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Critical Parameters Affecting the Reproducibility of Reversed-Phase Columns for Peptide Separations

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Abstract

Column-to-column variability has been referred to as the "Achilles Heel" in the development of rugged and reproducible HPLC methods for protein pharmaceuticals. Numerous column choices are available today to the separation chemist, with each manufacturer claiming to have the most reproducible product. How does one make the best choice? The critical factors affecting column-to-column reproducibility for small molecule separations have been the topic of numerous studies. These studies are typically performed using small molecule acidic, basic and neutral probes at various mobile phase pH's. However, very little information has been reported correlating the data generated using these small molecular probes to column-to-column reproducibility for peptide and protein separations.

We will present the results of our recent research efforts where we have successfully employed small molecule probes to aid in the design of a reproducible reversed-phase column for the separation of biopharmaceuticals. The discussions will center on the impact of critical parameters such as silica surface area and C_{18} surface concentration on column-to-column reproducibility using peptide maps as probes.

Outline

- Identify critical parameters affecting the retention behavior of small molecules
- Identify critical parameters affecting the retention behavior of biomolecules
- Conclusions

Definition of Chromatographic Parameters

- t_r = retention time
- t_0 = void time
- k = retention factor = $(t_r - t_0) / t_0$
- α = selectivity = k_2 / k_1
- separation = $t_r(2) - t_r(1)$

Critical Parameters of Chromatographic Packings

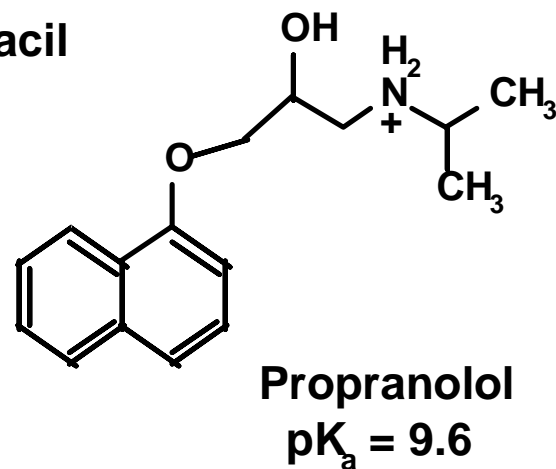
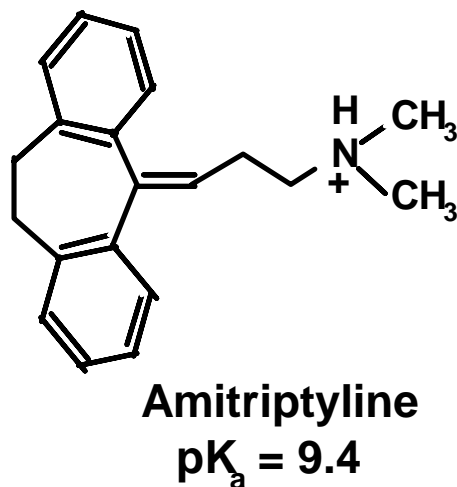
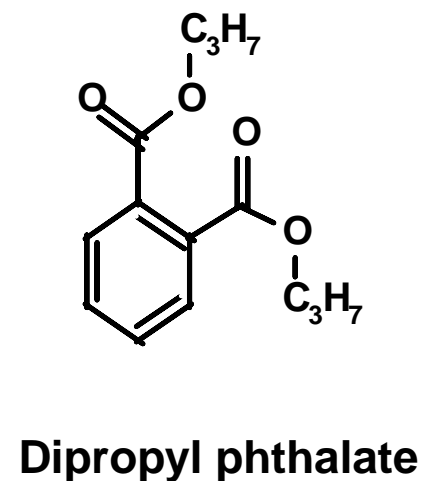
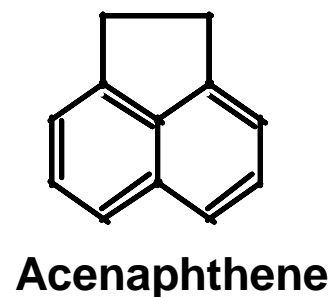
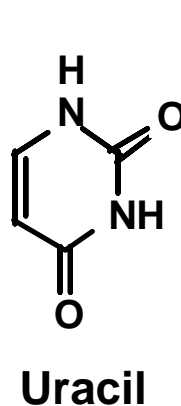
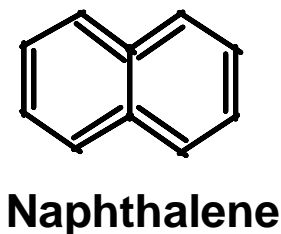
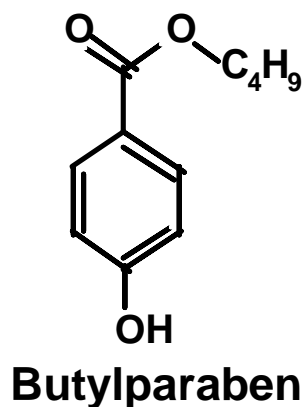
- Silica Specific Surface Area = area of surface (m^2) per gram of silica
 - ▶ determines the amount of chromatographic surface in a column, and hence **retention**
- C_{18} Surface Concentration = micromoles of C_{18} chains per m^2 of silica surface
 - ▶ determines the hydrophobic/silanophilic balance, and hence **selectivity**

