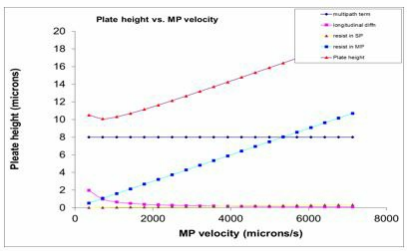
Basic Theory

Liquid Chromatography	MP → Liquid ▸ Actively participate in equilibrium process
	SP → Quasi/porous solid ▸ Most common do reverse-phase chromatography SP ▸ Film thickness → Very small (monolayer)
	$D_m \sim 10D_s$ ▸ B/U ~ 0
	$d_f \sim 0$ ▸ $C_sU \sim 0$
	Always carried-out in packed columns
Advantage	More versatile than GC
	Adaptable to needs
Disadvantage	Much less efficient than GC
	Diffusion coefficient of analyte is orders of magnitude smaller than in GC ▸ Bounced into other molecules →Diffusion rate is small
	In liquid phase (not gas)
Improve efficiency	Use small particle → Narrow range velocity ▸ Dependent on particle diameter (d_p) ▸ Smaller particle size = smaller plate height at any given velocity
	Compensate for travel distance of analyte to reach surface of SP ▸ Minimize the space between the particles that the analyte have to diffuse across
	Analytical LC → < 5μm
	HPLC → >3μm
	UHPLC → < 3μm

Theory Equations

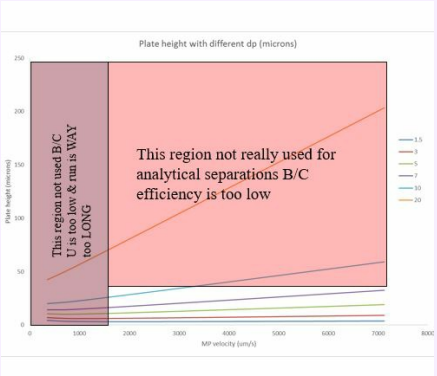


$$A = 2\lambda d_p \qquad \frac{B}{U} = \frac{2\gamma D_m}{U} \approx 0$$

$$C_sU = \frac{f_s(k')d_f^2}{D_s}U \approx 0$$

$$C_mU = \frac{f_m(k')d_p^2}{D_m}U$$

LC Velocity Range



Column

Packed Column	Packed full of particles
	Usually composed of stainless steel, etc.
	Length ▸ 2-20cm(analytical) ▸ Large column → Use for preparative scale ▸ Small column → Packaged inside a capillary (75-100um diameter) → Couple efficiently to MS

Column (cont)

Efficiency (N)

- 3000-20000
- Dramatically lower than GC
- Rule of thumb (GUESTIMATE ONLY): N~ 3500 *L(cm) / d_p(um)

Sample Capacity

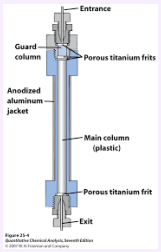
- Depending on size of column and packing
- ~5mg/g {[fa-arrow-right}} C18/silica
- ~10mg ➔ "vanilla" column

Thickness of SP

- ~ 1-2nm

Resistance to mass transfer in MP and multipath terms dominate

HPLC Column



HPLC System

MP reservoirs	Stores MP in inert glass bottles ▸ Platic coated Pyrex bottle (common) ➔ $300/pc
	Degas solvent ▸ Add element for filtering/degas ▸ Minimize amount of oxygen dissolved into MP ➔ Oxygen reactive in high pressure (increase oxidation of analyte) ▸ Small bubbles can form ➔ Result intensive undesirable peaks
	Connected to a computer(pump) ▸ Control mixing value to produces desired MP mixture

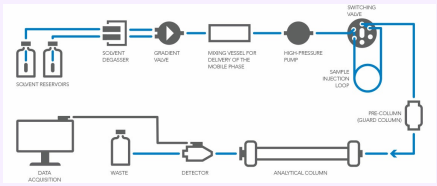
HPLC System (cont)

