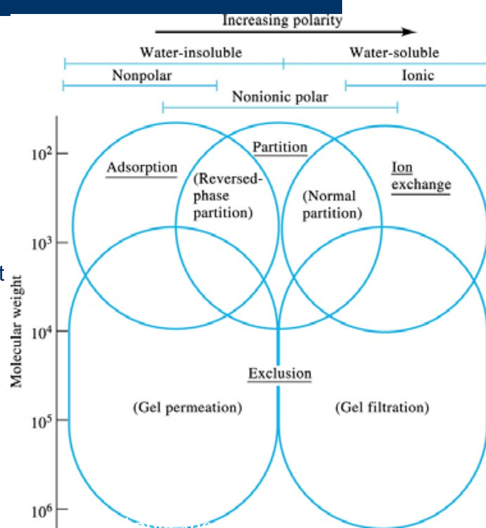
Chapter 28. Liquid Chromatography

- Scope of HPLC
- Column Efficiency in LC
- LC Instrumentation
- Partition Chromatography
- Adsorption Chromatography
- Ion Chromatography
- Size-Exclusion Chromatography
- Affinity Chromatography
- Thin-layer Chromatography

Scope of LC

- Liquid Chromatography (LC) : older
- **Advantages of LC vs. GC:**
  - Applied to the separation of any compound that is soluble in a liquid phase. → biological compounds, synthetic or natural polymers, and inorganic compounds
  - Liquid mobile phase → lower temperatures → compounds that may be thermally labile
  - Retention of solutes in LC depend on their interaction with both M and S phase. → more flexible in optimizing separations
  - Most LC detectors are non-destructive
- **Disadvantage of LC vs. GC:**
  - LC is subject to greater peak or band-broadening. → low R



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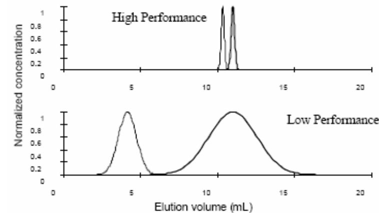
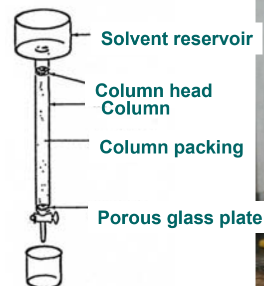
Traditional LC vs. HPLC

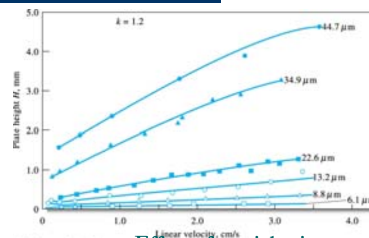
- Liquid chromatography
  - Use large, non-rigid support material
  - Particles size d_p: > 150 µm, column size d_c: 10 ~ 50 mm, column length L: 50 ~ 500 cm, flow rate F: < 1mL/min
  - Gravity. Large H, small N
  - Poor system efficiencies and large plate heights
- High-performance liquid chromatography (HPLC)
  - Use small, uniform, rigid support material
  - Particle size d_p < 40 µm, usually 3-10 µm in practice
  - Good system efficiencies and small plate heights, narrow peaks, shorter separation times



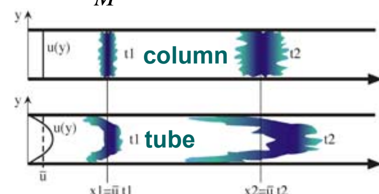


Column Efficiency of HPLC

- Zone spreading in HPLC
  - No minimal point for H vs. flow rate
  - Effect of particle size of packing:
    - Mobile phase mass transfer coefficient C_M →particle size d_p ↓ → Efficiency ↑
    - Diffusion term B/u is negligible
  - Extra column band broadening: $$H_{ex} = \frac{\pi r^2 u}{24 D_M}$$
    - Open tube in pipes, injection, detection system
    - Cross over rate difference
    - Serious for small-bore column
    - Not serious for GC for big D_M
    - Min. r and L



