Gradient Elution

• Why choose this separation mode?
• What HPLC parameters affect a gradient separation?
• How can I use these parameters to improve my gradient separation?
Is Gradient or Isocratic Elution the Preferred Separation Mode for RP-HPLC Assays?
Isocratic Elution
Separation occurs using a constant composition mobile phase

Substituted Anilines

Column: ZORBAX SB-C18
4.6 x 150 mm 5 µm
Mobile Phase: 42% Methanol
58% 25 mM phosphate buffer
pH 2.5
Flow Rate: 1 mL/min
Temperature: 22°C
Sample: 1. p-anisidine
2. m-toluidine
3. 3-aminobenzonitrile
4. p-chloroaniline
5. m-chloraniline
6. o-chloroaniline
Advantages of Isocratic Elution

- Simple HPLC instrumentation – single pump
- No column re-equilibration necessary
- Easy instrument and method transfer
- Method development better understood
Disadvantages of Isocratic Elution

- Peaks broaden with increasing retention time
- Sensitivity and LOD decrease with increasing retention time
- Peak broadening limits the number of compounds in a single chromatogram
- Peak broadening limits the polarity range of compounds eluted in a single chromatogram
Gradient Elution
Separation occurs by continuously increasing the solvent strength of the mobile phase.

Column: ZORBAX 300SB-C8
2.1 x 150 mm, 5 µm
Gradient: 2-62% B in 70 min.
Mobile Phase:
A: 100% H2O with 0.1% TFA
B: 0.1% TFA in 80% ACN, 20% H2O
Flow Rate: 0.2 mL/min
Temperature: 50°C
Sample: 50 pmol of BSA digest in 4M urea
Gradient Elution for Reversed-Phase HPLC

Increasing the solvent strength = Increasing the % organic in the mobile phase

Linear solvent strength gradient = % per min is a constant

For every 20% change in ACN, $\Delta t$ is 10 min.
Why Choose Gradient Elution?

• Case 1:
  • To separate samples having components that vary widely in polarity.
Separation of Herbicides on ZORBAX SB-C18

**Isocratic Elution**
70% aqueous/30% ACN

**Gradient Elution**
20 – 60% ACN in 30 min.

- Column: ZORBAX SB-C8 4.6 x 150 mm 5 µm
- Mobile Phase: A: H₂O with 0.1% TFA, pH 2
  - B: Acetonitrile
- Flow Rate: 1.0 mL/min
- Temperature: 35°C
- Sample:
  1. Tebuthiuron
  2. Prometon
  3. Prometryne
  4. Atrazine
  5. Bentazon
  6. Propazine
  7. Propanil
  8. Metolachlor

Sample retention times and peak order may vary depending on column and mobile phase conditions.
Why Choose Gradient Elution?

- Case 2:
  - To separate low molecular weight mixtures having a large number of components
Gradient Elution Analysis of Pesticides in Drinking Water

**Column**: ZORBAX SB-C18  
3.0 x 250 mm 5 µm

**Gradient**:  
10% B for 2 min.  
10 - 45% B in 70 min

**Mobile Phase**  
A: 2 mM Sodium Acetate  
pH 6.5 with 5% ACN  
B: Acetonitrile

**Flow Rate**: 0.35 mL/min  
**Temperature**: 40°C  

**Sample**: Pesticides

- 1. Desisopropylatrazine  
- 2. Metamitron  
- 3. Fenuron  
- 4. Chloridazon  
- 5. Desethylatrazine  
- 6. Metoxuron  
- 7. Carbetamide  
- 8. Bromicil  
- 9. Hexazinone  
- 10. Simazine  
- 11. Metribuzin  
- 12. Desethylterbutylazine  
- 13. Carbutilat  
- 14. Methabenzthiazuron  
- 15. Chlortoluon  
- 16. Atrazine  
- 17. Mondinuron  
- 18. Diron  
- 19. Isoxproxuron  
- 20. Metobromuron  
- 21. Metazachlor  
- 22. Buturon  
- 23. Propazine  
- 24. Dimefuron  
- 25. Terbutylazine  
- 26. Linuron  
- 27. Chlortromon  
- 28. Chloronur
Why Choose Gradient Elution?

