

Impact of Reversed-Phase Column Performance on Resolution and Selectivity for Well-Characterized Biopharmaceuticals

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ABSTRACT

Objective: To determine what column characteristics (i.e. pore size, pore volume, hydrophobicity and hydrolytic stability) affect the selectivity and the resolution of protein/peptide separations.

Methods: Tryptic digests of BSA and Cytochrome *c*, the Alberta Peptide mixture and Insulin, from various species, were used as probes to evaluate the column characteristics affecting the selectivity and resolution of protein/peptide separations. An increasing gradient of acetonitrile containing 1) 0.1% TFA and 2) 6 mM HCl in the mobile phase was used.

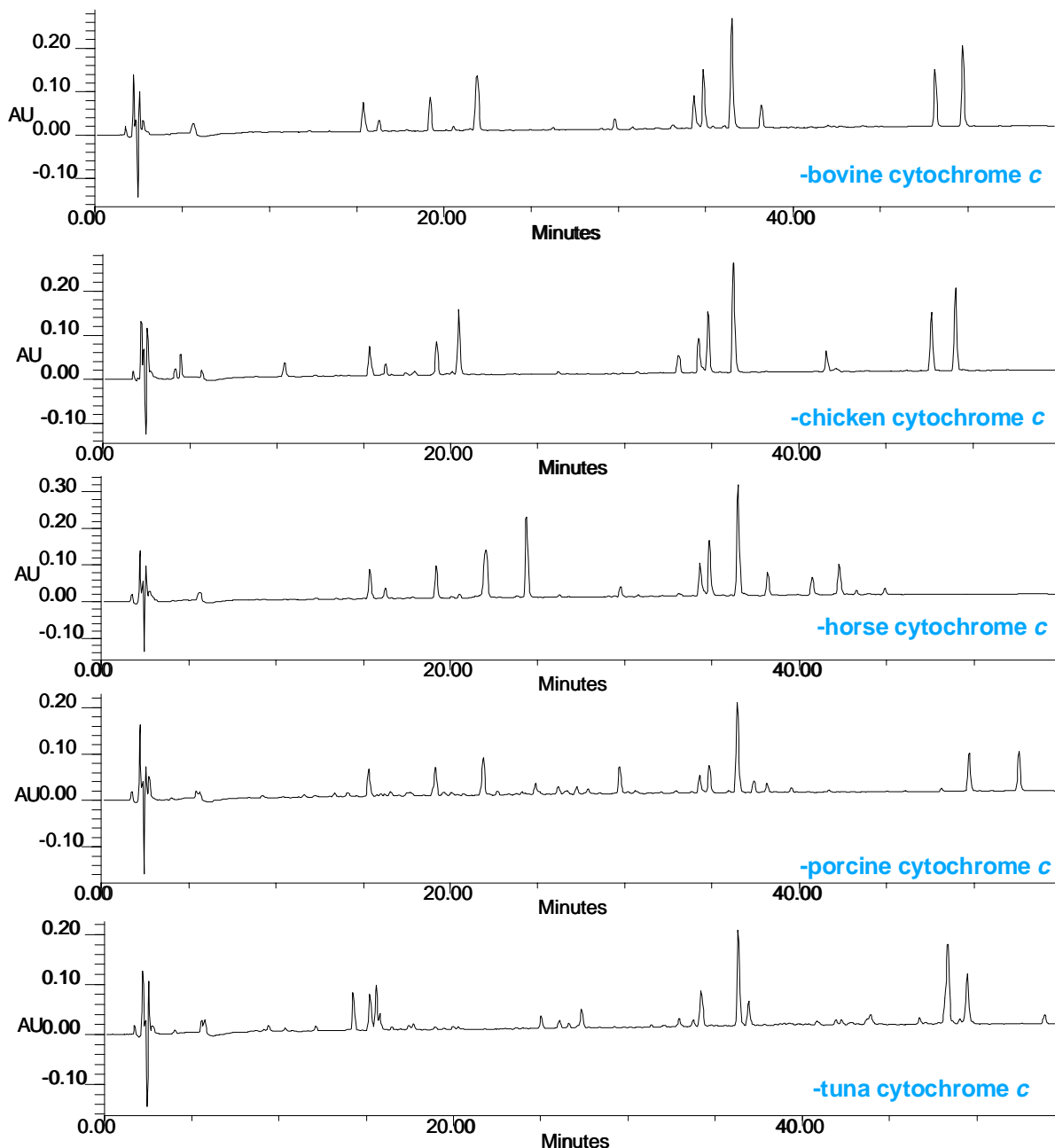
Results: Peptides and proteins must be purified for several reasons from a variety of mixtures. The selected method must separate these compounds over a wide range of sizes and chemical properties while, at the same time, resolve differences as small as a single amino acid. Thus there is a need to understand the effects of column characteristics and how these characteristics ultimately affect the selectivity and resolution of these types of compounds. Using protein and peptide probes, column characteristics of state-of-the-art, base deactivated 100 Å and 300 Å reversed-phase C₁₈ columns were evaluated. The studies revealed that notable differences in reversed-phase selectivity and resolution could be realized upon changing either the pore size of the packing material or the mobile phase composition.

Conclusion: Knowing the effect various column characteristics have on the selectivity and resolution protein/peptide separations allows us not only to determine which column characteristics are most important to us, but also allows us to be more educated in choosing the column which will better address our needs.

What Factors Influence RP-HPLC Separation...

- Gradient Slope
- Column Length
- Concentration of Modifier
- Temperature
- Pore Size of the Packing Material
- Flow Rate

