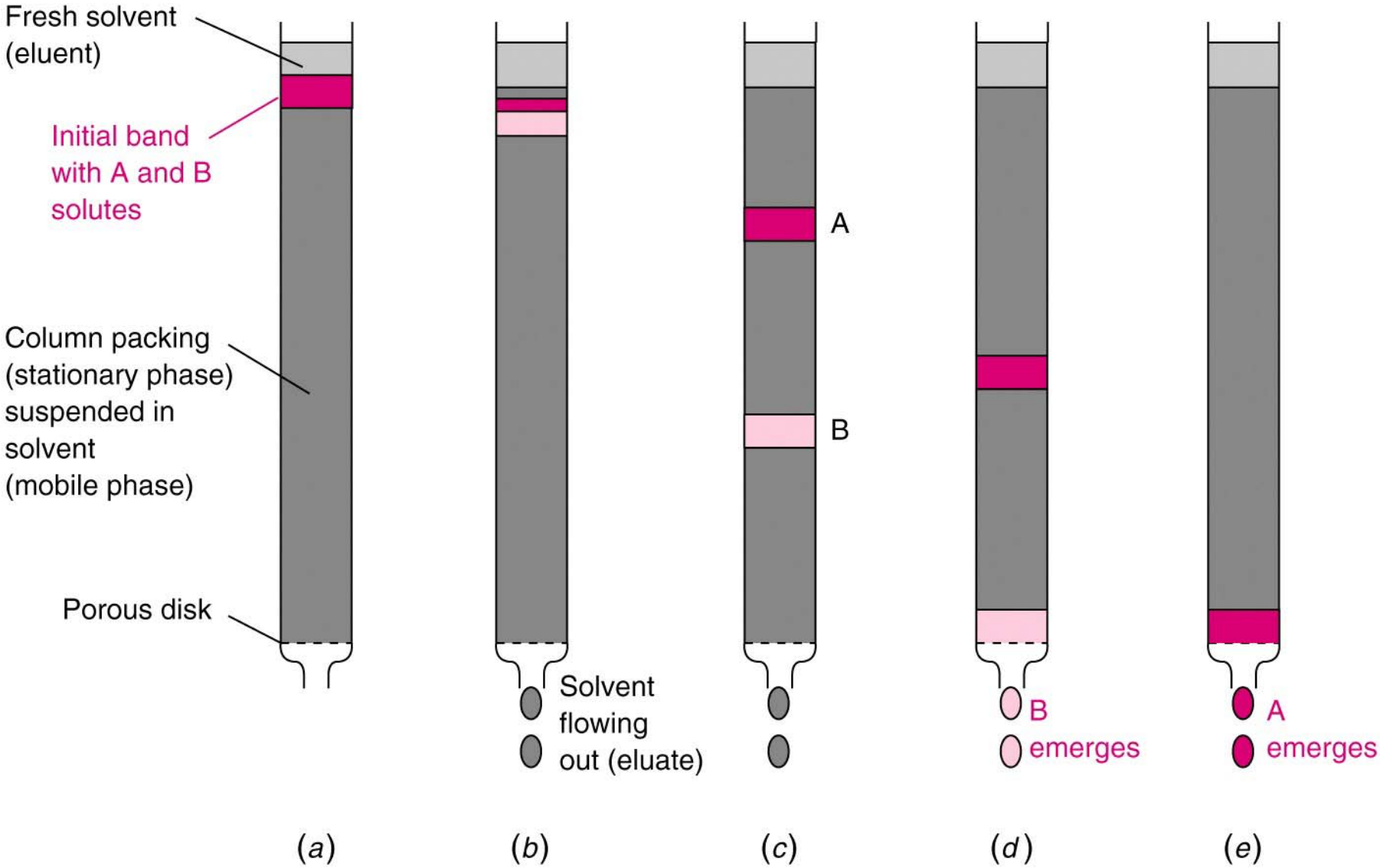
Chromatography and other Separation Methods

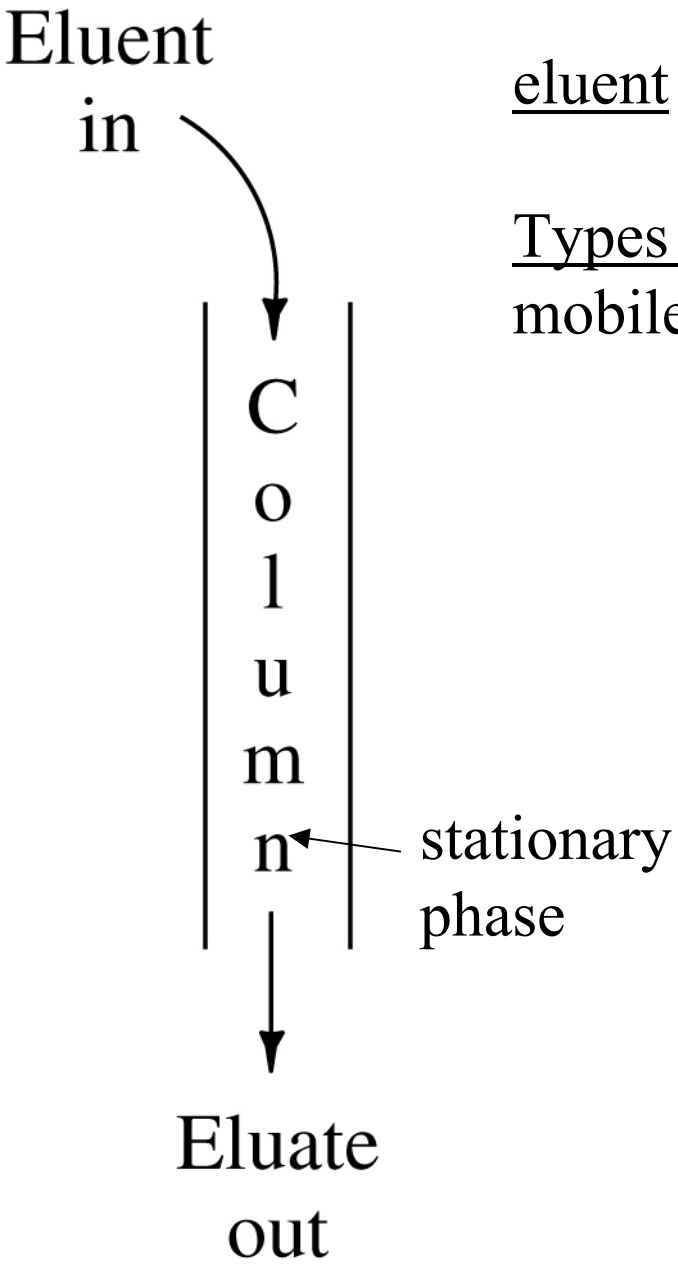
•Probably the most powerful class of modern analytical methods for analyzing mixture of components---and even for detecting a single component in a complex mixture!

•By first separating components (quickly if possible), it is easier to quantitate species with relatively non-selective detectors--- e.g., UV-vis absorbance, amperometric detectors, etc.).

