**Method Development in HPLC**

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**References:**
D A Skoog
Principles of instrumental analysis, 3rd Edition
 Chapters 25 and 27

D C Harris
Quantitative Chemical Analysis, 5th Edition
 Sections 25-3 and 25-4

Scope: Mainly Reversed Phase (RP-) HPLC

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**1. Stationary Phase Selection**

Match the polarity of solutes (in the sample) to that of stationary phase

- Non polar solutes separated on non-polar stationary phases.
  eg. Reversed phase (C18, C8 )

- Polar solutes separated on polar stationary phases.
  eg. Normal phase (Silica )

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**2. Mobile Phase Selection**

Polarity of the mobile phase is opposite to that of the stationary phase

- Polar Solvents for non-polar stationary phases
  eg. Methanol, Water, Acetonitrile, THF for Reversed phase (C18, C8)

- Non polar solvents for polar stationary phases
  eg. Hexane, Ethers for Normal phase (Silica)

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For Reversed phase, highly polar solvents are weaker solvents (i.e. lower eluent strength)

Polarity of water > ACN > methanol > THF

eg. Water is more polar than methanol thus a weaker solvent in RP HPLC
i.e. Water elutes the solute slower than methanol does. Therefore, when the percentage of water in the mobile phase is higher, the retention times are longer.
2. Mobile Phase Selection

Mobile phase solvents must be pure (HPLC grade solvents have only traces of contaminants. Water used is normally Milli Q grade).

Mobile phase must not produce a detector signal (i.e. a high back ground signal).

This is especially important with gradient elution

UV cut off for methanol is 205 nm
UV cut off for water is 190 nm
(See table 25-1 in Harris)

Question: Predict the shape of the background signal for the gradient of 10% methanol 15 min 90% methanol, detected at 200 nm.

How would you obtain a flat baseline at this detection wavelength?

3. Mobile Phase Preparation

1. Filter – To remove particulate matter (typically > 3 μM). Filtered under vacuum.

There are two different types of millipore filters, used depending on the composition of the mobile phase (organic or aqueous)

2. Degas – Dissolved oxygen and nitrogen cause:
   - Changes in flow rate
   - Signal disturbances
   - Base line noise
   - Fluorescence quenching (oxygen)

3. Mobile Phase Preparation

Methods of degassing:

- Refluxing – Boiling followed by condensing. Not safe with many solvents.
- Vacuuming – Creating a vacuum on solvent until it boils.
- Ultrasonification – Vibrating the solvent until dissolved gases are released.
- Helium Sparging – Bubbling He through solvent to replace dissolved gases. This is the most effective, safe and commonly used method.

4. Sample Preparation

Sample should be dissolved in the mobile phase or a solvent weaker than the mobile phase, before injection. Never in a stronger solvent.

eg. RP HPLC separation with 10% chloroform in acetonitrile as the mobile phase:
- Dissolve sample in mobile phase
- Dissolve sample in 5% chloroform in ACN
- Dissolve sample in pure ACN

Never dissolve in
- >10% chloroform in ACN or
- pure chloroform

4. Sample Preparation

Solute in stronger solvent give leading peaks

Gaussian band formed in the column due to mobile phase interactions

Band dragged by the stronger sample solvent

Detected peak shape

Resultant band shape
Other abnormal peak shapes

• Peak tailing- Due to extra active sites on the stationary phase
e.g. Polar silica sites on RP column

Some molecules retained longer than the others by the extra active sites

Tailed peak detected

Remedy: Using end capped RP columns or adding polar competing ions to the mobile phase (e.g. Et₄N⁺)

Other abnormal peak shapes

• Peak splitting- Due to column overloading

Split peak

Remedy: Reducing the sample size injected

5. Separation Optimisation

1. Increasing N:
   \[ N = \frac{L}{H} \]
   - Increase L by using a longer column
   - Reduce H by using smaller stationary phase particle size
   - Reduce H by increasing temperature
   - Reducing H by varying flow rate is not effective because the van Deemter curve is almost flat for LC.
   (An indirect way to improve resolution is to reduce peak asymmetry caused by peak tailing or leading)

5. Separation Optimisation

\[ R = \sqrt{N} \left( \frac{\alpha_s - 1}{\alpha_s} \right) \left( \frac{k'_s}{1 + k'_s} \right) \]

1. Increasing \( k' \):
   - Increase only up to ~10 because after that
   \[ \frac{k'_s}{1 + k'_s} \rightarrow 1 \]
   - Increase retention by
     - Reducing the eluent strength
     - Reducing temperature

5. Separation Optimisation

Optimisation of separation by mobile phase manipulation:

\[ R = \sqrt{N} \left( \frac{\alpha_s - 1}{\alpha_s} \right) \left( \frac{k'_s}{1 + k'_s} \right) \]

Target-
Increasing R (at least 1.5 for all peaks), while maintaining a constant \( k' \) (within the range 2-10).