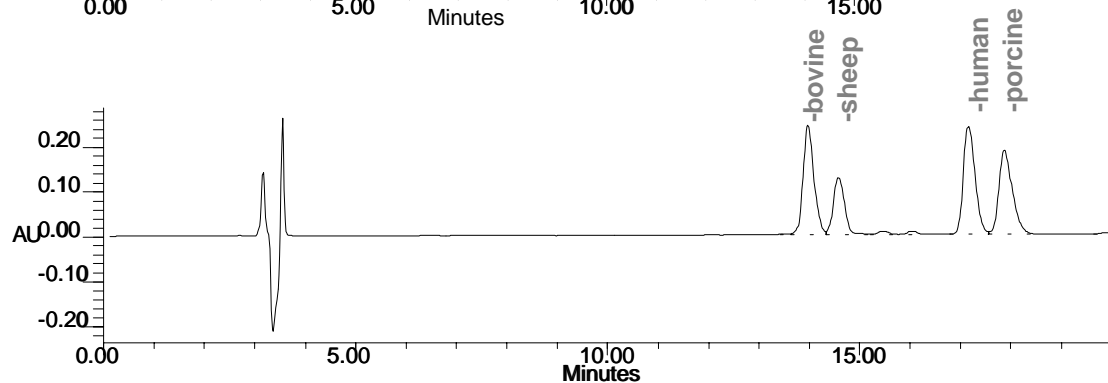
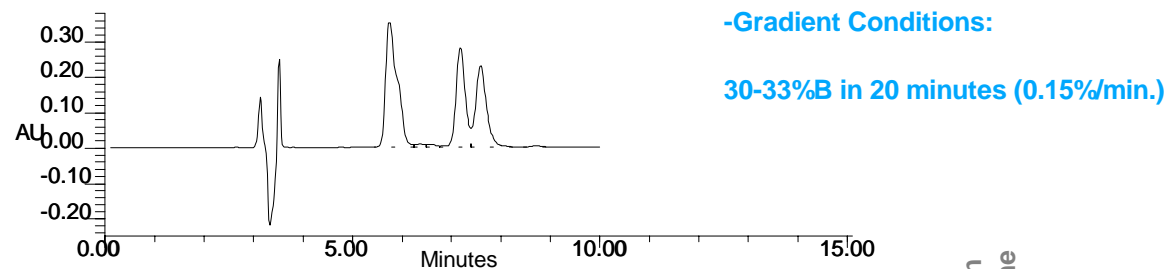
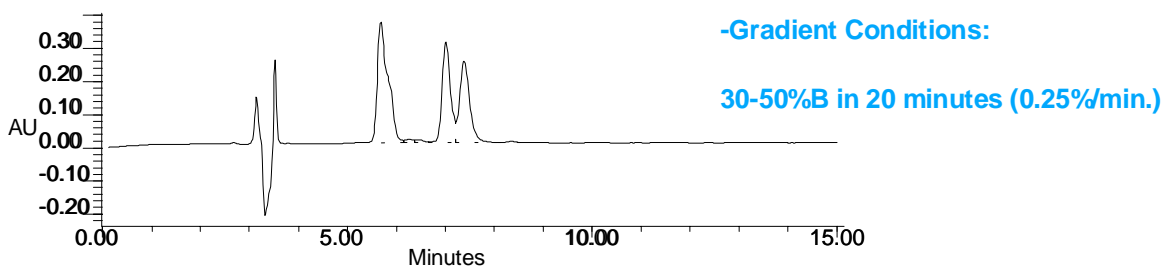
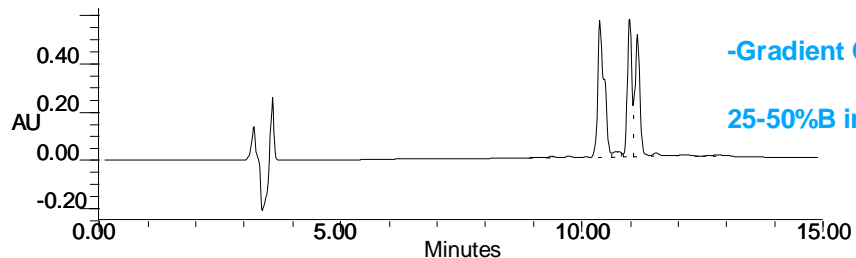
Tryptic Digests of Cytochrome c Variants



Conditions

- Column: Symmetry300™, 3.9x150mm
- Sample: Tryptic Digests of Cytochrome c Variants (bovine, chicken, horse, porcine and tuna from top to bottom)
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-50 min., 0-30%B
50-60 min., %B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm

Gradient Slope Effects on Resolution



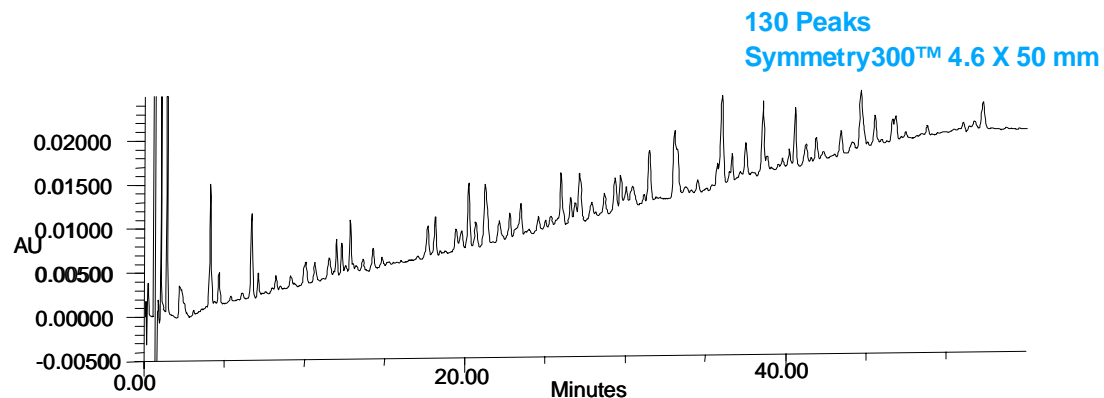
Conditions

- Column: Symmetry300™, 4.6x250mm
- Sample: Insulin Whole Protein Variants (bovine, sheep, human and porcine)
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Flow rate: 1.0 mL/min.
- Temperature: 35°C
- Detection: 214 nm

Column Length Effects on Resolution at a Constant Gradient

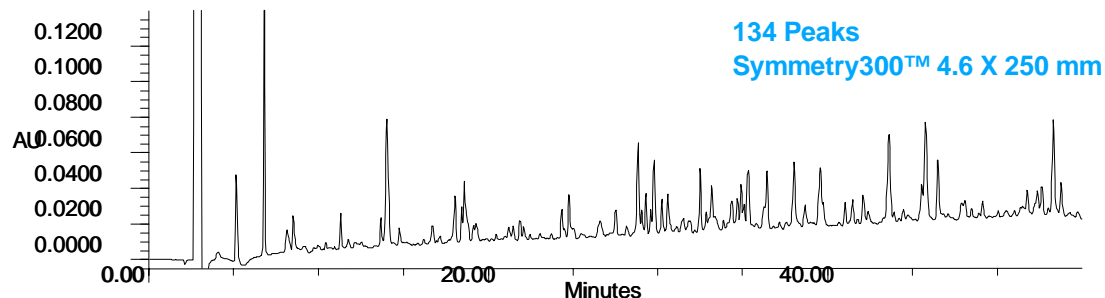
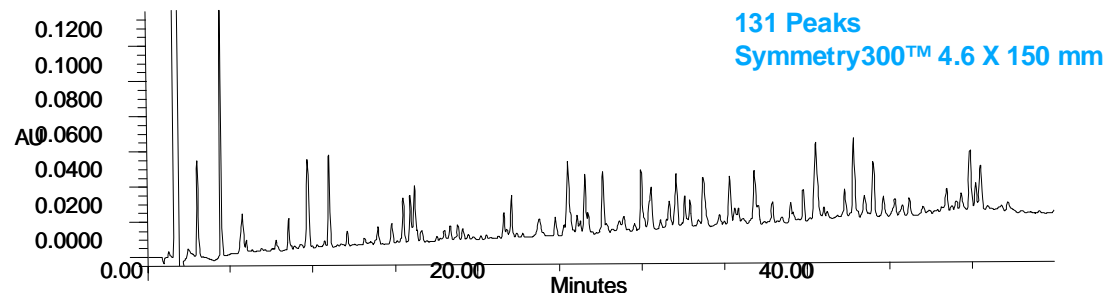
- Column length plays a small role in the separation of polypeptides when the gradient conditions are kept constant. This is due to an effective expansion of the gradient as the column length is shortened. The gradient expansion is a result of an increase in the number of column volumes the peptide fragment experiences.
- However, although the number of peptide fragment peaks remain relatively the same when column length is changed as the gradient is held constant, the resolution between certain pairs changes.