Small Molecule Separations

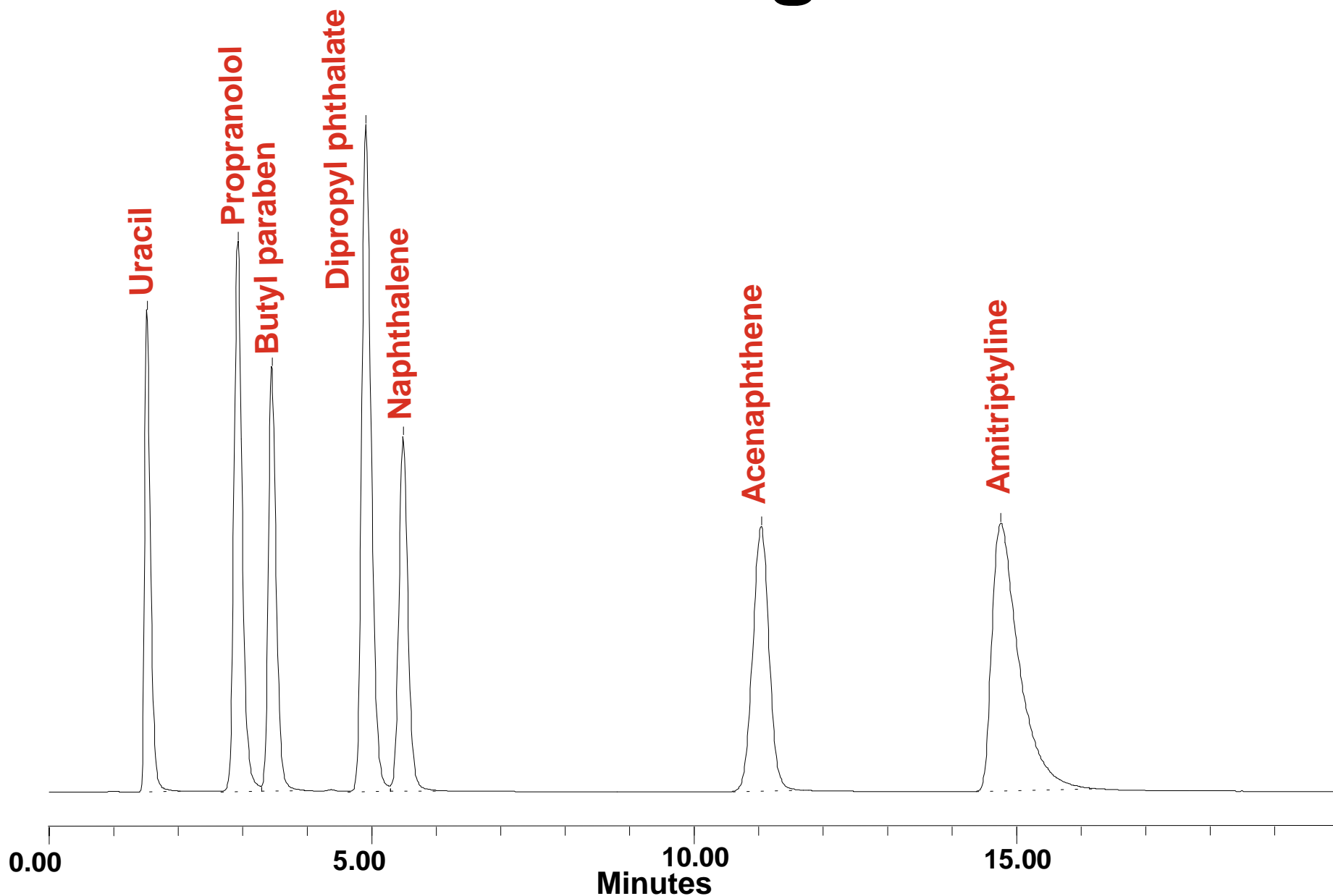
HPLC Conditions

- Instrument: Waters 600 Pump, 486 UV Detector, 717 Autosampler, Millennium Data System
- Column: Symmetry300™ C₁₈ 5 μm
3.9 x 150 mm
- Mobile Phase: 35 % 20 mM KH₂PO₄, pH 7.0
65 % Methanol
- Flow Rate: 1.0 mL/min
- Temperature: 23°C
- Detection: Absorbance at 254 nm

