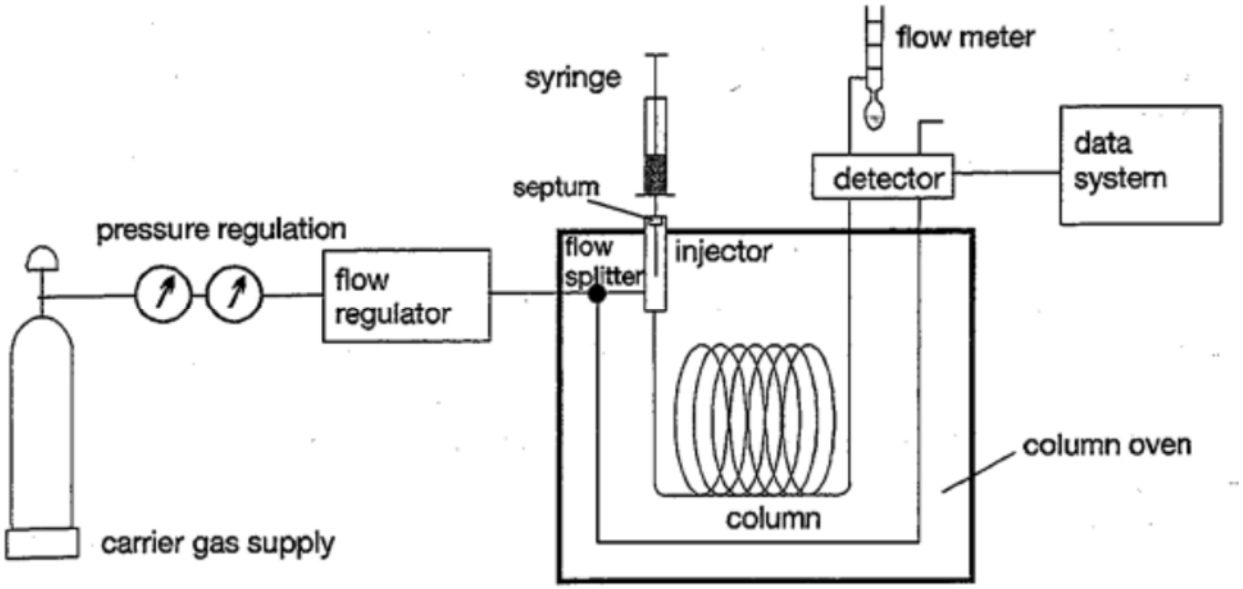
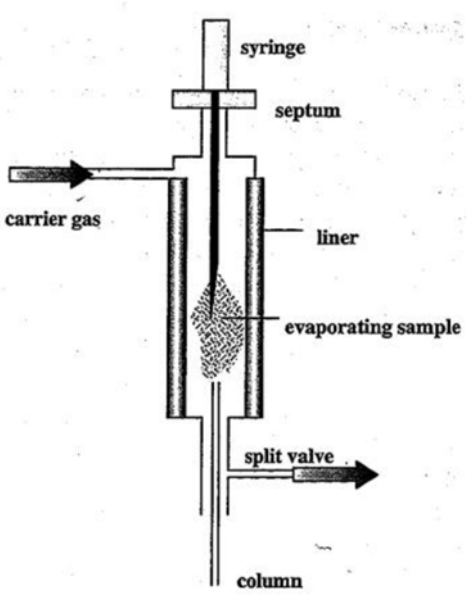
III. Chromatography techniques

- A. gas chromatography instrumentation



III.A. GC instrumentation

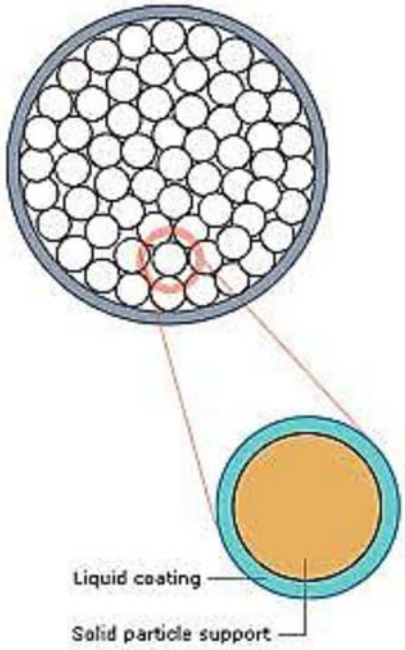
- major components of GC system
  - 1. carrier gas (mobile phase): high purity gas (99.99% or better)
    - usually He, H_2, N_2, or Ar
    - impurities can degrade column and cause false signals
    - traps are used to purify the mobile phase
  - 2. injector
    - sample injected into gas stream
    - sample vaporized in the injector
    - only useful for volatile compounds
    - 1-10 μL per injection
    - injector heated to vaporize sample
    - 30-50^o higher that highest oven T
    - samples often split before column



III.A. GC instrumentation

- components cont.
  - 3. columns
    - i. packed columns with solid stationary phase (charcoal, molecular sieves) used for purifying gases
    - ii. packed columns with inert solid support coated with liquid stationary phase
      - 1-8 mm in diameter
      - glass or stainless steel
      - 2-10 m in length

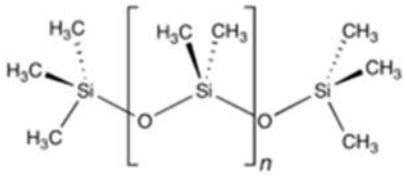
      - packing material: 5-10 μm in diam.
      - liquid stationary phases adsorbed
      - varying polarity
      - must be thermally stable
      - must be non-volatile
      - must not react with analyte

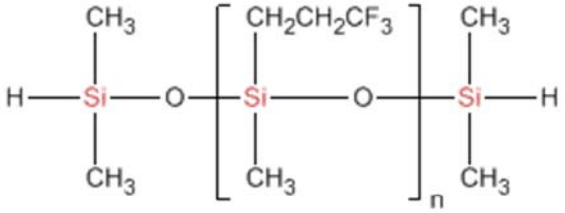


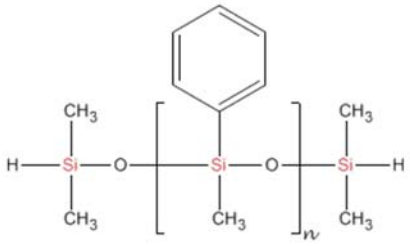
III.A. GC instrumentation

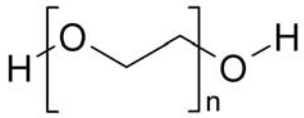
- liquid stationary phases for packed GC

stationary phase	T_{max}(°C)	uses
polydimethylsiloxane	350	general purpose nonpolar phase
5% phenyl-polydimethylsiloxane	350	esters, alkaloids, drugs, halogenated compounds
50% phenyl-polydimethlsiloxane	250	drugs, steroids, pesticides
50% trifluoropropyl-polydimethylsiloxane	200	chlorinated aromatics, substituted benzenes
polyethylene glycol	250	acids, alcohols, ethers, glycols



