
16.00	23.75	92	8
16.26	23.75	15	85
18.26	23.75	15	85
18.46	23.75	100	0
24.46	23.75	100	0

Gradient time: 16.00 – 3.26 = 12.74 min
Gradient volume: 12.74 min x 25 mL/min = 318.5 mL
Column volumes: 318.5 mL x 1 cv/23.804 mL = 13.38 cv
Gradient slope: 8%/13.38 cv = 0.6%/col vol

Loading pump flow rate during gradient = 1.25 mL/min
1.25 mL/min = 5% of the total flow
Total gradient method flow rate = 25 mL/min

At-Column Dilution with Temperature Control

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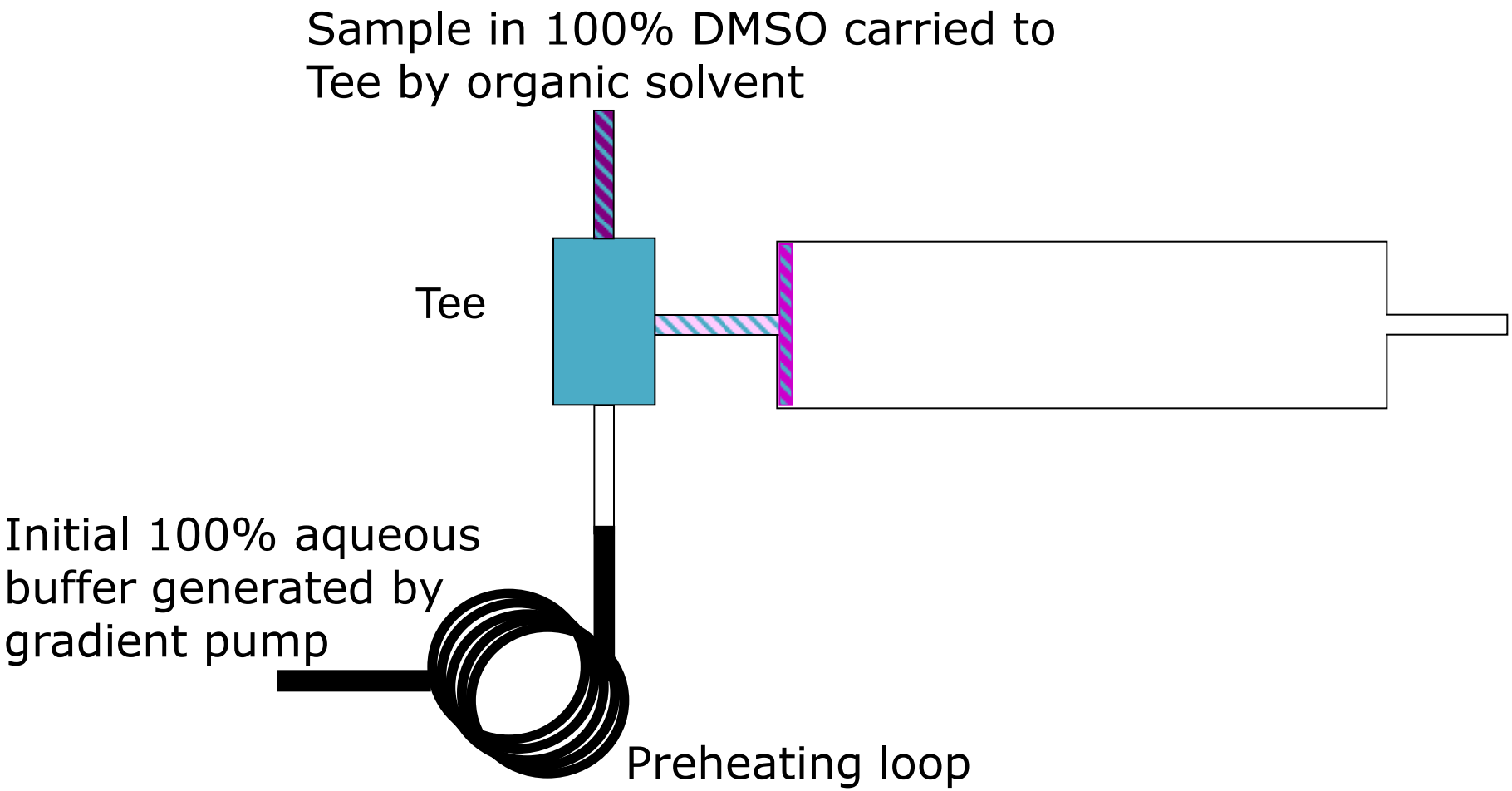

