The Theory of HPLC

Gradient HPLC

Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.
Aims and Objectives

Aims

- To explain and interactively illustrate how Gradient Elution HPLC works and how the retention and elution processes differ from Isocratic HPLC
- To demonstrate why peak shapes in Gradient HPLC are more efficient than those obtained from Isocratic HPLC
- To examine the effects of gradient steepness and show how the various gradient parameters can be practically determined
- To interactively illustrate the use of ‘Scouting’ Gradients in HPLC method development and optimisation
- Examine the pitfalls and advantages of Gradient Elution HPLC in a practical situation

Objectives

At the end of this Section you should be able to:

- To explain the problems that can be encountered when using Isocratic HPLC
- To demonstrate how these problems can be overcome using Gradient HPLC
- To define the parameters of Gradient Elution HPLC
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Isocratic HPLC Analysis

When the composition of the mobile phase does not change during an analysis (i.e. the composition is constant), the method is said to be 'Isocratic'.

Several potential problems are associated with isocratic analysis:

- When the range of analyte polarities is broad, some analytes may be poorly retained and resolution is lost with peaks eluting at or near the void volume ($t_0$)
- Alternatively, other analyte components may be significantly more hydrophobic and show unacceptably long retention times
- Due to the various band-broadening processes, these late eluting peaks will be broad and show reduced sensitivity due to reduced peak height
- It is possible that some components will be irreversibly adsorbed on the column and cause contamination

Potential problems encountered with Isocratic HPLC Analysis

Problems with Isocratic Analysis:

- Poor resolution or early eluting peaks
- Increase in peak width and decrease in peak height (reduced sensitivity) for later eluting peaks
- Long analysis time due to a wide sample polarity range (resulting in a wide range of $k$ values)
- Column contamination by strongly retained components
Gradient HPLC Analysis

Many of the problems associated with Isocratic HPLC analysis can be overcome using Gradient HPLC. In this mode of analysis, the mobile phase composition is altered during the analysis – normally by increasing the amount of organic modifier.

- The initial composition is chosen so that the strength is appropriate to retain and resolve early eluting analytes
- The elution strength is then increased in a predetermined way to elute compounds with optimum resolution
- The final mobile phase composition is chosen to ensure elution of all compounds of interest from the column within a reasonable time
- It is possible to increase the organic modifier concentration to ‘wash’ strongly retained, potentially contaminating components from the column

Gradient elution is best suited to analyses carried out using Reverse Phase, Normal Phase separations using bonded stationary phases and for ion-exchange chromatography. Particular pumps are required to carry out gradient HPLC analysis, which allow on-line mixing of the mobile phase components

<table>
<thead>
<tr>
<th>Table 1. Gradient elution</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Other uses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Improved resolution</td>
<td>• More expensive instrumentation</td>
<td>• Column Cleaning</td>
</tr>
<tr>
<td></td>
<td>• Increased detection</td>
<td>• Possible precipitation at interfaces, when using multiple Proportioning valves</td>
<td>• Scouting runs for method development</td>
</tr>
<tr>
<td></td>
<td>• Ability to separate complex samples</td>
<td>• Re-equilibration time adds to analysis time</td>
<td>(more later)</td>
</tr>
<tr>
<td></td>
<td>• Shorter analysis times</td>
<td>• Instruments vary in their dwell volume (Vd), which can cause method transfer problems</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Decrease in column deterioration due to strongly retained components</td>
<td></td>
<td></td>
</tr>
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</table>

Isocratic Elution 70% aqueous/30% ACN  
Gradient Elution 20 – 60% ACN in 30 min.

Separation of Herbicides on C8 column - Use of gradient elution to separate samples having components that vary widely in polarity
Gradient Elution Analysis of Pesticides in Drinking Water - Use of gradient elution to separate low molecular weight mixtures having a large number of components

\[ k^* = \frac{67t_0 F}{\Delta \Phi V_m S} \]

- Lysozyme is 15X more sensitive to changes in organic modifier than benzene and 4X more sensitive than leucine enkephalin.