Symmetry® HPLC Column Small Molecule Quality Control Test



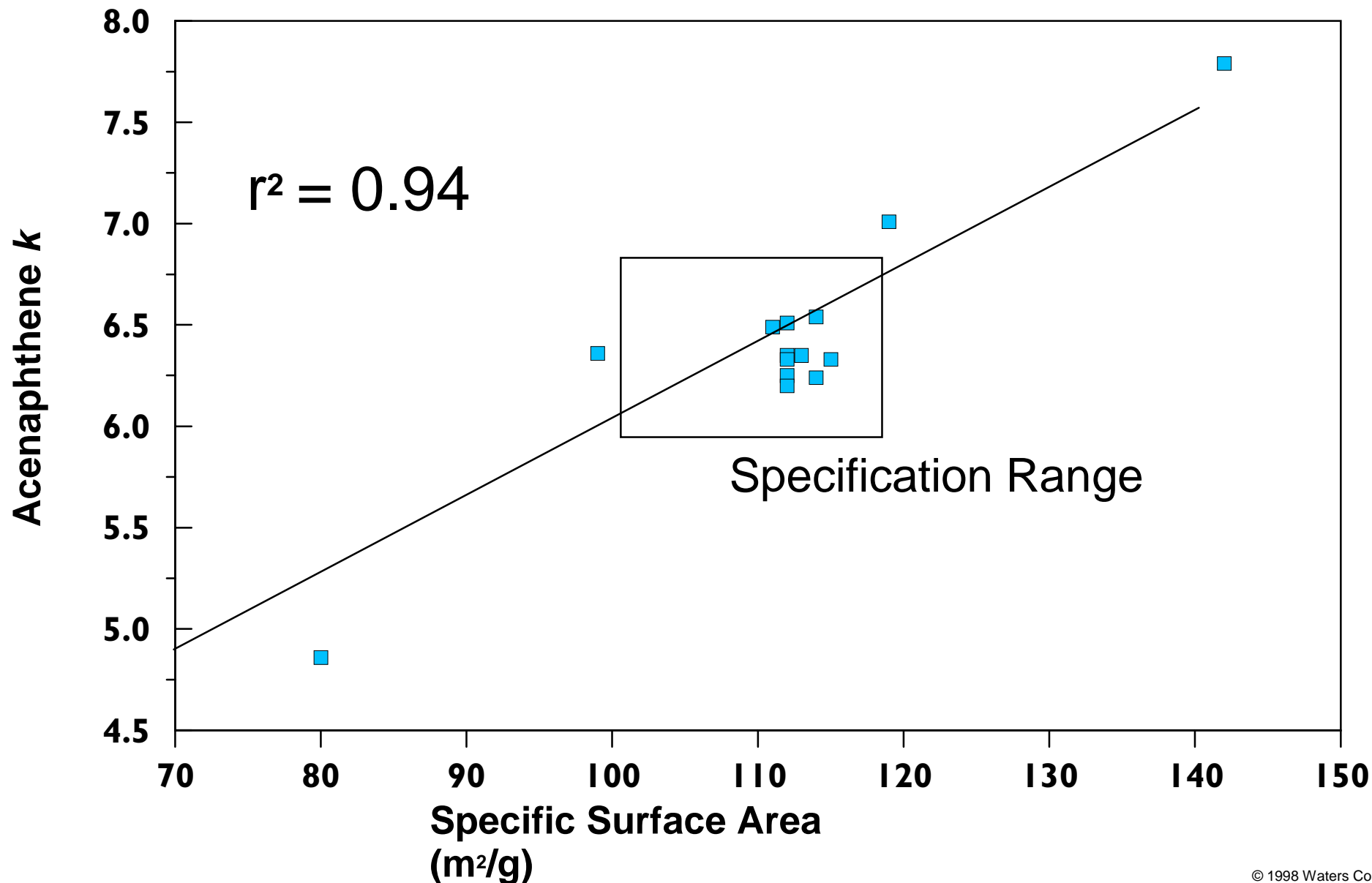
Typical Small Molecule Chromatogram



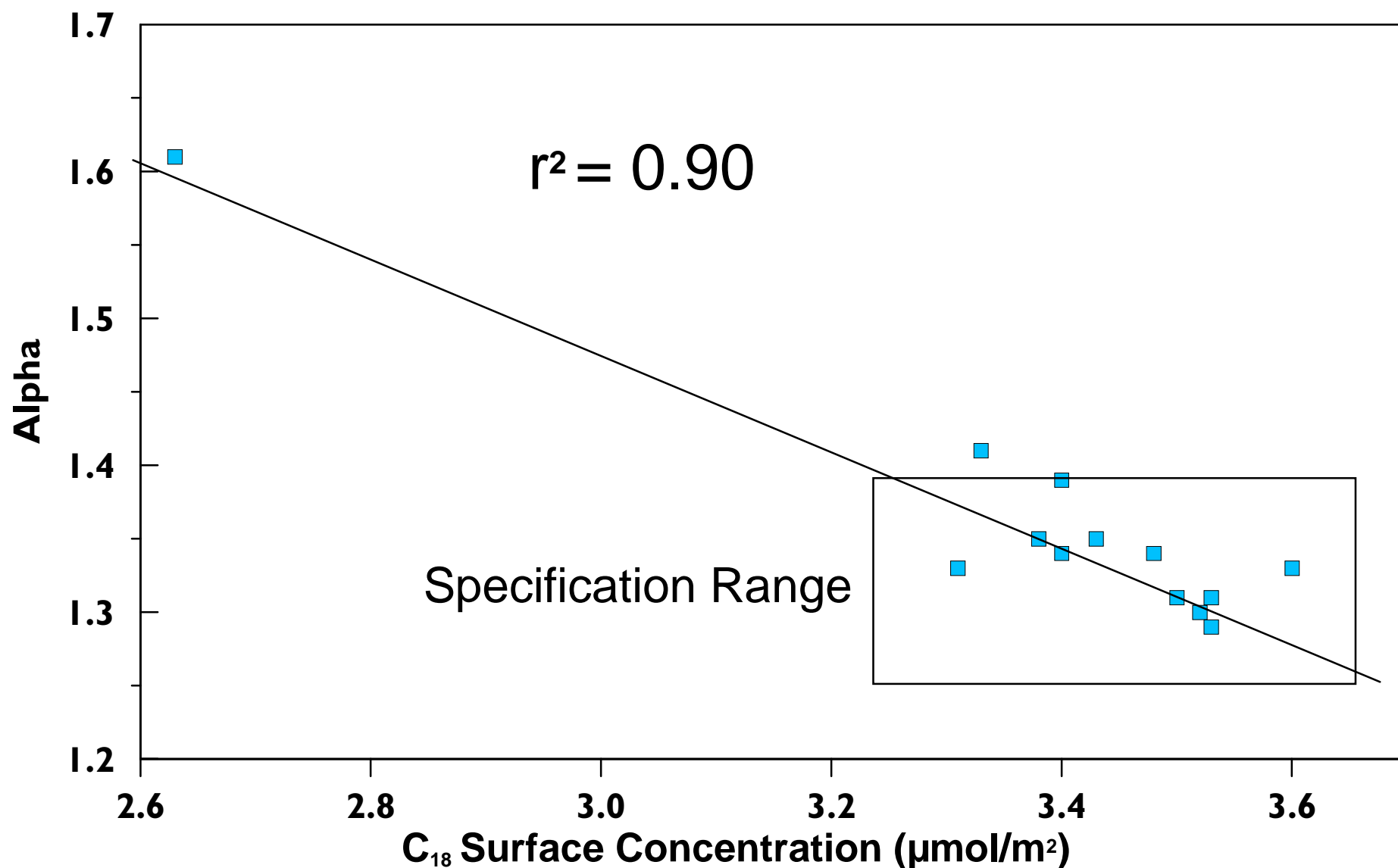
Typical Correlations - Small Molecules

- Graph 1 shows the retention factor (k) of the hydrophobic probe acenaphthene vs silica specific surface area
 - ▶ retention increases with increasing surface area (the amount of chromatographic surface increases)
- Graph 2 shows the selectivity (α) of one pair of analytes vs C_{18} surface concentration. This pair is particularly sensitive because the strong base amitriptyline is retained partly by silanophilic interactions.
 - ▶ selectivity decreases as C_{18} surface concentration increases (the concentration of unbonded silanols decreases)