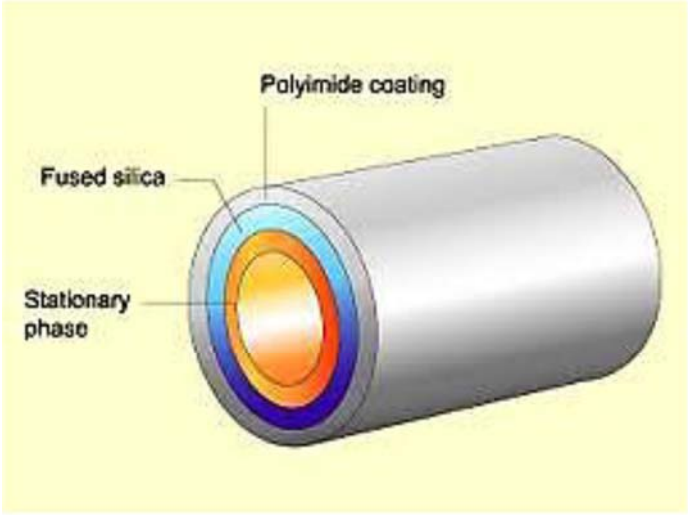


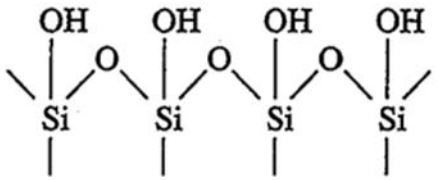


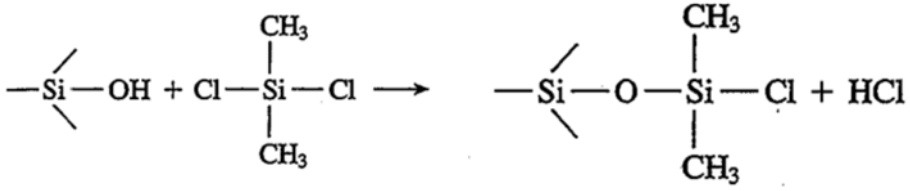


III.A. GC instrumentation

- components cont.
  - 3. columns cont.
    - open tubular columns
      - no solid support
      - s.p. bonded directly to interior surface of capillary column
      - columns made of fused silica
      - 100 – 500 μm i.d.
      - 10 – 100 μm s.p. thickness
      - 10 to 100 m long
      - different functional groups for different polarities
      - A term in V.D. equation v. small
      - more efficient separations





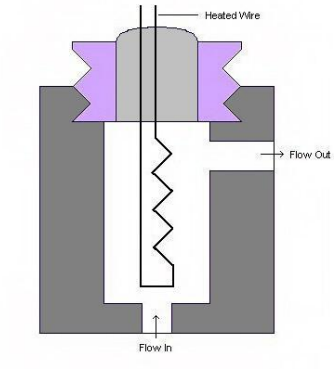


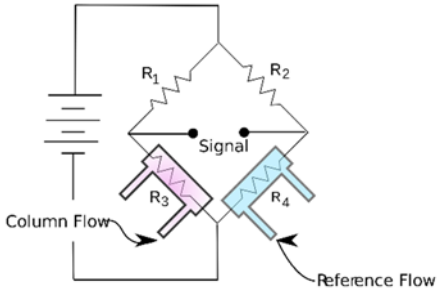
III.A. GC instrumentation

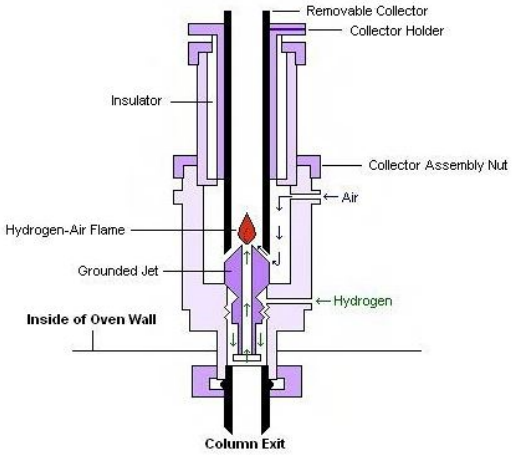
- components cont.
  - 4. detectors
    - <u>wish list:</u>
      - low limit of detection
      - wide linear response range
      - uniform response to a wide variety of compounds
      - simple calibration
      - rapid response and recovery (<sec)
      - small internal volume
      - low background signal and noise
      - inexpensive to operate and maintain
      - non-destructive to sample
    - NO current detector has all of these
    - choice will depend on problem to be solved and available funds

III.A. GC instrumentation

- components cont.
  - 4. detectors cont.
    - a. thermal conductivity detector (TCD)
      - measure change in resistivity of heated wire in gas stream
      - universal
      - non-destructive
      - poor sensitivity
      - requires reference flow (m.p. only)
    - b. flame ionization detector (FID)
      - most widely used detector
      - analytes burned in a H_2/air flame
      - analytes are ionized and the ion current is detected
      - more sensitive than TCD
      - almost universal
      - destructive

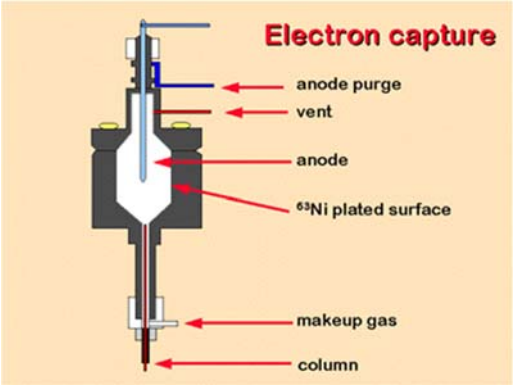


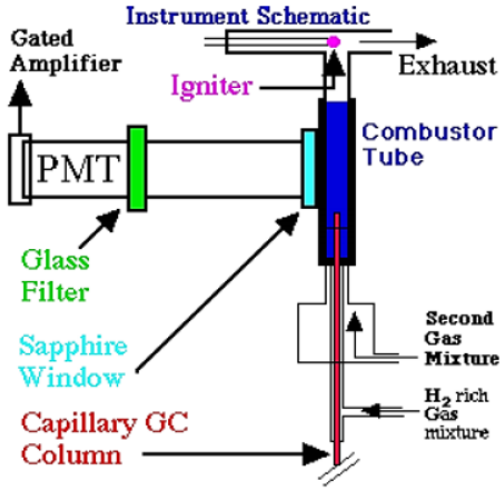




III.A. GC instrumentation

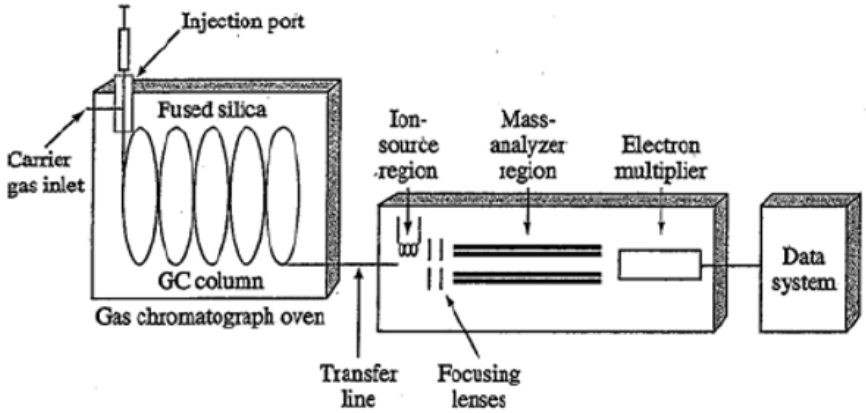
- components cont.
  - 4. detectors cont.
    - c. electron capture detector
      - radioactive (^{63}Ni) source ionizes sample
      - ions and electrons are generated
      - analytes containing electronegative atoms can "capture" the electrons, reducing the current
      - good for halogens, NO_2 or P-containing analytes
      - destructive
      - selective
    - d. flame photometric detector
      - analytes burned in flame
      - high T creates excited states
      - photons emitted from samples
      - selective for S and P compounds
      - destructive