- Gradient conditions are required for the separation of Leucine enkephalin (555 daltons) and Lysozyme (14,000 daltons). Larger Molecules are More Sensitive than Small Molecules to Changes in % Organic - Use of gradient elution to separate high molecular weight mixtures (i.e., peptides and proteins)
Gradient Elution Parameters

The chromatogram shown below is an example of a reversed-phase gradient separation of herbicides.

**Important Gradient Parameters:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial %B/Final %B</td>
<td>20% : 60% ACN in 30min.</td>
</tr>
<tr>
<td>Gradient Time (Steepness)</td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>C8 4.6 x 150mm 5μm</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>A: H2O with 0.1% TFA, pH2</td>
</tr>
<tr>
<td></td>
<td>B: Acetonitrile</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>35°C</td>
</tr>
</tbody>
</table>

**Sample**

1. Tebufluron
2. Prometon
3. Prometryne
4. Atrazine
5. Bentazon
6. Propazine
7. Proparil
8. Metolachlor

**Separation of herbicides – illustrating a typical parameter set for Gradient HPLC**

Typically, two solvents are used, as indicated by solvent reservoirs A and B in the chromatographic conditions shown. Reservoir A, usually contains the weaker solvent, in this case, water with 0.1% TFA (pH 2). Solvent reservoir B contains the stronger solvent, Acetonitrile.

The elution strength usually increases with time, as shown opposite, where the gradient starts at 20%B and ends at 60%B over 30 minutes. The gradient shown is a linear gradient that changes at a rate of 1.33% solvent B per min.

The mixing of the mobile phase composition is achieved using the HPLC pump, and two methods of mixing are common. The first is low pressure mixing, in which the solvents are proportioned on the low-pressure side of the pump using solenoid valves. The second approach is known as high pressure mixing and occurs when two or more pumps are used to deliver solvents at differing flow rates into a mixing chamber.

To aid with solvent mixing, sometimes solvents are premixed or ‘doped’ (i.e. Solvent A contains 5% solvent B and vice versa).
Gradient Elution Principles

Gradient elution is most useful for reversed-phase and ion exchange liquid chromatography.

Typical Gradient HPLC Elution Profiles for Different Analyte Types

The gradient is formed by increasing the percentage of organic solvent. Consequently – at the beginning of the analysis, when the mobile phase strength is low, the analyte will be partitioned wholly into the stationary phase (or ‘focussed’) at the head of the column.
As the mobile phase strength increases, the analyte will begin to partition into the mobile phase and move along the column. As the mobile phase strength is increasing continuously, the rate at which the analyte moves along the column accelerates.

At some point within the column elution, the analyte may be wholly partitioned into the mobile phase, and will be moving with the same linear velocity as the mobile phase.

One cannot assign a fixed k value to a compound when gradient elution is applied. k is the retention coefficient and changes during the elution. Calculating k using the formula $k = (t_r - t_0)/t_0$ is only correct during isocratic elution!

The relationship between the gradient retention factor and the mobile phase composition depends upon molecular properties. Band spacing may change as column length is altered!
Peak Shape in Gradient HPLC

In isocratic elution the peaks are relatively broad, the peak width increasing with retention time. In gradient elution, the peaks are narrow with almost equal peak widths.

The main reason for the narrow peak shape is the velocity of the peak as it leaves the column. During gradient elution, all compounds accelerate through the column and thus elute at a high velocity. The retention time difference between compounds is a consequence of the percent organic modifier at which each starts to accelerate. All compounds should have approximately the same speed when they leave the column.

Another minor reason for peak focusing is the fact that the front and tail of a peak are residing in different concentrations of organic modifier. The tail will experience a higher percentage of organic modifier than the heart of a peak. The velocity of the tail will thus be slightly higher than the heart of the peak and vice versa for the front. This results in peak focusing. Asymmetric peaks are less frequently a problem in gradient elution. In practicality, the narrow peaks obtained in gradient elution provide better detection limits and higher loading capacities.
As the gradient is applied, the moderately disperse analyte band begins to move down the column – the front and rear of the analyte band are in slightly different mobile phase compositions to the main middle region of the band. Analytes at the front of the band migrate in a slightly lower concentration of organic and therefore migrate slightly slower through the column relative to the main band. Analytes at the back of the band are migrating slightly faster in a marginally higher organic concentration. This has the effect of 'concentrating' or 'focussing' the analyte band - so improving peak shape.