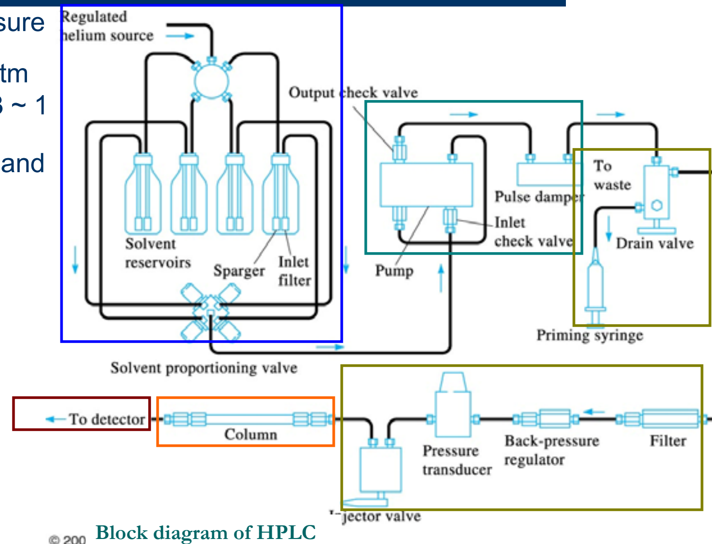
Effect of particle size on packing and flow rate on plate height in LC



HPLC Instrumentations

- High pressure several hundred atm
- Packing: 3 ~ 1 μm
- Elaborate and expensive

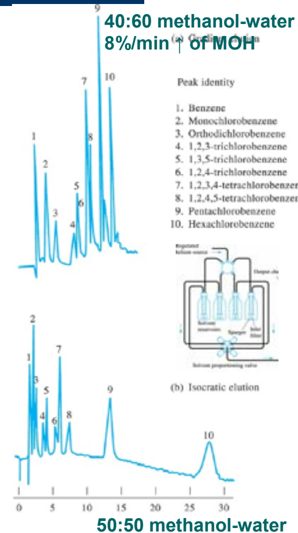
1. Solvent treatment system
2. Pumping system
3. Sample injection system
4. Column
5. detector



© 200 Block diagram of HPLC

Instruments – Solvent System

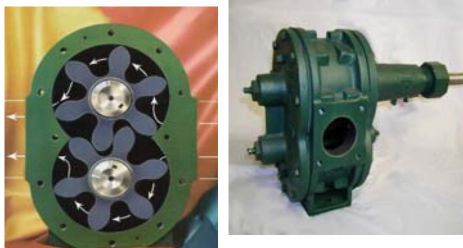
- Mobile phase reservoirs:
  - Several reservoirs (> 500mL)
  - Degassing: remove of dissolved gas → band spreading and interfering detection
    - Sparging: fine bubble of gas
    - vacuum pumping, distillation, heating
  - Dust removal: → interference with detection, column clogging, damage pumping system
    - Millipore filter under vacuum
  - Isocratic elution: constant composition
  - Gradient elution: different solvent systems during elution, continuous change or step wise, solvent proportion valve

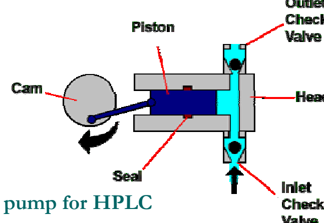


Instruments – Pumping System

- Pumping systems:
  - Requirement: high P (6k psi), pulse-free, constant F (0.1 ~ 10 mL/min.), reproducibility (0.5%), resistant to corrosion
  - Displacement pump (Screw-driven syringe pump): pulse free, small capacity (250 mL), no gradient elution
  - Reciprocating pump: Most widely used. Small internal volume (35 ~ 400 μL), high pressure (10^5 psi), gradient elution, constant flow. Need pulse damper