- Case 3:
  - To separate high molecular weight mixtures (i.e., peptides and proteins)
Larger Molecules are More Sensitive than Small Molecules to Changes in % Organic

- Lysozyme is 15X more sensitive to changes in organic modifier than benzene and 4X more sensitive than leucine enkephalin.
Gradient Separation of Peptides on 300SB

- Gradient conditions are required for the separation of Leucine enkephalin (600 daltons) and Lysozyme (14,000 daltons).
Advantages of Gradient Elution

- Complex samples are analyzed in a single HPLC run
- Analysis time is reduced
- All peaks elute with the same bandwidth
- More peaks can be baseline resolved per unit time
- Sensitivity and LOD are unchanged during a gradient run
Disadvantages of Gradient Elution

- More expensive instrumentation
- Possible precipitation at interfaces, when using multiple proportioning valves
- Re-equilibration time adds to analysis time
- Instruments vary in their dwell volume ($V_{dw}$), which can cause method transfer problems
What Parameters Maximize Gradient Resolution?
Factors that Maximize Isocratic Resolution Between Peaks

- Decrease % organic
- Change the chemistry of the mobile or stationary phase
- Change % organic
- Increase column length
- Decrease particle size or flow rate

- Increase retention
- Change relative peak position
- Reduce peak width

\[ k \]

\[ a \]

\[ N \]
To Maximize Gradient Resolution Between Peaks

- INCREASE one or more of the following:
  - $k^*$ Gradient retention
  - $\alpha$ selectivity$^1$
  - $N$ theoretical plates$^1$

$^1$similar to isocratic elution
Increasing Retention ($k^*$) in Gradient Elution is Difficult to Visualize
Which of the Following Increases Gradient Retention?

• A longer gradient time
• A shorter column
• A higher flow rate
• A shorter organic range
All of the Following Increase Gradient Retention ($k^*$)

- A longer gradient time $t_G$
- A shorter column $V_m$
- A higher flow rate $F$
- A shorter organic range $\triangle \Phi$

Because:

\[ 1/k^* \propto \text{Gradient steepness} = b = \frac{S \cdot \triangle \Phi \cdot V_m}{t_G \cdot F} \]
Use of a Longer Gradient Time Increases Gradient Retention

- Increased gradient retention improves resolution of several peak pairs – 1,2 and 4,5.
A Shorter Column:
Increases Gradient Retention, Increases Overall Resolution, Assumes Constant N

4.6 x 150 mm, 5 µm  
N = 12,000

4.6 x 75 mm, 3.5 µm  
N = 10,000

Column: ZORBAX SB-C8  
Gradient: 20 – 60%B  
Mobile Phase: A:H2O with 0.1%TFA, pH 2  
B: Acetonitrile  
Flow Rate: 1.0 mL/min  
Temperature: 35°C  
Sample: Herbicides
Gradient Elution

\[ 1/ k^* \propto \text{steepness} = b = \frac{S \cdot \Delta \Phi \cdot V_m}{t_G \cdot F} \]

This relationship also says:

If “b” is kept constant from run-to-run, peaks will elute in the same relative pattern.
Two Chromatograms Both Having the Same Gradient Steepness


Column: StableBond SB-C8
4.6 x 150 mm, 5 µm
Gradient
Time: 30 min.
Flow Rate: 1.0 mL/min
Analysis
Time: 24 min

Column: Rapid Resolution StableBond SB-C8
4.6 x 75 mm, 3.5 µm
Gradient
Time: 15 min.
Flow Rate: 1.0 mL/min
Analysis
Time: 12 min
Two Chromatograms Both Having the Same Gradient Steepness


Column: StableBond SB-C8
4.6 x 150 mm, 5 µm

Gradient Time: 60 min.
Flow Rate: 1.0 mL/min

Analysis Time: 40 min

Column: StableBond SB-C8
4.6 x 75 mm, 3.5 µm

Gradient Time: 15 min.
Flow Rate: 2.0 mL/min

Analysis Time: 7.2 min

Multiple gradient parameters can be changed to maintain gradient steepness and reduce analysis time.
Recommendations for Gradient Elution Method Development

- Before changing the stationary phase or mobile phase to generate changes in a, selectivity, explore increasing gradient retention, $k^*$, by
  - Increasing gradient time
  - Decreasing column length
  - Increasing flow rate
  - Shortening organic range

- Before finalizing a gradient method reduce column length (same $N$), and adjust the flow rate and gradient time to reduce analysis time and solvent waster while maintaining resolution.
Reversed-Phase HPLC Doesn’t Always Do the Job

- Not enough retention of ionized compounds, i.e., acids and bases
- Too much retention of hydrophobic compounds
- Peaks overlap no matter what you try
To Resolve Non-Retained Acids and Bases Ion-Pair Chromatography is Recommended
Ion-Pair Chromatography

This demonstrates a mechanism of ion-pair chromatography. The ion-pair reagent is embedded in the stationary phase and in solution where the analytes interact with it.
Ion-Pair Chromatography is Preferred Over Ion-Exchange

- Better column efficiency (N)
- C8 and C18 columns are more stable and reproducible
- More options to resolve bands
Suggested Experimental Conditions for Ion-Pair HPLC

- **Column:** C8 or C18
- **Mobile Phase:**
  - Organic Methanol preferred
  - Aqueous Buffered with appropriate IP-reagent
- **Temperature:** Controlled between 35° and 60°C