**Scouting Gradients**

When developing either gradient or isocratic separations – a scouting gradient analysis is a good starting point.

The scouting gradient is a linear gradient from 5-10% B to 100% B over a set time (20 minutes is standard). The elution strength is held at 100%B for a few minutes to make certain that all sample components have eluted. For reversed-phase gradient elution, water and acetonitrile are typically the eluents of choice.

**Typical Results from a Scouting Gradient Analysis**

\[ A: \text{NaH}_{2}	ext{PO}_{4} (20\text{mM}) \text{ pH 3.5} \]
\[ B: 5 - 100 \% \text{ Acetonitrile in 20 mins.} \]
\[ \text{Column: } 15 \times 4.6 (5\mu\text{l}) \text{ C18} \]
\[ \text{Flow: } 2\text{mL/min} \]

New initial gradient mobile phase composition = 54%B

\[ \Delta t_g < 0.25 \times t_g \quad (\Delta t_g = \text{gradient time, } \Delta t_g = (t_f - t_i)) \]

For the above scouting run – \( t_i \) (elution time of initial peak) = 12.8 mins \( t_f \) (elution time of final peak) = 21.2 mins

\[ \Delta t_g = 21.2 - 12.8 = 8.4 \text{ mins.} \quad 0.25 \times t_g = 0.25 \times 20 = 5 \text{ mins.} \]

\( \Delta t_g > 0.25 t_g \) therefore isocratic analysis is **NOT POSSIBLE** for this example.

**Typical Results from a Scouting Gradient Analysis**
The chromatogram obtained can reveal a lot about the required mobile phase composition for the separation.

If compounds are eluting more than 25% of the gradient time after the end of the gradient (i.e. after 25 mins. in our example), then a stronger B solvent is required for the analysis – or a less retentive column.

The equation shown can be used to determine if an isocratic separation is possible – this is always preferred to increase sample throughput (no re-equilibration time!) and simplify the analysis.

The mobile phase composition at which the first peak elutes, should be used as the initial mobile phase composition when developing a gradient.

**Estimating Isocratic Conditions from a Scouting Gradient**

![Diagram](image)

If isocratic analysis is found to be possible, the isocratic mobile phase composition can be simply estimated. Calculate from the scouting gradient the mobile phase composition for the average retention of the eluted compounds; that is:

$$t_{r_{(avg)}} = \frac{(b + b)}{2}$$

If isocratic analysis had been possible in this case then:

$$t_{r_{(avg)}} = \frac{(12.8 + 21.2)}{2} = 17.0\text{mins}.$$  
At 17.0 mins the mobile phase composition = 85.75% B

Therefore the mobile phase composition that should be used for the isocratic analysis is 14% Water (at the correct pH and buffer concentration) / 86% Acetonitrile

**Estimating Isocratic Mobile Phase composition from a Scouting Gradient Analysis**
Gradient Steepness

Gradient steepness is controlled by the mobile phase starting and ending composition and the gradient time. The steepness of the mobile phase gradient can have a significant effect on the separation – click the Gradient Steepness button opposite to see some examples.

The equation for the gradient retention factor \( (k) \) shown here can be found in many textbooks. Gradient retention factor is difficult to visualise as it differs from its isocratic counterpart and resembles more the profile of the gradient elution. It is effectively defined as the retention factor for an analyte that has migrated half way down the HPLC column.

\[
A: \text{NaH}_2\text{PO}_4 \ (20\text{mM}) \ \text{pH} \ 3.5 \\
B: \ 5 - 100 \% \text{Acetonitrile in 20 mins.} \\
\text{Column:} \ 15 \times 4.6 \ (5\mu\text{m}) \ \text{C18} \\
\text{Flow:} \ 2\text{mL/min.}
\]

\[
k = \frac{t_c F}{1.15 S \Delta \phi V_m}
\]

