Practical Aspects for the Development of a Reversed-Phase Analytical Method for Bio-Pharmaceuticals

Paul Kostel and Michael Li, Vydac/The Separations Group, Inc., 17434 Mojave St., Hesperia, CA 92345 USA

Reversed phase is solvent-eluted hydrophobic interaction.

Distinct from classic HIC, which is water-eluted hydrophobic interaction.

Macromolecule selectivity is more complex than small molecule selectivity.

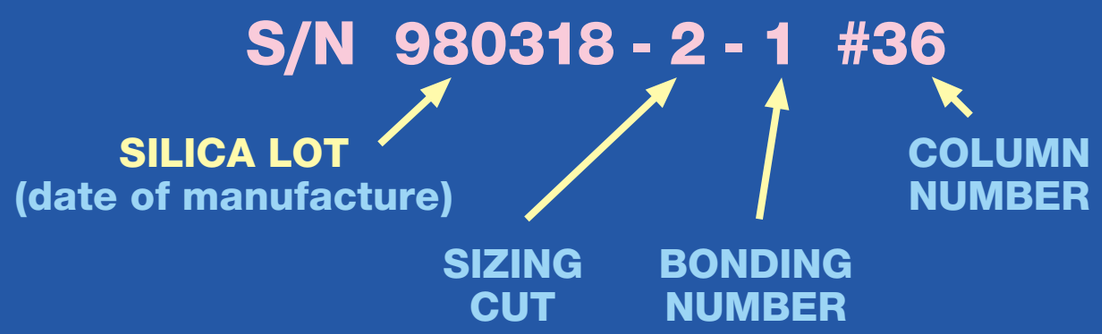
- Small molecules see the adsorbent primarily as a 2-dimensional surface chemistry.
- Macromolecule retention is more dependent on 3-dimensional internal pore structure.

Variations in reversed-phase silicas are not just chemical.

- Subtle physical variations from lot-to-lot, while not significant for research separations, can affect validated methods.
- When developing analytical reversed-phase methods for biopharmaceuticals, it is recommended to qualify the method across at least three different silica lots.

Vydac serial numbers indicate the silica manufacturing lot.

A typical Vydac serial number consists of four parts.



The interior of a reversed-phase silica is like a limestone cave.

Proteins, peptides, and other macromolecules are large enough to span the cave. They interact simultaneously with more than one wall. The size of the cave is important.

Their retention and selectivity are **"3-D"**.

Reproducible silica pore structure is essential for reproducible separations.

Small molecules do not span the cave. They interact with the reversed-phase adsorbent by simply hanging on the walls. The size of the cave matters little.

Their retention and selectivity are **"2-D"**.

Reproducible adsorbent chemistry is important. Silica structure has less effect.

Let your molecule design the mobile phase.

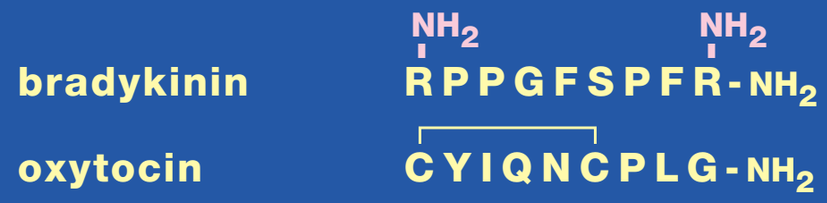
- Electrolytes like HCl and H_3PO_4 set pH and charge, but do not interact significantly with a reversed phase.
- Ion-pairing electrolytes attract charged groups on a macromolecule and bring them to the adsorbent surface.

  TFA is an ion pair for positive charges.

  TEAA is an ion pair for negative charges.