Column Length Effects on Resolution at a Constant Gradient

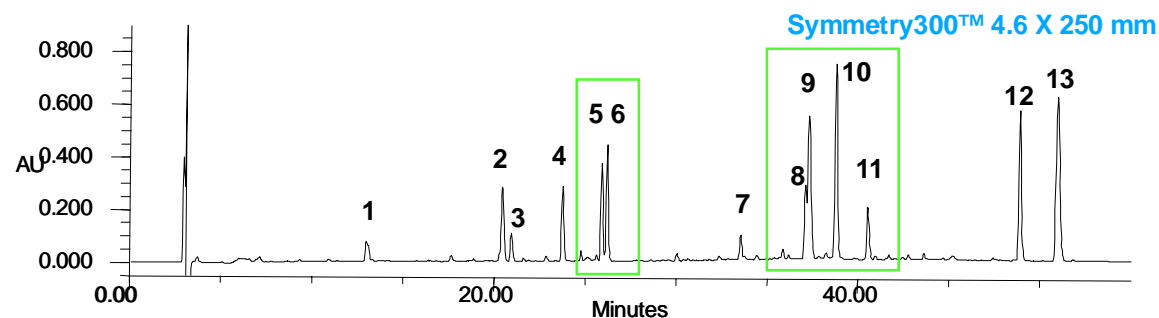
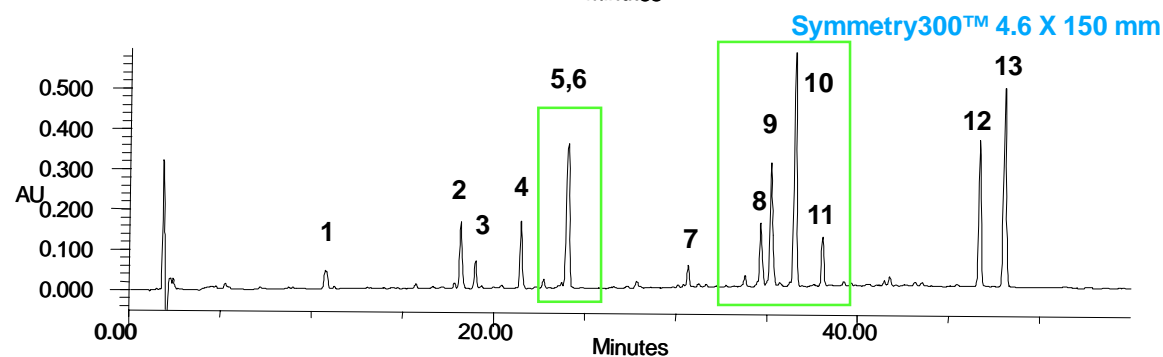
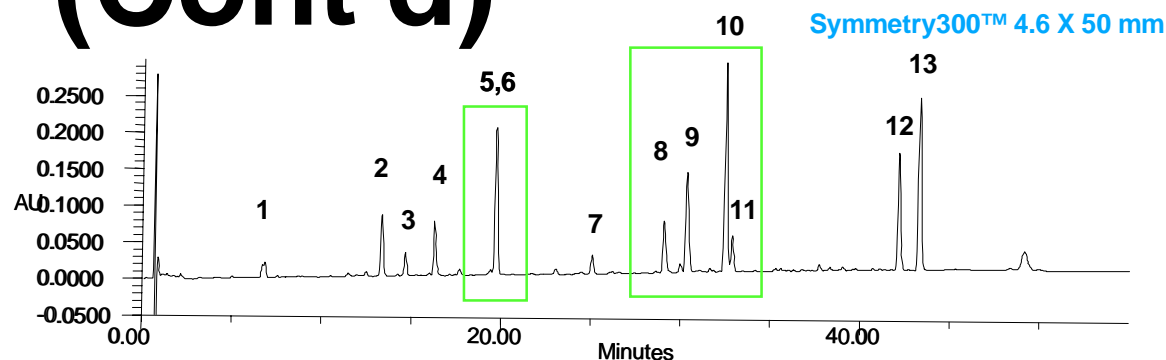


Conditions

- Sample: Tryptic Digests of Bovine Serum Albumin
- Injection: 20µL (7 µL for 4.6 X 50mm)
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm



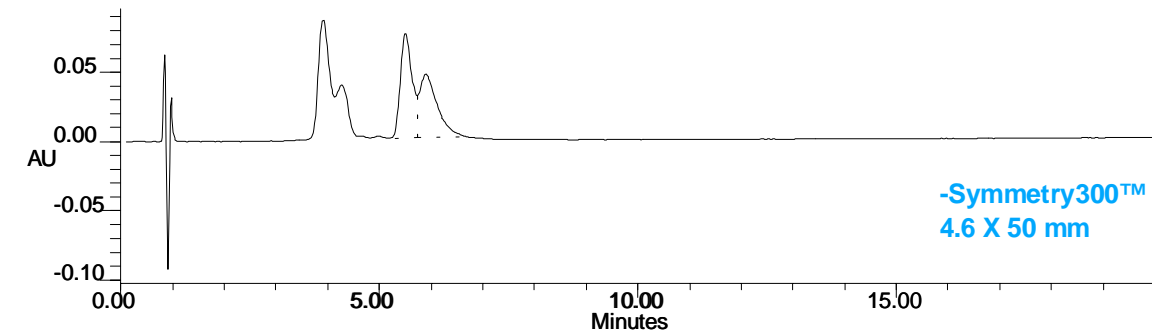
Column Length Effects on Resolution at a Constant Gradient (Cont'd)