•Also, can add additional element of selectivity---by using the detector to obtain more information about the species that elutes from the separation step (Mass Spectrometry as detector for chromatography, obtaining full UV-Vis spectrum with diode array spectrophotometer for each solute that elutes from column, etc., etc.)

Solutes---are species within sample that are separate!!





eluent = mobile phase!

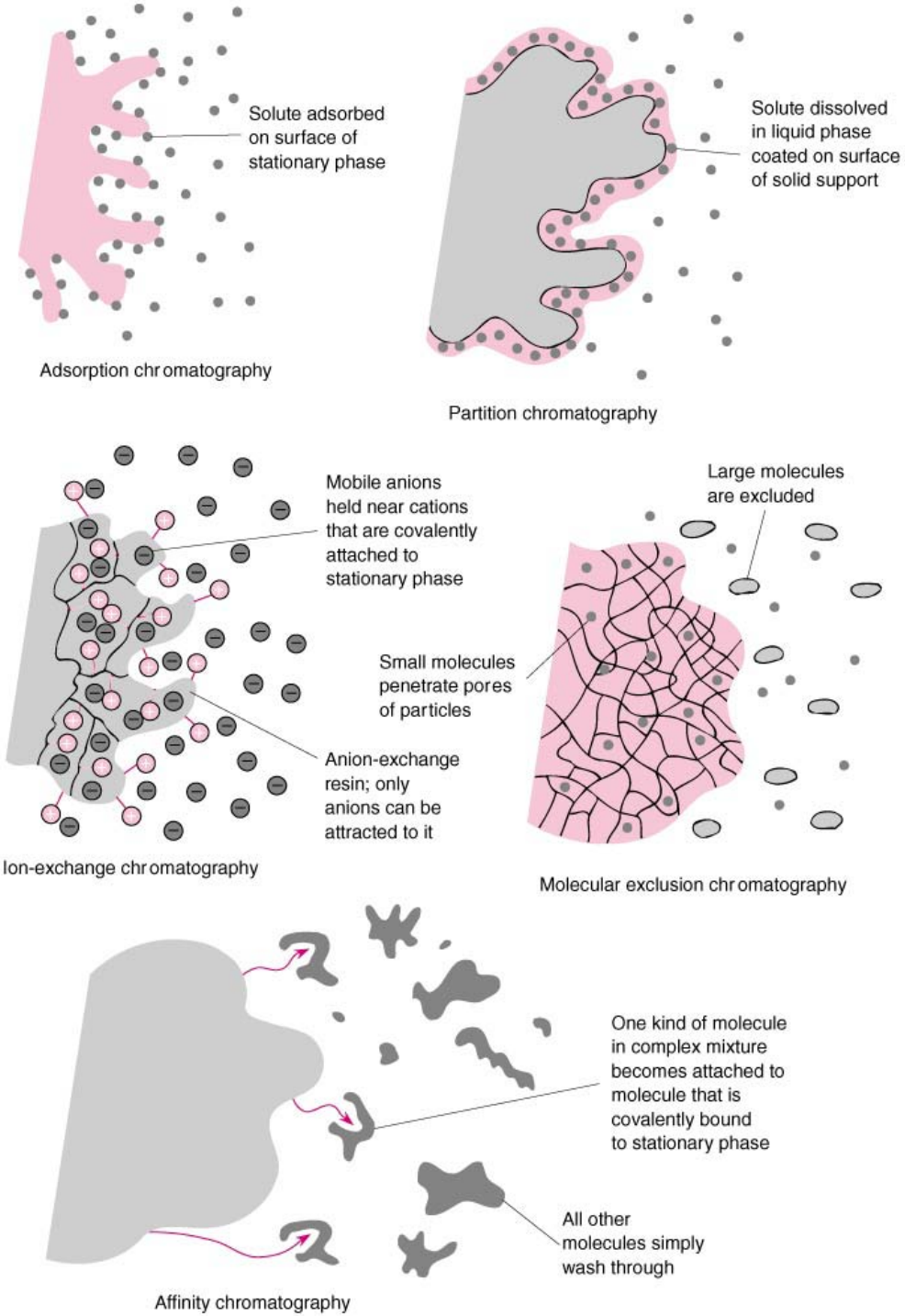
Types of chromatography--depends on nature of mobile phase and stationary phase!

mobile phases:

- liquid
- gas
- supercritical fluids

Stationary phases:

- adsorption
- partition phases
- ion-exchange (with liquid mobile)
- molecular exclusion
- affinity phases



adsorption--solutes stick to surface of stationary phase---some equilibrium constant for this interaction

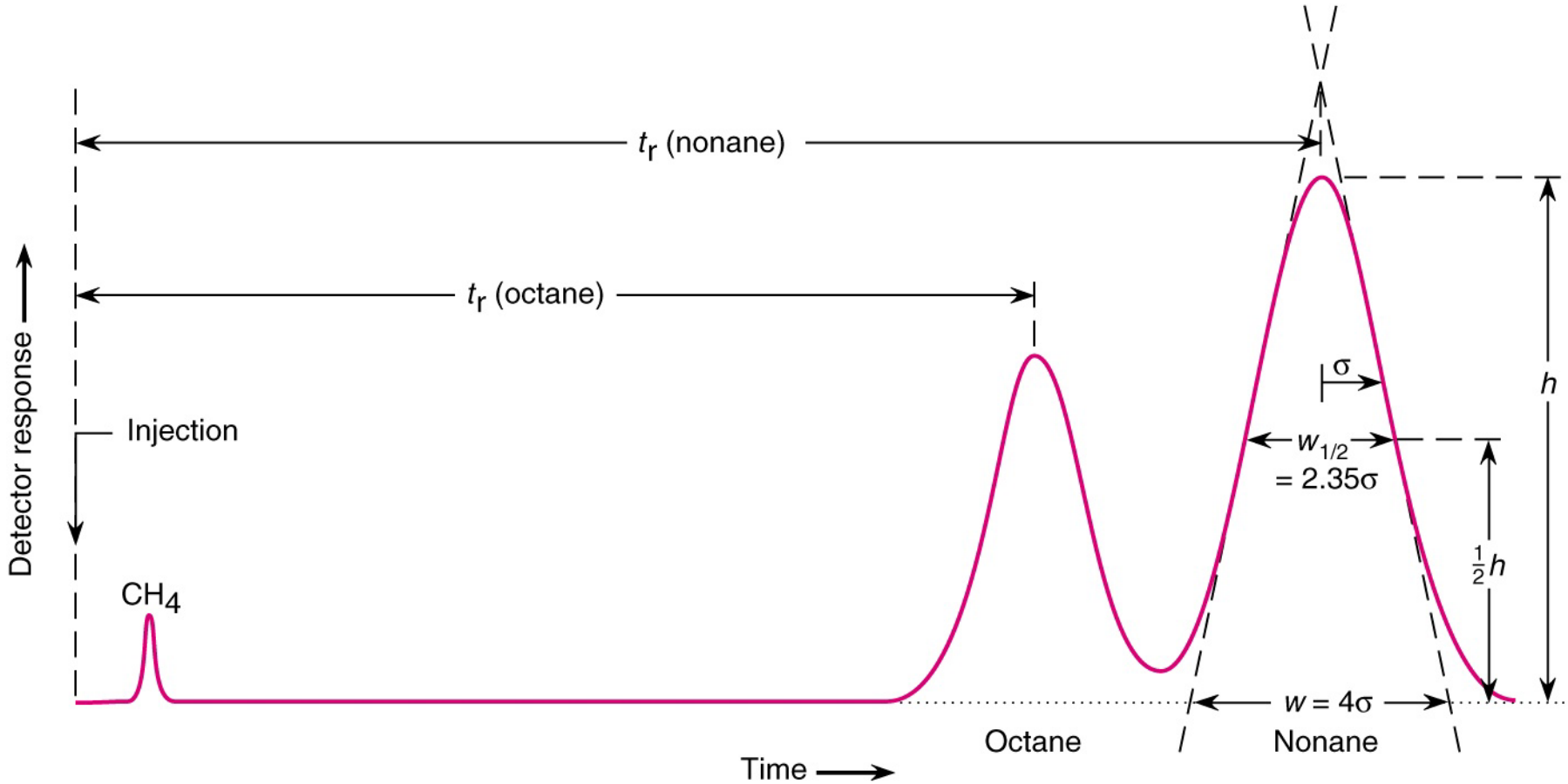
partition: stationary phase has finite volume, and solute species can equilibrate between two phases.

