A Summary of Analytical Separations

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December 6, 2018

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1.1 Introduction

- HPLC stands for "High Performance Liquid Chromatography" or "High Pressure Liquid Chromatography"
- **Advantages**
  - Analysis of thermally unstable compounds
  - Analysis of nonvolatile compounds
- **Major requirement of LC**
  - Solute solubility in mobile phase
  - This is in contrast to GC which require solute volatility

1.2 Scope of HPLC

Adsorption chromatography (LSC)

Ion chromatography (IC)

Size-exclusion chromatography (SEC)

Partition chromatography separation of analytes by partitioning, most commonly to a stationary phase bonded to a solid support
Note that this replaces liquid-liquid chromatography with its problems of stripping of stationary phase

Hydrophobic interaction chromatography (HIC) for separation of proteins without denaturation

Hydrophilic interaction chromatography (HILIC) for separation of very polar analytes

Chiral chromatography

Affinity chromatography

1.3 Column Efficiency in HPLC

- Recall the van Deemter equation for GLC (commonly called GC) for packed columns:
$$H = A + \frac{B}{u} + Cu \tag{1}$$
- For longitudinal diffusion in LC, as the $D_l \approx 10^{-5} D_g$, the peak broadening due to longitudinal diffusion in mobile phase (liquid) phase in LC is *negligible*.
- i.e., $\frac{B}{u} = \frac{2\gamma D_m}{u}$

1.4 Pumps

High Pressure Pumps required to force liquid through a densely packed column at constant flow rate, so that t_r is reproducible.

Requirements of a pump:

- output pressure all the way to 6000 psi, without any leaks; for UHPLC, the pressure is typically $\approx 18\,000$ psi
- have variable flow rates, from 0.1 mL/min to 10 mL/min; for UHPLC, the pressure can go all the way down to 0.01 mL/min
- have a pulse-free output
- have a reproducible flow rate (ours to $\pm 0.3\,\%$)

1.4.1 Reciprocating Pumps

These are the most common today, replacing pneumatic and displacement (syringe) pumps.

A single piston reciprocating pump:

- an eccentric (off-center) can drives a piston back and forth

- its motion is synchronized with operation of check valves which control direction of flow
- Fill stroke: piston cavity fills, drawing solvent through the inlet check valve via suction; the outlet valve is closed
- delivery stroke: mobile phase forced through column as inlet check valve closes and the outlet valve opens. Pulsed flow produces baseline noise that must be damped
- electronic pulse compensation: speeds up piston during refill cycle, reducing the time the piston is not delivering solvent ($\approx 200\,\text{ms}$); this reduces the noisy baseline
- note that the pulse flow pulsates, but the time-averaged flow rate is constant
- the regular pressure surge spikes in the baseline may still be seen at extreme sensitive detector
- an elliptical cam minimize pulsations

1.4.2 Dual Piston Reciprocating Pumps

- essentially 2 single-piston pumps driven by the same motor
- 2 pistons are 180° out of phase, i.e., they are synchronized to provide a continuous flow of mobile phase
- when one is in the fill stroke, the other is on deliver stroke

1.4.3 HPLC solvents (Mobile Phase)

Solvents need to be HPLC or of similar high-grade for a stable baseline and to extend the life of the column, 'cause those things are expensive!

Filtration to remove particulate matter

Degassing to remove dissolved gases, especially N_2 and O_2, by vacuum or He sparging

1.4.4 Pressure Drop Across Column (Backpressure)

- Pressure developed when the liquid mobile phase is pumped through the (packed) HPLC column:

  enter the equation for backpressure

- therefore, for much larger backpressure occurs with smaller particles, more tightly packed column and a more viscous mobile phase

- there is a tradeoff between better performance and higher pressure

1.5 Elution

- Unlike GC, the mobile phase important in LC separations

- Analyte molecules and solvent molecules can compete with each other for binding sites on the stationary phase

- The greater the mobile phase eluent strength, the more easily solvent molecules displace analyte molecules from the stationary phase

  - A stronger mobile phase results in lower k and t_r

- Layers of solvent at the interface affect separations

  - e.g., for reversed phase separations, methanol forms a monolayer on a C18 surface, while acetonitrile forms a pool 1.3 nm deep into which analytes can dissolve with different binding energies.
  - Net result: acetonitrile is a stronger solvent than MeOH

- The solvent choice also influences peak shape

1.5.1 Elution Techniques

- Two major types of elution techniques in HPLC:

  - Isocratic elution (one solvent)
  - elution with a mobile phase with an unchanging composition over course of separation

    * e.g., 80/20 CH_3OH:H_2O, 50/50 CH_3CN:H_2O

    - Analogous to isothermal GC
  - Gradient elution
  - technique in which mobile phase composition gradually changed over course of separation
    - Analogous to programmed temperature GC
    - Gradient can be ionic strength, pH, and/or organic modifier (e.g., CH_3OH in water)
    - Used to improve resolution and sensitivity
    - Done with proportioning valve in pump

1.5.2 Factors to Consider in Gradient Elution

- Be sure that solvents are miscible in proportions used AND that solutes are soluble in all solvent compositions used during gradient elution
- Column equilibration (i.e., solvent and stationary phase) is required prior to each injection, otherwise k changes and t_r is not reproducible
  - Requires 10-20 column volumes, therefore expensive
- Organics (usually phthalates) present in the water used as the solvent tend to interfere
  - Concentrate at the head of the non-polar column, then elute as the organic modifier (e.g., CH_3OH) percentage increases
  - Limits sensitivity
- Gradient elution less reproducible, more expensive than isocratic!

1.6 Injectors

- Although various techniques (e.g., septum/syringe, stop-flow injection) have been used, most common device is a sampling valve
- Rotary sampling valve: injects sample from a sample loop using flow switching without depressurizing system
  - LOAD: sample loop loaded with syringe at atmospheric pressure
    - Mobile phase by-passes loop

  - INJECT: turning rotor changes flow lines, bringing loop into line, and sample swept into column
    * No flow interruption, only a slight pressure surge (turn rotor quickly to prevent large pressure pulse)
    * For best reproducibility in quantitative work ($\pm 0.2\,\%$) use loop overload (precalibrated, in sizes from $0.1\,\mu$L to $1000\,\mu$L)

1.7 Columns

Learning objectives:

- Size (conventional vs. prep-scale vs. UHPLC)
- Particle packing size (prep-scale to conventional to UHPLC)
- Packing type (normal vs. reversed-phase, linking, etc.)

1.7.1 General Types of Analytical HPLC Columns

- HPLC columns usually straight, stainless steel tubing containing packing of 3-10 μm d_p (for UHPLC, its about 1.7 μm)
- Analytical columns: for quantitative analysis
  - Conventional: 2.1-4.6 mm i.d., 10-30 cm long, 0.5-2 mL/min (1 mL/min most common)
  - Fast HPLC: 2.1-4.6 mm i.d., 3-10 cm long, 2-4 mL/min
    * Shorter, faster flow acceptable
    * Very low extracolumn volumes required in instrument
  - UHPLC: 2.1 mm i.d., 2-5 cm long, $\approx 0.5\,$mL/min to $1\,$mL/min
  - Capillary HPLC: 0.15-0.32 mm i.d., 5-10 cm long, 0.01 mL/min
    * Provides similar linear flow rates as conventional columns

1.7.2 Guard and Preparative HPLC Columns

- Guard columns:

  - Short column containing similar packing as analytical packing
  - Larger d_p to avoid increasing backpressure (ΔP)
  - Removes impurities from sample/solvent, protecting the expensive ($500-$2000+) analytical column

- Preparative columns:
  - Larger i.d. (e.g., 22.4 mm) and length (e.g., 25-100 cm) to increase sample capacity
  - Used to isolate and purify chemicals e.g., pharmaceutical, biochemical applications
  - Flow rates to 20 mL/min
  - Requires specialty pump capable of handling high flow

1.8 Detectors

1.9 Types of Chromatography in HPLC

2 GC-MS

3 Important Tips

Isocratic elution one solvent, or constant solvent mixture.

Gradient elution continuous change of solvent composition to increase eluent strength.
Gradient elution in HPLC is analoguous to temperature programming in gas chromatography.
Increased eluent strength is required to elute more strongly retained solutes.

General elution problem for a complex mixture, isocratic conditions can often be found to produce adequate separation of early-eluting peaks or late-eluting peaks, but not both. This problem drives us to use gradient elution.

Note: Elution strength decreases as the solvent becomes more polar, correct???

Separation factor, α Also called relative retention; for two components, 1 and 2, it is the ratio of their adjusted retention times.

The greater the relative retention, the greater the separation between two components.

Relative retention is fairly independent of flow rate and can therefore be used to to help identify peaks when the flow rate changes. (show equation?)