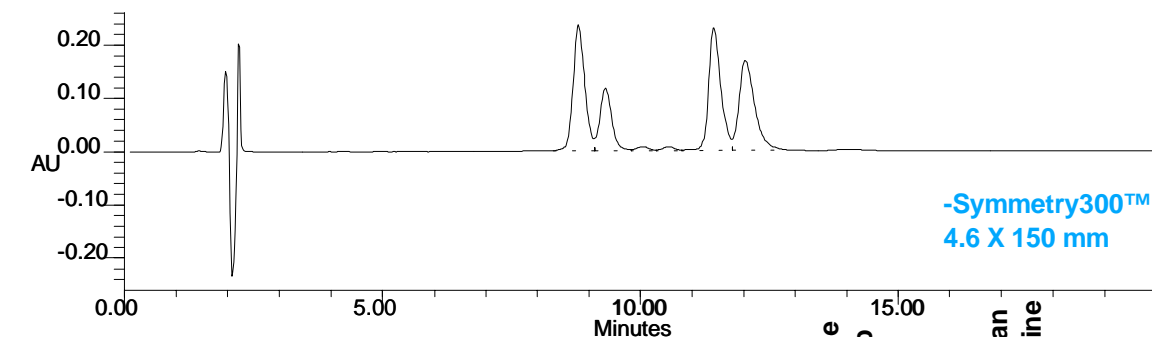
Conditions

- Sample: Tryptic Digests of Bovine Cytochrome c
- Injection: 20µL (7 µL for 4.6 X 50 mm)
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm

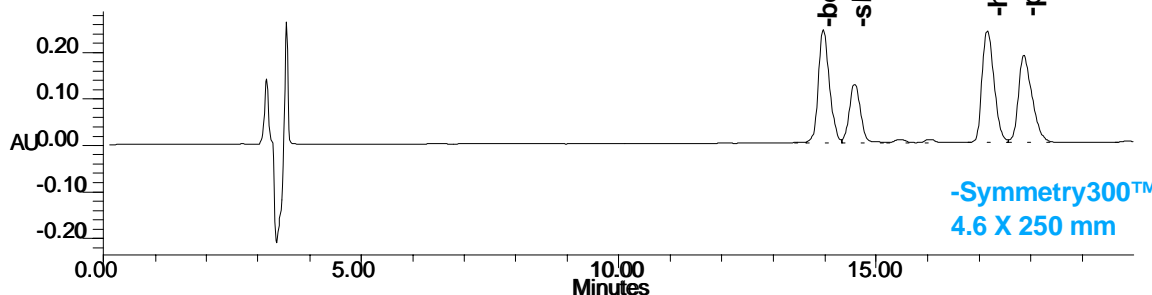
Column Length Effects on the Resolution of Small Proteins at a Constant Gradient



-Symmetry300™
4.6 X 50 mm



-Symmetry300™
4.6 X 150 mm



-Symmetry300™
4.6 X 250 mm

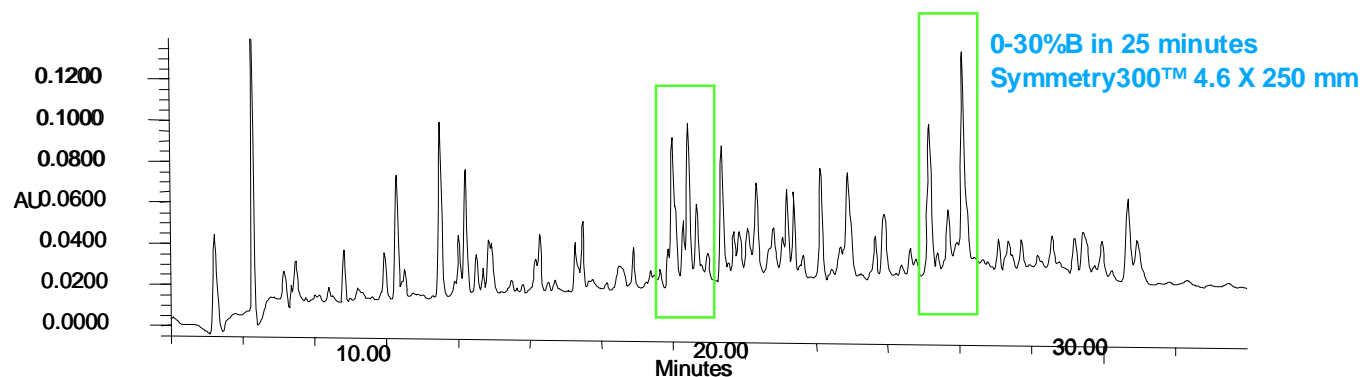
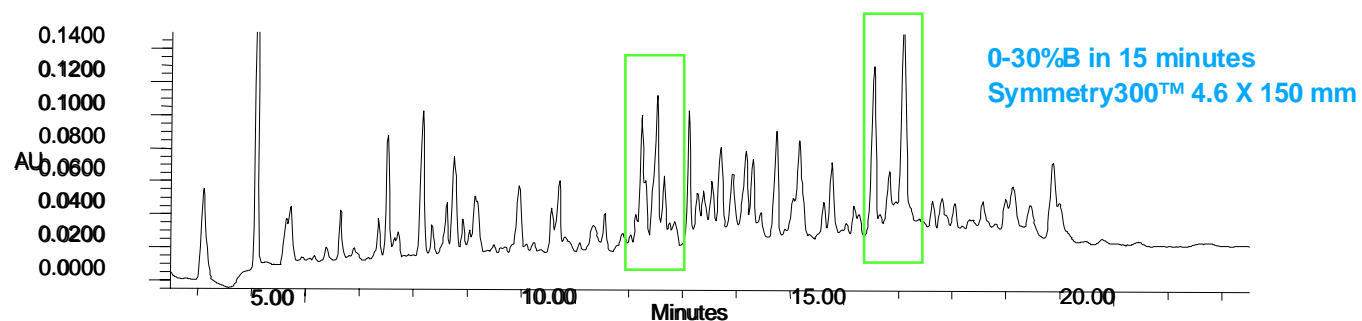
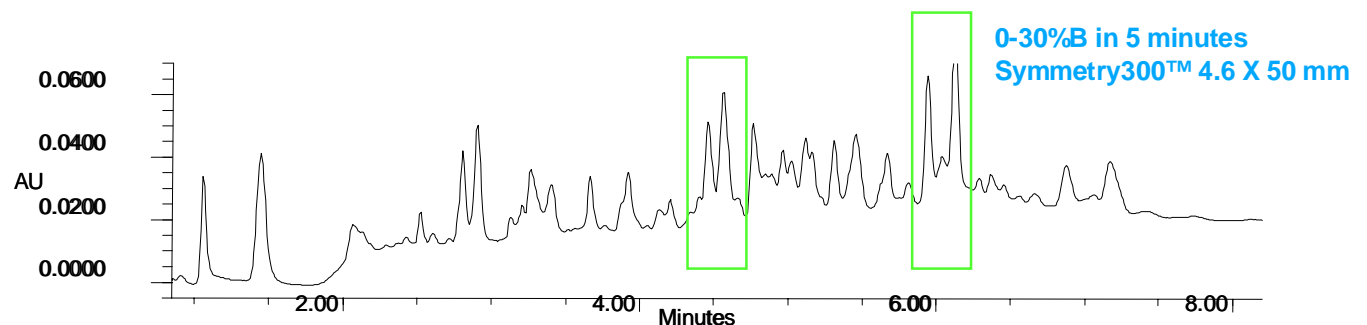
Conditions

