A Summary of Analytical Separations

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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

- Detector selection based on analytes to be detected
- Want a device that can provide a signal and operate in flow-through mode
- A wide variety of HPLC detectors are available, with different applications, advantages, drawbacks, sensitivities
- Optical detectors dominate market

1.8.1 HPLC Absorbance Detectors

- Measure changes in light absorbance as solutes swept through small (<10 μL) cell
- Thus, absorbance detectors measure a property of the analyte
- Characteristics:
  - Selective (response depends on molecular structure, i.e., $\epsilon(\lambda)$ = molar absorptivity)
  - Nondestructive, can collect effluent to isolate compounds manually or with an automated fraction collector

  - Good limits of detection (LOD): ng to as low as 1 pg when ϵ large (e.g., 10,000 to 20,000)
  - Compatible with gradient elution
    * But must not use solvents which don't absorb at wavelength of interest, i.e., are UV-transparent

1.8.2 Fixed Wavelength Detector (Filter Photometer)

- Uses low pressure Hg lamp, isolating intensive 254 nm emission line with filter
- Other Hg emission lines can be isolated with proper filters
  - 250, 313, 365 nm for example. There are others.
- Detection is restricted to compounds absorbing at 254 nm or other emission line used
- Quantification is based on Beer's law: absorbance (A) is directly proportional to the extinction coefficient (ϵ), path length (l), and concentration (c): $A(\lambda) = \epsilon(\lambda)lc$
- Note that different compounds have different $\epsilon(\lambda)$, so for a given chromatogram, peak heights don't reflect relative analyte concentrations
- Requires careful solute-by-solute calibration

1.8.3 Variable Wavelength Detector (Spectrophotometer)

- Uses continuous or broad-band light source e.g., D_2 lamp 190-400 nm
- Monochromator (grating optics) required for the dispersion of radiation and selection of a narrow bandpass for quantitative work
- Allows flexibility in choice of λ, which can be changed over course of run
- Operates otherwise like single wavelength detector, in that only 1 wavelength is measured at a time
- Wavelength switching is possible with appropriate software

1.8.4 Diode Array Detector (DAD)

- Uses an array of photodiodes (transducer which converts photon flow to electrical energy) to monitor many wavelengths simultaneously, rather than sequentially as with variable λ detector
  - Typical DAD has 328 diodes, each monitoring a 2 nm λ range, with circuitry on a 2 mm $\times$ 18 mm chip
- Obtains a complete spectrum in $<<$ 1 sec
- Overcomes slow λ scanning, too slow for HPLC with moving solutes
- Obtains spectra in 3D with appropriate software i.e., obtain complete spectra at specified sampling rate
  - A vs. λ: absorption spectra of each component as it passes through detector
  - A vs. t: chromatogram

1.8.5 Advantages of a Diode Array Detector (DAD)

- Increased sensitivity
  - Allows for quantitative determination of each solute at its λ_{max}
- More information is acquired as a full UV-vis absorption spectrum is obtained
  - Helps in analyte identification
  - Chemometrics
  - Note: Chemometrics is the use of mathematical and statistical methods to improve the understanding of chemical information and to correlate quality parameters or physical properties to analytical instrument data.
- Speed
- Disadvantage: cost compared to single and variable wavelength detectors

1.8.6 Refractive Index (RI) Detector

- A bulk-property detector, as monitors changes in the index of refraction of the column eluent with single or reference cells
- Monitors sodium D-line (589 nm) emitted for sodium lamp
- Advantages:
  - Universal detector as it responds to most compounds as long as they have different refraction index than mobile phase
  - Nondestructive
- Disadvantages:
  - Poor sensitivity (μg to ng in very favorable circumstances)
  - Very temperature sensitive, needs control to 0.001 °C for accurate work (i.e., sensitive to operating conditions)
  - Not compatible with gradient elution, as changing mobile phase composition also changes refractive index and therefore baseline. Even differential instrument can't keep up

1.8.7 Fluorescence Detector

- Responds to compounds that can be excited to fluoresce e.g., with a xenon lamp or more commonly with a laser
- A selective detector, extremely sensitive (pg to fg LODs)
- Very popular since many pharmaceutical products and environmental contaminants are fluorescent
  - Require rigid (e.g., fused ring) system for optimal fluorescence
  - Note that many coeluting analytes in the sample matrix aren't fluorescent and therefore won't interfere
  - Precolumn or postcolumn derivatization (on-line) can introduce a fluorescent label into a compound, e.g., dansylation of 1° or 2° amines
    - Note that this will change chromatography as a different chemical must be chromatographed!

1.8.8 Electrochemical Detectors

- Useful for analytes that can be oxidized/reduced
- Example: amperometric thin-layer detector
  - working electrode (e.g., glassy carbon) embedded in wall of flow channel and held at constant voltage (E_{app})
  - Current flows when a compound undergoes a redox reaction at the applied potential, with current $\propto$ C
  - Applied potential to electrode is adjustable, so detector may discriminate between different electroactive species
- Other detectors possible e.g., voltammetric, conductometric, coulometric
- pg detection limit for many analytes
- Mobile phase must be conductive AND isocratic, e.g., aqueous mobile phase ion chromatography or reversed-phase

1.8.9 Evaporative Light-Scattering Detector (ELSD)

- column effluent nebulized into fine aerosol using air or N_2 from nozzle
- Mobile phase aerosol is evaporated in a heated drift tube
- Fine particulates (the analytes) pass through a laser beam scatter the laser light
- Scattered light measured at perpendicular angle to flow
- Detector response is about the same for all nonvolatile analytes
- Detection limits more sensitive than refractive index detector
- Buffers in mobile phase must be used with care, as they must volatilize to avoid major interference with signal

1.9 Column Packings

There are two types:

- Microporous HPLC column packings
- Bonded phase HPLC packings

1.9.1 Microporous HPLC Column Packings

- Microporous HPLC packings currently used for columns:
  - small diameter (1.7, 1.8, 2, 3, 5, and 10 μm d_p)
  - fully porous
  - silica-based
- Characterized by:
  - High efficiency: small diffusional distances, so both stationary and mobile phase mass-transfer terms improved
  - High sample capacity, given high surface area since packing material is fully porous
  - Surface is fully hydrolyzed silica (silica heated with 0.1 M HCl for 1-2 days) with chemically reactive silanol groups

1.9.2 Bonded Phase HPLC Packings

- Bonded-phase packings have microporous silica support to which a liquid phase is covalently bonded
- No column bleed as with liquid-liquid columns
- Stationary phase produced by silanization
  - Choice of R determines functionality of stationary phase
    - e.g., R = $C_{18}H_{37}$ (bulky reagent reacting with <= 50% of surface silanol groups due to steric factors)
  - Many polar active sites left on surface which produce tailing problems in HPLC affecting performance

- To reduce this effect, end-capping with less bulky reagent to react with most free silanol groups
- A bulky i-butyl group can be used instead of a CH_3 group to increase stability (e.g., acid-catalyzed hydrolysis)

1.10 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?)