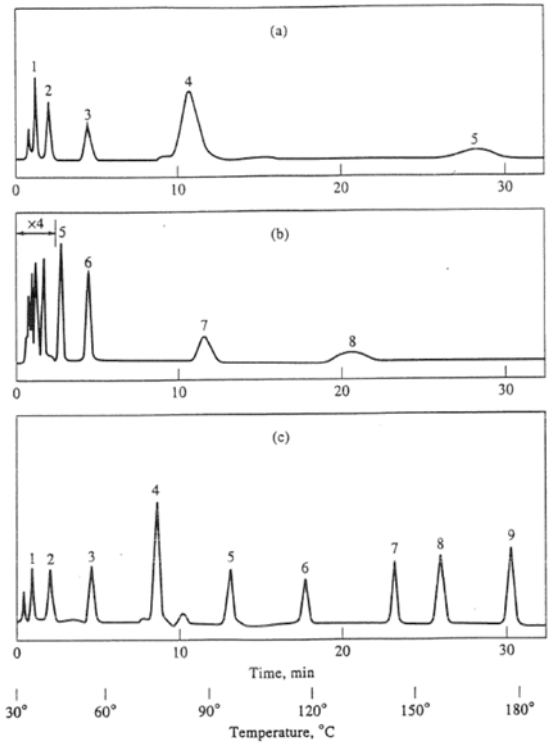
III.A. GC instrumentation

- components cont.
  - 4. detectors cont.
    - e. mass spectrometric detector
      - sample fed into a “jet separator” to remove majority of carrier gas.
      - analyte ionized by electron impact (more later)
      - ions detected by m/z ratio
      - gives structural information (qual) on analytes
      - destructive
      - mostly universal
      - expensive (>$30,000 for MS)



III.A. GC instrumentation

- components cont.
  - 5. oven and temperature selection
    - all GC runs take place at elevated temperature to keep the gases from condensing
    - two types of experiments
    - isothermal – oven remains at a single temperature
    - T gradient – temperature ramps during the run
      - more volatile things elute first (lowT)
      - less volatile things elute after

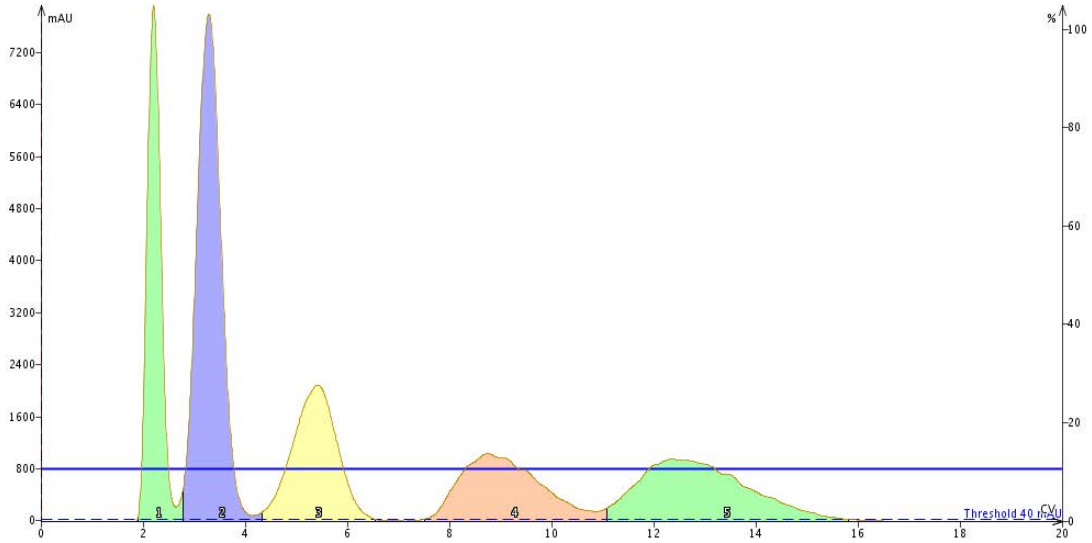


III.B. Liquid Chromatography Fundamentals

- B. liquid chromatography fundamentals
- fundamental difference between GC and LC
  - samples interact with both m.p. and s.p.
- m.p.: liquid – analytes dissolve in it
- s.p.: solid – (LSC) – column – analytes adsorb
  liquid – (LLC) – HPLC – analytes dissolve
- Two types of LLC
  - Normal Phase
    - m.p. is less polar than s.p.
    - order of elution: less polar ⟶ more polar
  - Reverse Phase
    - m.p. is more polar than s.p.
    - order of elution: more polar ⟶ less polar

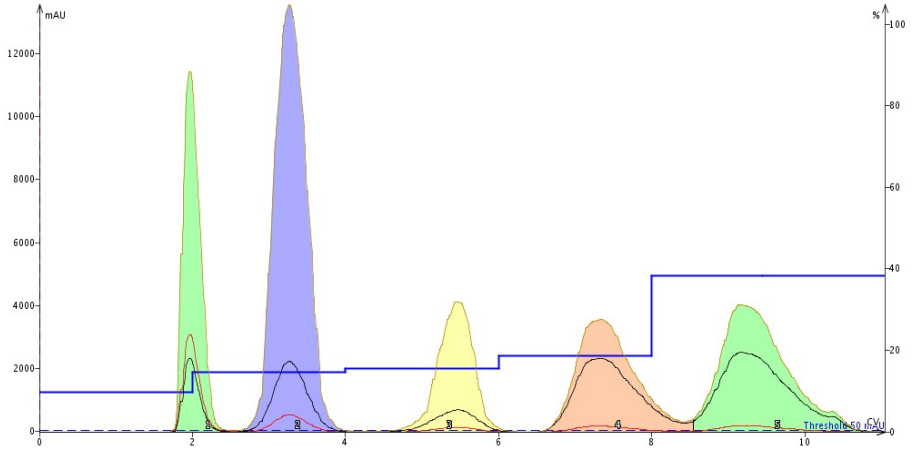
III.B. LC Fundamentals

- 1. mobile phases
  - one or more solvents mixed together
  - must be degassed (remove bubbles)
  - composition of solvent is an adjustable parameter
    - isocratic
      - solvent composition remains the same during entire run
      - can be a mixture
      - similar to isothermal in GC