- Sample: Insulin Whole Protein Variants (bovine, sheep, human and porcine)
- Injection: 20μL (7μL for 4.6x50mm)
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 27-31%B in 20 minutes
- Flow rate: 1.0 mL/min.
- Temperature: 35°C
- Detection: 214 nm

Variation in Column Lengths at Equal Ratio of Gradient Volumes to Column Volumes

- Resolution increases when the column length is increased in the same ratio as the gradient volume due to an increase in the number of plates.
- Selectivity remains the same as the column length and gradient volume changes.

Variation in Column Lengths at Equal Ratio of Gradient Volumes to Column Volumes



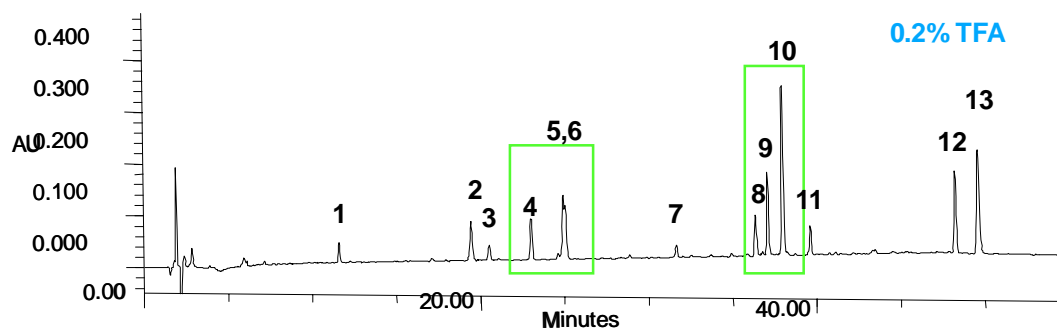
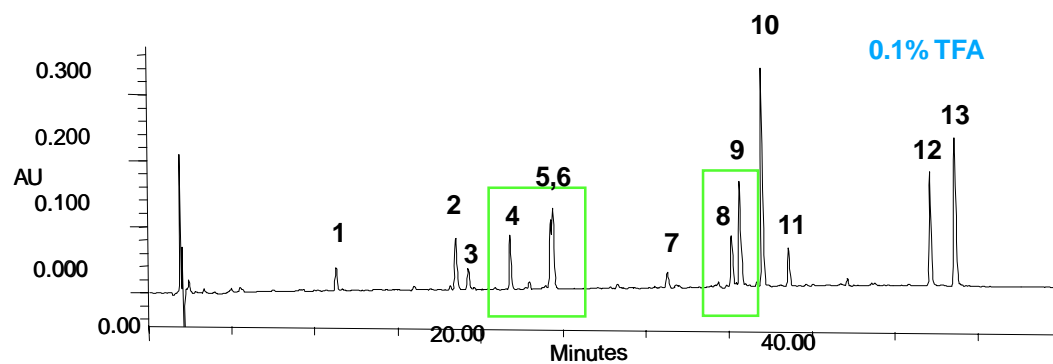
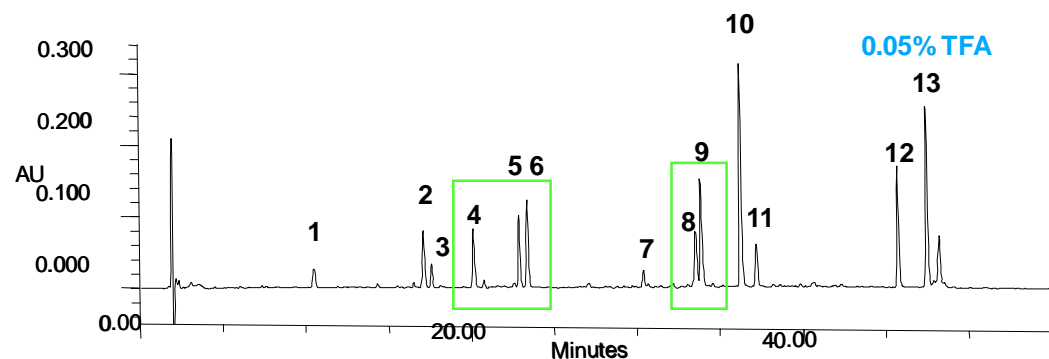
Conditions

- Sample: Tryptic Digests of Bovine Serum Albumin
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm

Effects of TFA Concentration on Resolution

- TFA is normally used at a concentration of 0.1%.
- The concentration of TFA can be optimized or adjusted to obtain the best separation between critical pairs.
- As shown, peaks 5,6 resolve upon lowering the TFA concentration.
- However, the resolution between peaks 2,3 and 8,9 is reduced upon lowering the TFA concentration