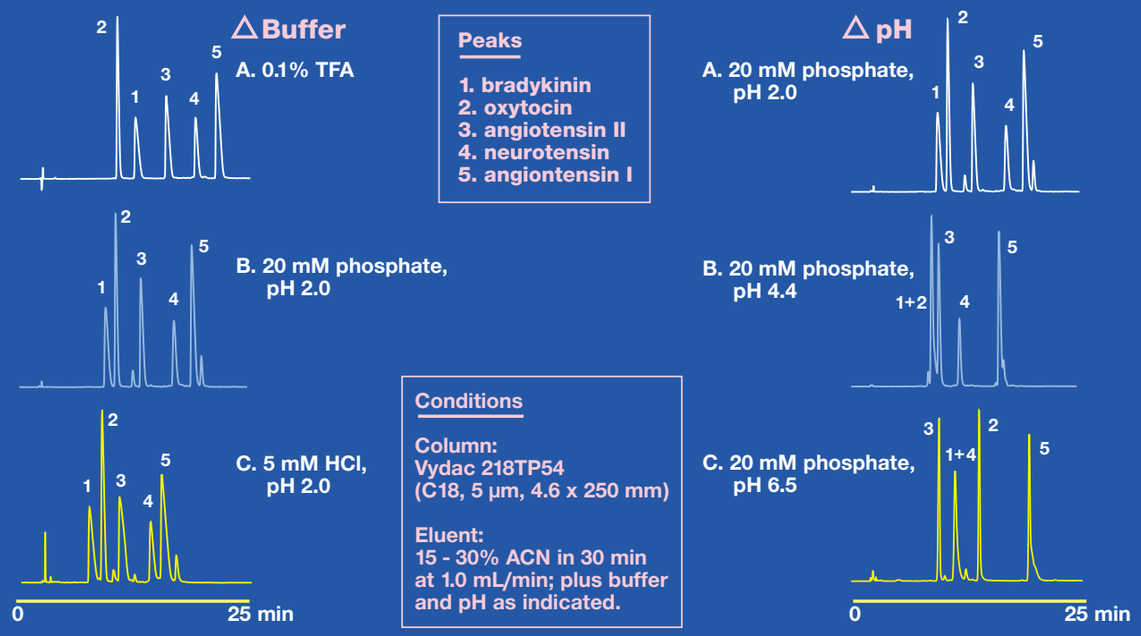
Charged substituents let pH and counterions control selectivity.

- Example: two nonapeptides



- Bradykinin has two arginines (+ charges), oxytocin does not. Note how selecting HCl or TFA as counterion can reverse the elution order.

Effects of buffer and pH



Peaks

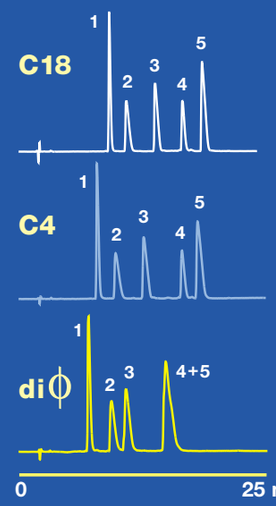
1. bradykinin
2. oxytocin
3. angiotensin II
4. neurotensin
5. angiontensin I

Conditions

Column:
Vydac 218TP54
(C18, 5 µm, 4.6 x 250 mm)

Eluent:
15 - 30% ACN in 30 min at 1.0 mL/min; plus buffer and pH as indicated.

Effects of reversed-phase ligand



Eluent: 15 - 30% ACN in aqueous 0.1% TFA over 30 min.

Peaks: 1. oxytocin 2. bradykinin 3. angiotensin II 4. neurotensin 5. angiotensin I

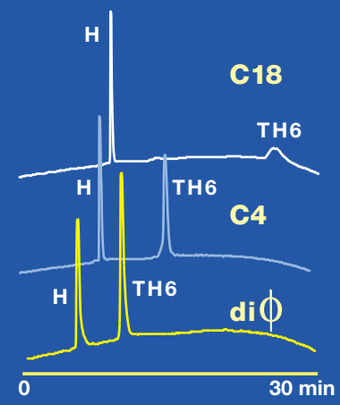
C18 = Vydac 218TP54
C4 = Vydac 214TP54
diphenyl = Vydac 219TP54

(all 300 Å, 5 µm, 4.6 x 250 mm)

For proteins and peptides, C4 is often nearly as retentive as C18.