Loading Method Comparison

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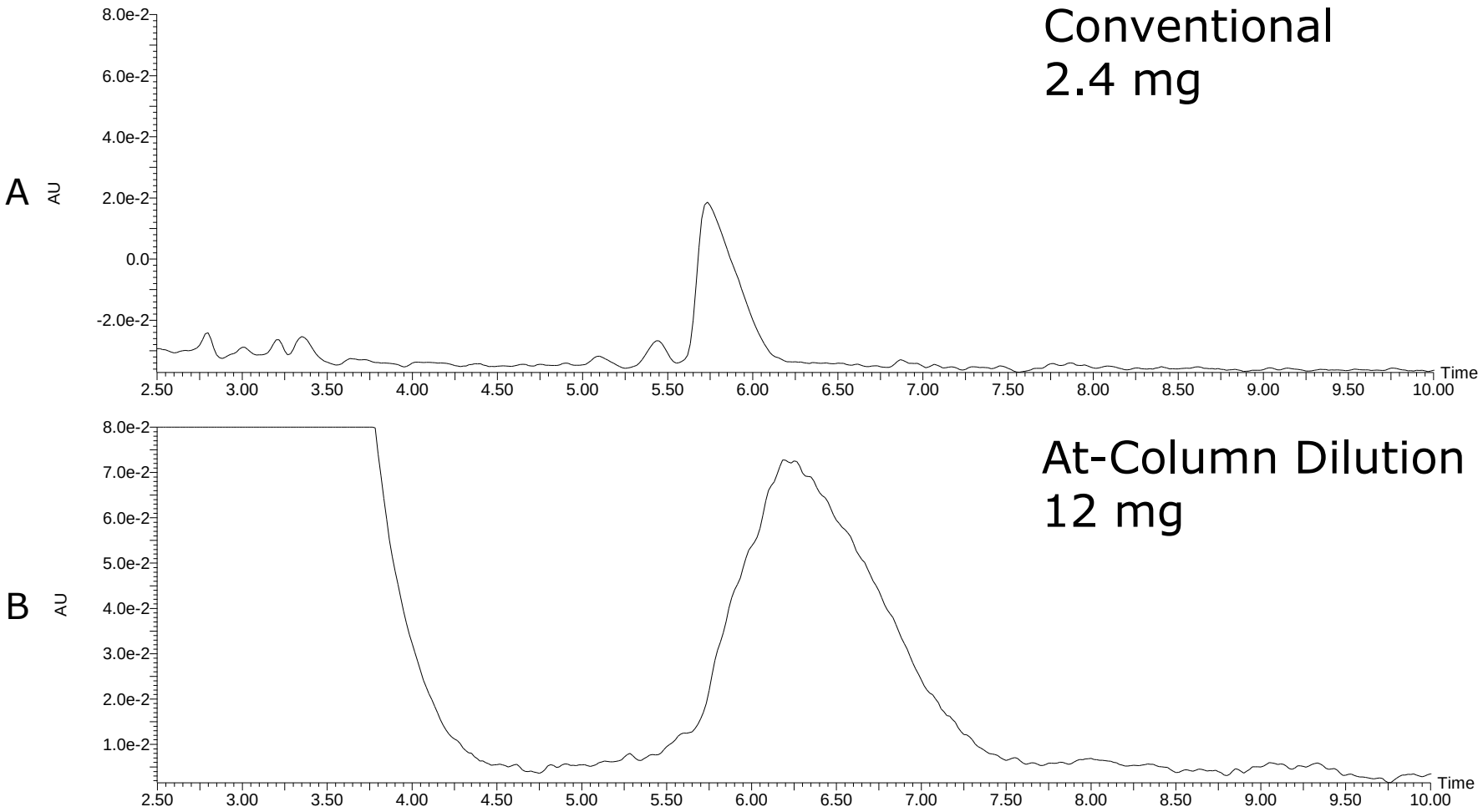