- **Cations – bases**
- **Buffer:** 25 – 50 mM phosphate, pH 2-3
- **Ion-pair – reagent:** 10 – 100 mM hexane sulfonate

- **Anions – acids**
- **Buffer:** 25 – 50 mM phosphate, pH 6 – 7
- **Ion-pair reagent:** 10 – 40 mM tetrabutyl ammonium phosphate
Comparison Separation of Catecholamines
Reversed-Phase and Ion-Pair HPLC

Reversed Phase HPLC
A: 97.5% H₂O with 0.1% TFA
B: 2.5% Acetonitrile

Ion-Pair HPLC
A: 90% 70 mM sodium phosphate, pH 3
10 mM hexane sulfonate
B: 10% methanol

Column: ZORBAX SB-C18, 4.6 x 150 mm, 5 µm
Sample: 1. Norepinephrine 2. Vanillyl Mandelic Acid 3. Tyramine
4. DOPAC 5. Homovanillic acid

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Critical Issues with Ion-Pair Chromatography

- Reproducibility
  - Temperature control
  - Column Equilibration
  - IP-reagent in the injection solvent
  - IP-reagent concentration in the mobile phase > 10 mM
  - Dedicate column to IP-HPLC

- Poor Peak Shape
  - 20 mM TEA may be added to the mobile phase to improve peak shape for basic solutes
  - Method may require routine washing with high percent organic
To Resolve Strongly Retained Hydrophobic Samples
Normal-Phase HPLC is Recommended
Other Reasons to Choose Normal-Phase HPLC

- Isomer separation
- Sample injection solvent is non-polar
- Recovery in non-polar solvents is desirable
Normal-Phase Chromatography

- Polar interactions between solute and stationary phase are most important.
- Retention decreases with increasing polarity of the mobile phase.
Normal-Phase HPLC
Stationary Phase Options

silica > amino > diol > cyano

Strongest

Cyano and Silica are Preferred

Weakest
Bonded Silica Preferred Over Silica for Analytical Work

- Cyano – CN
  - Faster equilibration
  - Control of water content not needed
  - Gradient elution is an option
  - Weaker phase
- Silica
  - Isomer separation
  - Preparative HPLC
## Suggested Normal-Phase HPLC Conditions

<table>
<thead>
<tr>
<th></th>
<th>Bonded-Silica</th>
<th>Silica</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column:</strong></td>
<td>ZORBAX CN</td>
<td>ZORBAX SIL</td>
</tr>
<tr>
<td></td>
<td>Packed in normal phase solvents</td>
<td>ZORBAX Rx-SIL – basic solutes</td>
</tr>
<tr>
<td><strong>Mobile Phase:</strong></td>
<td>Hexane with 1- or 2-propanol</td>
<td>Methylene chloride with 0.05% - 0.5% methanol</td>
</tr>
<tr>
<td><strong>Temperature:</strong></td>
<td>Ambient - 60°C</td>
<td>Ambient - 60°C</td>
</tr>
</tbody>
</table>
Comparison of Silica Types

- The low acidity, ZORBAX Rx-Sil improves peak shape of basic compounds.
Critical Issues in Normal-Phase HPLC

• Poor peak shape
  • Injection solvent stronger than mobile phase
  • Basic samples give better peak shape using high purity silica
  • Basic samples may require 20 mM TEA in the mobile phase
  • Acidic samples may require 20 mM acetic acid in the mobile phase
  • Strongly retained polar materials can build on the column and can be removed with water this is slightly acidic

• Reproducibility
  • Silica requires addition of water or methanol to maintain reproducibility
  • Use columns packed in normal-phase solvents
Hints
Isocratic Elution – Possible Column Contamination Problems

Double or split peaks for all components.

- Use of low organic mobile phases may necessitate routine washing with high % organic.
- Without these washes retained material building up on the analytical column or guard column can cause “peak splitting”.

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Gradient Elution

- Add low concentration modifiers (i.e., TEA, TFA) to both reservoir A and B to achieve better and consistent instrument mixing.
Gradient Elution

When using low wavelength detection, (i.e., 210 – 215 nm) with TFA and ACN, add slightly less TFA (10 – 15% less) to the organic reservoir to generate a flat baseline.

Equivalent TFA in reservoir A and B causes a rise in the baseline at low wavelengths.

Column: ZORBAX SB-CN, 4.6 x 150 mm, 5 mm
Gradient: 0-36% B in 30 min. Mobile Phase: A:H₂O with 0.1%TFA, pH 2
B: Acetonitrile with 0.1%TFA Flow Rate: 1.0 mL/min Temperature: 40°C Detection: UV 215 nm
Sample: Tripeptides