\( \Delta \phi \) = change in volume fraction of organic (final \%B – initial \%B) \\
\( S \) = constant determined by strong solvent and sample compound (small molecules (<500Da) the value is between 2 and 5; a value of 4 is used by convention when the value is not accurately known. Proteins have much higher values (typically between 50 and 100) and need longer gradient times for separation. \\
\( F \) = flow rate \\
\( t_g \) = gradient time (min.) \\
\( V_m \) = column void volume \((\pi r^2 L \times 0.68 \text{ - see section 2.4.6})\) \\
\( k \) = target value of 5 for average separation

Calculating Gradient Steepness (Gradient Time) from a Scouting Gradient Analysis

Usefully, the equation may be rearranged to obtain an expression that predicts the gradient time, based on a scouting gradient as previously described. Each of the terms in the equation are defined opposite. All terms in the equation are simply defined which makes the equation particularly useful in practical terms.

Gradient times above those estimated by the equation shown are unlikely to produce better resolution.
Theoretical Gradient Time

More gradient time will not considerably improve resolution.

\[ t_g = \frac{k(1.155\Delta\Phi V_m)}{F} \]

\[ t_g = 5 \frac{(1.15 \times 4 \times 0.95 \times 1.7)}{2} = 18.6 \text{ minutes} \]

- \( k = 5 \) for average separation
- \( S = 4 \) for small molecules
- \( \Delta\Phi (5\% - 100\%) = 0.95 \)
- \( V_m = 1.7 \text{ mL} \) (experimentally determined for 4.6 x 150 mm)
- \( F = 2 \text{ mL/min} \)

Gradient Steepness (Gradient Time) Adjustments

**Steep**

- \( t_g = 5 \)
- \( 0\% \text{B} \rightarrow 100\% \text{B} \)

**Shallow**

- \( t_g = 20 \)
- \( 0\% \text{B} \rightarrow 100\% \text{B} \)

The gradient steepness has a profound effect on the resolution of the analytes in the chromatogram. The same start and end compositions of mobile phase are used in these examples—the gradient time is the only parameter that is altered.

Effects of Altering Gradient Time (TG) to form steep and shallow gradients
Optimising Gradient Analyses

It is worthwhile taking a further look at the terms in the equation for the gradient retention factor, as there are several ways in which a gradient separation may be improved. Also, the manipulation of the gradient retention factor is counter intuitive from knowledge gained about isocratic HPLC.

\[
\bar{k} = \frac{t_0F}{1.15S\Delta\Phi\nu_m}
\]

- $\Delta\Phi$ = change in volume fraction of organic (final %B - initial %B)
- $S$ = constant determined by strong solvent and sample compound
- $F$ = flow rate
- $t_0$ = gradient time (min.)
- $\nu_m$ = column void volume ($\pi r^2L \times 0.68$ - see section 2.4.6)
- $k$ = gradient retention factor (k' value for analyte halfway down the column)
- $1/k$ = gradient steepness

From the equation it follows that in order to INCREASE the gradient retention factor you can:
- Use a longer gradient time (shallow gradient)
- Use a shorter column
- Use a higher flow rate
- Use a shorter Organic range ($\Delta\Phi$)

The use of shorter columns and higher flow rates to GAIN retention in reverse phase HPLC will be counter intuitive. You can click on some of the more important terms in the window opposite for a further explanation on how these changes will increase the gradient retention factor and how they may be usefully manipulated.

It should be highlighted that although $k$ is increasing, this will not necessarily improve the resolution – decreases in efficiency may counteract any benefit. This will depend upon the parameter being altered and the specific application type.

Adjusting Gradient Time (Steepness)

In this example the gradient time has been extended - so reducing the gradient steepness. This can affect the gradient retention factor as well as the selectivity of the separation.

*Increased gradient retention improves resolution of several peak pairs – 1,2 and 4,5.*

Improving a gradient separation of herbicides by adjusting the Gradient Steepness (TG)
Shortening Run Time in Gradient HPLC by keeping ‘b’ (1/K) constant – notice that the selectivity of the separation has not changed

Using a shorter column

Counter intuitively – the use of shorter columns can often improve separations using Gradient Analysis

This example shows the increase in resolution gained for this separation of proteins and peptides by decreasing Vm, using a shorter column, and therefore increasing the gradient retention term. It is counter intuitive compared to isocratic HPLC and something similar may happen with changes in flow rate. The effect may not improve resolution depending on changes in the efficiency of the separation.