C18 is more retentive for hydrophobic small molecules and therefore more prone to contamination. All else being equal, C4 is preferred.

A diphenyl column is less retentive than C4, therefore should be considered for very hydrophobic molecules.

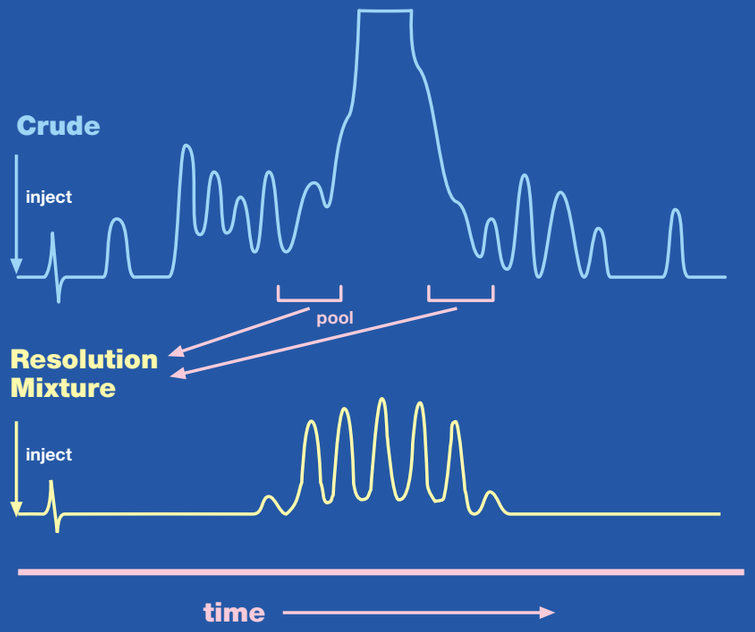


Eluent: 10 - 90% ACN in aqueous 0.1% TFA over 30 min.

H = 18-residue helical peptide
TH6 = 6-helix template-assembled synthetic protein

From V. Steiner, M. Schar, K.O. Bornsen, and M. Mutter, *J. Chrom.* **582** (1991)

The Concept of a Resolution Mixture



Definition

Mixture of target molecule, early and late contaminants – all at approximately the same concentration.

Source

Early and late side-fractions from a purification step.

Purpose

Use early in method development to screen parameters such as: optimum solvent, buffer and pH, column type and manufacturer.

Benefits of a Resolution Mixture

- SOPs written with resolution requirements and instructions for adjusting conditions to achieve the required resolution are inherently robust.
- Reduces the number of evaluations required downstream.
- Resolution differences are easier to detect and measure when all peaks are of similar size.
- Side fractions are also used for identification of contaminants by MS, NMR, etc. Early identification gives valuable insight for process development.
- Visual inspection of results.
- System suitability standard and lot qualification (packing, buffer, etc.) in validated method.
- Troubleshooting.

Rationale for Resolution Mixture in SOPs

- The need for rapid product development makes investigating all parameters difficult.
- Primary parameters investigated prior to the validation:
  - pH • column • flow rate
  - buffer • gradient • temperature
- Secondary parameters can affect results:
  - frits • column conditioning • solvent consistency
  - lot-to-lot variance in columns and packings • system hardware
- SOPs should be written to adjust to characteristic results. Allowing no flexibility essentially tests the equipment, column, and lab personnel without regard to the function of the method.
- Avoid single-source specifications.

Developing methods with the crude material requires collecting and analyzing fractions to compare different mobile phases and columns.

Developing methods with the resolution mixture only requires visual inspection of the chromatogram and resolution can be calculated automatically by the data system.

A resolution mixture provides a practical means for validating an analytical method.

- Resolution is important! A chromatogram with a single peak tells you nothing about resolution.
- A resolution mixture used for method development becomes a system suitability standard after validation.

System suitability standard: What is it?

- A mixture of target product and one or more identifiable closely eluting substances.
- Can be reproducibly prepared.
- Allows functional performance standard to be specified for the analytical separation.
- SOP allows flexibility for adjusting the chromatographic procedure to meet standard.
- A resolution mixture used for method development provides a good basis for definition of a system suitability standard.