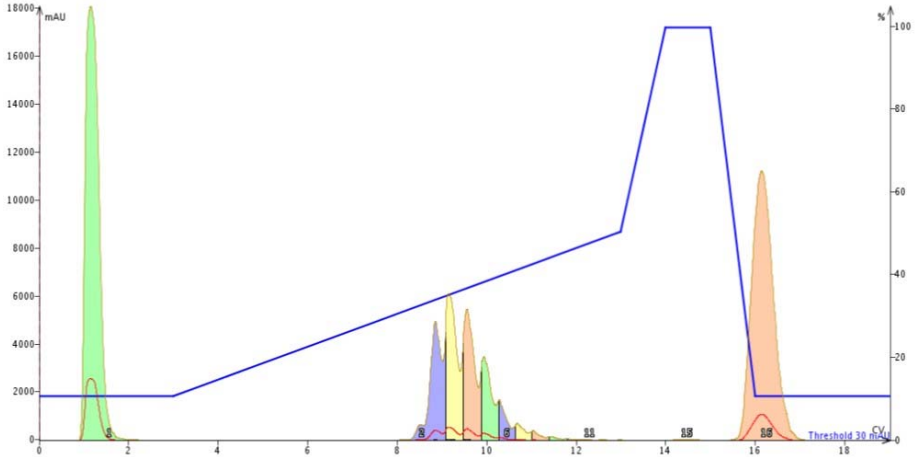


III.B. LC Fundamentals

- gradients
  - like T programing in GC
  - several types:
    - linear ramp

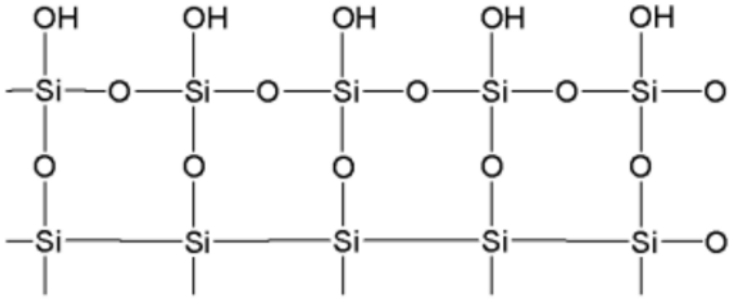


    - gradient

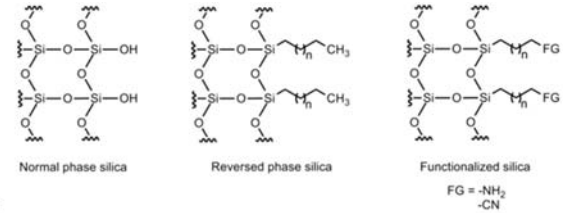


III.B. LC Fundamentals

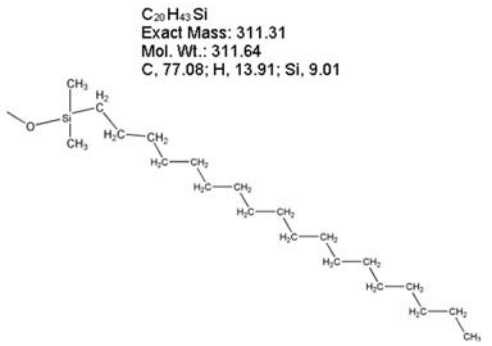
- 2. stationary phases
  - normally silica-based.



  - a. normal phase
    - s.p: SiO_2, Al_2O_3
    - m.p.: non-polar (hexane, pentane, benzene, etc)
    - non-polar analytes elute first (more affinity for the m. p.)
    - gradient: increase m.p. polarity to help elute more polar analytes
    - solvents must still be miscible!!

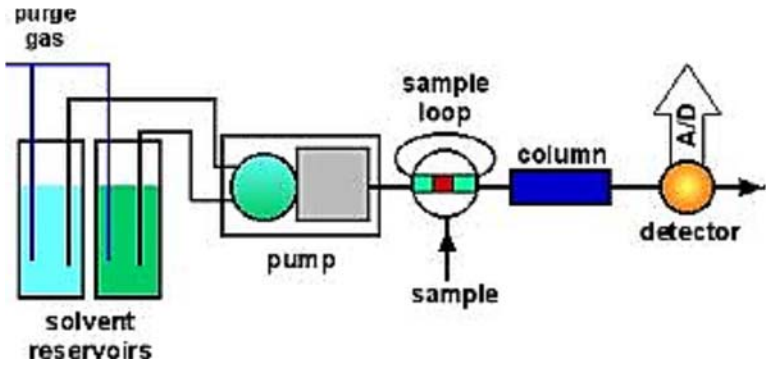


  - b. reverse phase
    - s.p.: silica based polymer
    - long-chain alkanes (C_8, C_{18})
    - m.p.: polar (H_2O, CH_3OH, CH_3CN, etc
    - polar analytes elute first
    - gradient: decrease polarity of m. p.



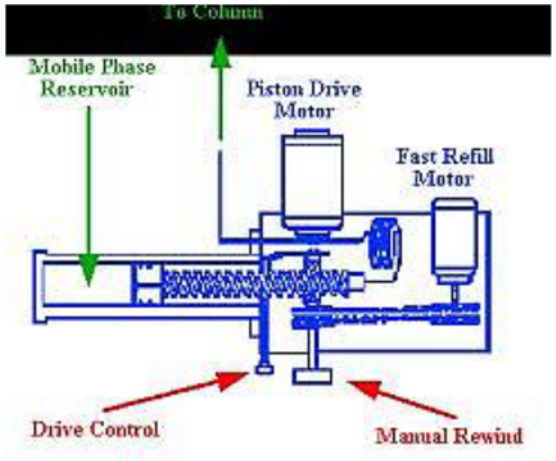
III.B. LC Fundamentals

- 3. high performance liquid chromatography (HPLC)
  - a. sample introduction
    - mobile phase supply system
    - need a way to mix several solvents in varying ratios
    - need a way to accurately control flow rate
      - 0.1 – 10 ml/min normal flow
      - 1-300 μL/min micro flow
      - 20-200 nL/min nanoflow
    - need a way to inject sample into flowing stream of m.p. without losing pressure
    - column assembly must be able to withstand high pressure (up to 1000 psi for normal flow and up to 5000 psi for Ultra PLC)

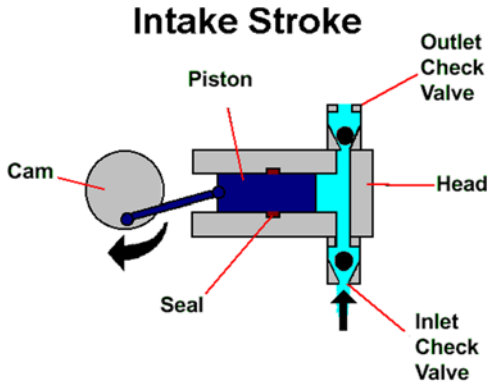


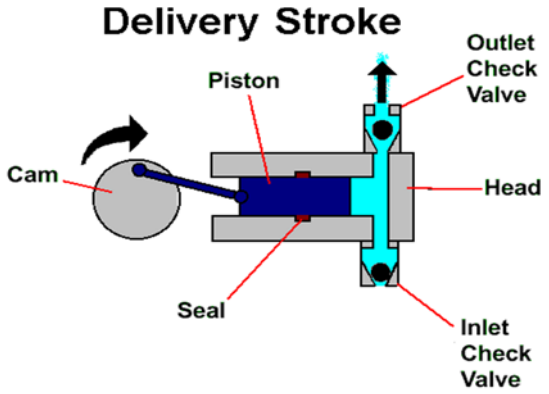
III.B. LC Fundamentals

- 3.a. sample introduction cont.
  - i. pumps:
    - syringe pump



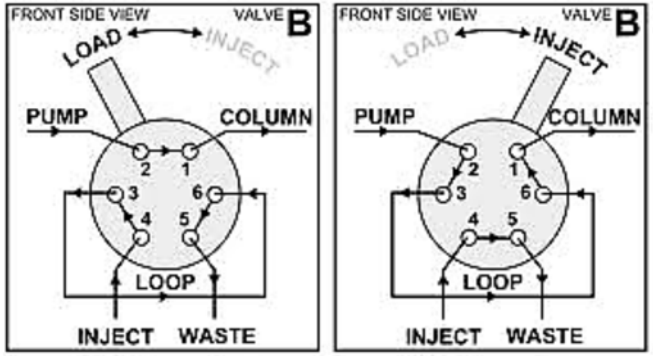
- reciprocating pump – more common, better control, higher flows



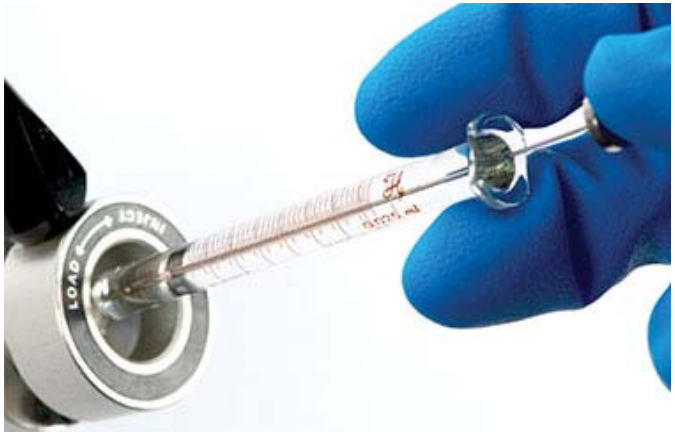


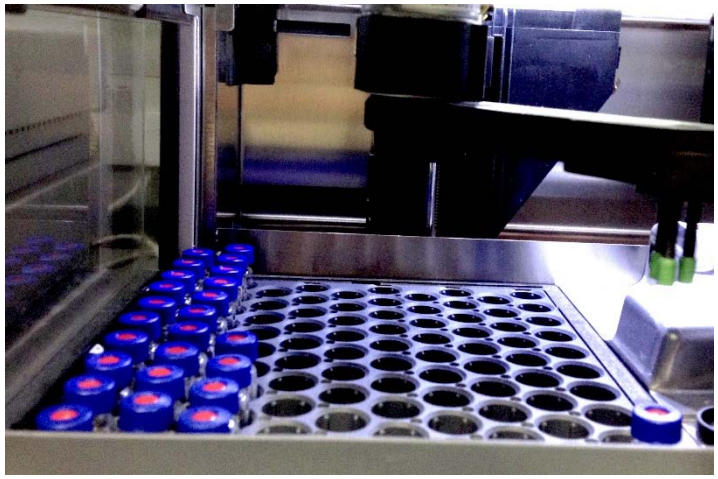
III.B. LC Fundamentals

- 3.a. sample introduction cont.
  - ii. injectors
    - need to deliver analyte into flowing stream without losing pressure
    - often makes use of a sample loop