Graph 1: Acenaphthene k vs Silica Specific Surface Area



Graph 2: α (Amitriptyline/Acenaphthene) vs C_{18} Surface Concentration

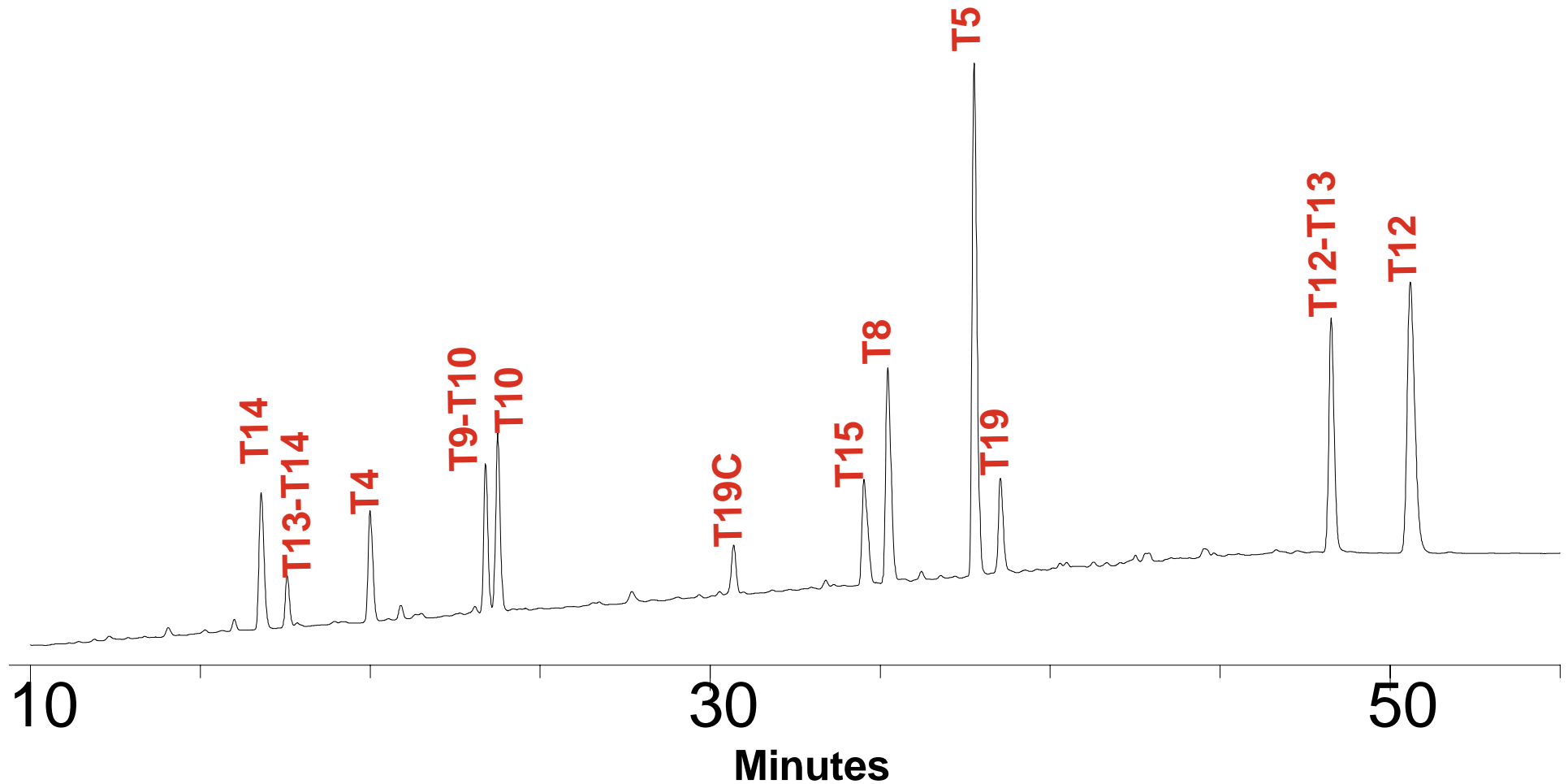


Separation of Peptides

HPLC Conditions

- Instrument: Waters Alliance 2690 XE, 486 UV Detector, Millennium Data System
- Column: Symmetry300™ C₁₈ 5 μm
3.9 x 150 mm
- Mobile Phase: A = 0.05 % TFA in Water
B = 0.1 % TFA in Acetonitrile
- Gradient: 0 - 28 % B in 45 min, linear
- Flow Rate: 1.0 mL/min
- Temperature: 35°C
- Detection: Absorbance at 220 nm