A displacement pump for HPLC

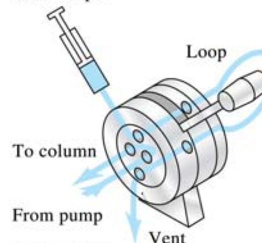




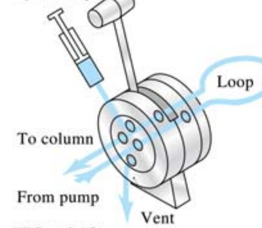
A reciprocating pump for HPLC

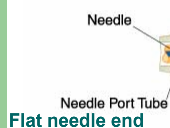
Instruments – Sample Injection System

- Sample Injection system:
  - Limit of precision of HPLC
  - Sample size: 0.5 ~ 500 μL
  - No interference with the pressure
  - Based on a sample loop, 1 ~ 100 μL, Reproducibility: 0.1%, P < 7000 psi
  - Auto sampler: inject continuously variable volume 1 μL – 1 mL
    - Controlled temperature environment for derivatization reaction

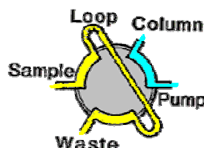


A sampling loop for HPLC



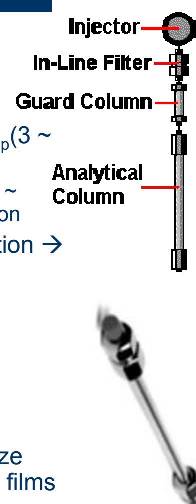


Flat needle end



Instruments - Columns

- Column: \$200 ~ \$1000
  - Stainless steel tubing for high pressure
  - Heavy-wall glass or PEEK tubing for low P (< 600 psi)
  - Analytical column: straight, L(5 ~ 25 cm), d_c(3 ~ 5 mm), d_p(3 ~ 5 μm). N (40 k ~ 70 k plates/m)
    - Microcolumn: L (3 ~ 7.5 cm), d (1 ~ 5 mm), d_p: 3 ~ 5 μm, N: ~ 100k plates/m, high speed and minimum solvent consumption
  - Guard column: remove particulate matter and contamination → protect analytical column, similar packing
  - T control: < 150 °C, 0.1 °C
- Column packing: silica, alumina, a polystyrene-divinyl-benzene synthetic or an ion-exchange resin
  - Pellicular particle: original, Spherical, nonporous beads, proteins and large biomolecules separation (d_p: 5 μm)
  - Porous particle: common used, d_p: 3 ~ 10 μm. Narrow size distribution, porous microparticle coated with thin organic films



Instruments - Detectors

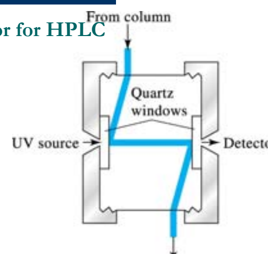
HPLC Detector	Available	(typical)	(decades)
Absorbance	Yes	10 pg	3–4
Fluorescence	Yes	10 fg	5
Electrochemical	Yes	100 pg	4–5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg–1 ng	5
Mass spectrometry	Yes	<1 pg	5
FTIR	Yes	1 μg	3
Light scattering	Yes	1 μg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4–5
Photoionization	No	<1 pg	4

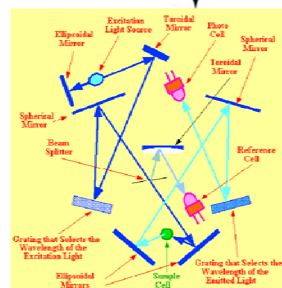
- Low dead volume: ↓extra-column band broadening
- Small and compatible with liquid flow
- No highly sensitive and universal detector as GC
- Types: Bulk-property detector and solute-property detector

Instruments – Detectors 1

- Absorption detectors:
  - UV-Vis: Most widely used
    - Z-shape, flow-through cell (V, 1 ~ 10 μL and b, 2 ~ 10 mm)
    - Photometer: Hg 254 nm and 280 nm line for organic, D_2 or W filament + interference filter
    - Spectrophotometer: more versatile
  - IR: filter instrument or FTIR
    - Similar cell (V, 1.5 ~ 10 μL and b, 0.2 ~ 1.0 mm)
    - Limit: no suitable solvent, special optics
  - Fluorescence: Hg or Xe lamp
    - Fluorometer and spectrofluorometer
    - Fluorescing species or fluorescent derivatives