    - manual or automatic sampling





III.B. LC Fundamentals

- 3.a. sample introduction cont.
  - iii columns
    - normal flow required stainless steel (1 to 4 mm in diameter) 5-150 cm long packed with 3 – 10 μm solid support.

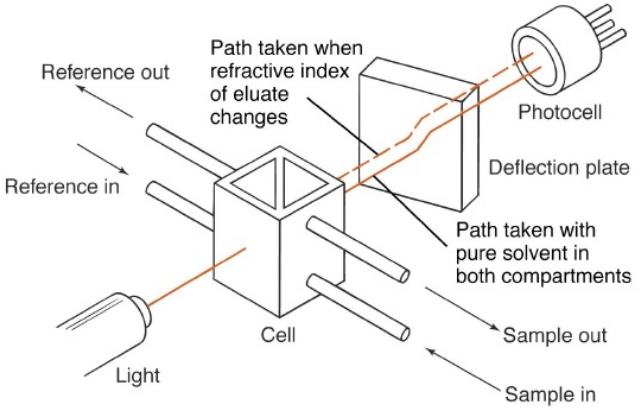
    - nano-bore columns can be much smaller (0.360 mm diameter) and can be made of fused silica.

III.B. LC Fundamentals

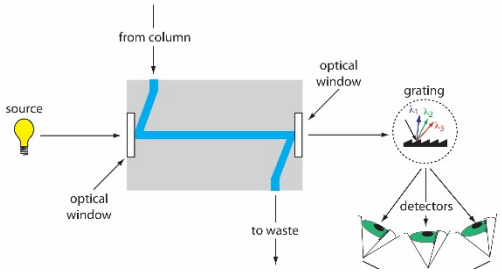
- 3.b. detectors
  - wish list:
    - low limit of detection
    - wide linear response range
    - uniform response to a wide variety of compounds
    - simple calibration
    - rapid response and recovery (<sec)
    - small internal volume
    - low background signal and noise
    - inexpensive to operate and maintain
    - non-destructive to sample
  - NO current detector has all of these
  - choice will depend on problem to be solved and available funds

III.B. LC Fundamentals

- 3.b. detectors
  - i. refractive index detector
    - compare refractive index of solvent vs. (solvent + analyte)
    - most universal detector
    - can't be used with solvent gradients
    - no structural info
    - non destructive

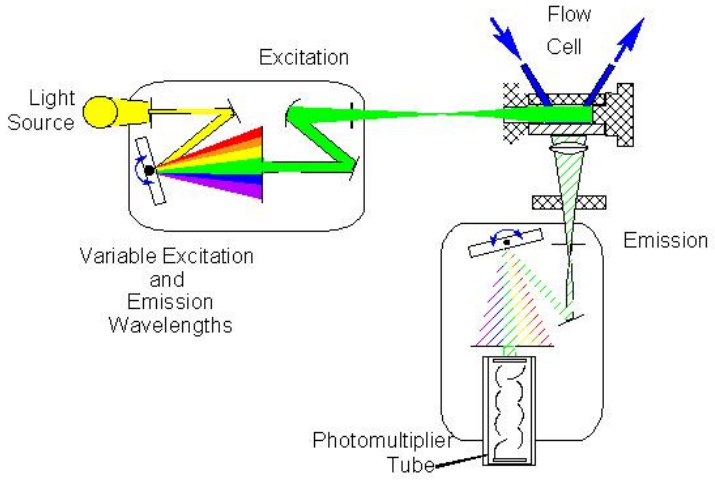


  - ii. uv-visible absorption
    - analytes can absorb light (more later)
    - absorption proportional to concentration
      - A = ebc, c = concentration
      - e=constant for molecule and wavelength of light, b = path length
      - sensitive and relatively universal
      - limited molecular information
      - non destructive



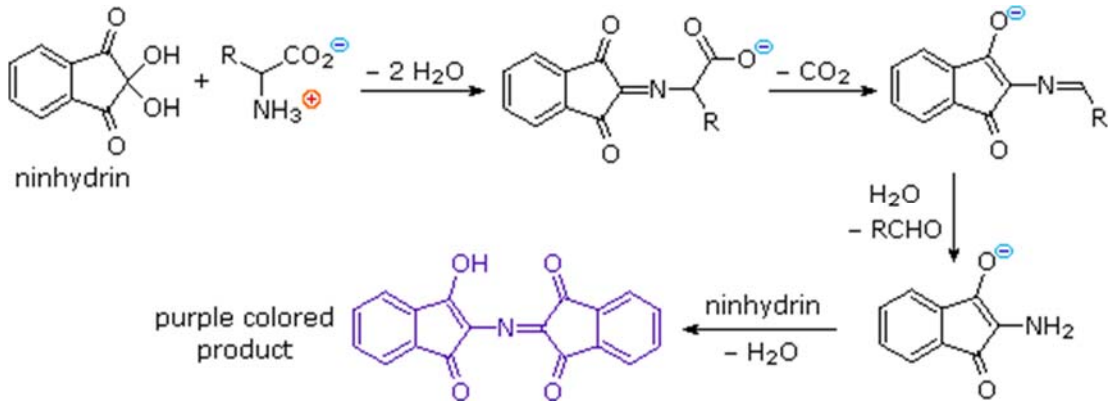
III.B. LC Fundamentals

- 3.b. detectors
  - iii. fluorescence
    - some molecules can absorb light at one wavelength and then re-emit at a different wavelength (more later).
    - this is called fluorescence
    - fluorescence is also proportional to concentration
    - very selective (most things don't fluoresce)
    - very sensitive (zero background)
    - non-destructive
    - fluorescence tagging is common



III.B. LC Fundamentals

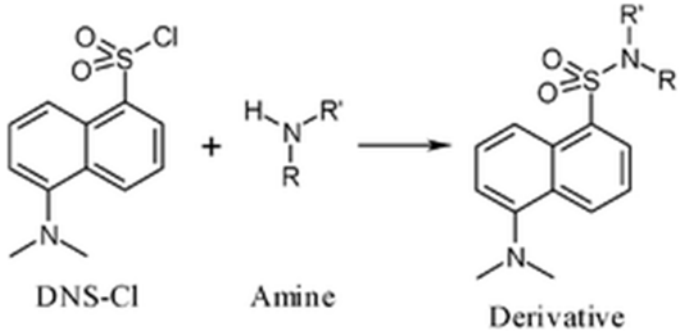
- 3.b.iii. fluorescence tagging
  - most things don't fluoresce, but many compounds can be derivatized to form fluorescent compounds (tagged)
  - two choices:
    - pre-column – before separation
    - post column – after separation but before detector
    - choice depends on tag
      - for example: ninhydrin reacts with primary amines, so it can be used to tag amino acids