Analytical Column	Wide variety ▸ Diameter ➔ ~0.5cm (general) ▸ Length ➔ 10-20cm (general)
Injector	Manual ▸ Syringe with sample ▸ Inject needle into port and release ▸ Liquid flow into loop (at atmospheric pressure) ▸ When rotate lever to 60 degree ➔ Rearrange injector (set of valves) ▸ Switches loop into flow path = swept down into analytical column
	Operates at very high pressure ▸ If inject sample into septum ➔ Shatter syringe
	Autosampler ▸ Prepare in vials ➔ Tightly seal for sample to not evaporate ▸ Program computer ▸ Runs separation overnight ▸ ~100 samples
Detector	Record data and integrate peak area ▸ Quantitation
High-Pressure Pump	Direct MP through system
	Use high pressure ▸ Analytical column is filled with fine particles
Dynamic Mixer	Needed to blend the different fluids (MP)
	Provides the correct percentages of fluids dynamically as the separation goes

HPLC System (cont)

Guard Column	Avoid column killers ▸ Species that strongly store in SP → Never eluted ▸ Contaminated column ▸ Can change separation → Destroy separation in terms of its analytical quality
	Very small column ▸ Contains same type of SP as analytical column
	Contamination is trapped inside ▸ Periodically replace cartridges → Preserves analytical column
	Optional
Narrow Bore Tubing	Tubes that connects components
	Has to be rated for HPLC ▸ Can handle high pressure ▸ Has to be narrow bore → Don't want MP to mixing + dilute sample peaks
	Use short length as much as possible
	If fitting not installed correctly ▸ Can result in dead volume → Analyte that gets trapped in dead volume = gets broaden
Thermostat Oven	Constant temperature ▸ 30-45 degrees ▸ For a given MP → Equilibrium is constant
Fraction Collector	Robots that periodically move tubes of the eluted species from detector
	Collect in vials ▸ Sophisticated → Deposit 1 peak per vial ▸ Less sophisticated → Periodically move from one vial to the next

HPLC System Diagram



Stationary Phase

Control Retention	Control retention → Control distribution constant(K)
	Control by: ▸ Adjust type of MP and SP ▸ Adjust "strength" of MP and/or SP ▸ Add additives to MP → Interact specifically with analyte, SP, MP ▸ MP velocity → Does not alter retention (K or K')
Stationary Phase	Most use silica support particles ▸ Not great in high pH → Use alumina(high pH resistance) ▸ Low pH → SP can come off of support (hydrolyzed) → Use polymera support
	Almost always a "pure" SP ▸ Not mixed
	Use chemistry reaction to anchor SP to wall/surface
	Almost always a "monolayer" SP ▸ $d_f \sim 0$
	Wide range of polarities ▸ Use chemical reactions to adjust the SP
	Can use chiral SP ▸ Separate enantiomers ▸ Reasonable environment conditions

Stationary Phase (cont)

Silane Reaction	Use to anchor/bond silicones to silica surfaces ▸ In packing materials (particles) ▸ FS capillaries
	Use to deactivate silanols
	Silanol ▸ Very reactive ▸ Highly polar ▸ Expose on surface of silica
	Deactivate silanol ▸ Use chloro silane ▸ Ex: C18 ▸ Result in silanization of surface
	Residual silanol ▸ SP is usually of a different polarity (non-polar) ▸ Results in tailing of analytical peak
	After reacting surface with SP ▸ Use a short chain alkyl ▸ Take care of residual silanol
	If silanol peaks present ▸ Column is old ▸ Molecules of SP are desorbs or removed from surface
Silanol Interactions	"Standard" silica → SP support particles ▸ Has silanols on surface → Si-OH ▸ ~50% of Si-OH are reacted to Si-O-Si-C18

Stationary Phase (cont)