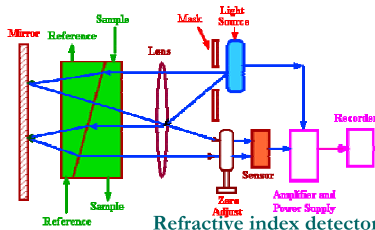
A UV-Vis detector for HPLC



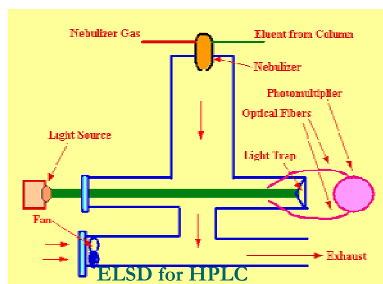


Instruments – Detectors 2

- Refractive index detectors (RI):
  - General, unaffected by flow rate
  - DA: limited sensitivity (1 ng/μL), highly T dependent (0.001°C), no gradient elution
  - Half solvent/eluant, η difference → bending of the incident beam
- Evaporative light scattering detector:
  - ELSD: new, laser beam
  - Nebulizer →fine mist in N_2 → solvent evaporation in drift tube → fine particles → scattered radiation
  - A: same for all nonvolatile solutes, more sensitive than RI, 0.2 ng/μL
  - DS: mobile phase must be volatile



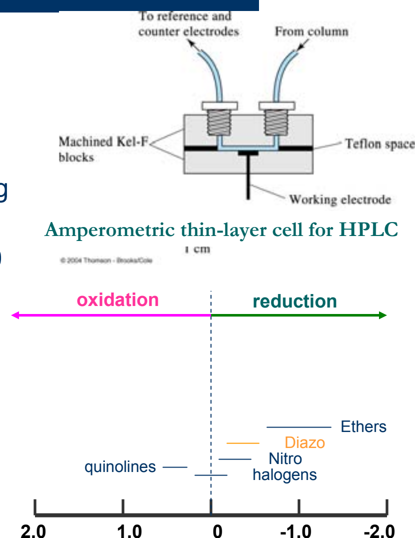
Refractive index detector



ELSD for HPLC

Instruments – Detectors 3

- Electrochemical detectors:
  - Amperometry, voltammetry, coulometry and conductormetry
  - A: simplicity, high sensitivity, convenience and wide-spreading application
  - Thin-layer flow cell of Teflon : 50 μm thick, 1 ~ 5 μL volume
  - Indictor E: Pt, Au, C
  - RE and CE: down stream
  - Multi-electrode: simultaneous detection or sample purity indication



Amperometric thin-layer cell for HPLC

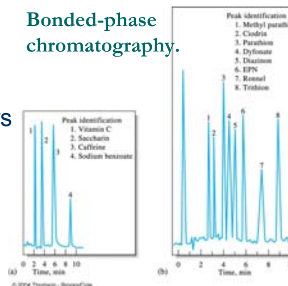
Partition Chromatography

- Stationary phase: liquid, immiscible with the mobile phase. Nonionic polar compounds (M_r < 3k), bonded (C_8 or C_{18})
- Normal-phase partition chromatography:
  - Highly polar stationary phase, triethylene glycol or water
  - Nonpolar solvent (mobile phase), hexane or i-propyl ether
  - Compound polarity ↓ or solvent polarity ↑ → t_R ↓
- Reversed-phase partition chromatography: common
  - Nonpolar stationary phase, hydrocarbon,
  - Polar mobile phase, water, ethanol, acetonitrile or tetrahydrofuran
  - compound polarity ↓ or solvent polarity ↑ → t_R ↑
  - Ion-pair chromatography: easily ionizable species
  - Longer chain → higher sample capacity
- Mobile phase polarity:
  - Water > acetonitrile > methanol > ethanol > tetrahydrofuran > propanol > cyclohexane > hexane