ion-exchange: stationary phase has charged functional groups on surface ($-N(CH_3)^+$, $R-SO_3^-$ etc.---species interact by electrostatic interactions!

molecular exclusion---packing acts like sieving phase--large species can't get in, while small species can--take longer to come out of column packing

affinity phases: immobilized species has very selective interaction with given solute!

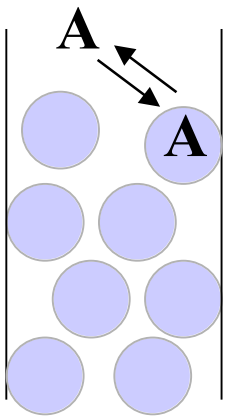
<u>chromatogram</u>---signal vs. time, as solutes elute off of the column!



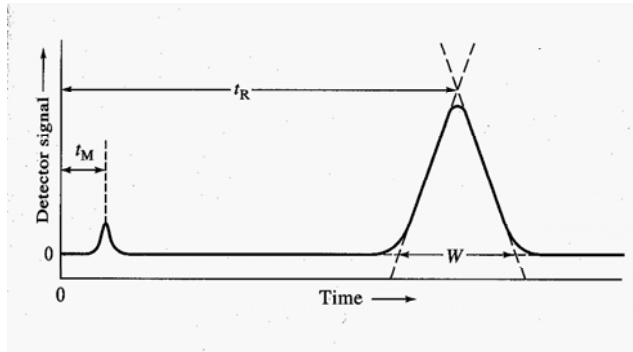
t_r = retention time of solute

t_m= void time--time it would take for solute to elute if it did not interact with stationary phase!

Theoretical Basis for Chromatography



$$A_{\text{startionary}} \overset{K}{\leftrightarrow} A_{\text{mobile}}$$



$$K = \frac{c_s}{c_M}$$

•The partition coefficient, K, is the ratio of concentrations the stationary and mobile phases.

•The analyte moves only when it is in the mobile phase.

$$\bar{v} = \frac{L}{t_R}$$

•Average analyte velocity, column length / retention time

This parameter is the key for qualitative analysis!

$$u = \frac{L}{t_M}$$

•Mobile phase velocity, u, column length / mobile phase transit time

Fundamental equation of Chromatography:

$$\mathbf{V_r = V_m + KV_s}$$

K = partition coefficient of solute between stationary and mobile phase

V_m = void volume of column; volume of mobile phase in column- and volume that solute would come out in after injection even if it did not interact with stationary phase!

V_r = retention volume; = volumetric flow rate x retention time (t_r)

Rely on differences in K values for two different solutes in order to separate them--so they have different V_r and t_r values!

Theoretical plates: imaginary discrete sections of a chromatography column in which solute species equilibrate between the stationary phase and mobile phase.

The retention of a solute on the column can be described by the number of theoretical equilibration steps that occur between injection and elution. The more "apparent" equilibration steps the narrower the width of the solute band when it elutes!

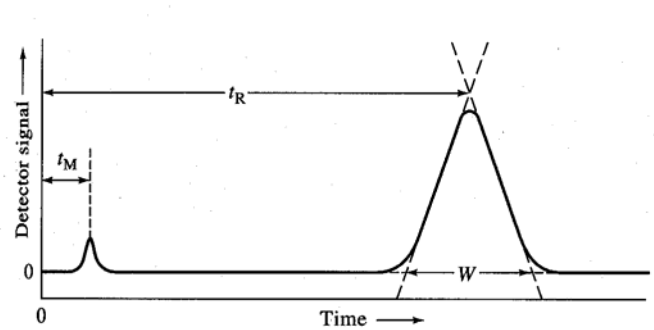
Number of Theoretical plates = N

The more theoretical plates in a given column---the more difficult separations that can be done (solutes with very similar partition coefficients).

HETP=height equivalent to theoretical plate = plate height = L / N

L = column length!

Resolution -depends on N theoretical plates



$$H \equiv \frac{L}{N}$$

- N: number of theoretical plates
- L: column length
- H: theoretical plate height (column length)

$$w = 4 \cdot \sigma_A$$

$$w_{1/2} = 2.35 \cdot \sigma_A$$

•H= the column length over which A(stationary phase) and A(mobile phase) are in equilibrium.
•N =the number of phase transfers that occurred during the complete separation.

$$H = \frac{\sigma_t^2 L}{t_R^2}$$

•Gaussian width measured with respect to column length
•actually measure peaks with respect to migration time, t_r