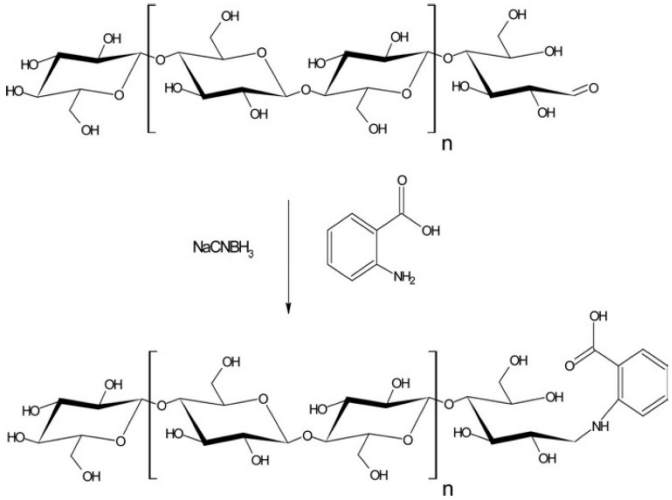
      - note that the R group from the amine does not end up in the product, so ninhydrin must be used post-column after separation of the amines

III.B. LC Fundamentals

- other tags
  - dansyl chloride – since “R” group is retained in derivative can be used pre or post-column

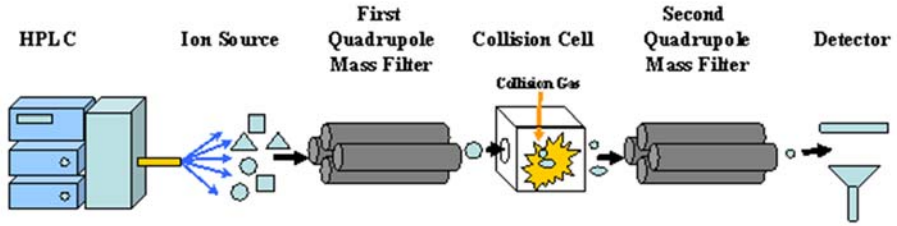


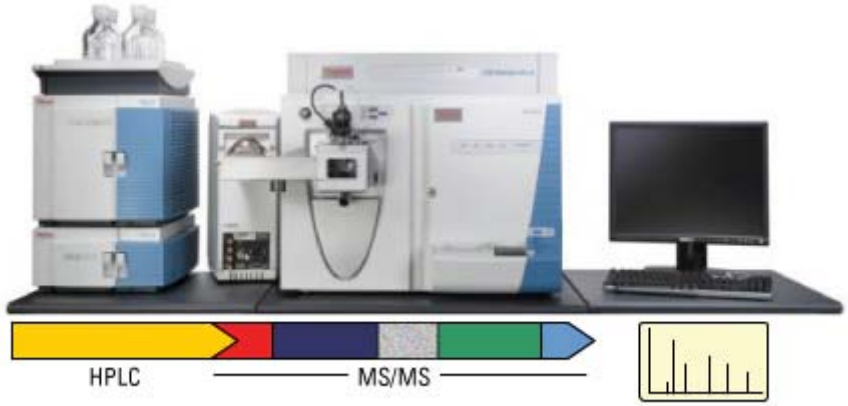
  - anthranilic acid –tag for carbohydrates – pre or post



III.B. LC Fundamentals

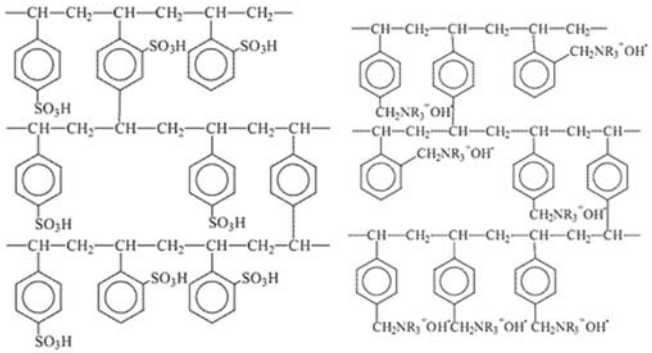
- 3.b. detectors
  - iv. mass spectrometer
    - universal (though better for polar molecules)
    - compatible with electrospray ionization (more later)
    - lots of structural information
    - destructive





III.C. Other types of LC

- III.C. other types of LC
  - 1. ion exchange
    - s.p. is a polymer with either positively or negatively charged side groups.
    - substituted polystyrenes often used



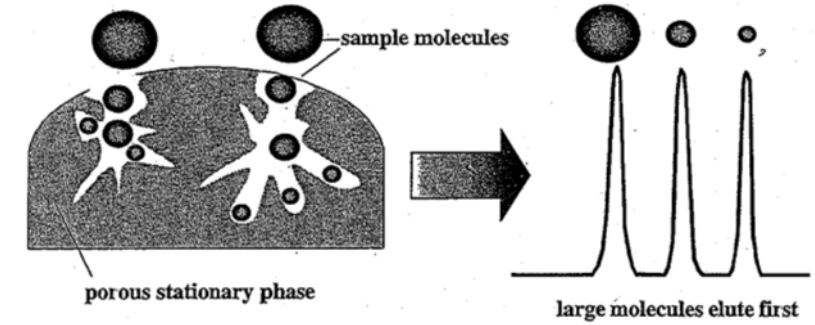
A strongly acidic sulphonated polystyrene cation exchange resin

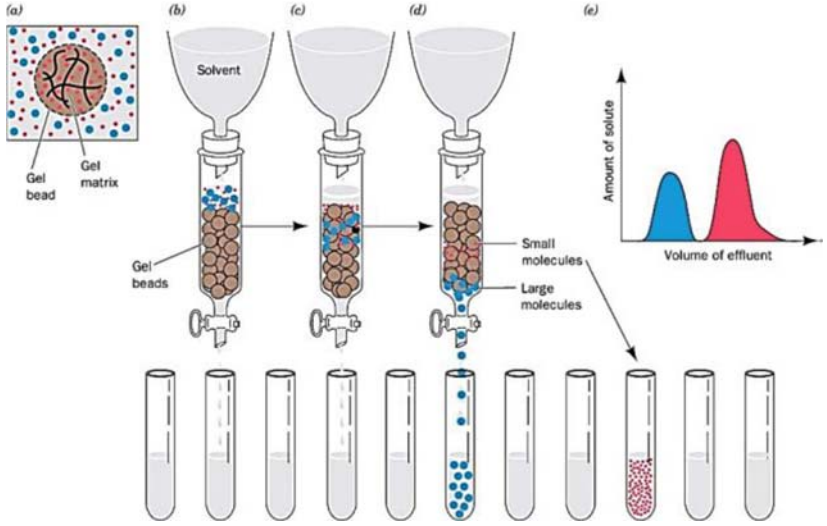
A strongly basic quaternary ammonion anion exchange resin

    - separation depends on charge density of analytes
      - small compact ions bind more tightly that larger more diffuse ions
      - dications bind more strongly than mono-cations, etc.
    - example: amino acids and peptides
      - low pH: cations
      - medium pH: zwitterions (neutrals)
      - high pH: anions

III.C. Other types of LC

- 2. size exclusion chromatography
  - s.p. is a cross-linked polymer with very large, uniform pores.
  - smaller molecules become trapped in the pores, whereas large molecules are “excluded” from the pores
  - retention inversely proportional to MM