The trade off between flow rate, column length and time is similar for isocratic and gradient elution. When separation time is a vital issue use a short column (5-15cm) and run at high flow rate. When maximal resolution is crucial it is advisable to use a long column (20-30 cm) at normal flow rate.
Compounds with a very steep velocity curve (e.g. proteins) do not benefit from long columns. These compounds quickly reach (after a few centimetres of acceleration) a $k' = 0$ i.e. no stationary phase interaction.

When applying steep gradients it is actually counter-productive to use a long column as the compounds reach $k' = 0$ (no chromatography) before leaving the column. The last part of the column then only adds band broadening.

**Increasing the Mobile Phase Flow Rate**

![Graph showing the effect of increasing mobile phase flow rate on gradient separations.](image)

Use of Mobile Phase flow rate to Optimise Gradient Separations

Usually gradient slope is expressed as “% / min”. Gradient slope for a given column dimension is however more correctly given as “% / mL”. It is flow and not time that transports a compound through the column. An increase in flow rate consequently means a decrease in gradient slope, i.e. typically an improvement in resolution and vice versa. The change in plate number with flow rate may counteract this. The net effect is case dependent. However, changes in band spacing may occur as with any alteration of a gradient slope.

The elution times are not very flow rate sensitive. This is best understood by considering a simplified case: Take a molecule that does not migrate when the organic modifier percentage is below 30% but when it is above 30% it move at the same velocity as the mobile phase. The gradient reaches 30% at 20 minutes.

At a flow rate of 1 mL/min the compound elutes at 22 minutes, i.e. it does not move for 20 minutes and then it goes through the column in two minutes.

Increasing the flow rate to 2 mL/min will results in an elution volume of 21 minutes – a much less significant change in retention behaviour compared to isocratic analysis!
Practical Gradient HPLC

A few practical considerations for running gradient elution chromatography:

- Due to long column equilibration times, applications using strongly retained components such as triethylamine are not suitable. For the same reasons, ion pair applications are not suited to gradient elution.
- Normal-phase HPLC applications utilizing bare silica columns are not suitable either. Long equilibration times lead to retention time imprecision.
- Solvents utilized in gradient elution must be pure. Water quality is of particular importance. Impurities are retained on the column while the composition of the mobile phase is weak. As the elution strength is increased, the impurities appear as peaks in the chromatogram. The chromatogram shown is a real-life example of these ghost peaks – the impurities are from the water, which has been inappropriately stored.
- To avoid precipitation problems within the instrument, test the buffer to make certain it is soluble in the final mobile phase composition.
- Finally, to increase retention time precision, make certain that adequate re-equilibration time is allowed between each chromatographic run. This is usually around 10 column volumes but may vary widely with different columns and applications. To be certain, retention time reproducibility should be verified during the analytical development.

Gradient Elution may not be suitable for:

- Applications utilizing strongly retained additives.
- Ion Pair Applications.
- Normal-phase liquid chromatography on bare silica due to water adsorption and irreproducibility.

Ghost Peaks in Gradient analysis can be caused by impurities in the solvents used - water is particularly susceptible to this phenomenon.
Importance of Solvent Cleanliness

Gradient-elution chromatograms generated using water stored in:
(a) polyethylene container,
(b) laboratory glassware,
(c) open containers

Column: C18
Gradient: 100% water to 100% acetonitrile in 20 min.
Flow rate: 1 ml/min.

Estimating Gradient Parameters

A: Na2HPO4 (20mM) pH 3.5
B: 5 – 100 % Acetonitrile in 20 mins.
Column: 10 x 4.6 (5μm) C18
Flow: 1 ml/min.

19.851 mins.

phenol / 4-nitrophenol / 2,4-dinitrophenol / 2-nitrophenol / 2,3-dimethylphenol / 4-chloro-3-methylphenol / 2,4-dichlorophenol / 2,4,5-trichlorophenol / pentachlorophenol

\[ t_g = \frac{k(1.15S\Delta\Phi V_m)}{F} \]

Assume \( S = 4, \bar{K} = 5 \)

Interactive Tutorial Exercise on Gradient Analysis – Important to Access Online material to get access to the full benefit of this exercise!