	Residual Si-OH ▸ When close to a metal in the silica ▸ Are "acidic" and deprotonate easily leaving a $Si\text{-}O^-$ on the surface
	Act as ion-exchange sites for basic analyte ▸ Reverse-Phase MP is not suited to ion-exchange separation ▸ Very poor peaks are obtained for basic analyte → Tailing peak
	Alternative options ▸ Use a high purity silica column → Less acidic silanol ▸ Purchase a deactivated silica column
	Affects only basic compounds ▸ Neutral and acidic compounds does not show tailing
Particle and Surface Area	Terms that dominate: ▸ Overall plate height ▸ Overall plate number ▸ d_p → VD eq.: A and C_mU
	Spherical Particle ▸ Surface area/Volume scales with $1/d_p$ ▸ ↑ A/V = ↓ Retention ▸ More SP packed = ↑ Retention (K') = ↑ Resolving Power (R')
	Size ▸ Nearly all SP are um scale silica particles ▸ Impacts VD equations

Stationary Phase (cont)

	Porous Particles ▸ ↑ Surface area per particle ▸ ↑Amount of SP inside column ▸ ↑ Retention and sample capacity = Better R' ▸ Smaller the pore→ the larger the surface area/g of support
	Diffusional Trap of small pores ▸ Loss of analyte → Tailing peaks ▸ Large MW analyte go into small pores → Never gets eluted
	Separation molecules ▸ Small molecules ~ 80Å ▸ Large proteins ~120-300Å
Normal Phase (NP)	Developed initially ▸ Used raw silica as SP → Polar silanol
	Separation based ▸ Polar-polar interactions with silanol ▸ Non-polar elute earlier ▸ Polar analyte elute later
	In general ▸ MP is opposite polarity to SP ▸ Works well for polar species only
Reverse Phase (RP)	Use non-polar SP → Silane reaction ▸ Use polar MP (Water based)
	Separation based ▸ Nonpolar-Nonpolar interactions ▸ Reverse separation of normal phase ▸ Polar species elute first ▸ Non-polar species elute later

Stationary Phase (cont)

More popular
- Organic solvents used for MP → Expensive/dangerous
- Most analytes are made our of biological origin → Soluble in water-based MP

Controlling Retention

SP Polarities	Retention depends on ▸ Mass of SP ▸ Type of SP
	Mass ▸ Control by chain length ▸ Density of SP on silica→ % of silanol reacted ▸ Surface area →Porisity
	Selectivity depends on ▸ Type of SP ▸ Chain length ▸ Linker type/length
Chain Length	↑Chain length and/or % organic (carbon) load of SP = ↑ Retention (K')
	Example ▸ C4 → C8 chain ▸ Double chain length → Double volume of SP → Double retention
	If within the same type of SP ▸ Ex: Alkyl chains ▸ No significant changes in selectivity ▸ Only shrinking/expanding the chromatogram about t m ▸ Shrink c-gram = reduce carbon ▸ Stretch c-gram = Increase carbon load

Controlling Retention (cont)

	If analyte is not retained ▸ Increase % carbon of SP/chain length
	If analyte is excessively retained ▸ Reduce % carbon
	Significant changes in selectivity → Resolving power ▸ By changing the type of SP ▸ Overall retention should remain roughly constant ▸ Keeping the % carbon constant
Effect of MP Strength	MP plays an active role in retention ▸ Distribution constant
	Common solvents can be sorted according to their polarity
	Polarity of MP ▸ Main factors of controlling K → K'
	When changing MP strenght ▸ Can calculate the retention under new MP
	Equation (Only for RP): ▸ K'new/K'old= 10((P'new-P'old)/2)
	Equation (Polarity) P'MP= Weighted Polarity = %A*PIA + %B* PIB ▸ PI= Polarity
	Equation (Only for NP): ▸ K'old/K'new= 10((P'old-P'new)/2)
Example	1. Look at K' of first and last peak ▸ K'old = (last peak - first peak)/first peak →K' = (2.8-1.8)/1.8 = 0.5 ▸ K'new → Want it at 10
	2. Replace terms in equation ▸ K'new/K'old = 10/0.5 = 20

Controlling Retention (cont)