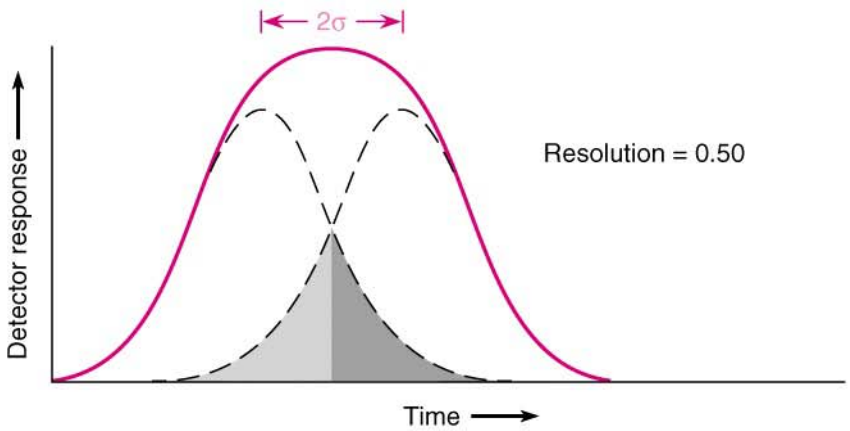
$$H = \frac{w^2}{16}\frac{L}{t_R^2}$$

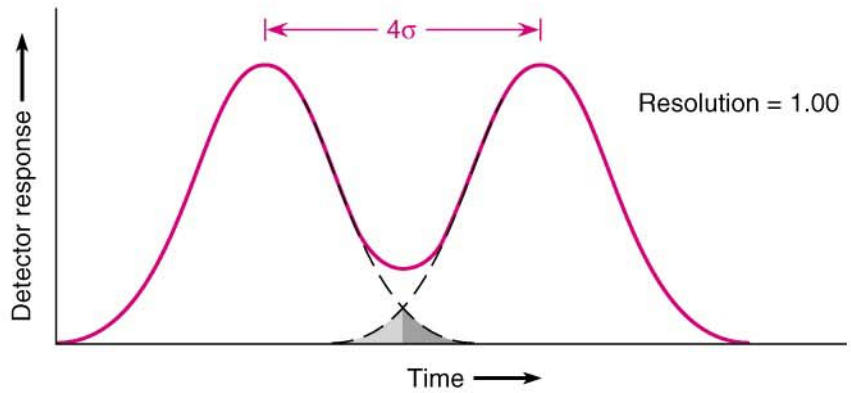
$$N = 16\left(\frac{t_R}{w}\right)^2 \quad \text{or} \quad N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Resolution = Δt_r / w_{avg}

Δt_r = peak separation in time

w_{avg} = average width of the peak at the base



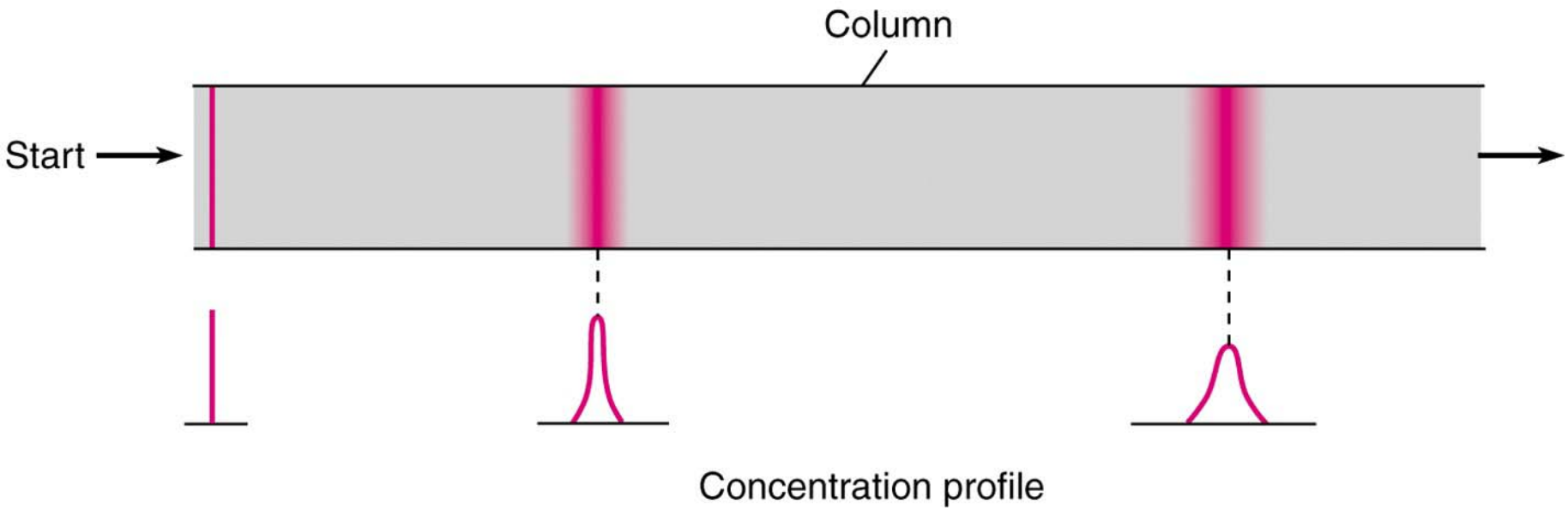


With an R= 1.0 there is 2.3 % overlap!--

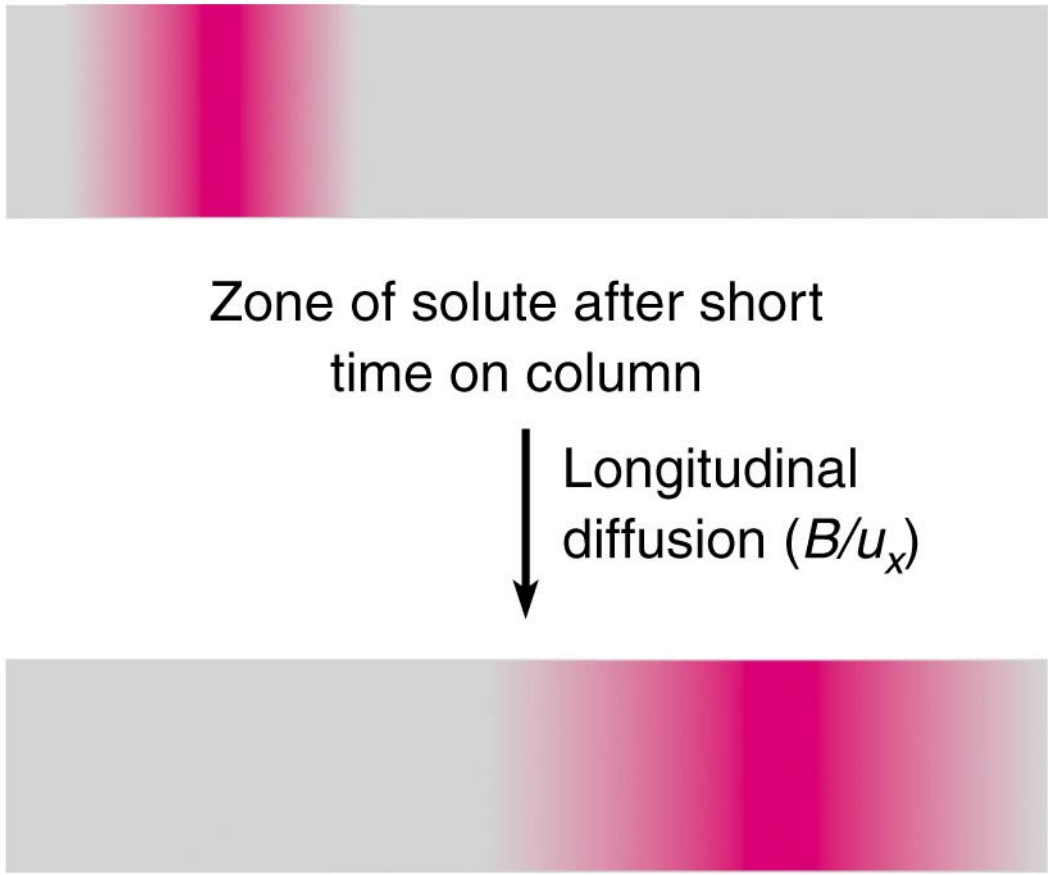
to improve resolution--want more N per given length

or increase length of column (with given stationary phase)!

