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

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)

1. Stationary Phase Selection

Usually separation of low molecular weight (<2000), neutral molecules is carried out with RP HPLC.

eg. Separation of homologues series

However, isomers are better separated by normal phase such as silica because solutes have stronger, more specific interactions with the stationary phase.

Analytical separations are usually carried out on 3-10μM porous micro particle stationary phases.

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)

2. Mobile Phase Selection

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

Polarity of water > ACN > methanol > THF

e.g. 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:

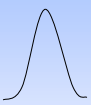
- Dissolve sample in mobile phase
- Dissolve sample in 5% chloroform in ACN
- Dissolve sample in pure ACN

Never dissolve in

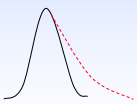
- >10% chloroform in ACN or
- pure chloroform

4. Sample Preparation

Solutes in stronger solvent give leading peaks

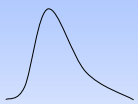


Gaussian band formed in the column due to mobile phase interactions



Band dragged by the stronger sample solvent

4. Sample Preparation

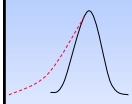


Resultant band shape

Detected peak shape

Other abnormal peak shapes

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



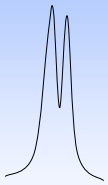
Some molecules retained longer than the others by the extra active sites

Tailed peak detected

Remedy: Using end capped RP columns or adding polar competing ions to the mobile phase (eg. Et_4N^+)

Other abnormal peak shapes

- Peak splitting- Due to column overloading



Split peak

Remedy: Reducing the sample size injected

5. Separation Optimisation

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha_{a,b}-1}{\alpha_{a,b}}\right)\left(\frac{k'_b}{1+k'_b}\right)$$

1. **Increasing N:**
   N = 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

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha_{a,b}-1}{\alpha_{a,b}}\right)\left(\frac{k'_b}{1+k'_b}\right)$$

1. **Increasing k':**
   Increase only up to ~10 because after that

$$\left(\frac{k'_b}{1+k'_b}\right) \rightarrow 1$$

Increase retention by
- Reducing the eluent strength
- Reducing temperature

5. Separation Optimisation

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha_{a,b}-1}{\alpha_{a,b}}\right)\left(\frac{k'_b}{1+k'_b}\right)$$

1. **Increasing α:**

- Change the stationary phase to increase α.
- Change the mobile phase to increase α.
  This is the most effective practical option thus in more detail →

5. Separation Optimisation

Optimisation of separation by mobile phase manipulation-

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha_{a,b}-1}{\alpha_{a,b}}\right)\left(\frac{k'_b}{1+k'_b}\right)$$

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

Optimisation of separation by mobile phase manipulation-

1. Isocratic elution-

- **Select the best solvent composition**

  eg. 25% methanol in water

- **Maintain that composition throughout the run**

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

5. Separation Optimisation

I. Isocratic elution-

Calculation of eluent strength for each composition is achieved by:

$$\phi_c = \phi_b \frac{\delta_b}{\delta_c}$$

Where

ϕ = solvent composition

δ = solvent strength parameters

(See fig. 25.24 in Harris for an example of selecting equivalent eluent strengths for RP separations)

5. Separation Optimisation

Optimisation of separation by mobile phase manipulation-

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.

eg. 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

eg. 10% THF for 5 min.

15% THF for the next 10 min.

20% THF for the next 8 min.

5. Separation Optimisation

Types of gradients-

2. Linear gradient

Vary the solvent composition in a linear fashion. This is the most commonly used method

eg. 10% methanol 10 min 30% methanol

i.e. 2% per minute linear rate

5. Separation Optimisation

Types of gradients-

3. Non-linear gradient

The rate of variation of the solvent composition is changed with time.

eg. 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

5. Separation Optimisation

Dwell volume –

- A gradient can be reproduced on a different instrument system, only if the dwell volume of the original system is known
- Determining the dwell volume can be done by:

– Determining the volume of tubing from the reservoir to column (using the internal diameters of the tubing)

OR

– Running a steep gradient without the column, after adding a detectable substance to the reservoir containing the strong solvent. The detector signal will be a sharp rise after a lag time (fig. 25.26 Harris) that corresponds to the dwell volume (flow rate x lag time)

5. Separation Optimisation

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

5. Separation Optimisation

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

5. Separation Optimisation

Using gradient elution in method development-

– 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

(Read 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.

$$HETP = A + \frac{B}{u} + C\bar{u}$$

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:

σ^2_{inj} or $\sigma^2_{det} = (\Delta t)^2/12$ where Δt is the time taken by the peak to pass through the injector/detector.

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

$$N = \left(\frac{t_r}{\sigma_T}\right)^2$$

Variances are additive. Therefore:

$\sigma^2_T = \sigma^2_{inj} + \sigma^2_{column} + \sigma^2_{det}$

σ^2_T is obtained from the peak width

(At base W = 4σ and at half height W1/2 = 2.35σ)

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)**