	3. Old MP polarity ▸ If old MP is 20% water/80% Acetonitrile ▸ P'old= (0.2)(10.2)+(0.8)(5.8) = 6.68
	4. New MP polarity ▸ P'new = 2log(K'new/K'old) + P'old ▸ P'new= 2log(20) +6.68 = 9.28
	5. Solve new MP components ▸ P'new = (x)(10.2) + (1-x)(5.8) = 9.98 ▸ 79.1% water and 20.9% ACN
	Rule of 3 ▸ Tool to check/estimate results (not used in calculations) ▸ Change of 20% water ~ 3x change in K'
MP Gradient	Dynamically adjust MP
	Some sample contain wide range of analytes ▸ Low or high retention ▸ no single MP that will elute them all in a satisfactory range of k’
	MP gradient ▸ MP strength is initially "weak"→Analyte well retained ▸ Those with low retention→Elute at reasonable K' ▸ Strengthen MP over the course of separation ▸ Strongly retained species can be eluted→At a reasonable K" and R'
	MP≠constant → Changing strength ▸ K and K' ≠ constant ▸ Can no longer be predicted

Controlling Retention (cont)

MP Selectivity

Alter selectivity (α) by changing type of solvent

Resolving power

- R= (α-1)(K'/(1+K'))(√N/4)
- Sensitive to selectivity → Critical pairs in peaks

Selectivity

- Depends on nature of MP
- Change selectivity = change type of MP

Try and keep

- P'old~P'new
- Overall retention is roughly the same
- Selectivity of peaks change

Selectivity changes cannot be predicted

Chiral Separation

Basic Theory

Important to bioanalyses and pharmaceutical separation

Separation of chiral species

- Enantio selective

Chiral SP or chiral additives to MP → Separation of enantiomers

Possible to separate structural isomers → Strength of interaction changes as a function of isomer

Ion-Exchange

Ionic Species

Small "hard" ions

- Inorganic ions
- Cannot use ion pairing
- Ions can interact with appropriate SP → Ionic SP

Ion-Exchange (cont)

Basic Theory

Ion-exchange

- Equilibrium-based separation
- Discreet soption and displacement process
- Carry throughout column

Column

- Does not use silica particles
- Use polymer resin
- Attach with a strong anion or cation

Example

Using a strong anion SP

- Cation exchange column

1. SP sulfonic acid (IEX resin) → Anion surface particle (SO_3^-)
   - Wash column with acid solution {{fa-arrow-right}} Cations (H^+)
   - Sulfonic acid is protonated (SO_3H)

2. Inject sample with cation analytes → Metal ion (M^{2+})
   - Metal ions interact with SP
   - Metal ions displace some of H^+ from resin

3. Unbind analyte from SP
   - Introduce a higher concentration of protons behind analytes → MP gradient
   - H^+ displace weakly bound analytes the move onto strongly bound analyte (cation) → Exchange process
   - Cation analytes is displace off of surface and solubilize in MP

Ion-Exchange (cont)

4. Analytes move down the column in strength of MP
- Each type of analyte elute as a peak
- MP ahead of each analyte is too weak → Bound
- MP behind each analyte is too strong → Fully displace

Equilibrium Constant

If it behaves like an equilibrium → There is an equilibrium constant
- Expect to behave like an LC → Produce peaks

Ion-exchange equilibrium constant would behave like a distribution constant
- Obtain similar result of chromatogram peaks
- MP controls retention

Equation
- K_{iex}=[exchange&analyte]$_s$/[analyte]$_m$ = C_s/C_m

Optimization

Process of Separation

1. Carry out initial separation
- Choose a strong MP
- Ensure everything is eluted and fast separation

2. Adjust MP strength
- Retention of last peak is within the right region
- Depending on the complexity of sample
- Simple sample → K' ~ 10
- Complex sample → K'~ 20
- Do calculations for an estimate adjusting needed

Optimization (cont)

3. Examine if peaks are within the acceptable region
- Examine if all analytes are well resolved

4. Consider if a gradient is required
- Presence of large area of empty baseline

5. If needed
- Switch MP type to alter selectivity
- Gradient to reach acceptable retention and resolution

6. Consider using additives in MP
- Help alter selectivity

If MP type/mix strength does not achieve required separation
- Change SP type
- May consider type of separation

Summary of MP Effects

Very powerful tool → Versatile
- Control retention and selectivity

Directly affects distribution constant

↑ MP strength = ↓ K'