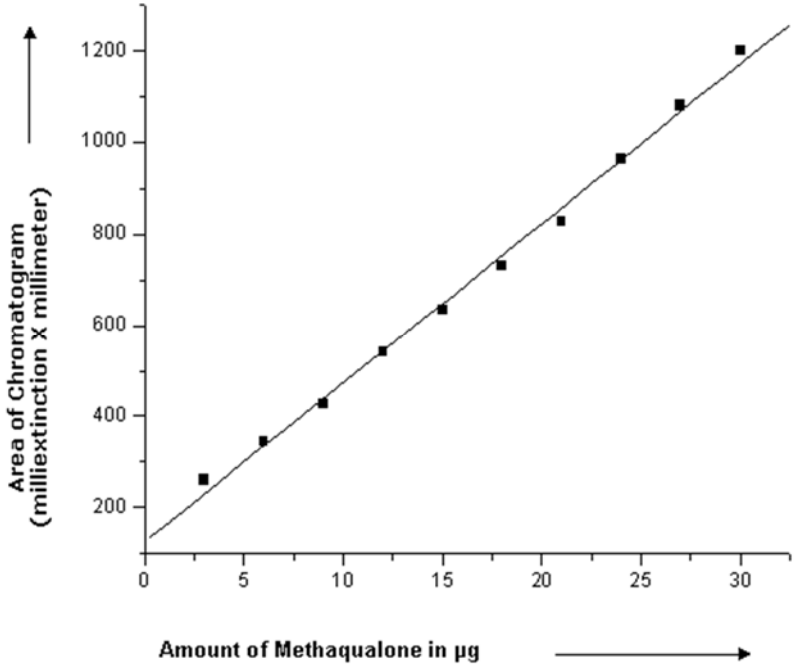


III.D. Qual. and Quant. Analysis

- D. qualitative and quantitative analysis
- qualitative analysis is performed by comparing retention times of analyte to those of known standards under the same conditions
  - requires pure reference compounds
  - more than one compound can have the same t_R
  - NOT the best for true qual., though use of MS improves this dramatically
- quantitative analysis is performed by comparing peak heights (or areas).
- two methods generally used
  - calibration curve
  - standard addition (later)

III.D. Qual. and Quant. Analysis

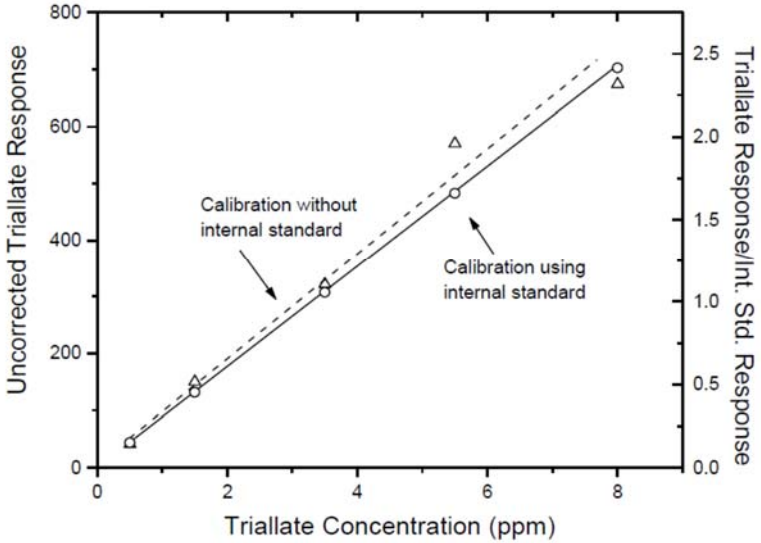
- quant cont.
  - calibration curves:
    - create standards of known concentration
    - run on instrument under known conditions
    - use peak height or peak area to create curve
    - run unknown under same conditions and interpolate
  - concerns
    - linearity of Beer's law
    - injection volume
  - approach
    - dilutions
    - internal standards

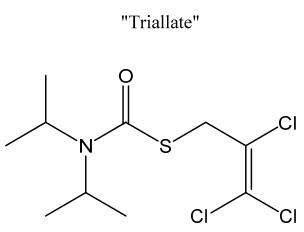


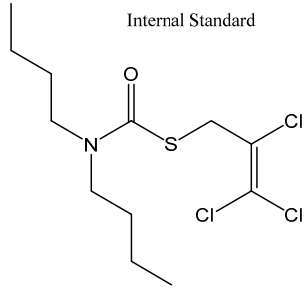
III.D. Qual. and Quant. Analysis

- quant cont.
  - internal standards
    - compound that has similar structure to analyte can be added in known concentration to each standard and unknown
    - ratio of peak areas or peak heights eliminates sample to sample variation in injection volume

Triallate conc. (ppm)	Internal std. conc. (ppm)	Triallate response	Internal std. response	Corrected Triallate response
0.50	5.00	41.2	273.1	0.1509
1.50	5.00	150.1	331.8	0.4524
3.50	5.00	321.5	304.4	1.0562
5.50	5.00	570.3	343.6	1.6598
8.00	5.00	673.7	279.0	2.4147
unknown	5.00	226.1	284.4	0.7950

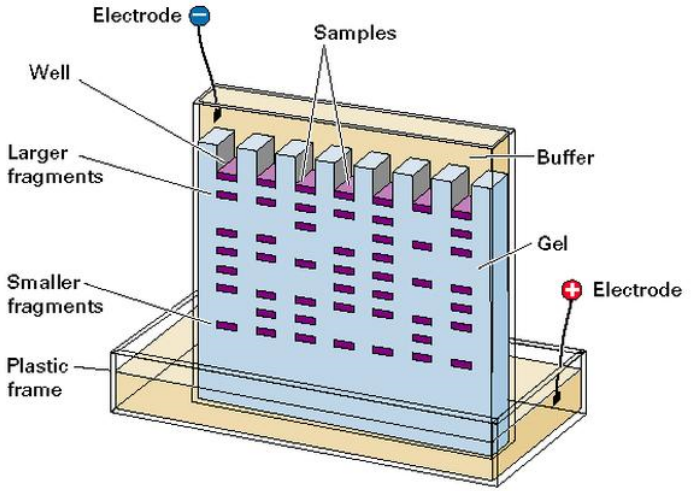


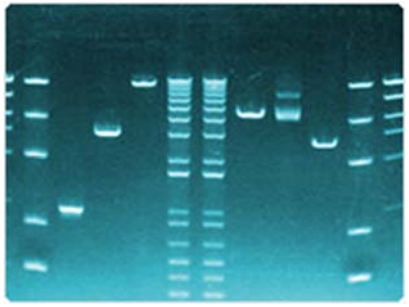




III.E. Electrophoresis

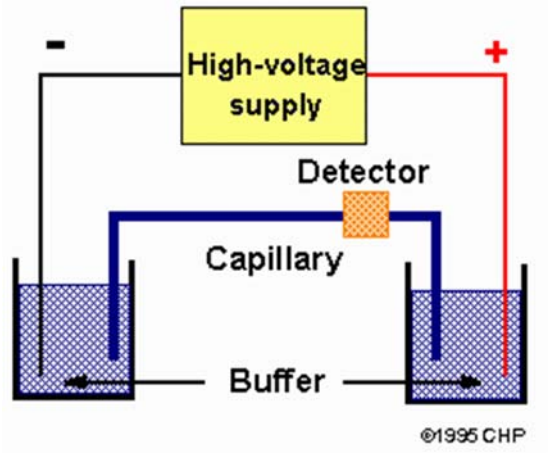
- E. <u>electrophoresis</u>
  - different approach to separation
  - two major types
    - gel and capillary
  - 1. gel electrophoresis
    - gel is a x-linked polymer
      - agarose
      - polyacrylamide
    - voltage applied across gel
    - anions migrate towards cathode (down)
    - only works for charge species
    - separation based on charge density (size)
    - useful for DNA and protein separations
    - separation proportional to applied voltage, but resistive heating limits V. (< ~500 V)