Preparative Chromatography At-Column Dilution

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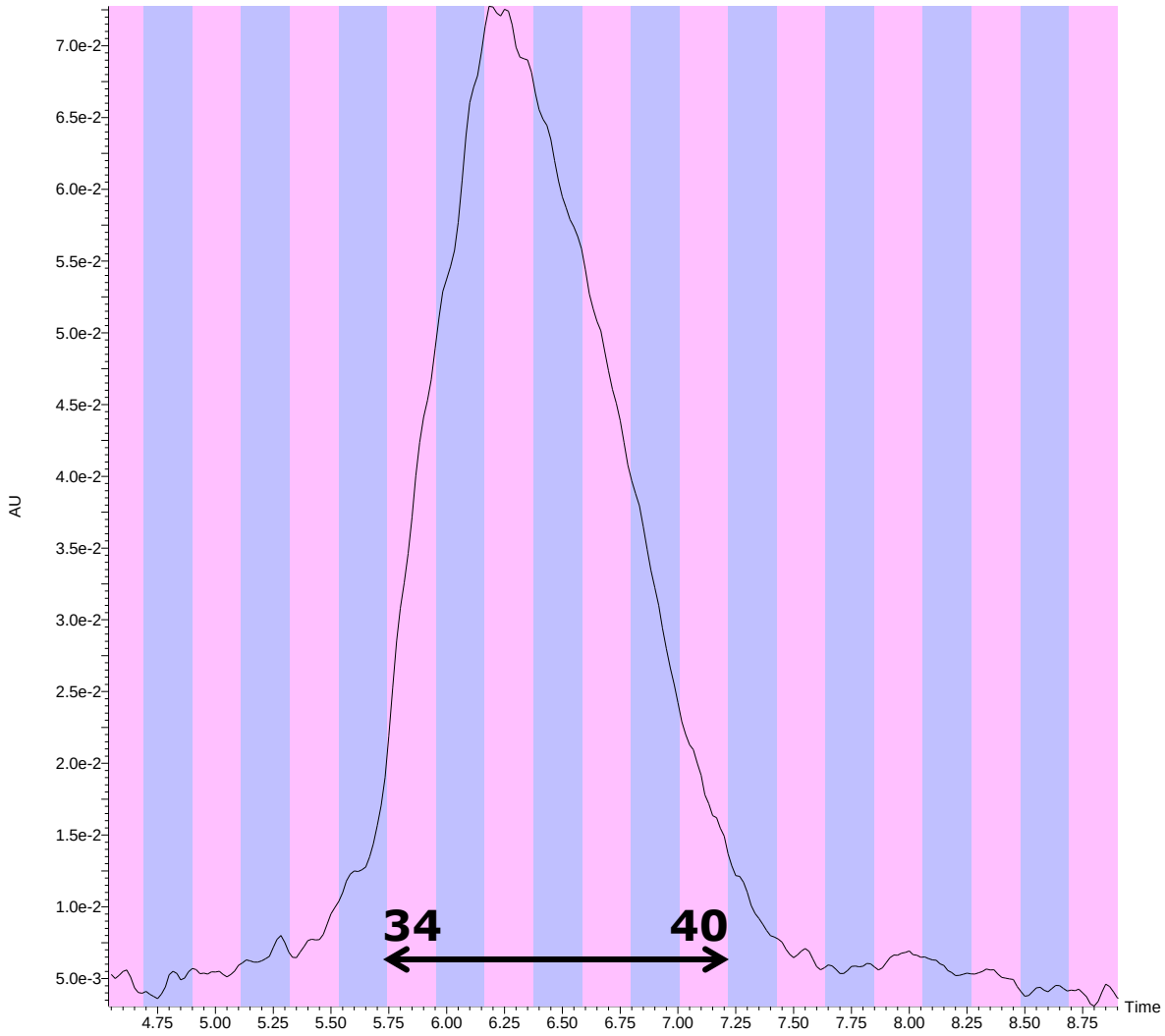

Fraction Analysis

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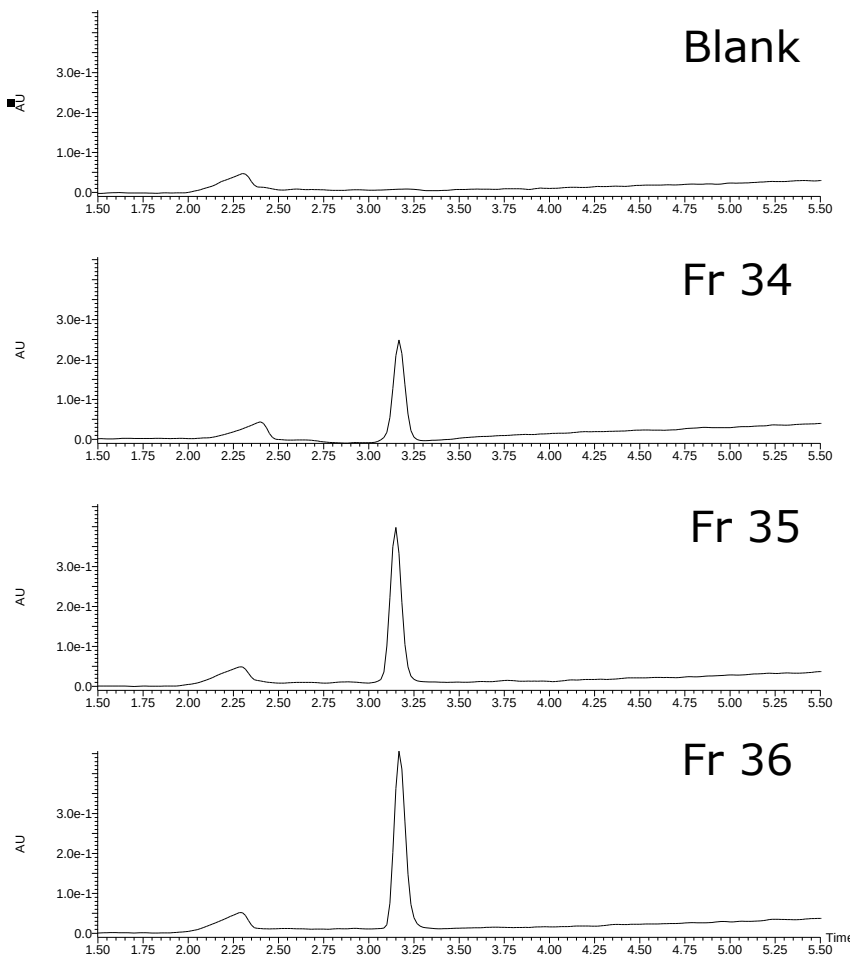

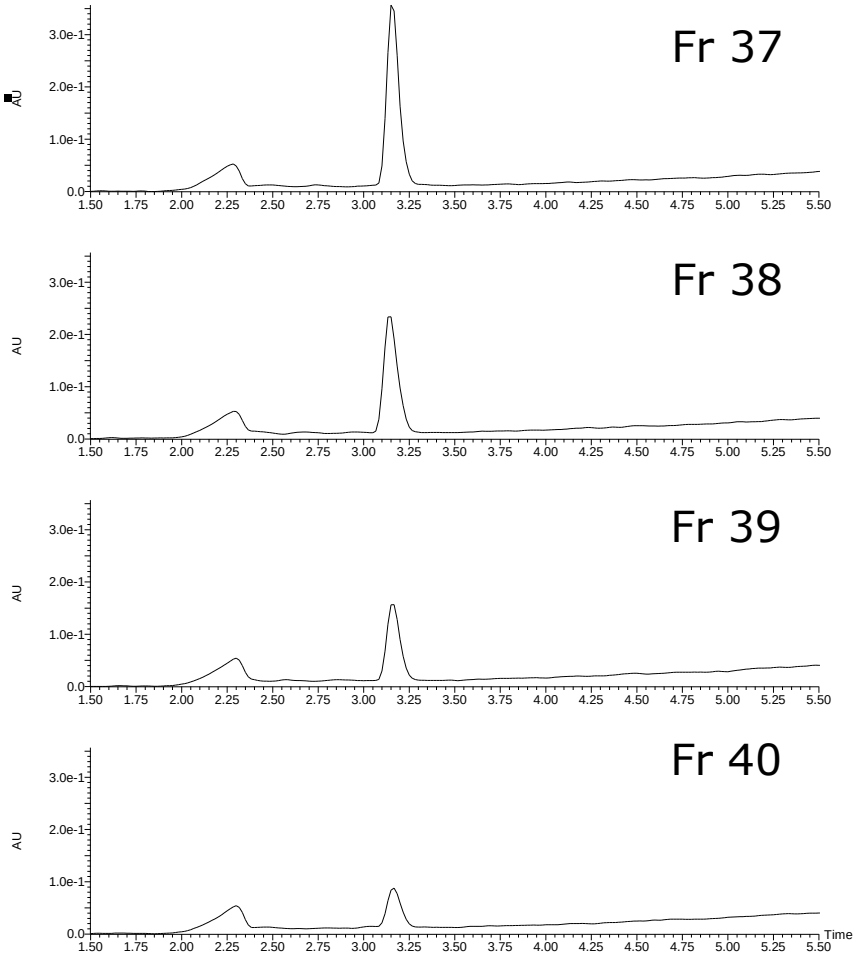

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- Background
- Techniques
  - Scaling a separation
  - Focusing the gradient
  - At-column dilution
  - Temperature control
- Practical example
- **Summary**

Summary

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- Focusing the gradient improves resolution and load
- Temperature control is a useful tool for improving peptide detection, peak shape, and resolution
- At-Column Dilution permits injection of large volumes of strong solvents, improves mass capacity and resolution, increases system ruggedness, and extends column life

Supplementary Information

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- Developing Focused Gradients for Isolation and Purification
  - Waters Technical Note, 720002955EN
- Effective Use of Temperature Control in Compound Isolation
  - Waters Technical Note, 720002954EN
- At-Column Dilution Application Notes
  - Waters Application Notes, 71500078010
- Prep 150 LC System: Considerations for Analytical to Preparative Scaling
  - Waters Application Note, 720005458EN
- Peptide Isolation Using the Prep 150 LC System
  - Waters Application Note, 720005455EN
- Topics in Liquid Chromatography Part 2, Optimum Bed Density [OBD™] Columns
  - Waters White Paper, 720001939EN