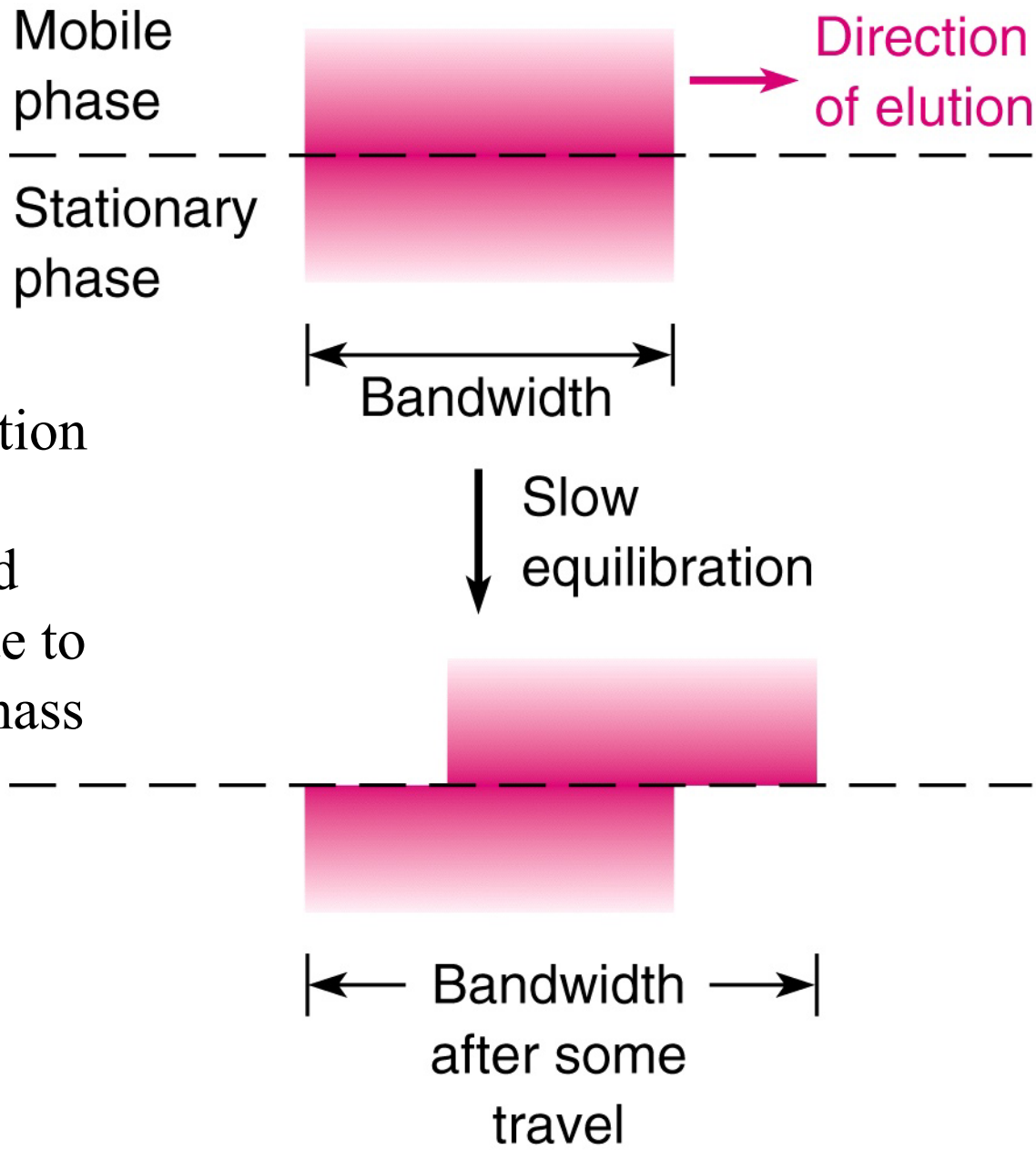
Why do solute bands spread?



longitudinal diffusion---diffusion of solute from region of high concentration to low concentration in mobile phase!---causes band broadening! Always present!!---worse in gas phase--due to much higher diffusion coefficients!--can decrease if you speed up the separation (higher flow rate!).

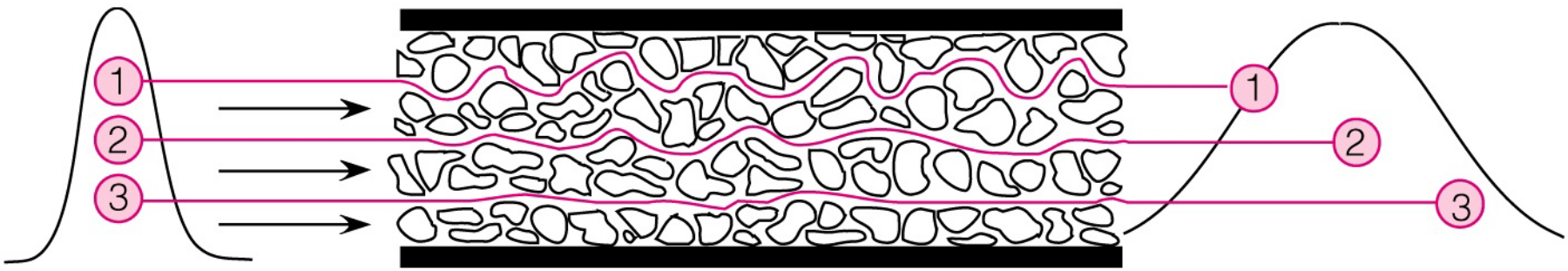

Zone of solute after short time on column
Longitudinal diffusion (B/u_x)
Zone of solute after longer time on column
Direction of travel

fast and slow equilibration between two phases---called broadening due to finite rate of mass transfer!



Band broadening due to multiple flow paths of solute through packing of column!

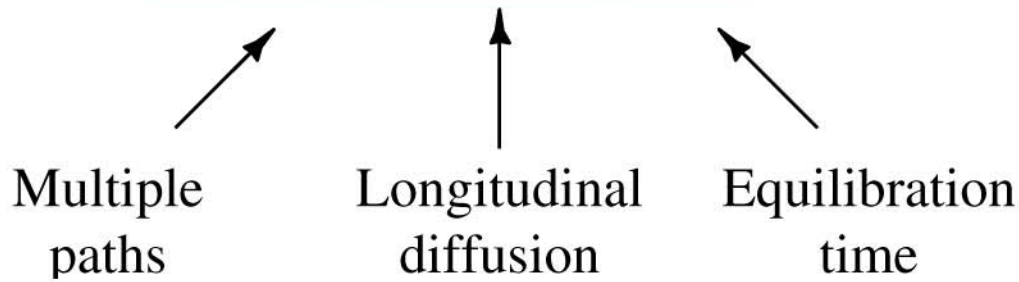
depends on size and homogeneity of stationary phase packing material!