MP "strength" is polarity
- Effect are opposite in RP vs NP
- RP → Non-polar solvent (organic) = Stronger solvent
- NP → Polar solvent = Stronger solvent

Ramped MP → Gradient
- Helps dynamically adjust K'

Useful to make separation less intuitive → R↓ = K' ↓
- Gradient runs → R ↑ = K' ↓

LC Detectors

Ideal Detectors	High sensitivity ▸ Steep slope
	High stability ▸ Minimal drift ▸ Minimal noise on baseline
	Very low DL
	Long LDR
	Can accept MP over wide range ▸ Need reference to null out MP gradients
	Fast response ▸ Independent of MP
	Easy to use, maintain and repair
	Inexpensive
	Selective/universal ▸ Can be either depending on properties
	Non destructive ▸ Can collect fractions
1λ: UV- Vis Detector	Volume ▸ ~1-10 uL (very small) ➔If V is too large, the signal becomes constant and we see a square shaped peak
	Pathlengths ▸ ~5-10mm ▸ Longer = better Bigger absorbance for same concentration (beer--lambert law)
	Window material ▸ Quartz

LC Detectors (cont)

	D_2 lamps ▸ Good broad UV source ▸ 185-400nm ▸ Spectrometer to isolate narrow band of wavelength ▸ More simple than FAA ➔UV does not have to compensate for a flame
	2 sensors ▸ Sample diode (I) ➔Intensity coming through the sample ▸ Reference diode ($I_○$) ➔ Intensity from the light source ▸ Equation ➔A= -log(I/$I_○$)= -log(T)
	Chromatogram ▸ Abs vs time ▸ Use peak area for quantitation
Many λ: Photodiode array (PDA) Detector	Chromatogram ▸ Collect many chromatograms across many wavelength (a spectrum)
	Sensitivity ▸ Can choose/use chromatogram that provides the greatest sensitivity for each analyte ▸ Find wavelength where analyte has least interference from neighbouring peaks

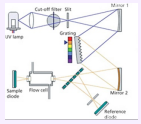
LC Detectors (cont)

	Application ▸ Useful to verify which peaks is which when MP is changed
Refractive Index (RI) Detector	Uses refractive index of analyte compared to MP ▸ Snell's law ➔ The rays will bend if there is a mismatch in refractive indices of the outside and the inside ▸ When refractive indices match ➔ Rays not refracted
	Chromatogram ▸ If RI match (only MP) ➔ Full intensity reaches sensor ▸ If RI does not match (analyte eluting) ➔ Reduced intensity reaches sensor ▸ Plot signal vs time
	Properties ▸ Universal ▸ Sensitivity ➔ ~3 orders of magnitude less sensitive than UV ▸ Absorbance ➔ Optically silent ▸ Reference flow ➔ Limited gradient capability

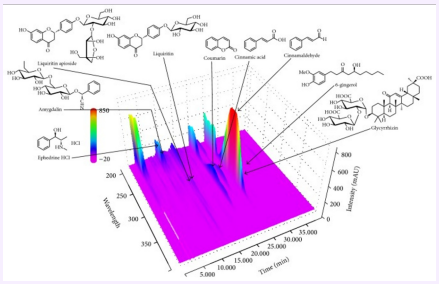
LC Detectors (cont)

Evaporative Light Scatter (ELS) Detector	How it works ▸ Uses nebulizer to produce aerosol ▸ MP evaporates ➔ Leaves behind analyte fine crystals ▸ Scattering of light (usually laser) ➔ Only when crystals are present
	Analyte ▸ Needs to produce crystals ➔ Very low volatility ▸ Can work for non-absorbing analytes ▸ Response is nearly uniform for all analytes
	Buffers (MP) ▸ Must be volatile ➔ Restricts choices ➔ Can't use inorganic buffers: Leads to buffer salts
	Better than RI detector ▸ Higher sensitivity ▸ Longer LDR