Effects of TFA Concentration on Resolution



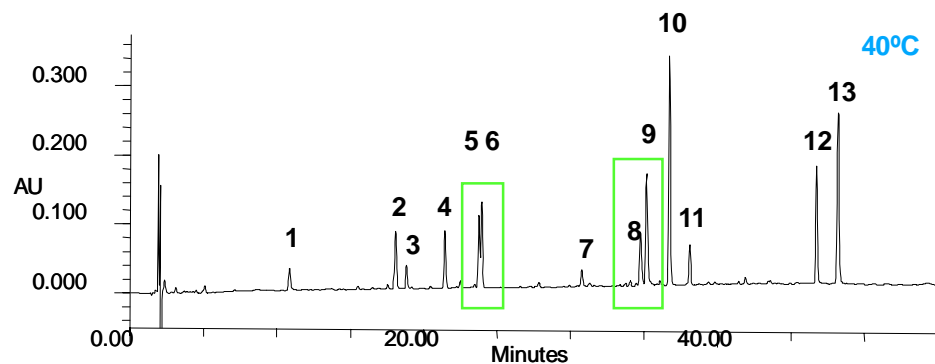
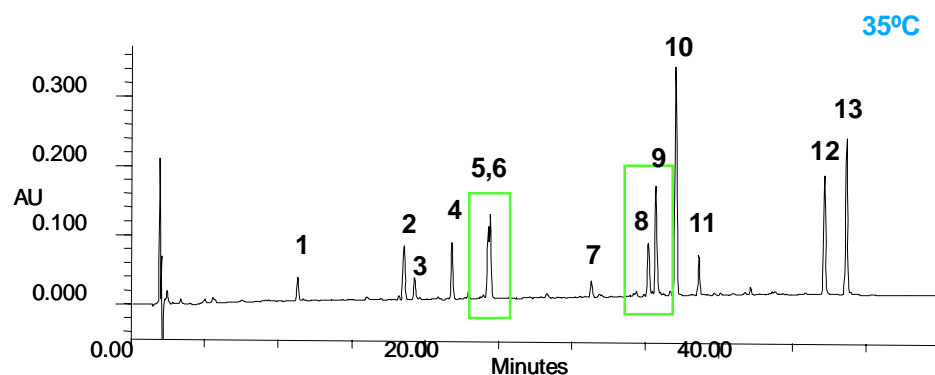
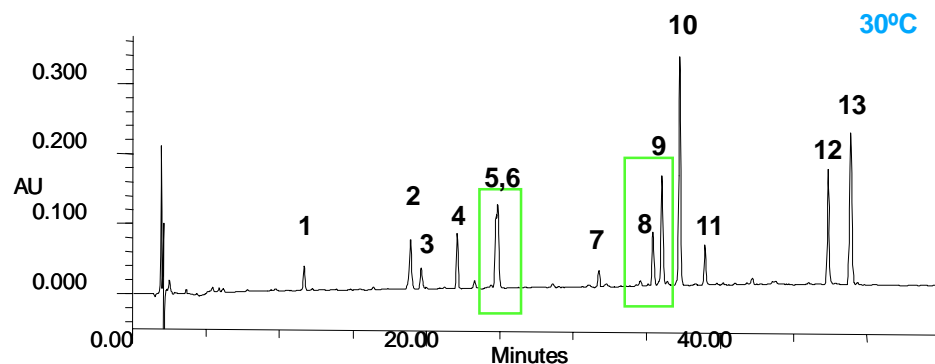
Conditions

- Column: Symmetry300™, 3.9x150mm
- Sample: Tryptic Digests of Bovine Cytochrome c
- Injection: 20µL
- Mobile Phase:
Solvent A: water
Solvent B: acetonitrile
- Gradient: 0-45 min., 0-30%B
45-55 min., %B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm

Temperature Effects on Resolution

- Temperature can have similar effects to TFA concentration. In the tryptic map of bovine cytochrome *c*, the peptide fragments elute earlier as the temperature increases from 30 to 40°C.
- Peptide fragments (5,6 and 8,9) move relative to each other increasing and decreasing the resolution between certain pairs.

Temperature Effects on Resolution

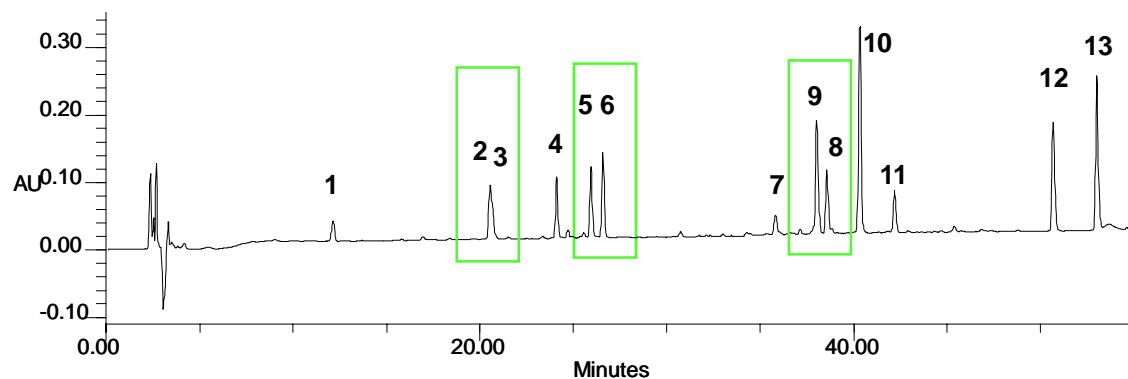


Conditions

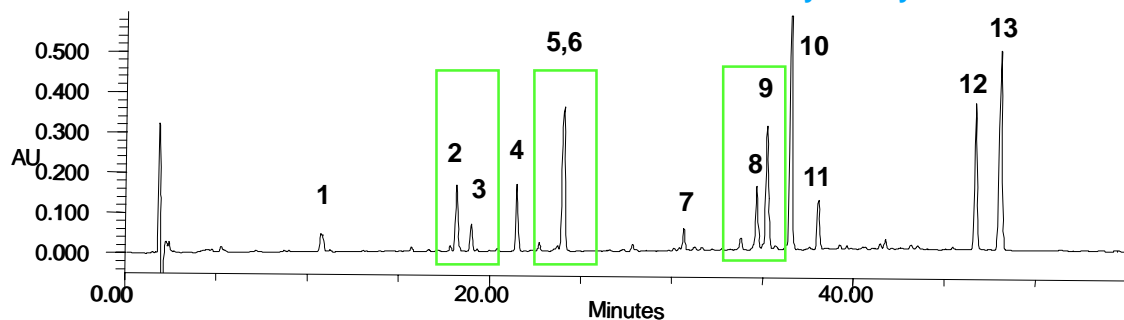
- Column: Symmetry300™, 3.9x150mm
- Sample: Tryptic Digests of Bovine Cytochrome C
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
45-55 min., %B
- Flow rate: 0.75 mL/min.
- Detection: 214 nm