Heeding stability data can help.

A counterion that is detrimental to stability at a pH where others engender stability may be a good candidate for the analytical mobile phase.

REASON:
Counterions that interact with a biopolymer in such a way as to stress bonds, thereby decreasing long term chemical stability, tend to hold the macromolecule in a more rigid configuration. Rigid molecules produce sharper reversed-phase peaks.

(Because analytical chromatography is fast, subtle long-term destabilization is usually not a problem for the analytical method.)

System suitability standard example

USP 23 method for Human Insulin provides example of system suitability standard:

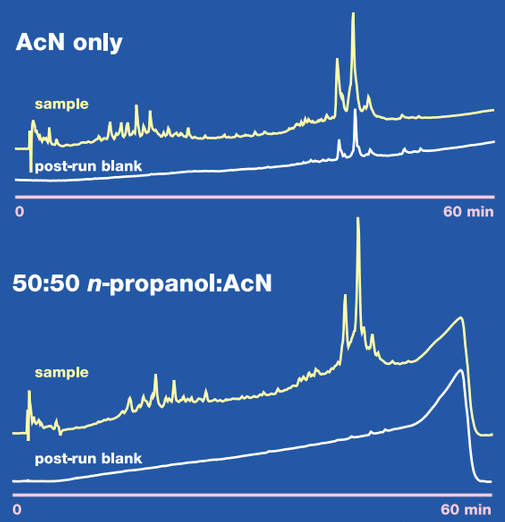
"*System suitability preparation* – Dilute an accurately measured volume of *Test preparation A* quantitatively with 0.01 N hydrochloric acid to obtain a solution having a known concentration of about 1 mg per mL, and allow it to stand at room temperature for 16 to 24 hours to enable desamido insulin to be produced."

"To determine the suitability of the chromatographic system, chromatograph the *System suitability preparation,* and record the peak responses as directed under *Procedure:* the tailing factor for the insulin peak is not more than 1.8, the resolution, *R,* between the insulin and desamido insulin peaks is not less than 1.8..."

"*Mobile phase* – Prepare a filtered and degassed mixture consisting of 74 volumes of 0.1 M monobasic sodium phosphate, adjusted with phosphoric acid to a pH of 2.0, and about 26 volumes of acetonitrile. [NOTE – Proportions of the *Mobile Phase* components may be adjusted to achieve satisfactory resolution and retention times.]"

Always check for memory.

A blank gradient run after analysis should be free of ghosts.



A lipid peptide, at first glance, appeared to give a suitable analytical chromatogram with a gradient of acetonitrile. However, a post-run blank revealed severe memory effects.

Incorporating a stronger solvent, *n*-propanol, into the mobile phase solved this problem.

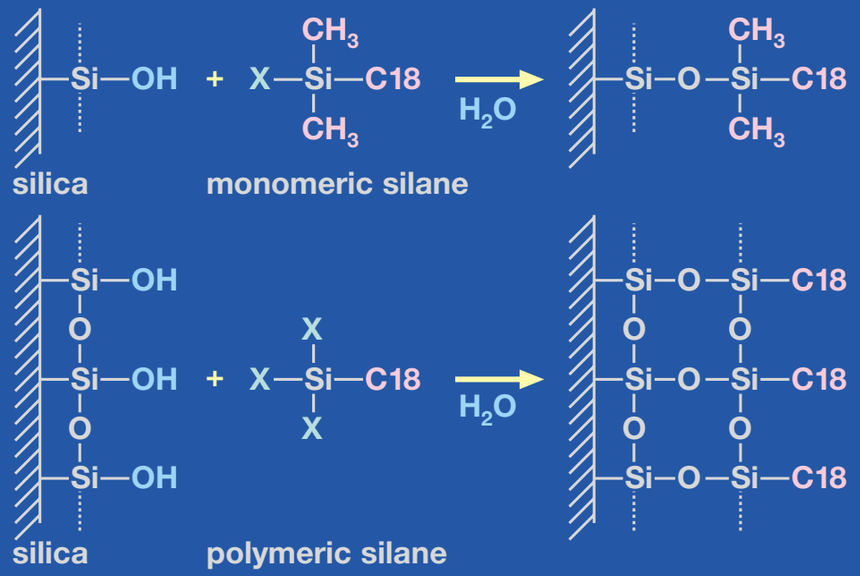
TIPS:
If column memory is encountered, try higher temperature, stronger solvent, stronger wash between analyses, and starting the analysis with higher solvent concentration in both sample and column.