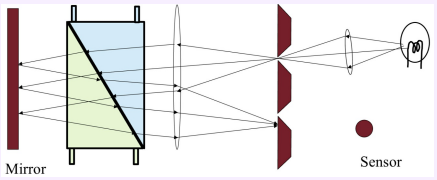
UV-Vis Detector Diagram



Photodiode Array Chromatogram



Refractive Index Detector Diagram



LC-MS

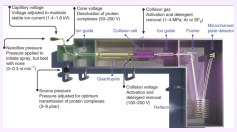
Properties	Electrospray ▸ Sample goes through nebulizer ▸ High voltage is applied → Produces charged droplets
	Fine metal capillary tube ▸ ~0.5-1mm ▸ Connected to the outlet ▸ Charged with high voltage
	Signal ▸ MP is pumped ▸ Charged droplets are attracted to MS interface ▸ Droplets dry down in flight → charge density ↑ until charge repulsion causes coulombic explosion
Single Quadrupole MS	Mass spectrum ▸ Simple ▸ MP evaporates away → Leaves $[M+H]^+$ ions → no fragments
	Problem ▸ Difficult for definitive ID ▸ Potential m/z overlap
Triple Quadrupole MS	Allows the production of fragments ▸ Contains Q1, Q2(CID) and Q3
	Q1 → Parent ions are selected Q2(CID) → Collision induced dissociation → Selected ions collide with Ar/He/N2 (Creates fragments) Q3 → Fragment ions are filtered/scanned → Detected to produce mass spectrum

LC-MS (cont)

	Mass spectrum ▸ Scanning mode → Produce full spectrum → For method development ▸ Multiple reaction monitoring (MRM) → Only selected fragments are measured → For quantitation
	Better than single quadrupole ▸ Lower DL → Less interferences ▸ Longer LDR ▸ Allows positive ID of analyte ▸ Better selectivity with MRM
	Problem ▸ Q3 scans across m/z range pretty slowly (1-30 spectra/s) ▸ Lowering resolution allows faster scanning → Can't get a full detailed spectrum
QTOF MS	Quadrupole time of flight MS
	Advantage ▸ Can scan 10000 spectra/s → Many are averaged together to improve quality (better than QQQ) ▸ Allows more analytes to be measured simultaneously ▸ Higher mass accuracies and resolution → Permits greater ID power ▸ LDR>5 orders of magnitude
	Problem ▸ Not as precise as QQQ ▸ Expensive

Triple Quadrupole Detector Diagram

QTOF MS Detector Diagram



Summary and Applications

Advantage	MP plays a critical role in controlling separation ▸ Retention ➔ "Strength" ▸ Selectivity ➔ "Type"
	Wide range of MP available ▸ Diverse set of separation conditions ▸ Within the same SP and column ▸ Allow to quickly try different separation conditions ▸ Allow to quickly arrive to a newer optimization separation
	No requirements of volatile analyte ▸ Needs to be soluble in MP
	Wider range of SP available ▸ Can choose type ▸ Can change particle size ▸ Can choose the amount of SP/unit of column
	Easy to collect purified analyte
Disadvantage	Much lower N compared to GC-FSOT ▸ Degrades R and ↑ Overlapping peaks ▸ Many LC have low N ➔ 1000-5000
Detector Comparison	Selective or universal
	DL
	LDR

Summary and Applications (cont)

	Cost ▸ Purchase ▸ Maintenance
	Sample capacity
	Immune from MP gradients?
	Amendable to using IS?
Key Factors if LC is useful	1. Analytes soluble in liquid MP
	2. Concentration of analytes are high enough ▸ Can load larger volumes/concentraton on columns ▸ Combine with sensitive detectors
	3. Does sample require a high R' separation ▸ GC favored over LC
	4. Need to recover analyte ▸ LC > GC
	5. Slower than GC
Applications	Anti-dopping and forensics
	Pharmaceutical ▸ Process control ▸ Quality control ▸ R&D ▸ Metabolic ▸ Proteomic
	Food and Beverages ▸ Vitamins ▸ Pesticides ▸ Contaminants
	Environmental ▸ Pesticides ▸ Industrial materials

Summary and Applications (cont)

R&D

- Organic synthesis
- Catalysis

Industrial

- Feedstock

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