Pore Size Effects on Resolution

Symmetry® C18, 5μ,
4.6 X 150 mm



Symmetry300™ 4.6 X 150 mm



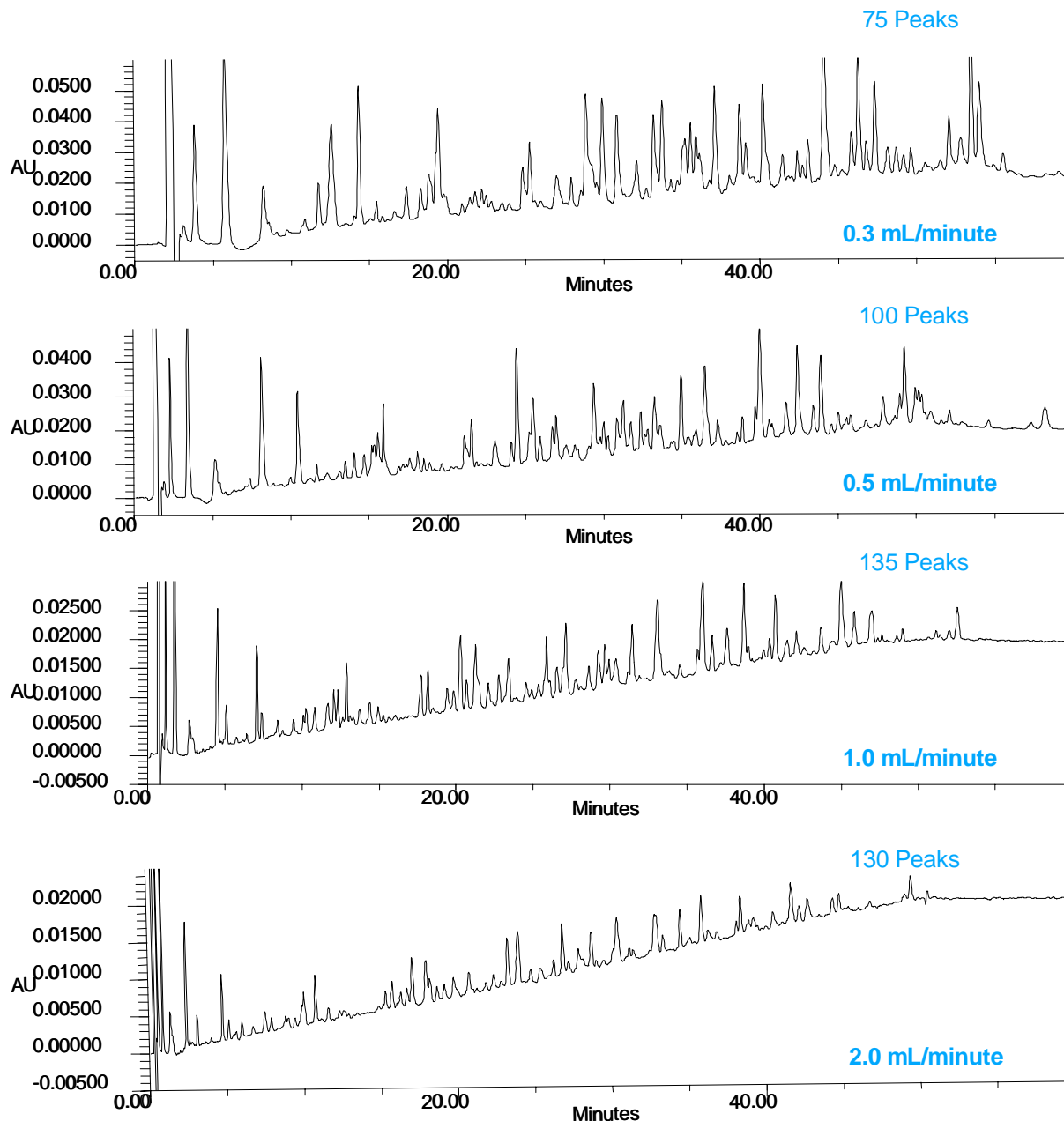
Conditions

- Column: Symmetry® C18, 5μ, 4.6 X 150 mm
- Sample: Tryptic Digests of Cytochrome c (bovine)
- Injection: 20 μL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-50 min., 0-30%B
50-60 min., %B
- Temperature: 35°C
- Flow Rate: 0.75 mL/min.
- Detection: 214 nm

Flow Rate Effects on Resolution at a Constant Gradient

- Under constant gradient conditions, flow rate affects resolution and position of peaks in the chromatogram.
- As the flow rate is increased the volume of the gradient is increased. Effectively, the gradient is expanded resulting in an increase in the number of plates which yields an increase in resolution.
- The increase in resolution occurs until the mass transport is deleteriously effected causing a decrease in resolution.

Flow Rate Effects on Resolution at a Constant Gradient



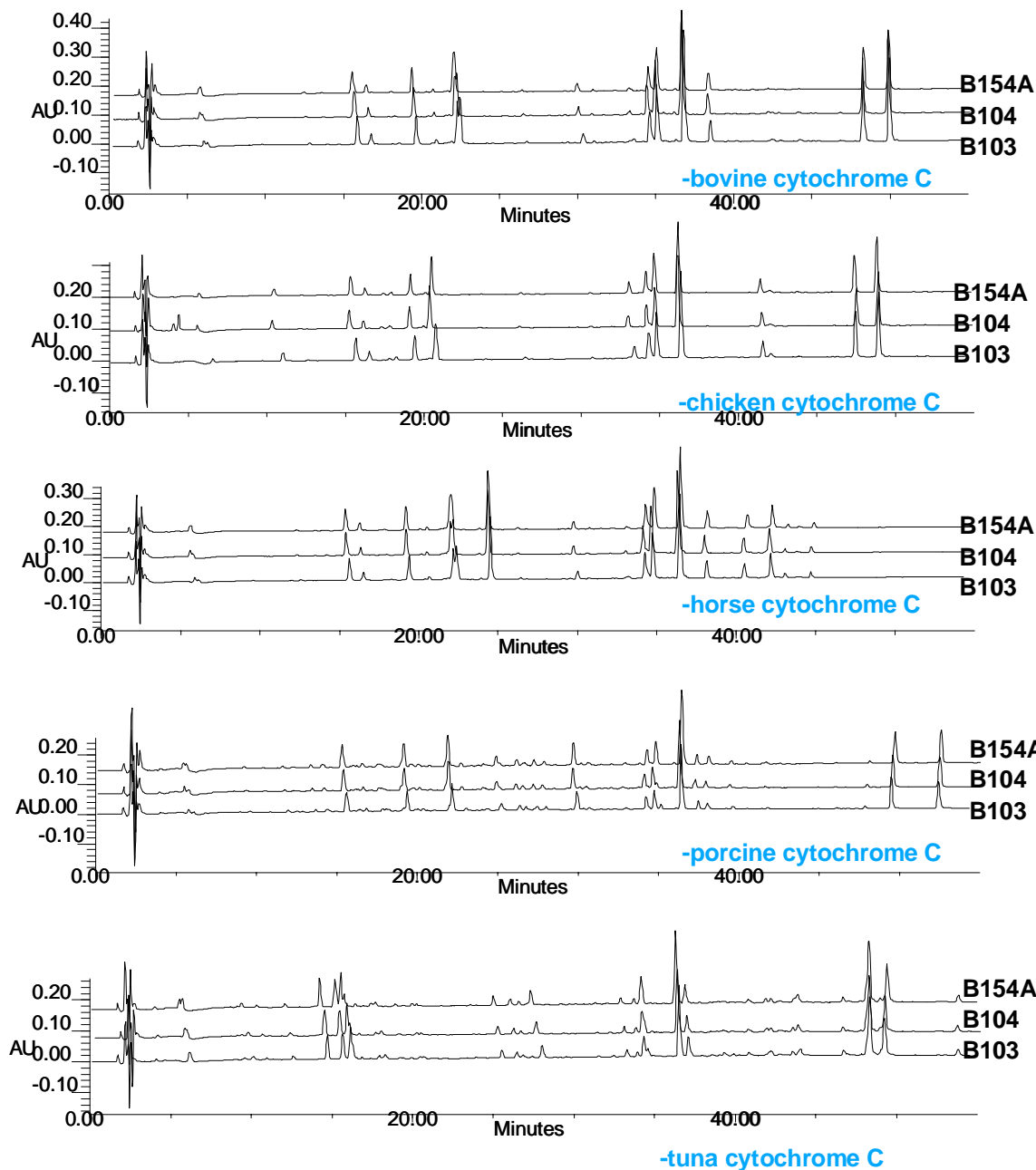
Conditions

- Column: Symmetry300™, 3.9x50mm
- Sample: Tryptic Digests of Bovine Serum Albumin
- Injection: 7 µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
45-55 min., %B
- Temperature: 35°C
- Detection: 214 nm