Note: there is also broadening of band due to diffusion that takes place before and after column---must try to minimize ---e.g., use small “dead” volume detectors!!

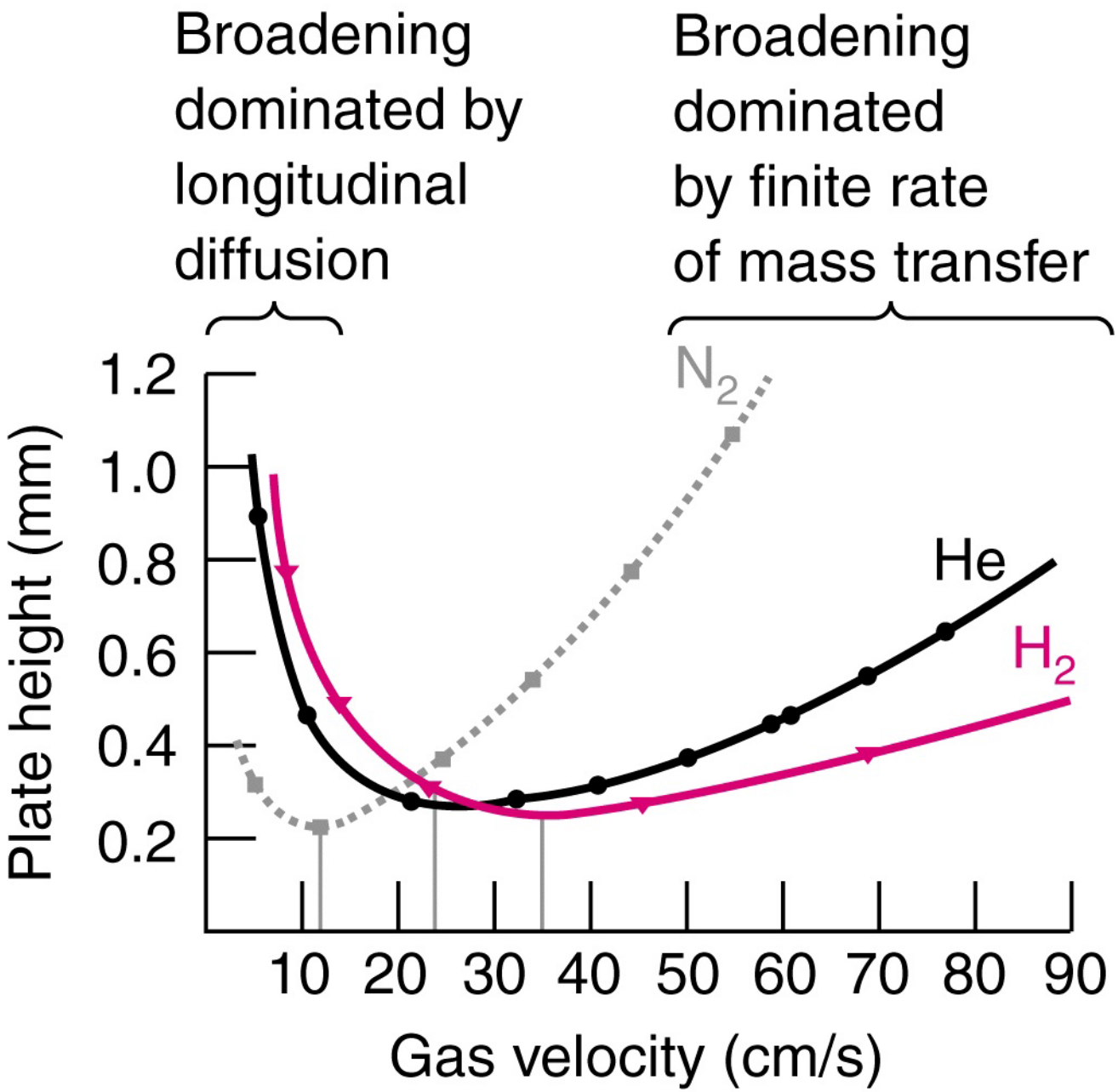
van Deemter equation for plate height:

$$H \approx A + \frac{B}{u} + Cu$$



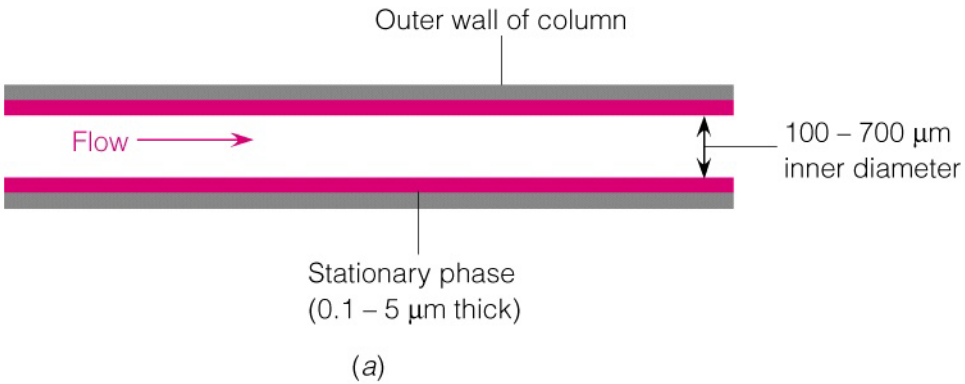
B--term---depends on diffusion in mobile phase---much greater value in GC then in LC

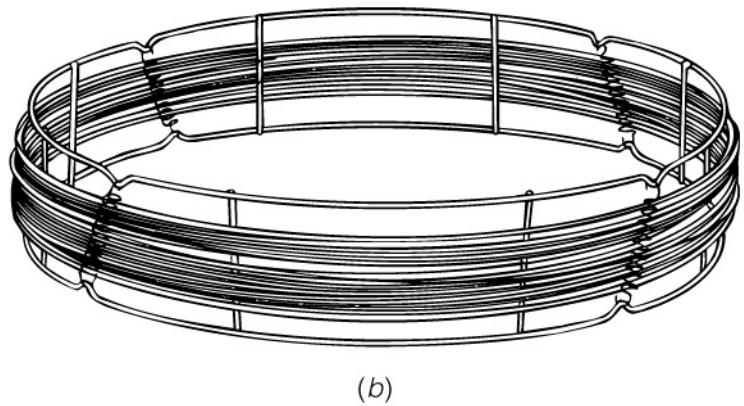
C--term---depends on size of packing material---and thickness of stationary phase layer!----origin of HPLC!!