Typical Bovine Cytochrome c Tryptic Map



Sample Probe: Bovine Heart Cytochrome c

T1 **T4**
Ac GLY ASP VAL GLU LYS/ GLY LYS/ LYS/ ILE PHE VAL GLN LYS/ **CYS**

T5
ALA GLN **CYS** HIS THR VAL GLU LYS/ GLY GLY LYS/ HIS LYS/ THR GLY

T8 **T9**
PRO ASN LEU HIS GLY LEU PHE GLY ARG/ LYS/ THR GLY GLN ALA PRO

T10
GLY PHE SER TYR THR ASP ALA ASN LYS/ ASN LYS/ GLY ILE THR TRP

T12 **T13**
GLY GLU GLU THR LEU MET GLU TYR LEU GLU ASN PRO LYS/ LYS/ TYR

T14 **T15**
ILE PRO GLY THR LYS/ MET ILE PHE ALA GLY ILE LYS/ LYS/ LYS/ GLY

T19
GLU ARG/ GLU ASP LEU ILE ALA TYR/ LEU LYS/ LYS/ ALA THR ASN GLU

T19C

***BONDED TO HEME GROUP**

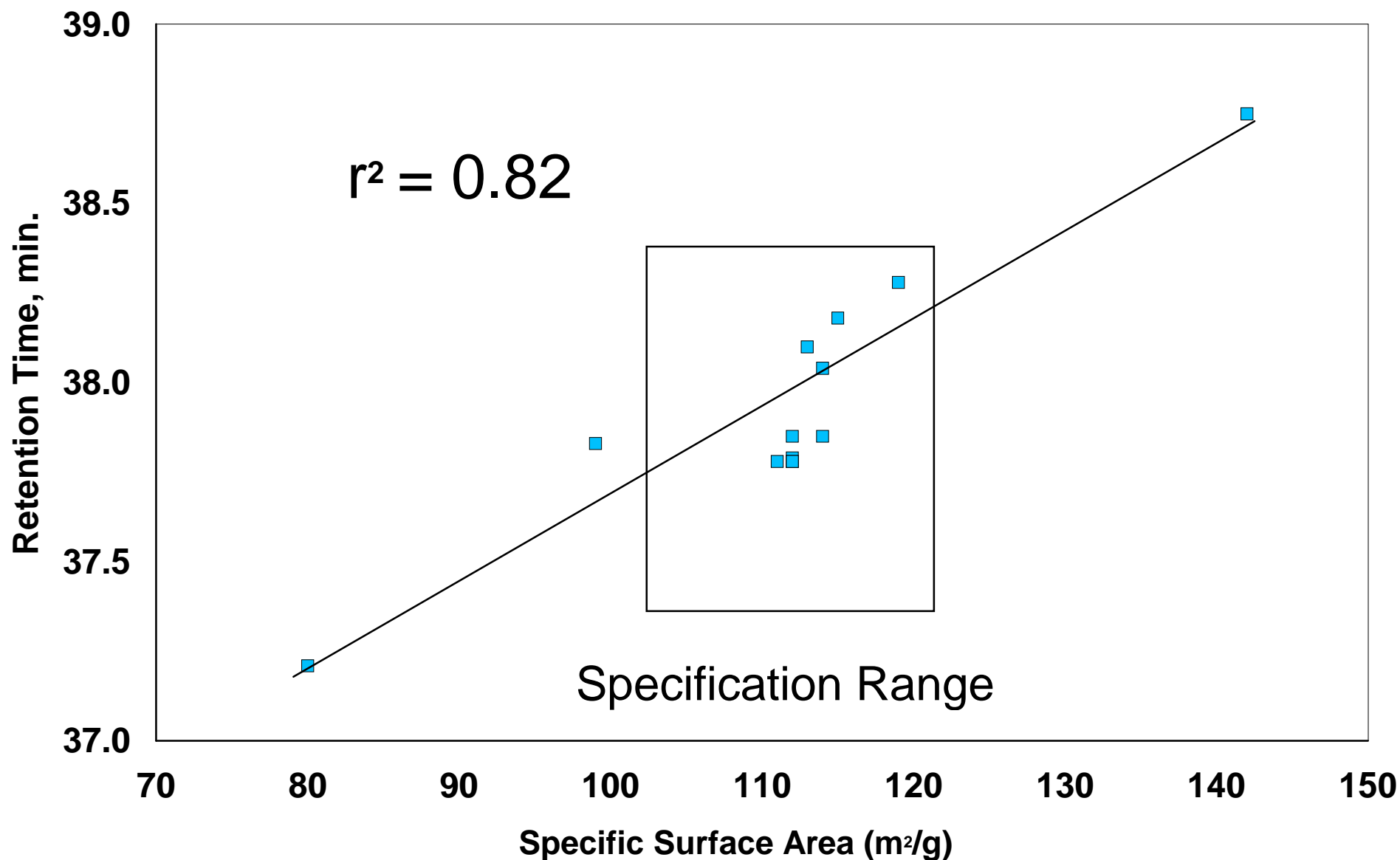
Digestion Conditions

- Suspend 8 mg of protein in 900 μL of pH 8 ammonium bicarbonate buffer.
- Add 0.8 mg of trypsin in 100 μL of buffer.
- Heat at 35°C for 24 hrs.
- Remove sample from heating block and cool to room temperature.
- Add 9 mL of deionized water and 65 μL of TFA