Batch-to-Batch Reproducibility

- The most important aspect of developing a superior RP-HPLC separation is the development of a reproducible separation.
- Symmetry300™ is part of the Symmetry® family and is held to the same high standards of our other Symmetry® products.
- And although the reproducibility obtained using a standard system was excellent....

Batch-to-Batch Reproducibility



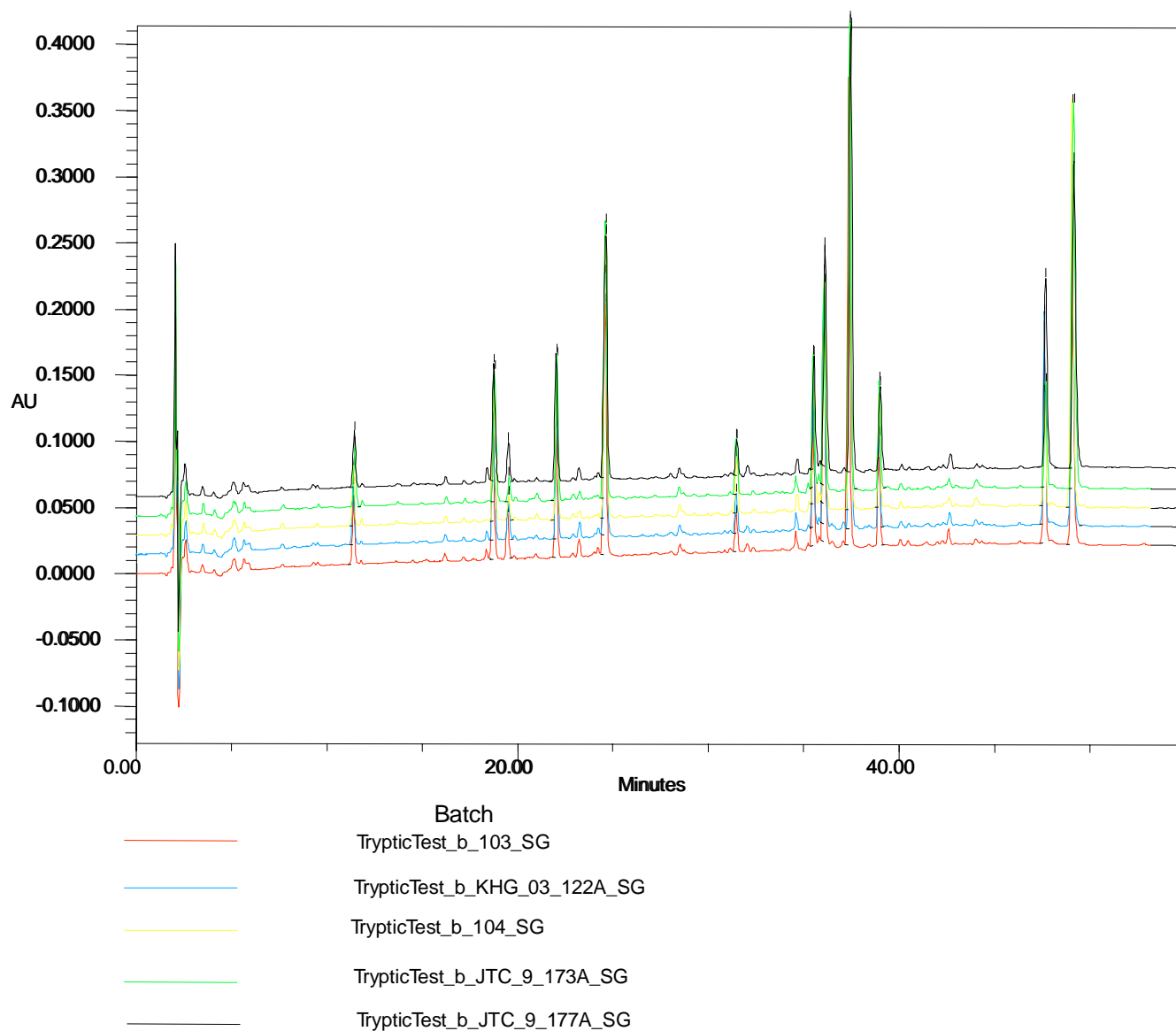
Conditions

- Column: Symmetry300™, 3.9x150mm
- Sample: Tryptic Digests of Cytochrome C Variants (bovine, chicken, horse, porcine and tuna)
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-50 min., 0-30%B
50-60 min., %B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm
- Instrument: Traditional HPLC system

Batch-to-Batch Reproducibility

**...look at what you can achieve
when the system "noise" is
removed...**

Batch-to-Batch Reproducibility



Conditions

- Column: Symmetry300™, 3.9x150mm
- Sample: Tryptic Digests of Cytochrome C (bovine)
- Injection: 20µL
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
45-55 min., %B
- Flow rate: 0.75 mL/min.
- Temperature: 35°C
- Detection: 214 nm

- Instrument: Waters Alliance™ 2690 HPLC

Conclusion

- There are several factors to assess when developing/optimizing a RP-HPLC method for peptides.
 - Consider using column length and gradient slope as the major tools for optimizing your separation. Start with a short column.
 - Once the method has been basically optimized, fine tune using temperature, modifier type, and modifier concentration.
- Batch-to-batch reproducibility of the column is of the utmost importance to consider when developing a validated/transferable method. Symmetry300™ has unsurpassed batch-to-batch reproducibility.

Conclusion (Continued)

- Symmetry300™ is the only column in the industry where the batch-to-batch reproducibility is monitored by not only a small molecule test but also a protein tryptic digest (shown above).
- Using an HPLC with a high level of performance allows the "system noise" to be removed, thereby refocusing the attention on the column performance and optimizing the separation.
- To obtain superior results, one must consider system effects such as: pump efficiency and delay volume. The Waters Alliance™ 2690 has a delay volume of only 600 µL and delivers a seamless, reproducible gradient every time.