i.e. Improving separation but not sacrificing the run time

5. Separation Optimisation

1. Increasing \( \alpha \):
   - Change the stationary phase to increase \( \alpha \).
   - Change the mobile phase to increase \( \alpha \).
   This is the most effective practical option thus in more detail →
5. Separation Optimisation

Optimisation of separation by mobile phase manipulation -

I. Isocratic elution -
- Select the best solvent composition
e.g. 25% methanol in water
- Maintain that composition throughout the run

Mixing solvents for isocratic run and limitations of isocratic elution method - See handout

Where
\[ \phi_c = \phi_b \frac{\delta_b}{\delta_c} \]

II. Gradient elution -
- Vary the solvent composition during the run so that the eluent strength gradually increases to achieve the optimum separation for all peaks.
e.g. 15% methanol 10 min 40% methanol

The column must be equilibrated with the initial composition before sample injection and subsequent gradient run.

5. Separation Optimisation

Types of gradients -

1. Step gradient (or segmented gradient)
- Vary the solvent composition in steps. Each step is similar to an isocratic run
e.g. 10% THF for 5 min.
15% THF for the next 10 min.
20% THF for the next 8 min.

2. Linear gradient
- Vary the solvent composition in a linear fashion. This is the most commonly used method
e.g. 10% methanol 10 min 30% methanol
i.e. 2% per minute linear rate

3. Non-linear gradient
- The rate of variation of the solvent composition is changed with time.
e.g. Logarithmic or exponential increase in eluent strength during the run
5. Separation Optimisation

**Dwell volume**
- This is the volume that the solvent has to travel from the reservoir to the beginning of the column.
- A gradient starts after a lag time from the injection, which corresponds to the dwell volume.
  
  eg. If dwell volume is 2 ml and the flow rate is 2 ml/min, the gradient starts 1 min after injection.

**Optimisation of separation by mobile phase manipulation**

Using gradient elution in method development:
- A scouting gradient is a good way to start method development for isocratic as well as gradient elution methods.
- A scouting gradient is a steep linear gradient.
  
  eg. 10% Methanol  20 min  100% methanol.

**Using gradient elution in method development**

- The chromatogram obtained for a mixture of compounds using a scouting gradient contains important information for further method development.
  
  - If all compounds elute close together, an isocratic elution method can be developed. The composition at the first peak is a good starting point for isocratic method development for the mixture.

- If the peaks are far apart, gradient elution is better suited. The initial gradient can be designed based on the position of peaks on the scouting run.
  
  eg. If the first peak elutes at 40% methanol and the last peak elutes at 70% methanol, start the gradient development from 40% methanol  20 min  70% methanol.

(Red section 25-4 Harris, p 739-742, for an example. Hands on practice at the compulsory HPLC practical session)

**Band Broadening in Liquid Chromatography**

Two factors which contribute to the separation of two compounds by chromatography are:
- differences in retention times
- broadness of peaks

Peak width is related to band broadening. Band broadening is measured as N and S of the peak.

S is due to specific problems such as peak tailing. N is due to:
  1. On column effects
  2. Extra column effects
1. Band broadening within the column

The van Deemter equation summarises on-column effects leading to band broadening.

\[
HETP = A + \frac{B}{u} + Cu
\]

Multiple paths  Longitudinal diffusion  Equilibration time

2. Band broadening outside the column

Band broadening occurs during the passage through the injector, detector and the tubing as they all have a finite volume.

Band broadening due to injector or the detector is expressed as:

\[
\sigma_{\text{inj}}^2 \text{ or } \sigma_{\text{det}}^2 = \left( \Delta t \right)^2/12 \quad \text{where } \Delta t \text{ is the time taken by the peak to pass through the injector/detector.}
\]

The observed variance, or the total variance (\(\sigma_T\)) is used to calculate N of the peak:

\[
N = \left( \frac{t}{\sigma_T} \right)^2
\]

Variances are additive. Therefore:

\[
\sigma_T^2 = \sigma_{\text{inj}}^2 + \sigma_{\text{column}}^2 + \sigma_{\text{det}}^2
\]

\(\sigma_T^2\) is obtained from the peak width
(At base \(W = 4\sigma\) and at half height \(W_{1/2} = 2.35\sigma\))

Problem

A band from a column eluted at a rate of 1.35 ml/min gives a peak with a half height width of 16.3 s. The sample was injected as a sharp plug with a volume of 0.30 ml. The detector volume is 0.20 ml.

Find the variance caused by the injector, the column and the detector.

What is the percentage of band broadening introduced by the injector and detector? (i.e. extra column band broadening)

Reducing the extra column band broadening:

1. Minimise the column dead volume
eg. Add more packing material

2. Minimise tube lengths (especially between injector-column and column-detector)

3. Apply the sample uniformly in a narrow zone into the column (sample must enter the column before mixing with the mobile phase)