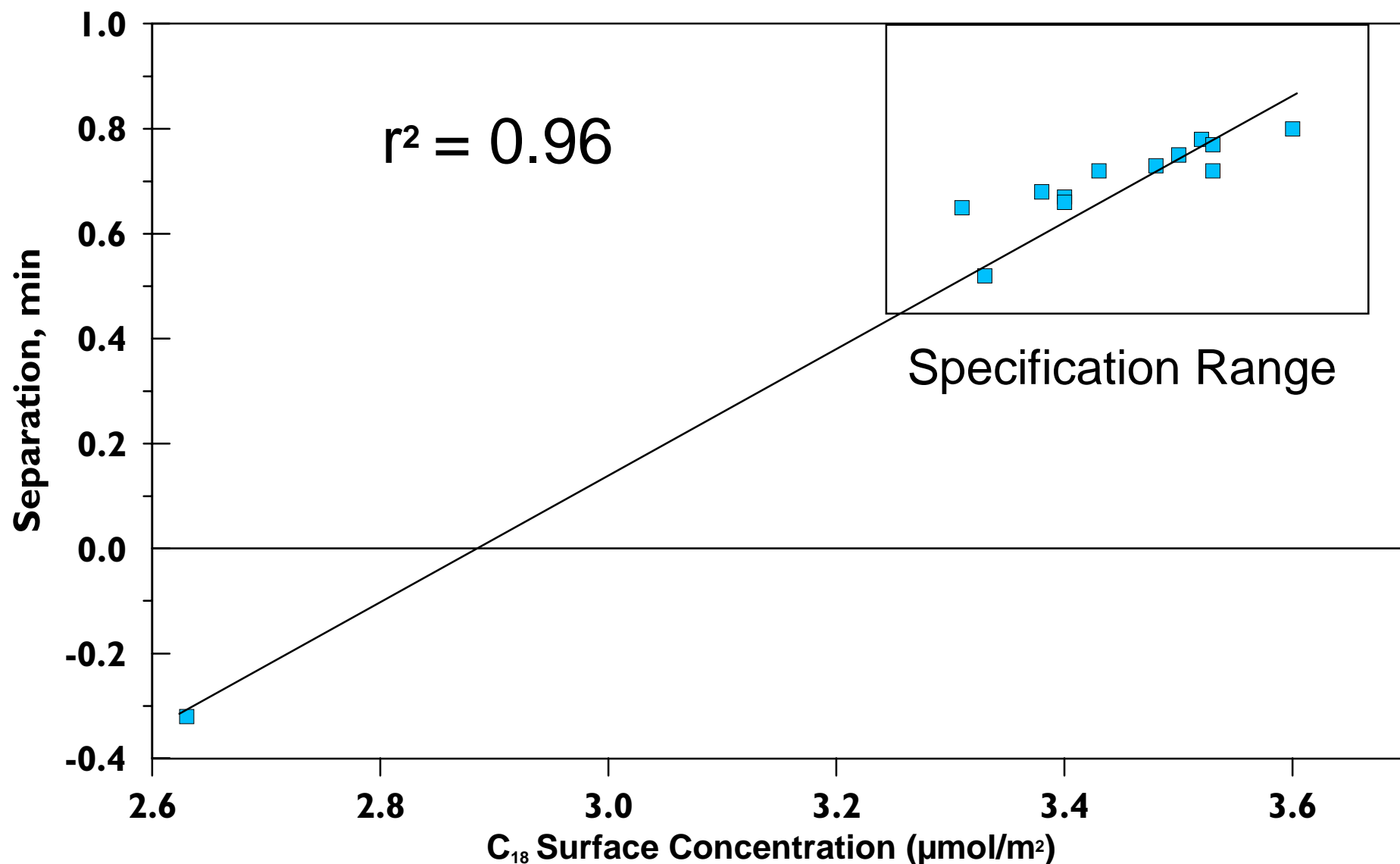
Typical Correlations - Peptides

- Graph 3 shows the retention time of the T5 (heme-bearing) peptide vs silica specific surface area
 - ▶ retention increases with increasing surface area (the amount of chromatographic surface increases)
- Graph 4 shows the separation of the T19 / T5 pair vs C₁₈ surface concentration. This pair is particularly sensitive because the unusually hydrophobic T5 (heme-bearing) peptide is retained largely by hydrophobic interactions, whereas the T19 peptide is retained partly by silanophilic interactions.
 - ▶ separation increases as C₁₈ surface concentration increases