Partition Chromatography

- Method development: balance the polarity of analyte, mobile and stationary phase
- Column selection:
  - Functional group: Hydrocarbon < ethers < esters < ketones < aldehydes < amides < amines < alcohols
  - Stationary phase polarity ~ analyte, different from mobile phase
- Mobile phase selection: N, k and α
  - Effect of solvent strength on k: easiest way,
    - Polarity index, P': -2 to 10.2 (water)
    - Retention factor in normal phase:
  - Mobile phase on selectivity:
    - Reverse: water + acetonitrile and tetrahydrofuran

Bonded-phase chromatography.



$$P'_{AB} = \phi_A P'_A + \phi_B P'_B$$

$$\frac{k_2}{k_1} = 10^{(P'_1 - P'_2)/2}$$

Mobile Phase Properties in LC

Solvent	Refractive Index	Viscosity (cP)	Boiling Point (°C)	Polarity Index (P)	Eluent Strength (ε^o)
Fluoroalkanes	1.27-1.29	0.4-2.6	50-174	<-2	-0.25
cyclohexane	1.423	0.90	81	0.04	-0.2
N-hexane	1.327	0.30	69	0.1	0.01
1-chlorobutane	1.400	0.42	78	1.0	0.26
Carbon tetrachloride	1.457	0.90	77	1.6	0.18
i-propyl ether	1.365	0.38	68	2.4	0.28
toluene	1.494	0.55	110	2.4	0.29
Diethyl ether	1.350	0.24	35	2.8	0.38
tetrahydrofuran	1.405	0.46	66	4.0	0.57
chloroform	1.443	0.53	61	4.1	0.40
ethanol	1.359	1.08	78	4.3	0.88
Ethyl acetate	1.370	0.43	77	4.4	0.58
dioxane	1.420	1.2	101	4.8	0.56
methanol	1.326	0.54	65	5.1	0.95
acetonitrile	1.341	0.34	82	5.8	0.65
nitromethane	1.380	0.61	101	6.0	0.64
Ethylene glycol	1.431	16.5	182	6.9	1.11
water	1.333	0.89	100	10.2	large

Partition Chromatography

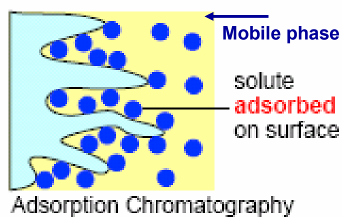
- Ion-pair chromatography
  - Organic counter-ion (ion-pairing reagent) in mobile phase: NR_4^+, RSO_3^-
  - Partition of neutral species or retention by charges
- Chiral chromatography
  - Chiral resolving agent, stationary phase
  - Attractive force: hydrogen bond, π bonds or dipole

Typical Applications of High-Performance Partition Chromatography

Field	Typical Mixtures Separated
Pharamaceuticals	Antibiotics, sedatives, steroids, analgesics
Biochemicals	Amino acids, proteins, carbohydrates, lipids
Food products	Artificial sweeteners, antioxidants, aflatoxins, additives
Industrial chemicals	Condensed aromatics, surfactants, propellants, dyes
Pollutants	Pesticides, herbicides, phenols, polychlorinated biphenyls (PCBs)
Forensic chemistry	Drugs, poisons, blood alcohol, narcotics
Clinical medicine	Bile acids, drug metabolites, urine extracts, estrogens

Adsorption Chromatography

- Liquid-solid chromatography: adsorption of analyte on a solid surface
- Stationary phase: polar solid, finely divided silica SiO_2(more common higher sample capacity) or alumina Al_2O_3
- Analyte polarity ↑→ t_R↑
- Mobile phase: the variable
- Application: relatively nonpolar, water-insoluble organic compounds (M_r < 5000)
- Decreased application due to normal phase LC

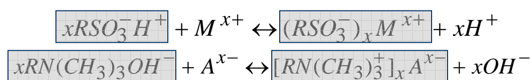


A: retain and separate special compounds like geometrical isomers

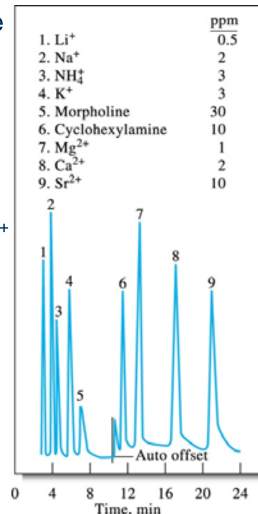
DA: – very strong retention of some solutes
– may cause catalytic changes in solutes
– solid support may have a range of chemical and physical environments → non-symmetrical peaks and variable retention times