Broadening dominated by longitudinal diffusion
Broadening dominated by finite rate of mass transfer
Plate height (mm)
1.2
1.0
0.8
0.6
0.4
0.2
N_2
He
H_2
10 20 30 40 50 60 70 80 90
Gas velocity (cm/s)

Open Tubular Columns----tend to have lower H values (greater N per given column length) since there is no A term in H equation (Van-deemter)---non packed column--

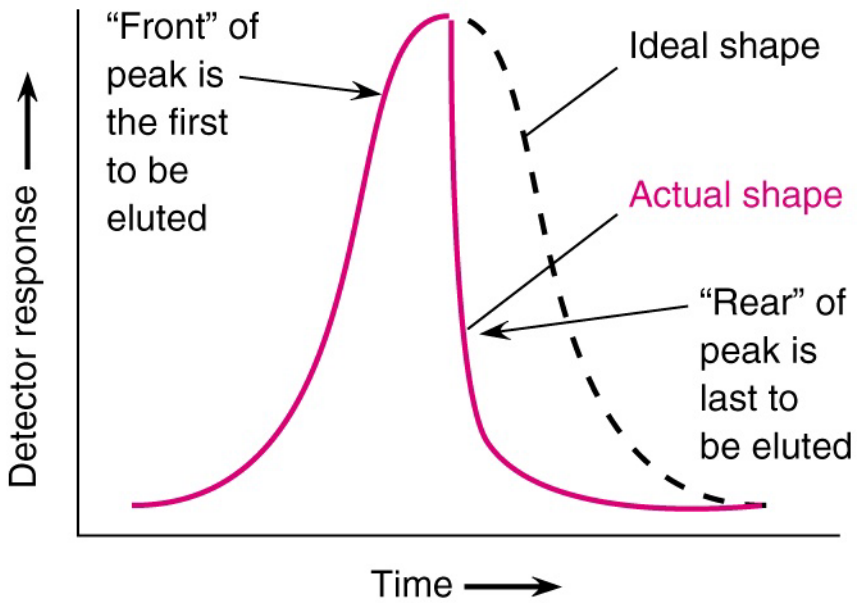
However, C term is highly dependent on radius of tube!! (why?)



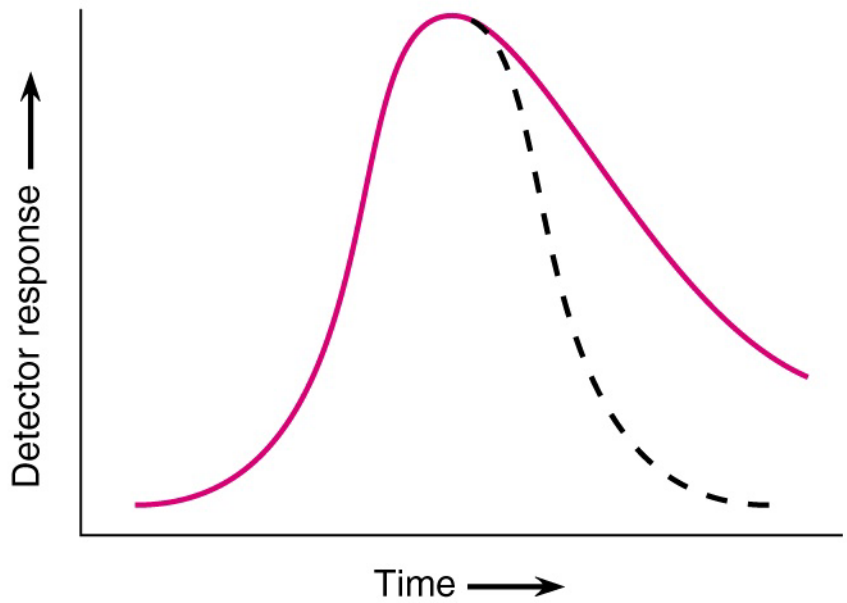


Such columns are often used in GC---to achieve very high # of plates by having very very long column (less backpressure--due to tubular nature; ---can't used very long packed column to increase N--since back-pressure would be too great to get decent gas flow!

Peaks can have strange shapes---often not Gaussian!



(*a*) Overloading



(*b*) Tailing

concentration of solute injected is too high---stationary phase cannot handle--

tailing---occurs when there are some other sites on stationary phase--that have strong interaction with solute

Qualitative and Quantitative chemical analysis with chromatography---

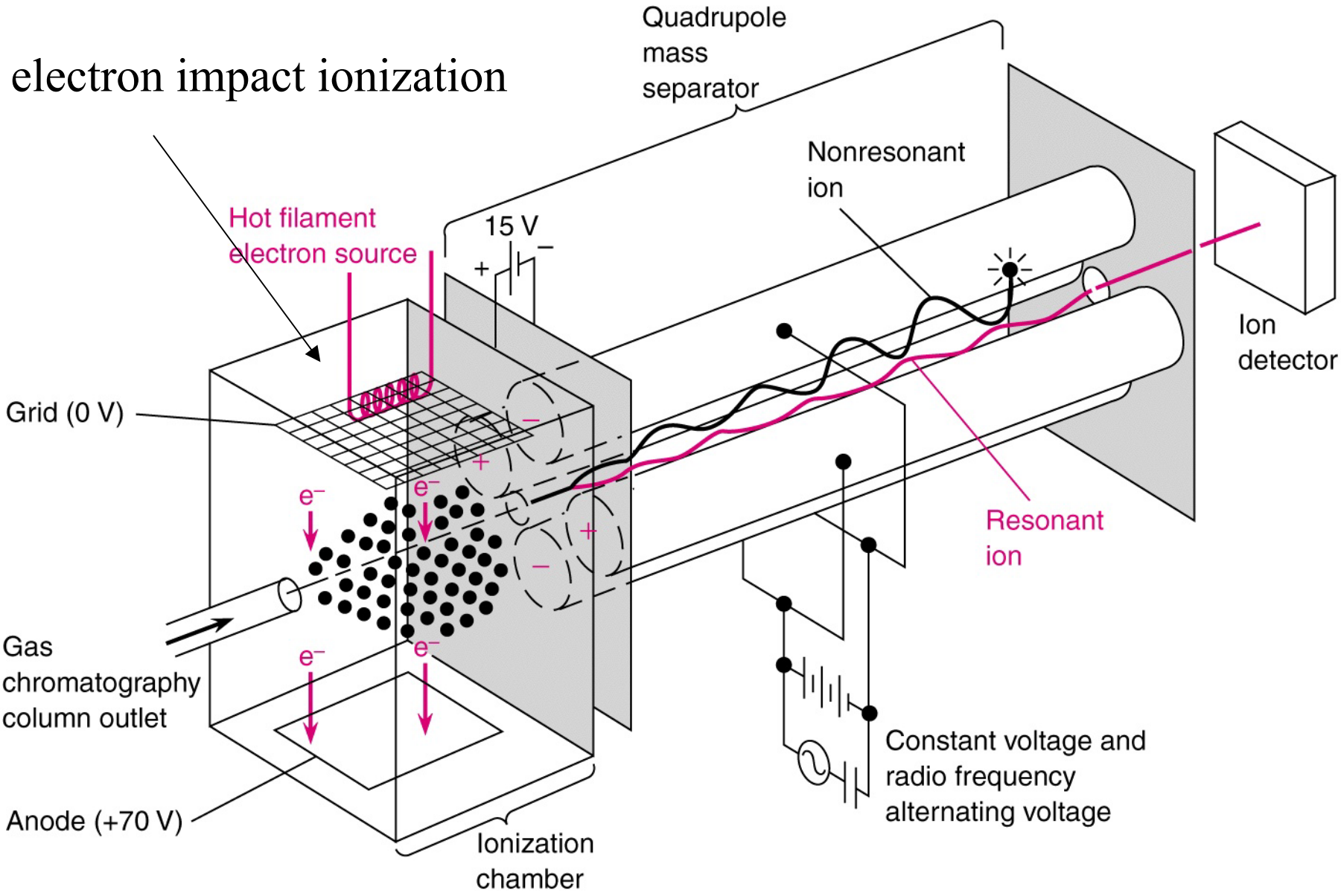
Must first know that the peak that is being detected is indeed the analyte---t_r should be unique for given analyte---but sometimes if you do not have enough N---solutes will have overlapping peaks! (means that two or more solutes have similar K_{part} values!)