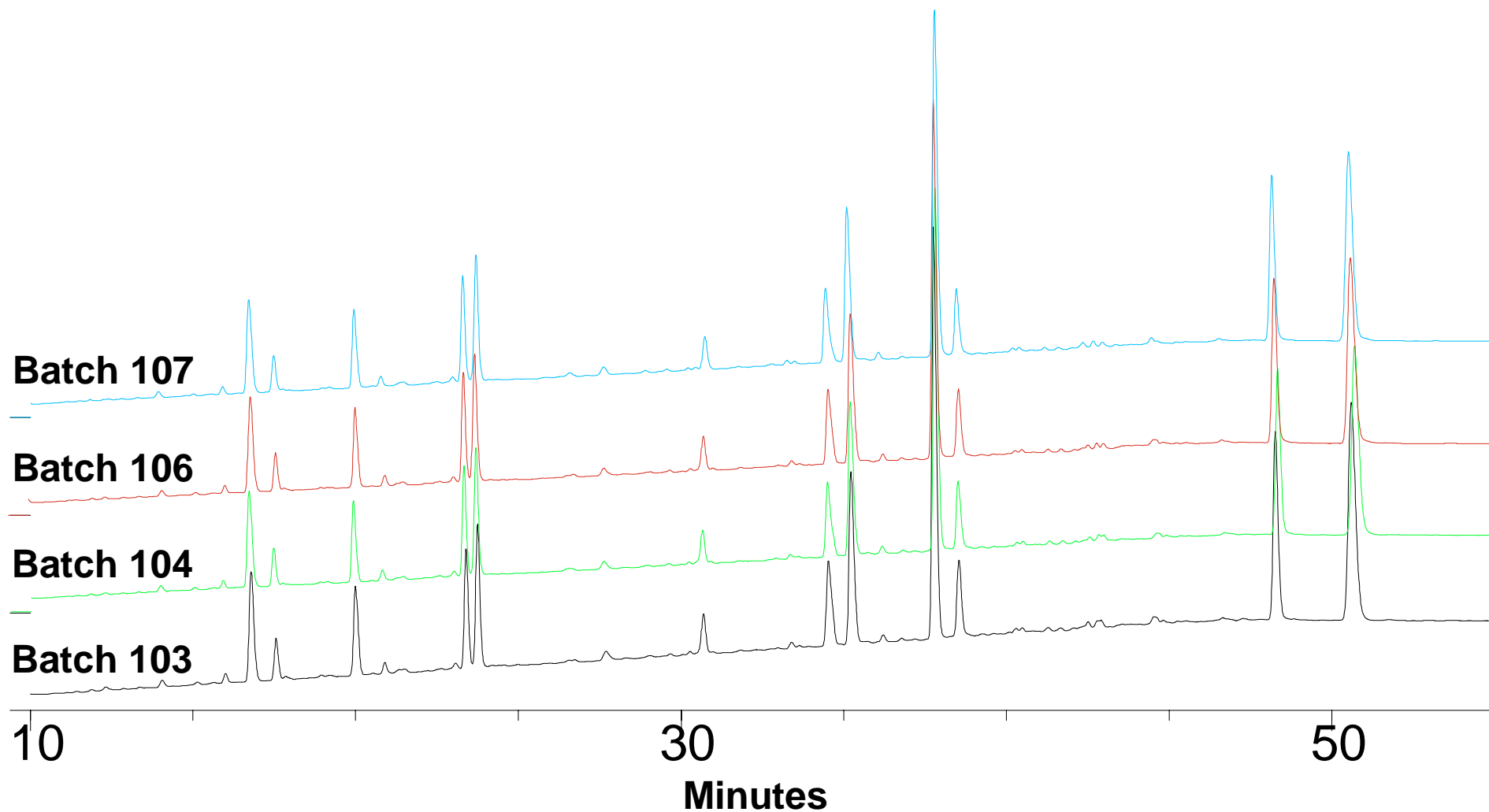
Graph 3: T5 Retention Time vs Silica Specific Surface Area



Graph 4: T19 / T5 Separation vs C_{18} Surface Concentration



Batch-to-Batch Reproducibility



Conclusions

- The factors affecting column-to-column reproducibility for both biomolecules and low molecular weight analytes are identical
- Peptide fragment retention times and critical pair separation show strong correlations with silica surface area and C₁₈ surface concentration
- The column-to-column reproducibility problems previously associated with biomolecule separations have been dramatically reduced