Ion-Exchange Chromatography

- Separate and identify charged ions: small sample capacity
- Conductivity detector: simple, $\propto$ c, easy to maintain, general for charged ions
- Cation exchange resin: sulfonic acid or –COOH
- Anion exchange resin: tertiary or primary amine
- $K_{eq} \uparrow \rightarrow t_R \uparrow$:
- $Tl^+ > Ag^+ > Cs^+ > Rb^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$
- $Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+}$
- $SO_4^{2-} > C_2O_4^{2-} > I^- > NO_3^- > Br^- > Cl^- > HCO_2^- > CH_3CO_2^- > OH^- > F^-$



$$K = \frac{c_S}{c_M} = \frac{[(RSO_3^-)_x M^{x+}]_S}{[M^{x+}]_M} = \frac{[(RN(CH_3)_3)_x A^{x-}]_S}{[A^{x-}]_M}$$



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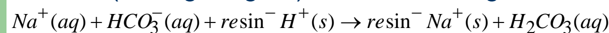
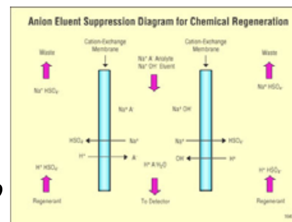
Ion-Exchange Chromatography

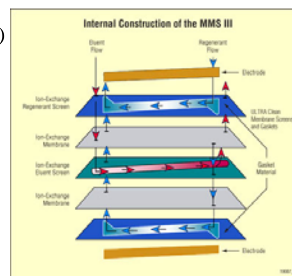
- Suppressor-based IEC:
  - Eluent suppressor column: converts the high c of eluent ions to species of limited ionization
  - Cation separation: HCl (eluting reagent), anion-exchange resin

  - Anion separation: $NaHCO_3$ or Na_2CO_3 (eluting reagent), cation-exchange resin



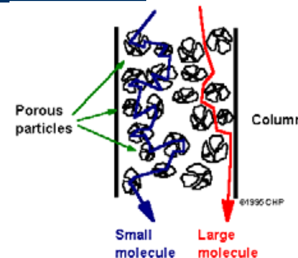
  - Regenerate periodically (10 h)
  - Micromembrane suppressor: no need for regeneration
    - Ultra-thin ion-exchange membranes
    - Analyte elution exchange in one direction
    - Regeneration from a stream in opposite direction

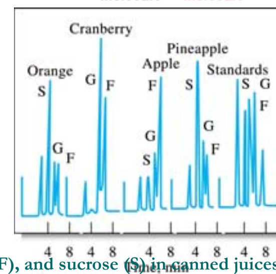




Size-Exclusion Chromatography

- Based on effective molecular size: high M_r
- Stationary phase: 10 μm particles with uniform pore
  - Silica: rigid → high P, stable at high T, interacts with analytes
  - Polymer: cross-linked, different pore size, no interaction
- Molecule effective size and shape: $d_{analyte} > d_{pore}$: no trap. $d_{analyte} \downarrow \rightarrow t_R \uparrow$
- No chemical or physical adsorption, only diffusion
- Gel filtration: hydrophilic packing, sulfonated divinylbenzenes or polyacrylamides
- Gel permeation: hydrophobic packing

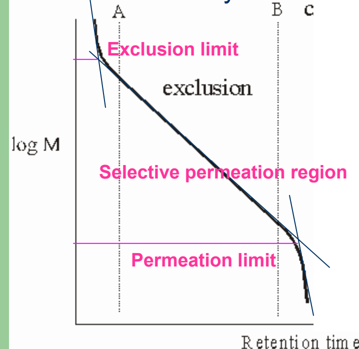




Gel filtration chromatogram of glucose (G), fructose (F), and sucrose (S) in canned juices