can confirm solute bands by spiking sample with known amount of analyte--and then making sure the peak that you want to use for quantitation actually increases!!

More often, many LC and GC systems are now linked to mass-spectrometer detectors-----which provides powerful information to unambiguously determine which band is due to which species!---
---called LC-MS or GC-MS; very very powerful analytical tools!!

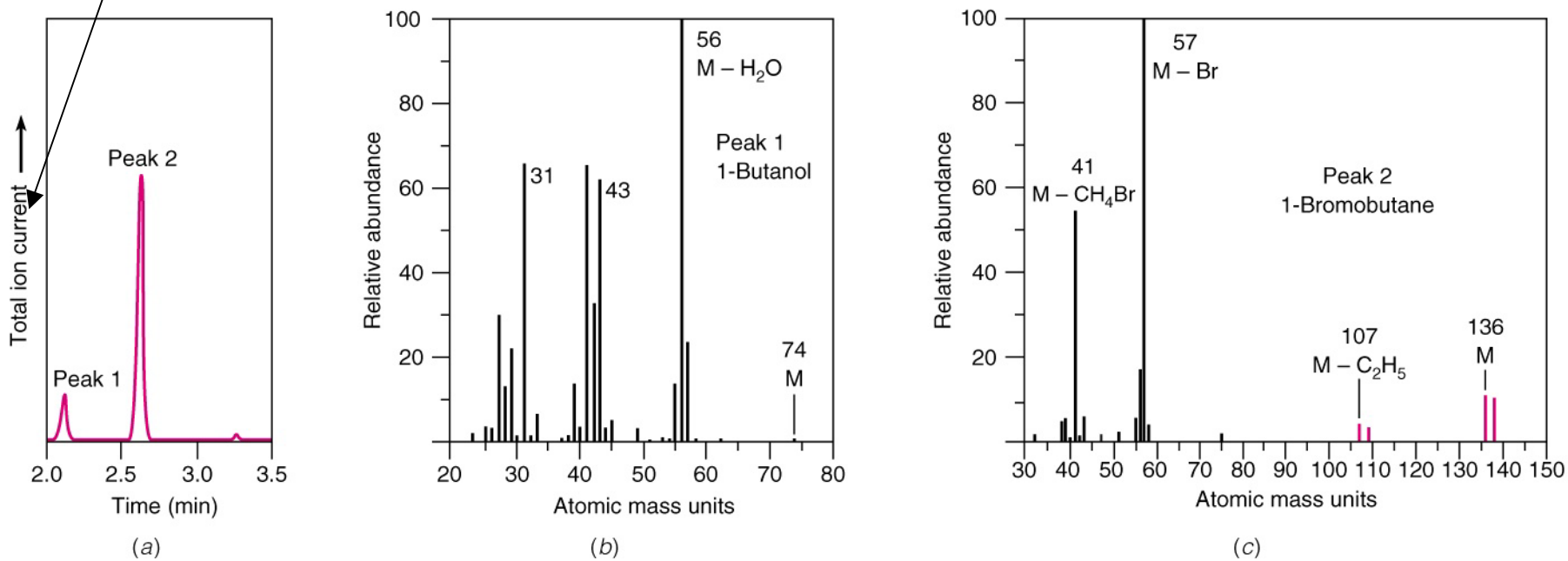
Quadrupole Mass Spectrometer---often used as detector for chromatography---ionize eluting solutes---separate based on m/z ratio

electron impact ionization



With electron impact ionization---you get considerable <u>fragmentation-can be very helpful in identifying species eluting (many species can have same m/z for parent ion---but few will have same fragmentation pattern---determined by structure of species)</u>

total ion current is integration of all peaks from MS of eluting solute---will be proportional to concentration or amount injected



$$CH_3CH_2CH_2CH_2OH + Br^- \longrightarrow CH_3CH_2CH_2CH_2Br + OH^-$$

1-Butanol 1-Bromobutane

Quantitation----usually will determine area of eluting band from chromatography---this will be proportional to amount of analyte injected on column---

If injection method allows very reproducible volumes to be injected (e.g., HPLC---special injection valve with well defined injection volume)--then can use calibration curve method (plot peak area or peak height--vs. concentration of injected standards)---still need to worrry about changes in flow rate that can effect peak height, etc.

However, for GC---hard to inject exact same microvolume each time--
Also--if using Mass Spectrometer as detector---ionization efficiency can change with time---hence amount of total ion current can be different even for same amount of analyte injected! (instrument drift!!)

In these instances---use method of Internal Standard---to quantitate analyte of interest!! (this method is also used often in straight Mass Spectrometry methods---and in some atomic emission methods)

method of Internal Standard (not same same as standard addition!)

compare signal from analyte--to signal from another species--that is similar to analyte---but is not present in the original sample!---

e.g., for analysis of ethanol in alcoholic beverages----you can spike samples with known amount of isopropanol!, etc.

Steps--1) determine the response factor, F, for analyte and internal standard; make up mixture with same concentration of X (analyte) and Y (internal standard). Inject into chromatography system--and record the peak areas for each species;

F = area (x) /area (y) ---measure of relative response of detector toward each species.

2) spike unknown sample with known amount of internal standard (y) to yield given concentration of y is sample [y].

then use following equation to calculate [x] (analyte concentration)

$$\frac{A_x}{[x]} = F\frac{A_y}{[y]}$$

A_x and A_y are measured peak areas (can also use peak heights-if bands are symmetrical-gaussian shape)--obtained from chromatogram of spiked sample!

[y] is known---since you spiked sample with known amount of y
F--is known--since you determined this value---

therefore---can determine [x] ---unknown analyte concentration

Isotope dilution mass spectrometry---one of the most accurate and definitive analytical methods of all---is based on this "internal standard" principle---but instead of spiking sample with different species---you spike sample with isotopically enriched version of analyte species (I.e., deuteriums replace all Hydrogens, etc.)

Isotope Dilution Mass Spectrometry