Column: Vydac 219TP54 (diphenyl, 300 Å, 5 µm, 4.6 x 250 mm)
Flow: 1.5 mL/min. Temperature: 45°C. Gradient: 27- 82% ACN in 55 min. 25-70% *n*-propanol:ACN in 45 min.

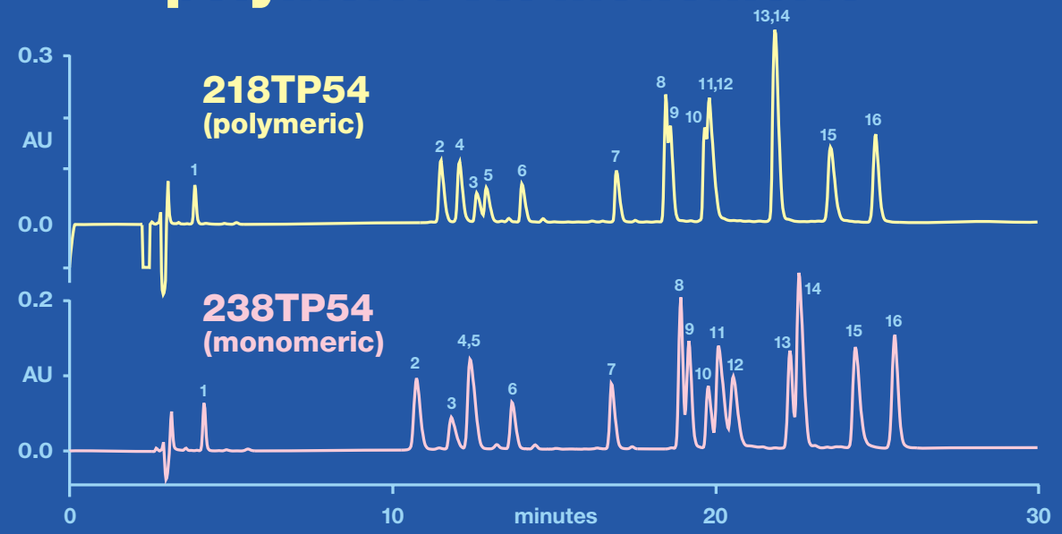
Ref: Vydac Application Note #9802, "Designing Purification Methods for Hydrophobic Peptides"

Poster design by Seaview Scientific

Reversed-phase bonding type... polymeric vs. monomeric



Effect of bonding type... polymeric vs. monomeric



sample = mixture of synthetic peptides

Bonding type changes selectivity in subtle ways.

Reading List

Monograph (64 pages)

The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC

Application Notes

#9701: The Influence of C18 Ligand Type on the Peptide Selectivity of Silica-Based Reversed-Phase Columns
#9703: Developing a Robust Reversed-Phase Method for the Analysis of Polypeptides
#9802: Designing Purification Methods for Hydrophobic Peptides
#9804: What is 0.1% TFA?
#9809: The Concept of a Resolution Mixture in Developing and Validating Process Chromatographic Methods
#9811: Assessing Polypeptide Integrity: Reversed-Phase HPLC vs. PAGE

The publications above are available on request from Vydac or may be downloaded in electronic form from Vydac's web site.

VYDAC®

The Separations Group, Inc.
17434 Mojave Street
Hesperia, CA 92345

Tel: (760) 244-6107 *(800) 247-0924*
Fax: (760) 244-1984 *(888) 244-6610*

Email: experts@vydac.com

http://www.vydac.com