Size-Exclusion Chromatography

- SEC: molecular mass or mass distribution
- Universal calibration curve: log[M_r] vs. V_r
- Permeation limit (most retention) < M_r < exclusion limit (no retention)
- Calibrated by standards

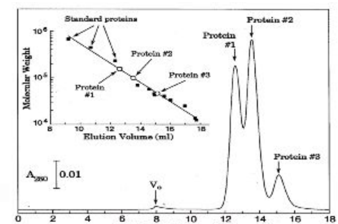


$$V_t = V_g + V_i + V_o$$

$$V_e = V_o + KV_i$$

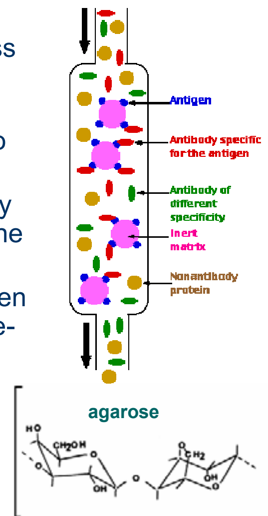
$$K = \frac{V_e - V_o}{V_i} = \frac{c_S}{c_M}$$

V_t : total volume
V_g : volume of gel
V_i : solvent volume in pore
V_o : free volume
V_e : elution volume
K : distrubution constant



Affinity Chromatography

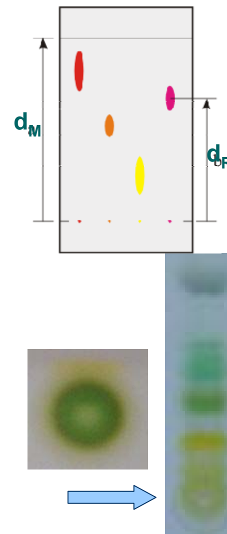
- Stationary phase: covalently bonding affinity ligands to a solid support (agarose, porous glass bead)
- Affinity ligands: antibodies, enzyme inhibitors, molecules that reversibly and selectively bind to analytes
- Analyte molecules are retained by the stationary phase → change the mobile phase to release the analyte
- Mobile phase: 1. support strong bonding between analyte and the ligands. 2. eliminate the analyte-ligand interaction. Change of pH or μ
- A: Extraordinary specificity
- Application: rapid isolation of biomolecules



Thin-layer Chromatography

- Planar chromatography
  - Flat thin layer (200 μm or 100 μm) of finely divided particle (20 μm or 5 μm) adhering on glass, plastic or metal surface
  - Force: gravity, capillary force or electrical potential
  - Similar to LC, wide application in industry
  - Preparation: spreading aqueous slurry of finely particles (w/o binder) on the surface (5×20, 10×20 or 20×20 cm^2)
  - Sample: 0.01 ~ 0.1%, small diameter (< 5 mm), at 1 ~ 2 cm away from the edge, using capillary tube
  - Plate development: when dry, in covered chamber

$$R_F = \frac{d_R}{d_M}, k = \frac{d_M - d_R}{d_R} = \frac{1 - R_F}{R_F}$$

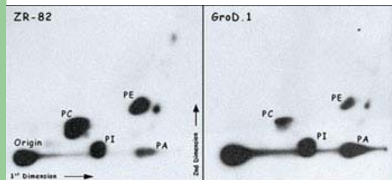


Thin-layer Chromatography

- Plate height
- Application of TLC

$$N = 16\left(\frac{d_R}{W}\right)^2, H = d_R / N$$

  - Qualitative TLC
    - R_F variable with sample size → not enough for identification
    - Adding pure species → absence of analyte
    - Scraping-and-dissolution technique: identification using other methods
    - Two-dimensional TLC
  - Quantitative analysis
    - Area under the spot
    - Scraping-and-dissolution technique
    - Scanning densitometer → fluorescence or absorbance → quantitative information



Parent cells (ZR-82) and GroD1 cells were labeled with 32P-inorganic phosphate for 3 hours. The radioactive lipids were extracted, separated on 2D-thin-layer chromatography & the TLC plates were exposed to x-ray film. Note the loss of signal in PC & PE in GroD1 with an increase in phosphatidic acid (PA) and no change in phosphatidylinositol (PI).