III.E. Electrophoresis

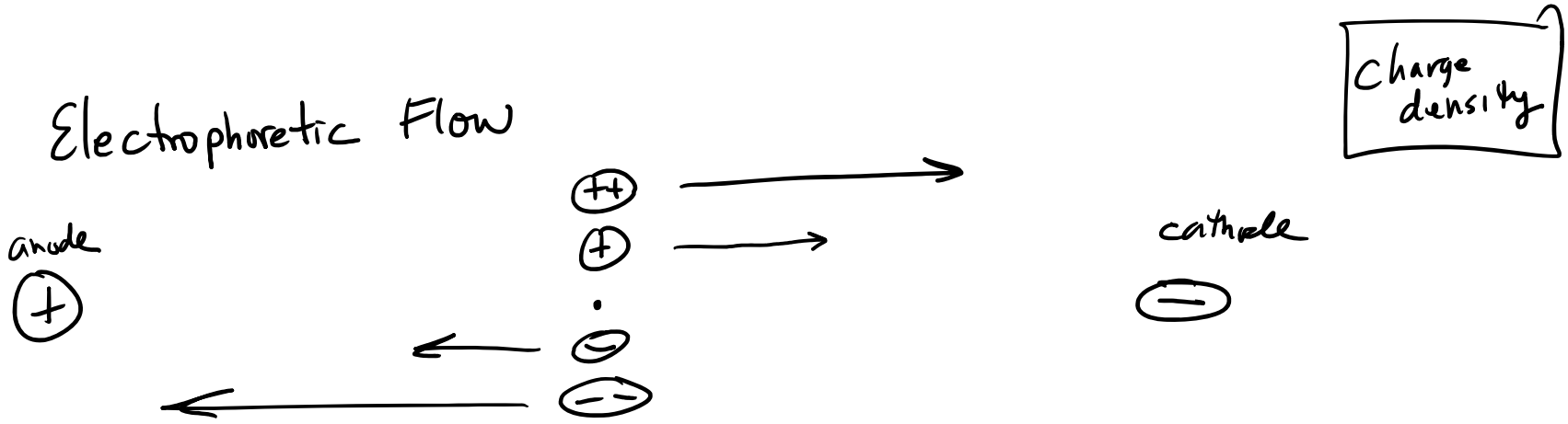
- 2. capillary electrophoresis
  - replace gel with capillary filled with buffer solution



  - capillary is fused silica (10-100 μm id, 40- 100 cm long)
  - small sample volumes (nL – μL)
  - higher voltages (10s of kV) leads to VERY efficient separation (N can be 10^6 theoretical plates)
  - doesn't work for neutrals (CEC)
  - many detector available (uv/vis, fluorescence, MS)

III.E. Electrophoresis

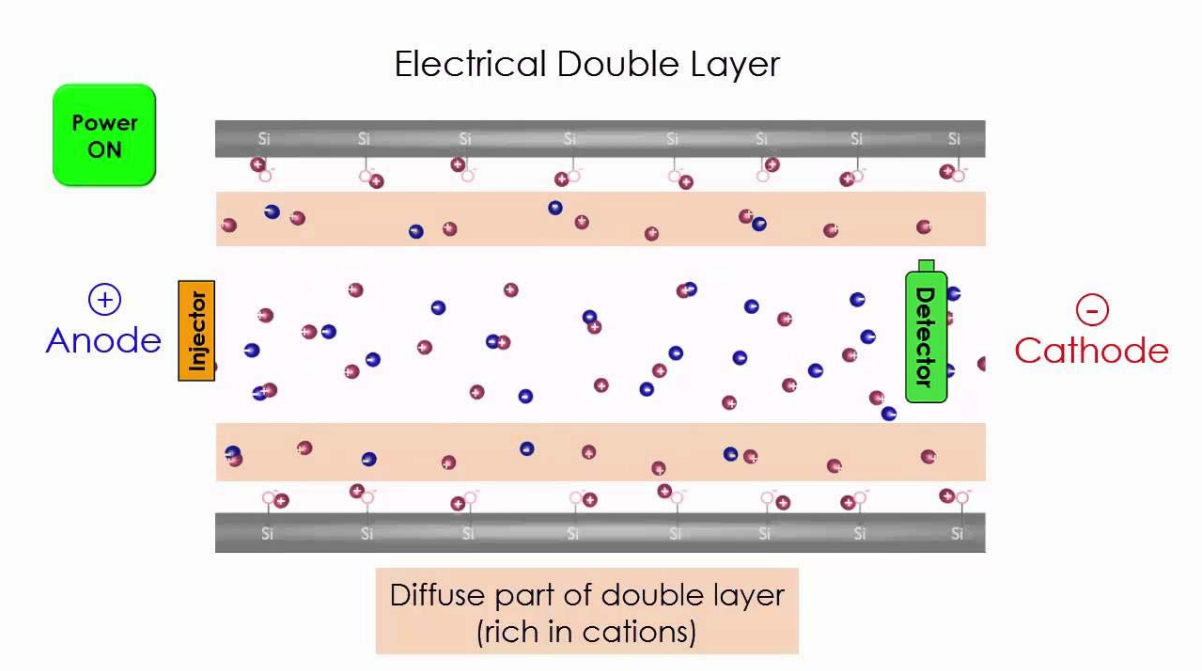
- 2. CE cont.
  - two different flow in capillary
    - 1. electrophoretic flow (ep)
      - cations attracted to cathode, anions attracted to anode
        - $v = \mu_\varepsilon \vec{E}$ v = velocity, E is electric field (V/L), μ_ε is mobility



- only gain with capillary is the ability to apply larger voltages without worrying about melting the capillary

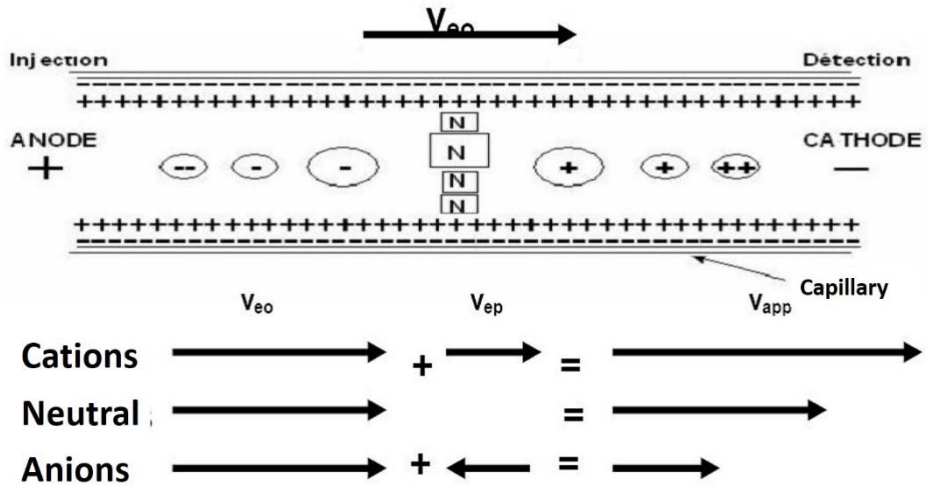
III.E. Electrophoresis

- 2. CE cont
  - in CE, you also have electro-osmotic (eo) flow:
  - silica capillary has anionic side groups above pH 1-2
    - cations in buffer attracted to wall of capillary forming an electrical double layer
    - next layer is rich in cations and is attracted to cathode
    - results in net buffer flow towards cathode.



III.E. Electrophoresis

- 2. CE cont.
  - eo flow generally larger than ep flow
  - combination of ep and eo flows results in separation of anions from cations (and neutrals)



  - separation within charge classes is by charge density

III.E. Electrophoresis

- 2. CE cont.
  - other things to consider:
    - retention time inversely proportional to V: $t_r = \frac{L^2}{\mu_\varepsilon V}$
    - no stationary phase!!!
    - only contribution to broadening is long. diffusion (B)
    - large numbers of theoretical plates:
    - $N = \frac{\mu_\varepsilon V}{2D_m}$, D_m is diffusion coefficient. independent of L
    - $R = \frac{1}{4\sqrt{2}}(\mu_{\varepsilon 1} - \mu_{\varepsilon 2})\left[\frac{V}{D_m(\bar{\mu}_\varepsilon + \mu_{eo})}\right]$
    - additional gain due to flow profile: