FOR414W
Chapter 3
Method Development

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Method Development

- Definition: Optimize analytical experimental conditions for detection of target compounds in specific samples.

  - Sample preparation condition (dirty samples to clean samples)
  - Instrumental conditions: e.g. Oven Temperature, Choice of Detector and Temperature, Column, Injection Amount and Temperature, ETC
  - Data analysis condition

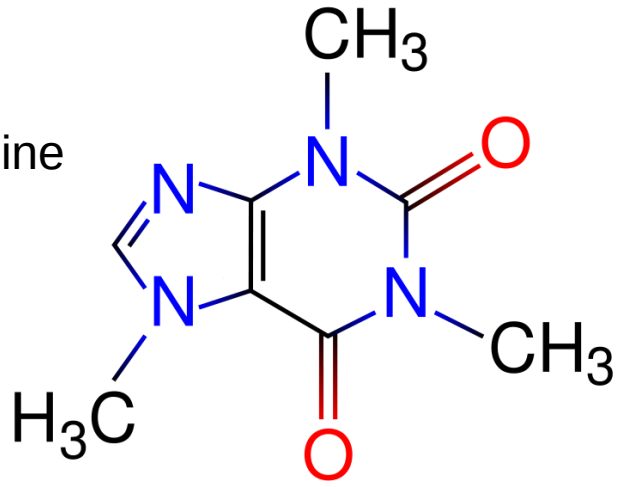
- For a specific target compound, many methods are possible.

- Developed Methods should be validated (proved).

Problem:
How much caffeine in a chocolate bar?

Physical and Chemical Properties of Caffeine

Caffeine



Molecular formula	$C_8H_{10}N_4O_2$
Molar mass	194.19 $g \cdot mol^{-1}$
Appearance	Odorless, white needles or powder
Density	1.2 $g \cdot cm^{-3}$, solid
Melting point	237 °C (non-equilibrium, superheated)
Boiling point	178 °C (sublimes)
Solubility in water	22 $mg \cdot mL^{-1}$ (25 °C) 180 $mg \cdot mL^{-1}$ (80 °C) 670 $mg \cdot mL^{-1}$ (100 °C)
Acidity (pK_a)	−0.13 – 1.22

What's the best analytical technique? and why?

- We know targets already
- We need to know quantity (concentration)
- Chromatography: separation and quantification
- Gas Chromatography? Why?
- Liquid Chromatography? Why?
- Other Chromatographic Techniques?

Three Steps

- Sample preparation (including references)
- Optimize experimental conditions
- Data analysis

Sample Preparation

1. Raw (dirty) samples are often complex **matrix** (e.g., alcohol in blood, cocaine in urine, caffeine in chocolate bar, pesticides in fruit).

2. Raw samples (e.g., blood, chocolate, urine, fruit, etc) can't be injected to instrument directly (damage to instrument, signal interference, signal reduction (matrix effect), etc).

3. Clean up is REQUIRED.

4. Target compound(s) can be separated or interference(s) can be removed from samples.

5. Separation via the differences in physical or chemical properties of the individual components.

6. Solubility, boiling point, pK_a, and vapor pressure, etc.

Partition Theory

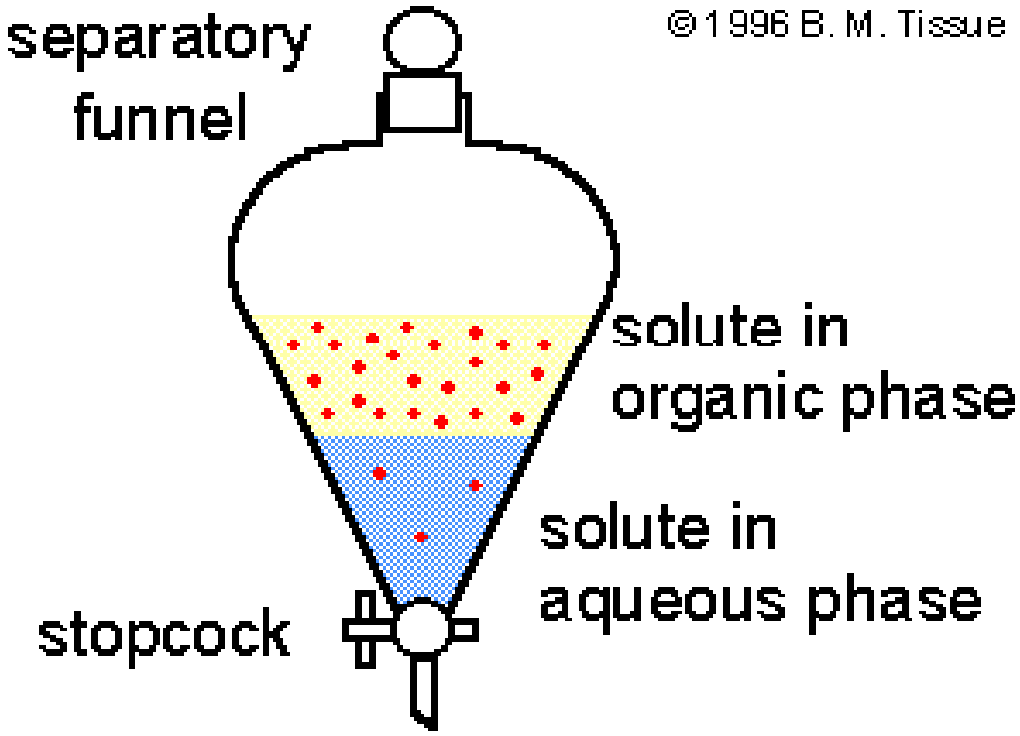
Distribution of a solute, *S*, between two immiscible solvents (such as aqueous and organic phases) is an equilibrium condition that is described by the following equation:

$S_{aq} <==> S_{org}$

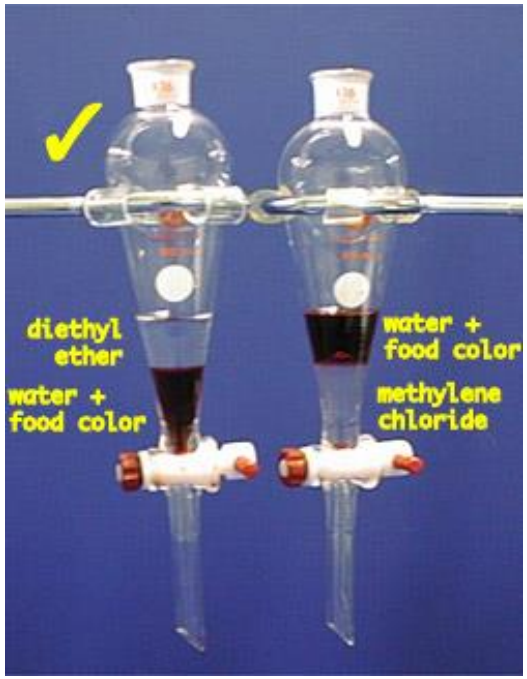
The equilibrium constant :

$K = [S_{org}]/[S_{aq}]$

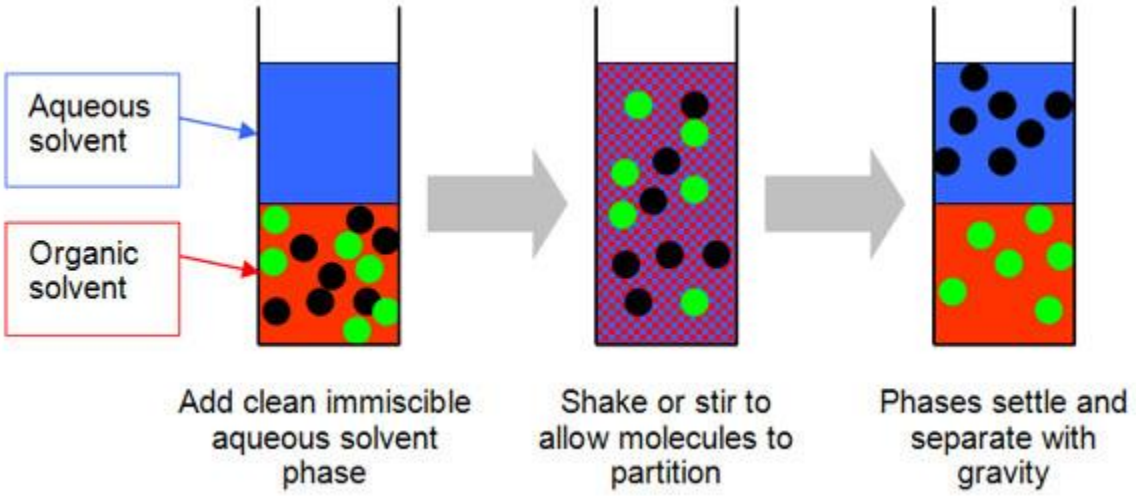
K: the partition
(or distribution) coefficient.



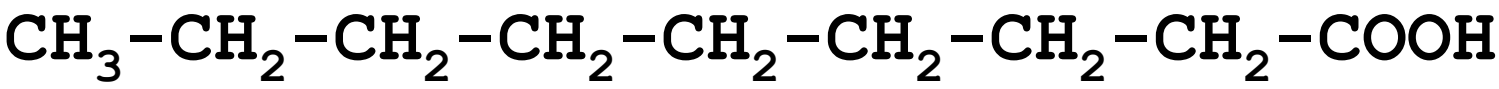
Liquid/Liquid Extractions



Solids are usually dissolved or digested in caustic solution and liquids are sometimes extracted to separate the analyte from interferences.



pH Controls Extraction



- At low pH: neutral, soluble in organic solvent
- At high pH: ionization of COOH group, soluble in water
- Effects of pK_a

pK_a vs. pH

$$HX(aq) \rightleftharpoons H^+(aq) + X^-(aq)$$

$$K_a = \frac{[H^+][X^-]}{[HX]}, then\ [H^+] = K_a \frac{[HX]}{[X^-]}$$

$$-log[H^+] = -log\ K_a - log \frac{[HX]}{[X^-]}$$

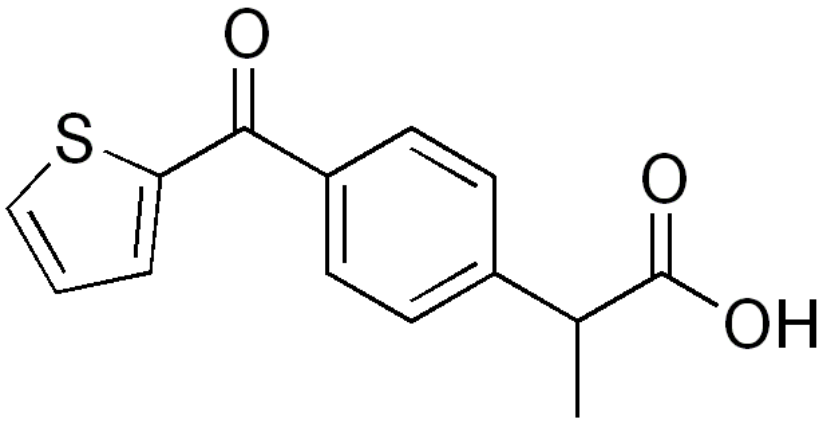
$$\therefore pH = pK_a + log \frac{[X^-]}{[HX]}$$

$$\therefore pH = pK_a, if\ [HX] = [X^-]$$

- pK_a is the pH where 50% of acids is deprotonated.
- If pH > pK_a, more than 50% of acids are deprotonated
- If pH < pK_a, less than 50% of acids are deprotonated

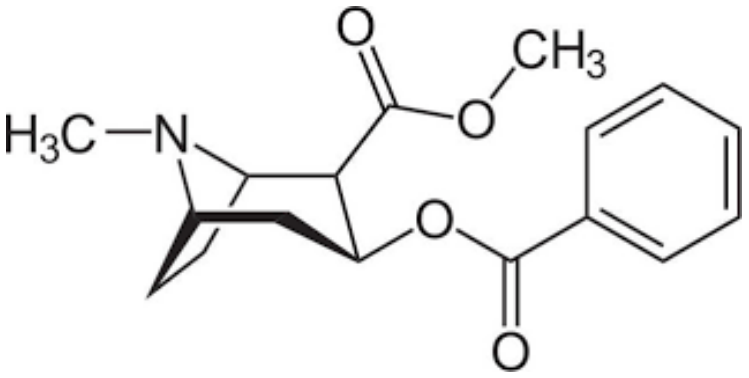
Many Drugs are Either Acids or Bases

suprofen



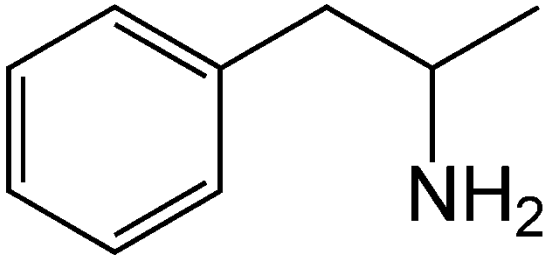
pK_a = 3.9

cocaine



pK_a = 8.6

amphetamine



pK_a = 9.8

composition at pH = 7.2?

Solid Phase Extractions (SPE)

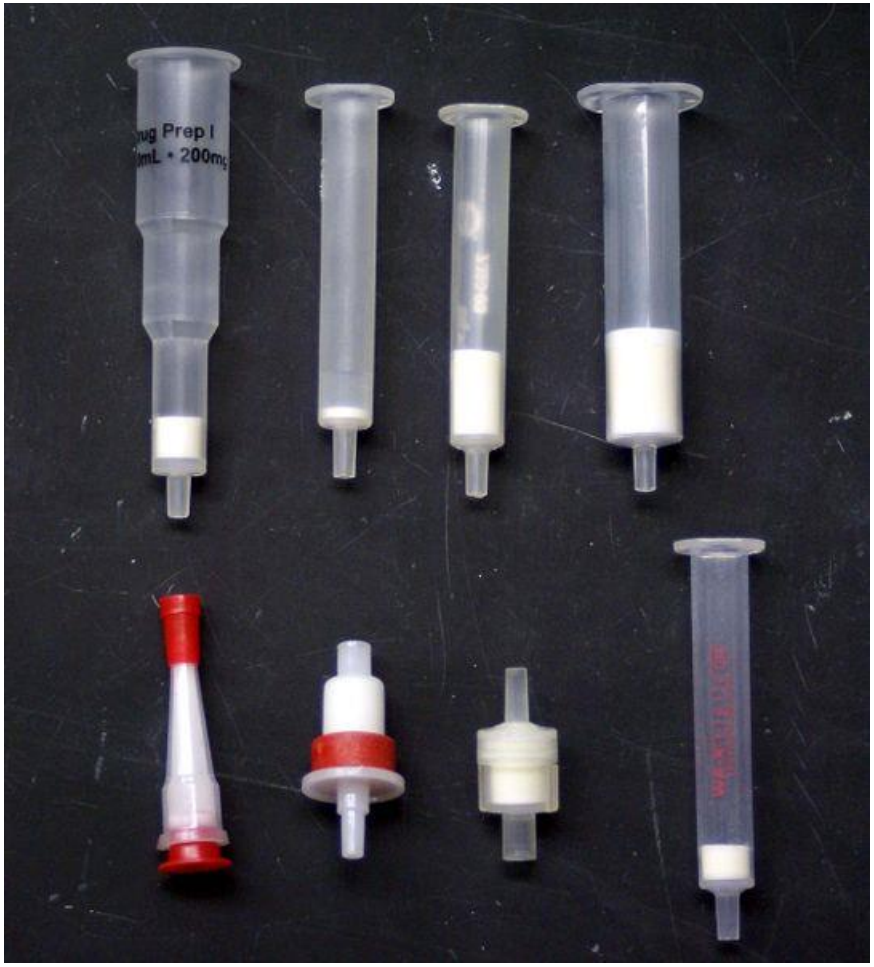
1. Considered as a pre-liquid chromatographic process

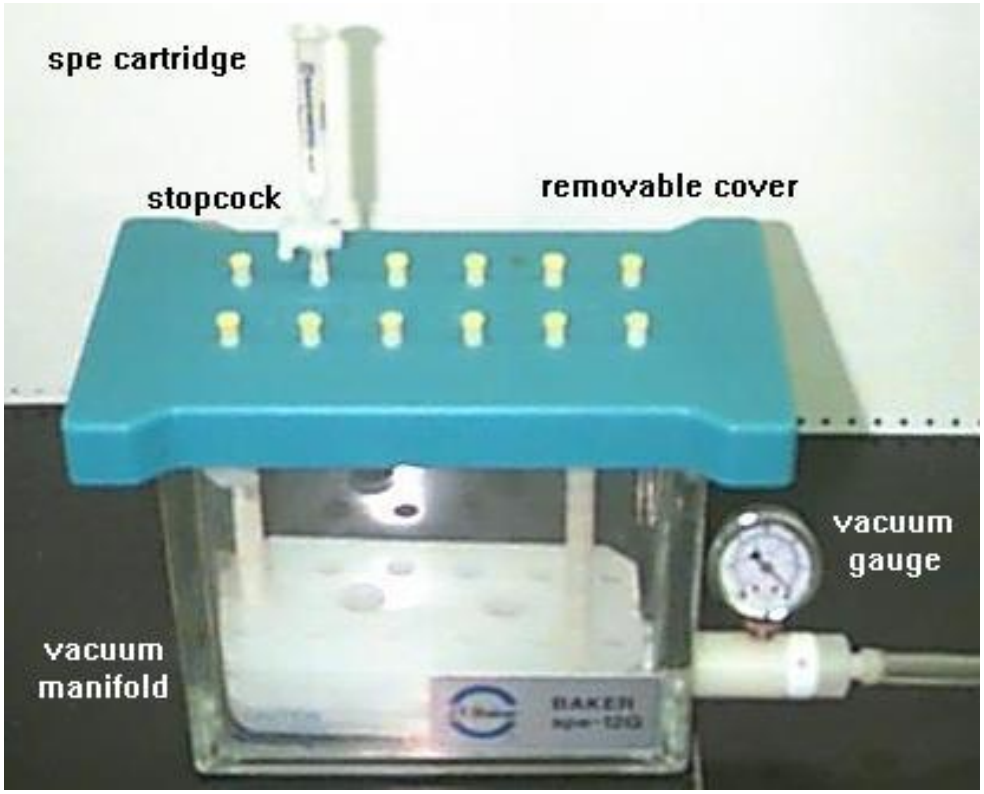
2. SPE is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution.

3. The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube.

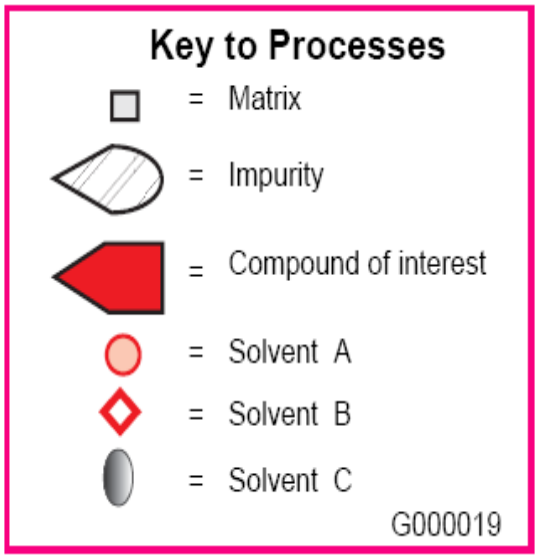
4. SPEs use the same type of stationary phases as are used in liquid chromatography columns. The column might have a frit on top of the stationary phase and might also have a stopcock to control the flow of solvent through the column.

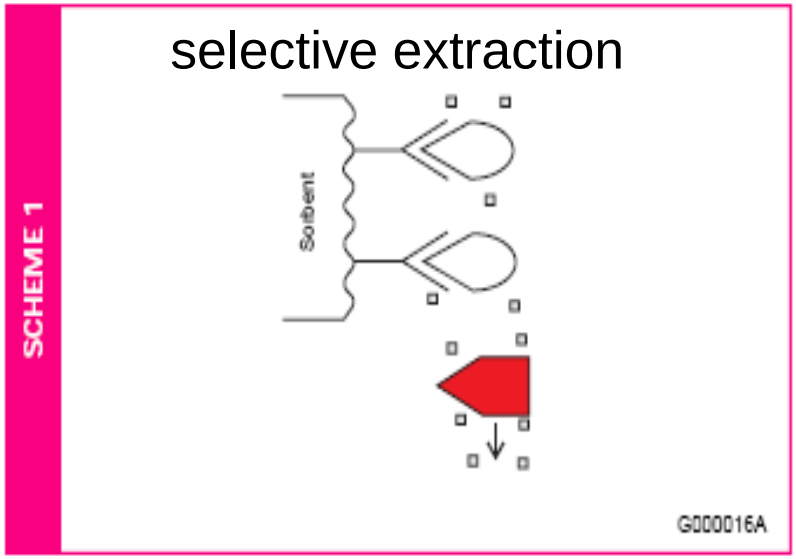
SPE Cartridges

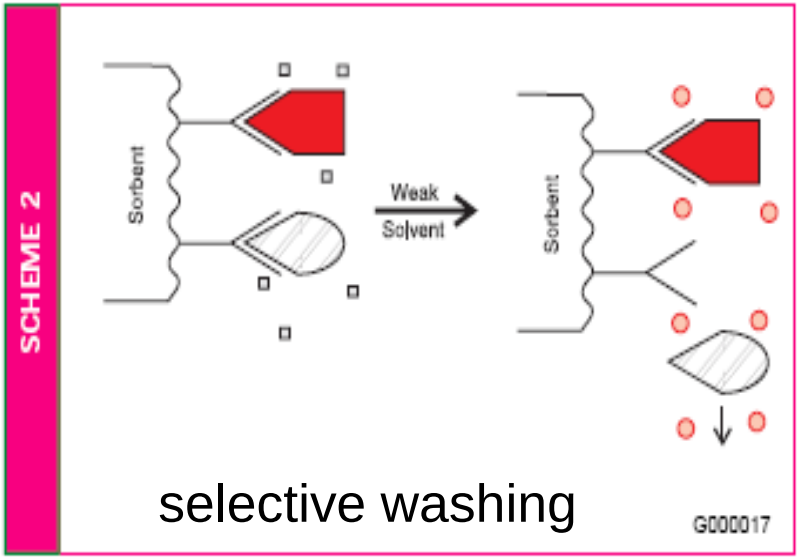


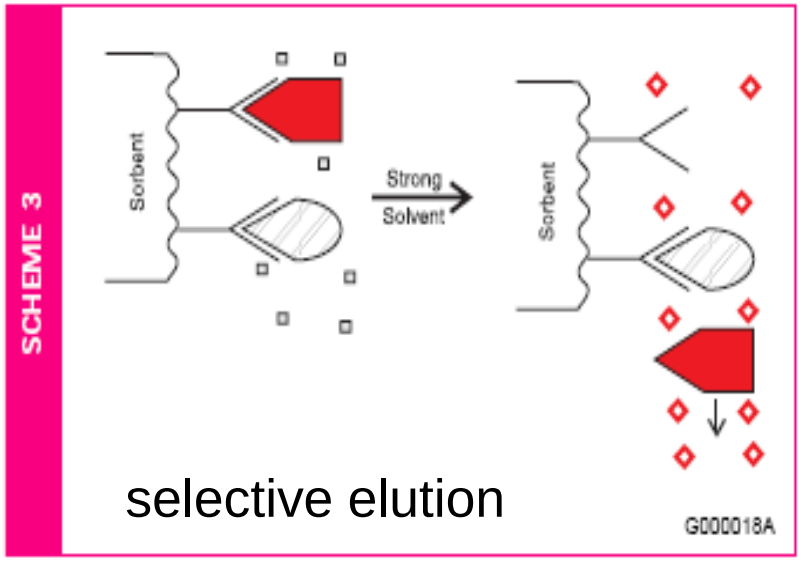


Three SPE Schemes









Characteristic of Solvents Commonly Used in SPE

Polarity			Solvent	Miscible in Water?
Nonpolar	Strong Reversed Phase	Weak Normal Phase	Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Methylene chloride (dichloromethane)	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
	Weak Reversed Phase	Strong Normal Phase	Methanol	Yes
			Water	Yes
Polar			Acetic acid	Yes

Issues in SPE Procedures

- Is the separation perfect?
- Or are all target compounds separated (or collected)?
- Or all interference (matrix) removed?
- Any loss of target compounds?
- If not, how do you know the real amount of target compounds in a sample?

Recovery Test and Standard Addition

- Recovery test:
  1. Prepare sample using a known amount of target and same (or similar) matrix.
  2. Repeat same SPE and estimate how much target was recovered

- Standard addition: Addition of a known amount of standard to samples

SPE Procedure Tips

1. Find appropriate literatures

2. Compare results and tune the experimental conditions by changing solvent compositions (polarity of solvents), pH, etc

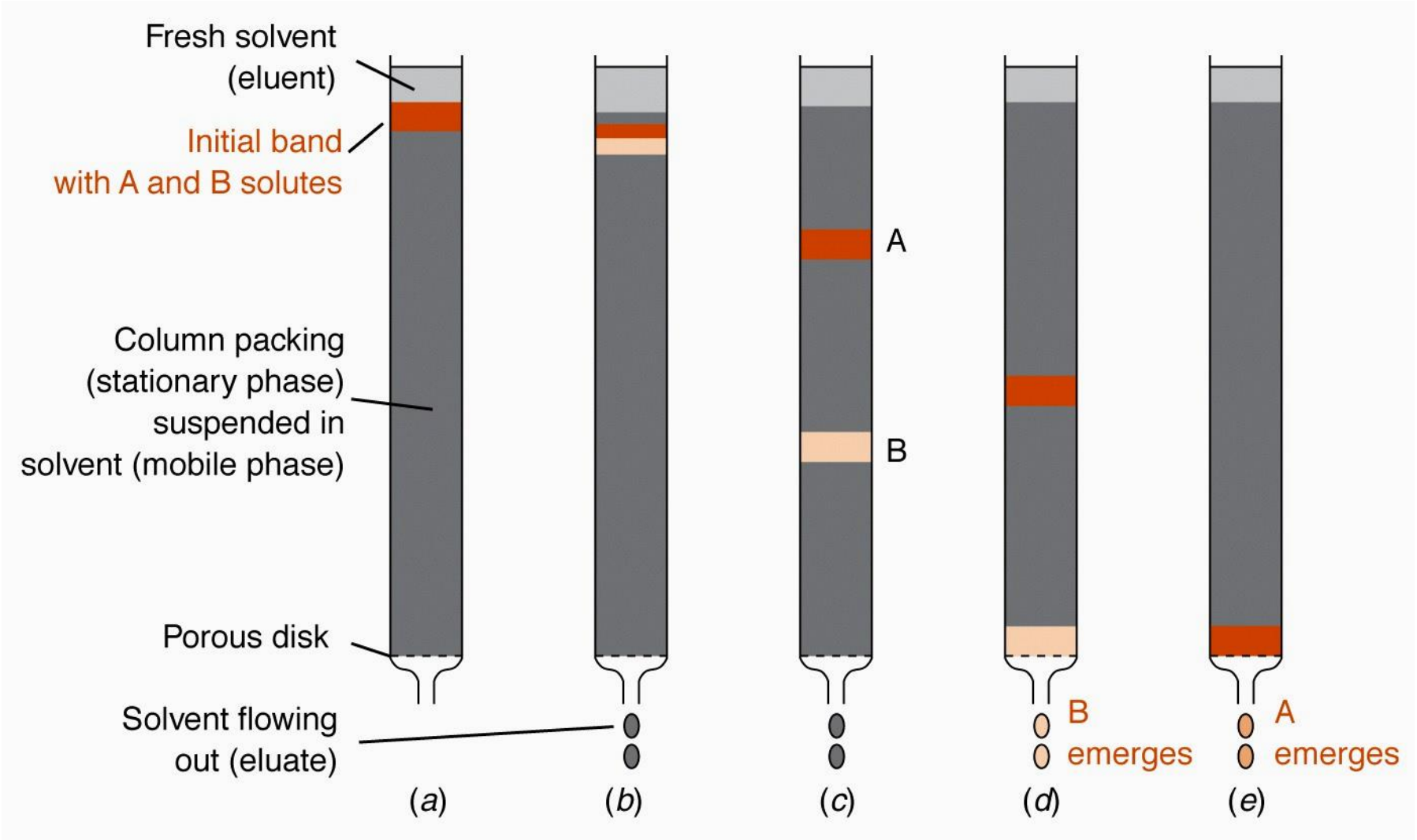
Gas Chromatography

- http://www.youtube.com/watch?v=dffeiLgeKx8
- Samples (gas or liquid or solid) should be vaporized.
- Sample is transported through the column by the flow of inert and gaseous mobile phase.
- Components in a sample are separated and pass the detector with different time.

Basic Principles of Chromatography

1. Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (gas or liquid), which is then forced through an immobile, immiscible *stationary (solid) phase*.
2. The stationary phases are chosen such that components of the sample have different solubilities (or affinity or interaction, etc) in each phase.
3. A component which is quite soluble (more interactions) in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase.
4. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.
5. Techniques such as HPLC (High Performance Liquid Chromatography) and GC (gas ghromatography) use *columns* - narrow tubes packed with stationary phase, through which the mobile phase is forced.

Chromatography (e.g. LC)



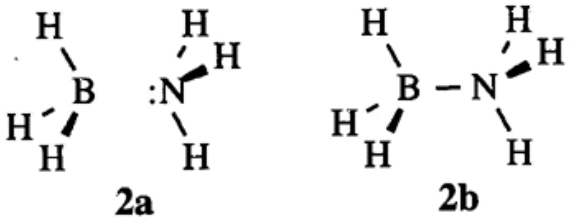
Interactions Between Surfaces and Molecules

1. Charge-charge interactions (electrostatic interactions): These are interactions between cations and anions with formal charge. The electrostatic force between two point charges is given by $F = k\,[q1 \cdot q2]/r^2$

2. Acid-base interaction: electron pair donor Lewis bases "react with" or "complex with" or "interact with" electron pair acceptor Lewis acids.

3. Dipole-dipole interactions: Water and methanol

4. Dipole-ion interaction: NaCl (Na^+ and Cl^-) in water



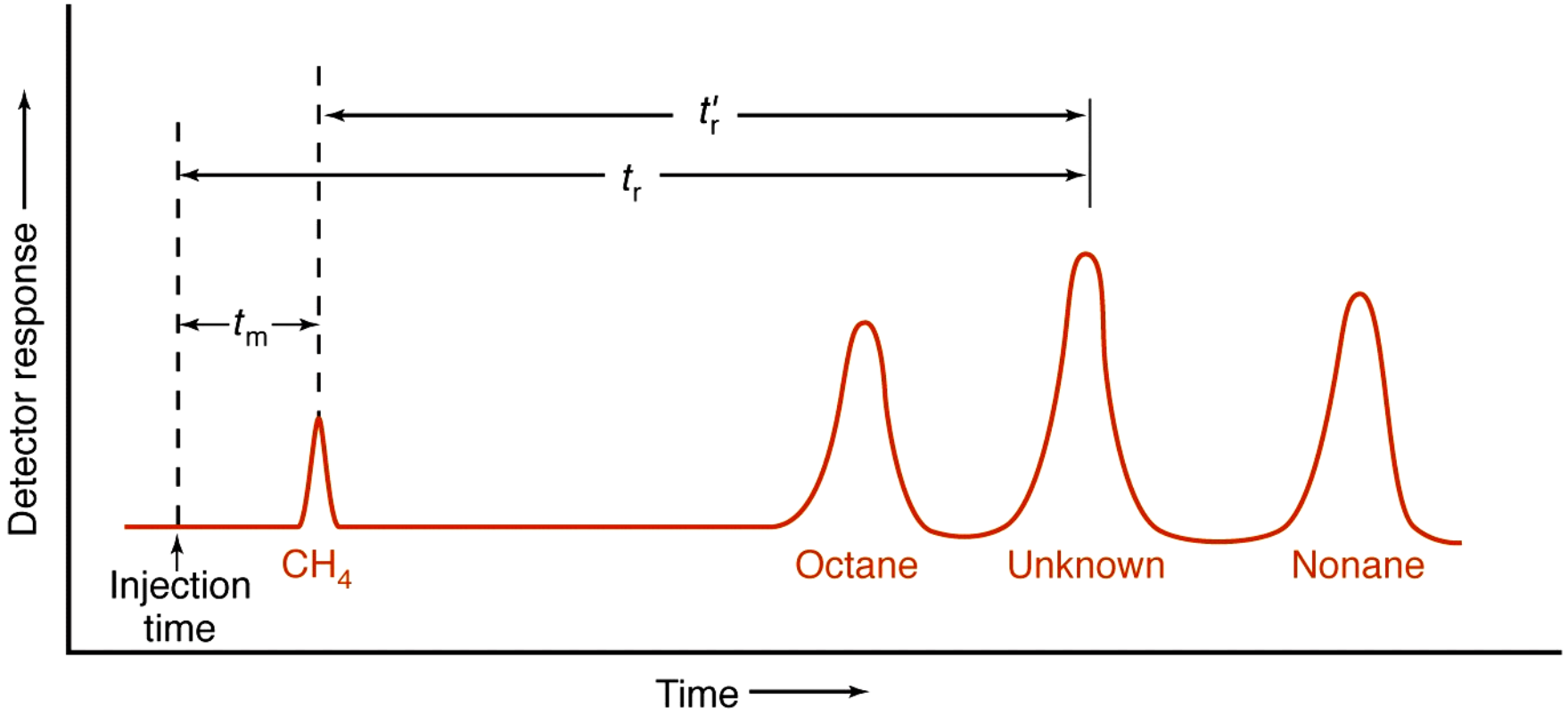
2a 2b

5. van der Waals interactions

6. Hydrogen bonding

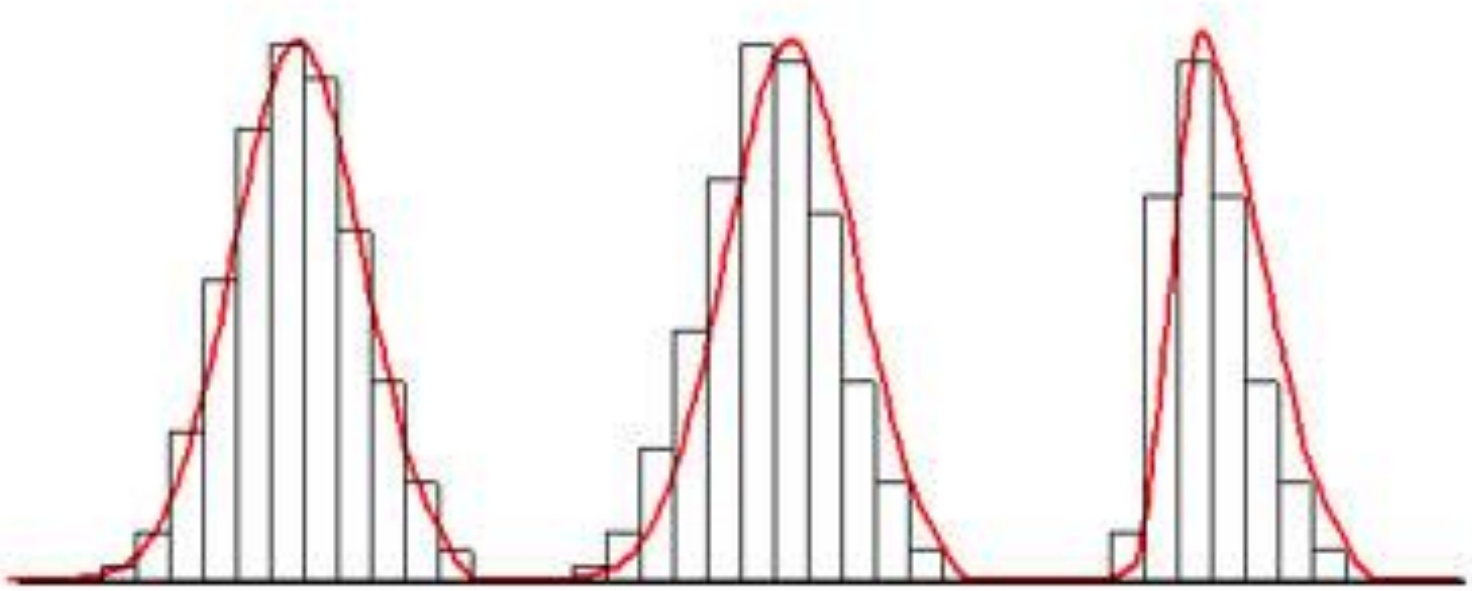
7. Hydrophobic interaction: Two areas of hydrophobic substances will encounter one another, combine and form one larger hydrophobic region that is excluded from the water matrix.

Retention Time: Qualitative Analysis



Accuracy of Quantification: Peak Shape

Effect of summation algorithm on recorded peak area



rectangles symmetrical around data points optimal estimate of true peak area (left)

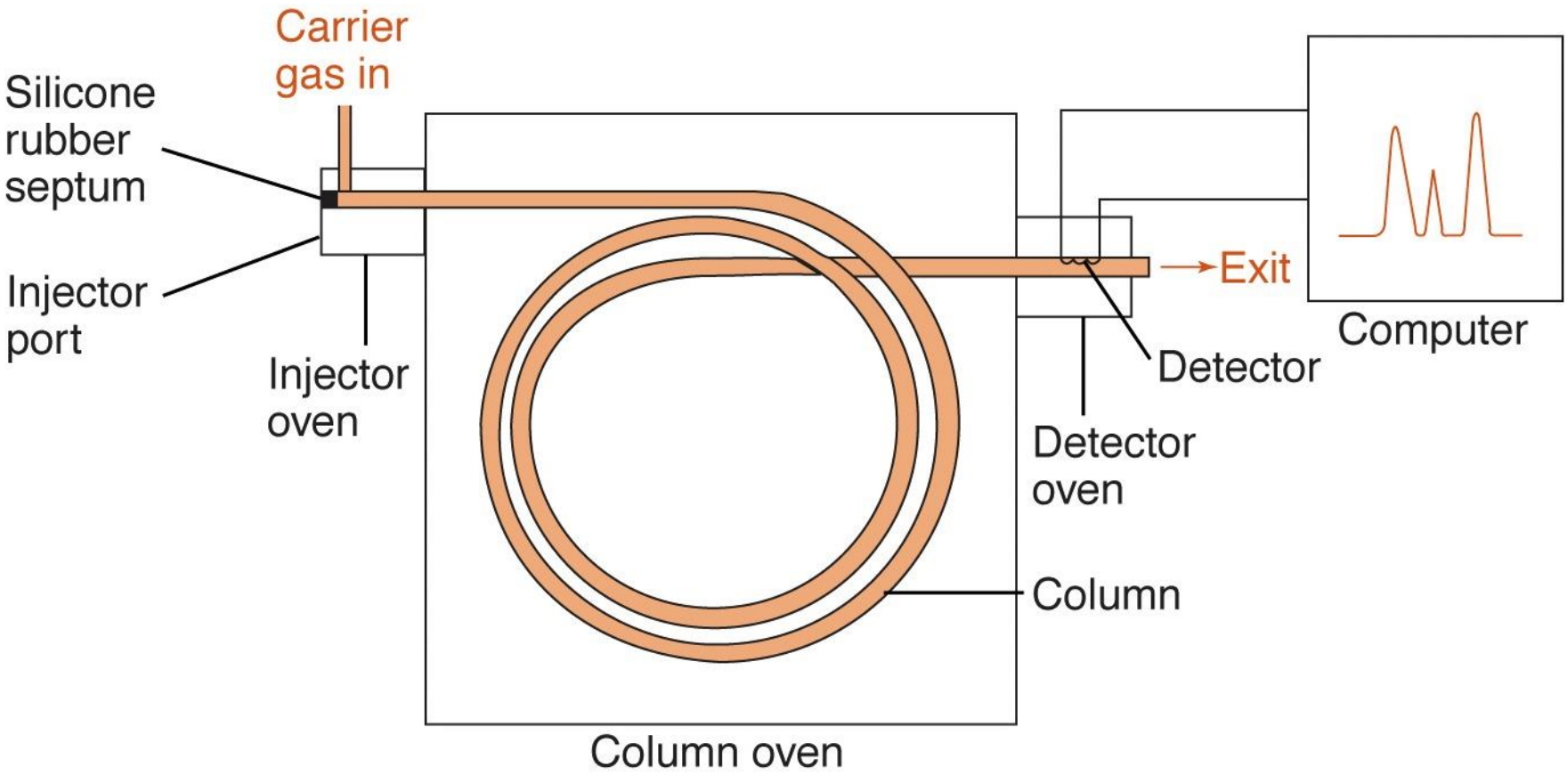
rectangles asymmetrical with respect to curve front overestimate compensated by tail underestimate for symmetrical peaks (center), but not for asymmetrical peaks (right)

What's the method?

- Analytical procedure to detect and quantify target compounds from sample preparation, chemical analysis, and data analysis.
- GC: choice column, inlet temperature, detector, oven temperature, flow rate, etc.
- For a given target compound, different methods are developed depending on samples (caffeine in coffee, caffeine in urine, caffeine in blood, etc).
- For a given compound in a same sample (caffeine in coffee), many methods are developed.
- For some compounds, methods are not flexible (should flow specific methods).

GC Method

- For a give compound, experimental conditions are specified (choice column, inlet temperature, detector, oven temperature, flow rate, etc).
- For a new method development, these conditions should be optimized to get the best results (peak shape, resolution, etc).
- Methods should be validated using a standard sample.
- First step: (a) understand components in GC and their functions and (b) effects of experimental conditions on analytical data (chromatogram)

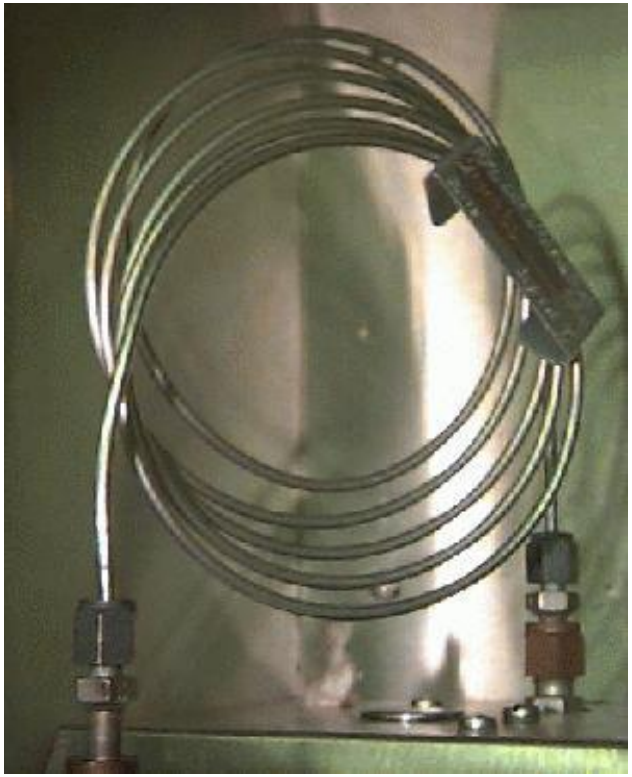

Carrier gas in
Silicone rubber septum
Injector port
Injector oven
Exit
Computer
Detector
Detector oven
Column
Column oven

Carrier Gas

- The carrier gas must be *chemically inert*.
- Commonly used gases include *nitrogen, helium, argon, and carbon dioxide*.
- The choice of carrier gas is often dependant upon the type of detector which is used.
- The carrier gas system also contains a molecular sieve to remove oxygen, water, and other impurities.

Columns

- There are two general types of column, *packed* and *capillary* (also known as *open tubular*).

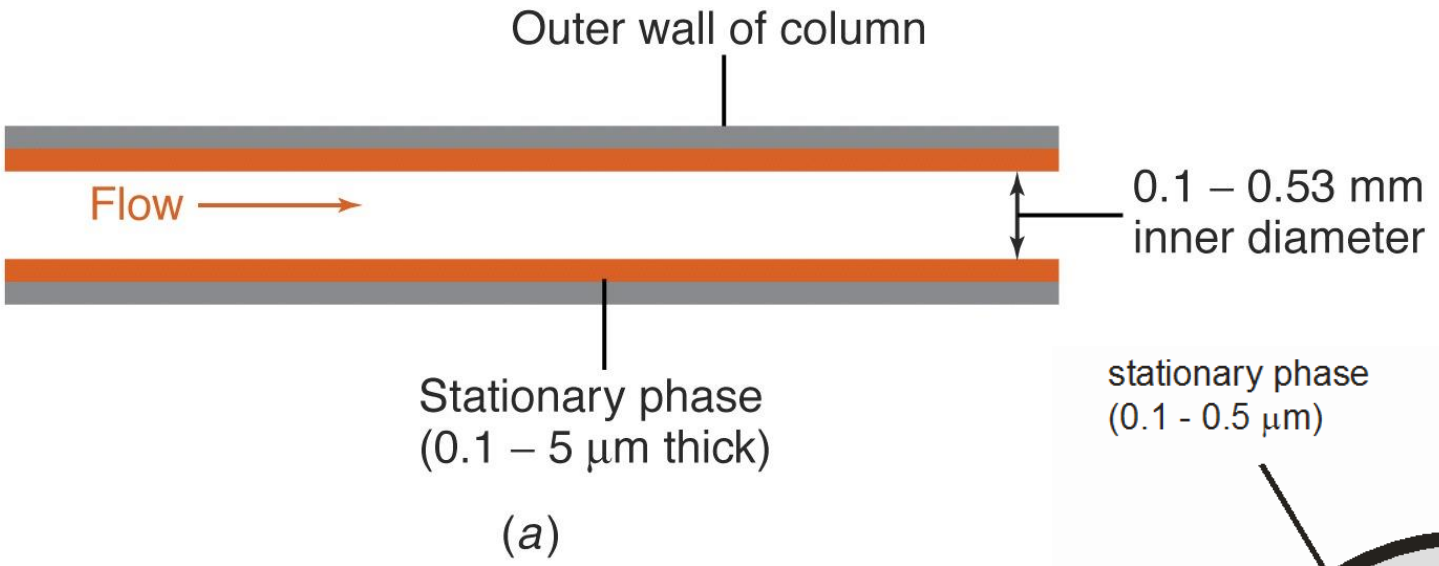


Packed Columns (Limited Use)

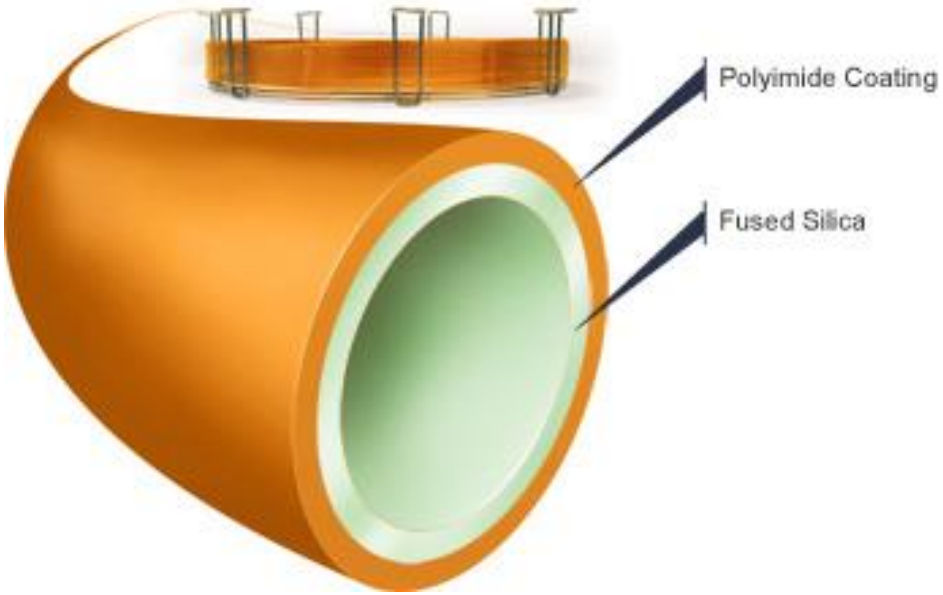
- Packed columns contain a finely divided, inert, solid support material (chromosorb, commonly based on natural product, *diatomaceous earth*) coated with various stationary phase.

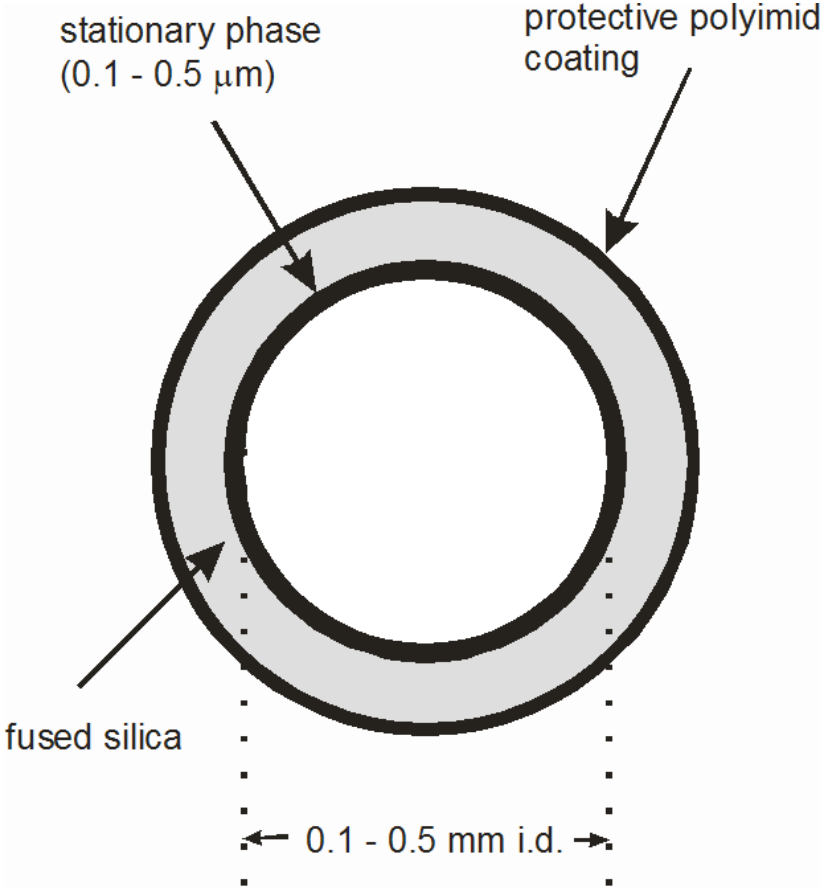
- Most packed columns are 1.5 – 10 m in length and have an internal diameter of 2 – 4 mm.

Capillary Columns (Popular)



(*a*)





Capillary Column's Labels

Manufacturer
Stationary Phase
Dimension
Film thickness
Operation Temperature

J&W Scientific Inc

Cat. No. 1241574	DB-5
75 m X 0.45 mm	2.55 micron
-10 to 260 C	SN 0517

Stationary Phase

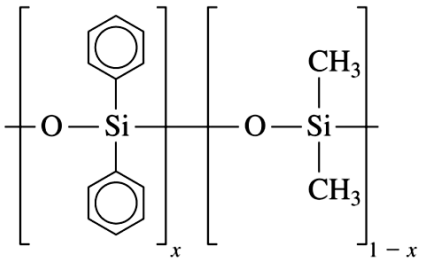
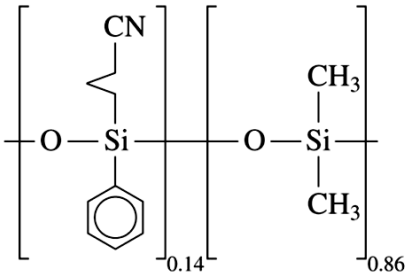
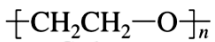
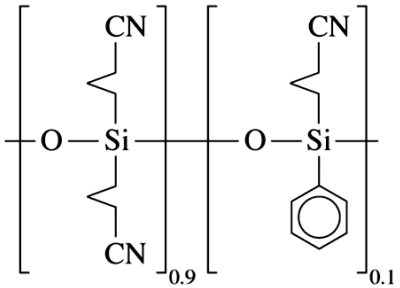
Chemically inert

Non-volatile

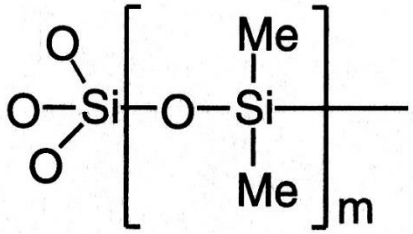
Thermal stablility

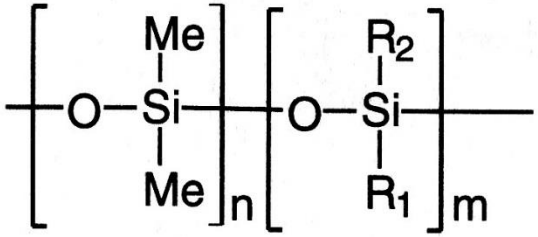
Appropriate physical sorption of analyte

Table 24-1 **Common stationary phases in capillary gas chromatography**

Structure	Polarity	Temperature range (°C)
 (Diphenyl)$_x$(dimethyl)$_{1-x}$ polysiloxane	$x = 0$ Nonpolar $x = 0.05$ Nonpolar $x = 0.35$ Intermediate polarity $x = 0.65$ Intermediate polarity	−60°−320° −60°−320° 0°−300° 50°−370°
 (Cyanopropylphenyl)$_{0.14}$ (dimethyl)$_{0.86}$ polysiloxane	Intermediate polarity	−20°−280°
 Carbowax (poly(ethylene glycol))	Strongly polar	40°−250°
 (Biscyanopropyl)$_{0.9}$ (cyanopropylphenyl)$_{0.1}$ polysiloxane	Strongly polar	0°–275°

Bonded polysiloxanes (examples) :

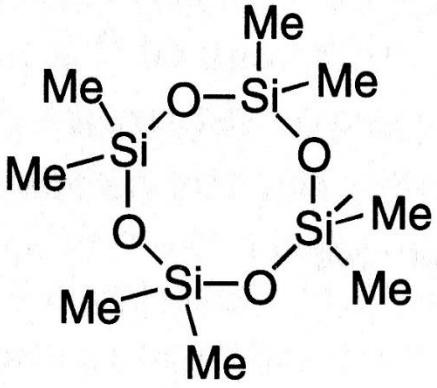




and numerous other phases with diverse chains $(CH_2)_3$-CN, C_2H_4-CF_3 ...

ex. R_1 and R_2 = Ph m = 95% and n = 5%

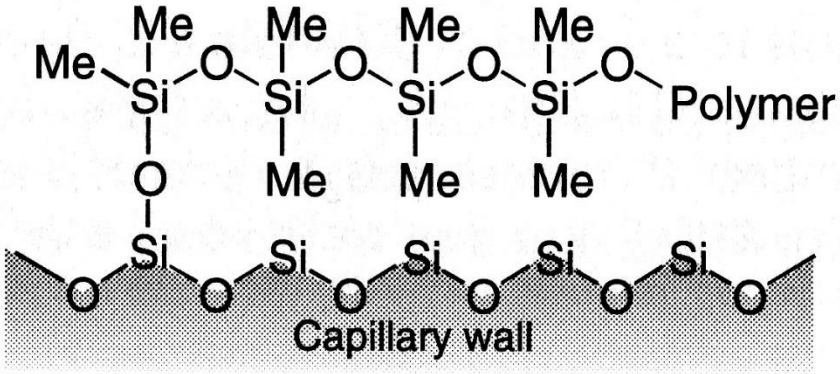
Method of formation of a bonded phase



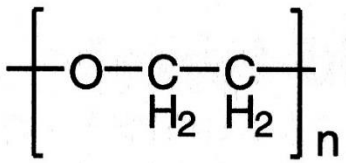
T > 400□C

Capillary wall

(-CH_4)



Polyethyleneglycols

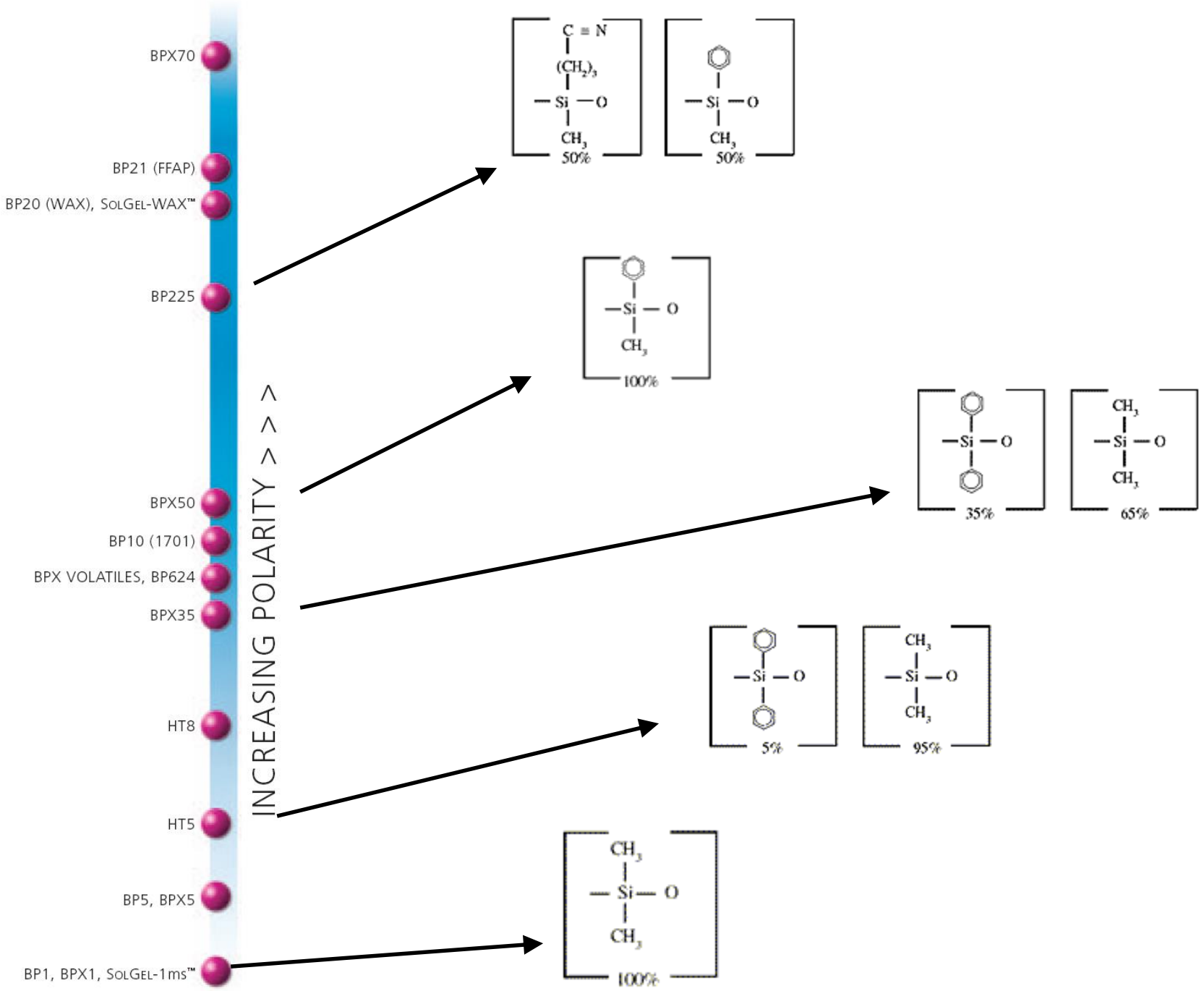


"carbowax"

Table 24-2 **Polarity of solutes**

Nonpolar	Weak intermediate polarity
Saturated hydrocarbons	Ethers
Olefinic hydrocarbons	Ketones
Aromatic hydrocarbons	Aldehydes
Halocarbons	Esters
Mercaptans	Tertiary amines
Sulfides	Nitro compounds (without α-H atoms)
CS_2	Nitriles (without α-atoms)
Strong intermediate polarity	**Strongly polar**
Alcohols	Polyhydroxyalcohols
Carboxylic acids	Amino alcohols
Phenols	Hydroxy acids
Primary and secondary amines	Polyprotic acids
Oximes	Polyphenols
Nitro compounds (with α-H atoms)	
Nitriles (with α-H atoms)	

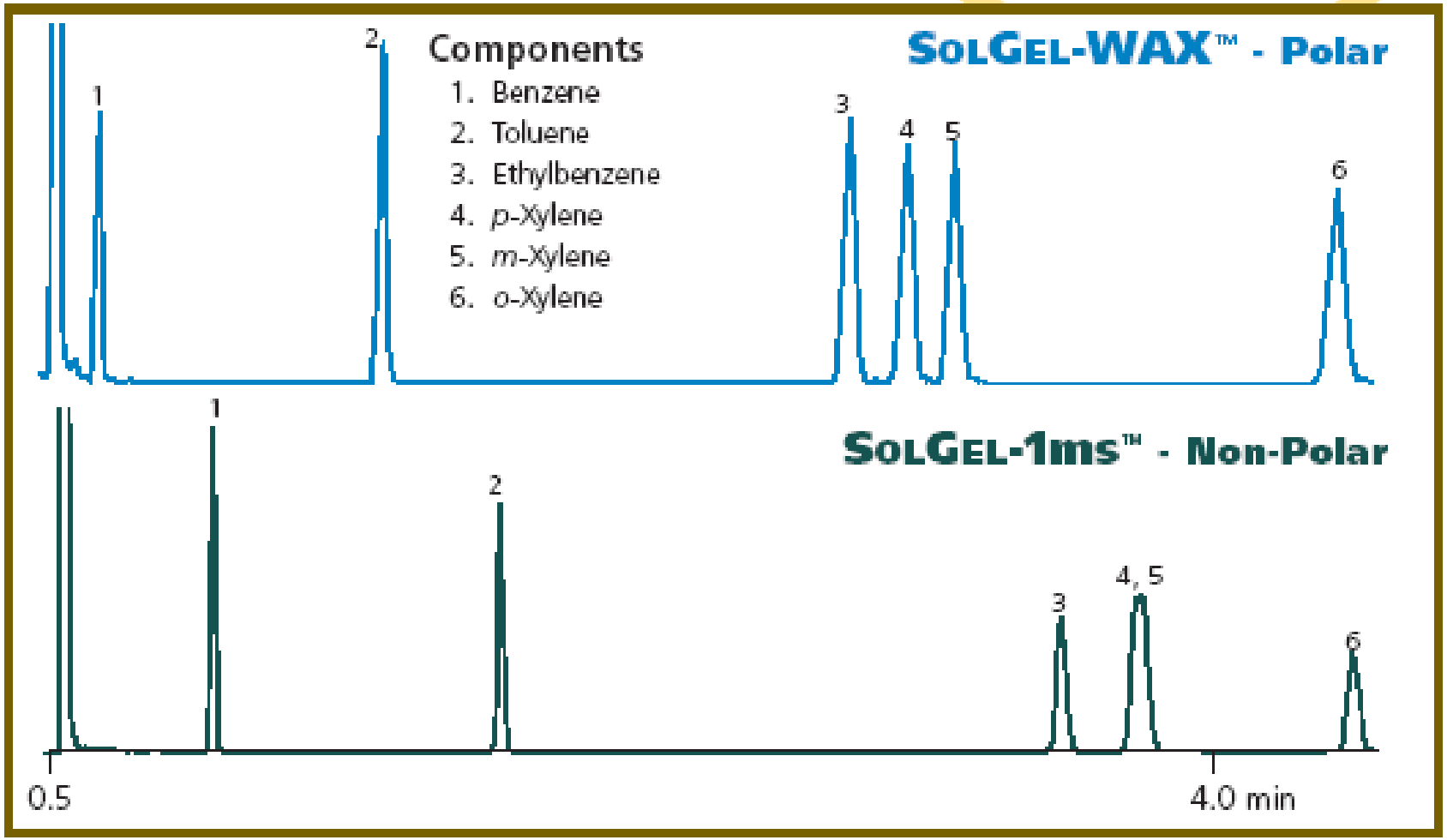
SOURCE: Adapted from H. M. McNair and E. J. Bonelli, *Basic Gas Chromatography* (Palo Alto, CA: Varian Instrument Division, 1968).


BPX70
BP21 (FFAP)
BP20 (WAX), SolGel-WAX™
BP225
BPX50
BP10 (1701)
BPX VOLATILES, BP624
BPX35
HT8
HT5
BP5, BPX5
BP1, BPX1, SolGel-1ms™
INCREASING POLARITY > > >
50%
50%
100%
35%
65%
5%
95%
100%

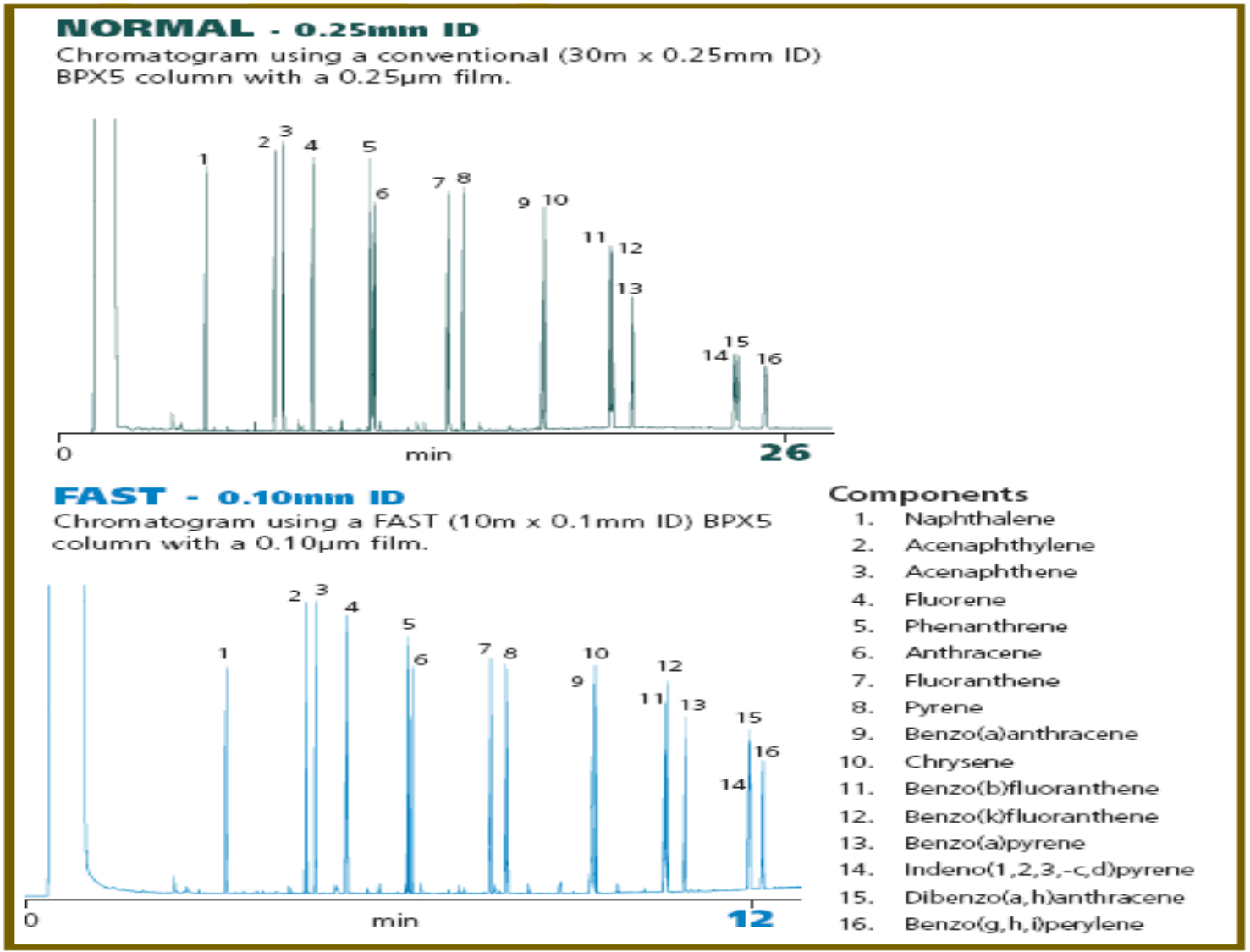
Selection of Columns

- Column internal materials (stationary phase)
- Stationary phase thickness
- Column internal diameter (ID)
- Column length
- etc

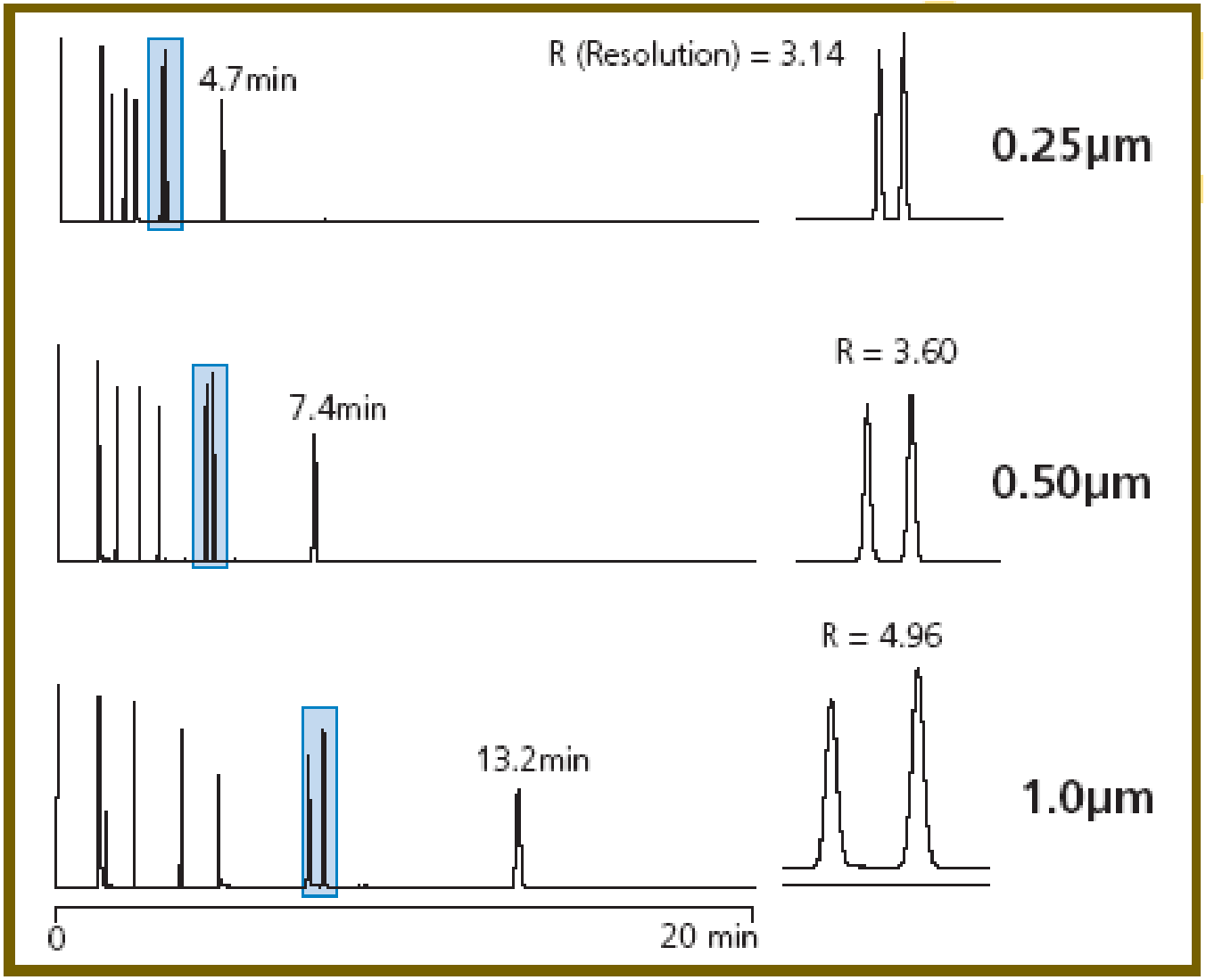
- Choose the least polar phase that will perform the separation you require.
- Choose BP-WAX™ to separate compounds such as alcohols, ketones, aldehydes, and esters.
- The 5% phenyl columns (BP5 or HP5 or DB5) can be used in 90% of all separations.



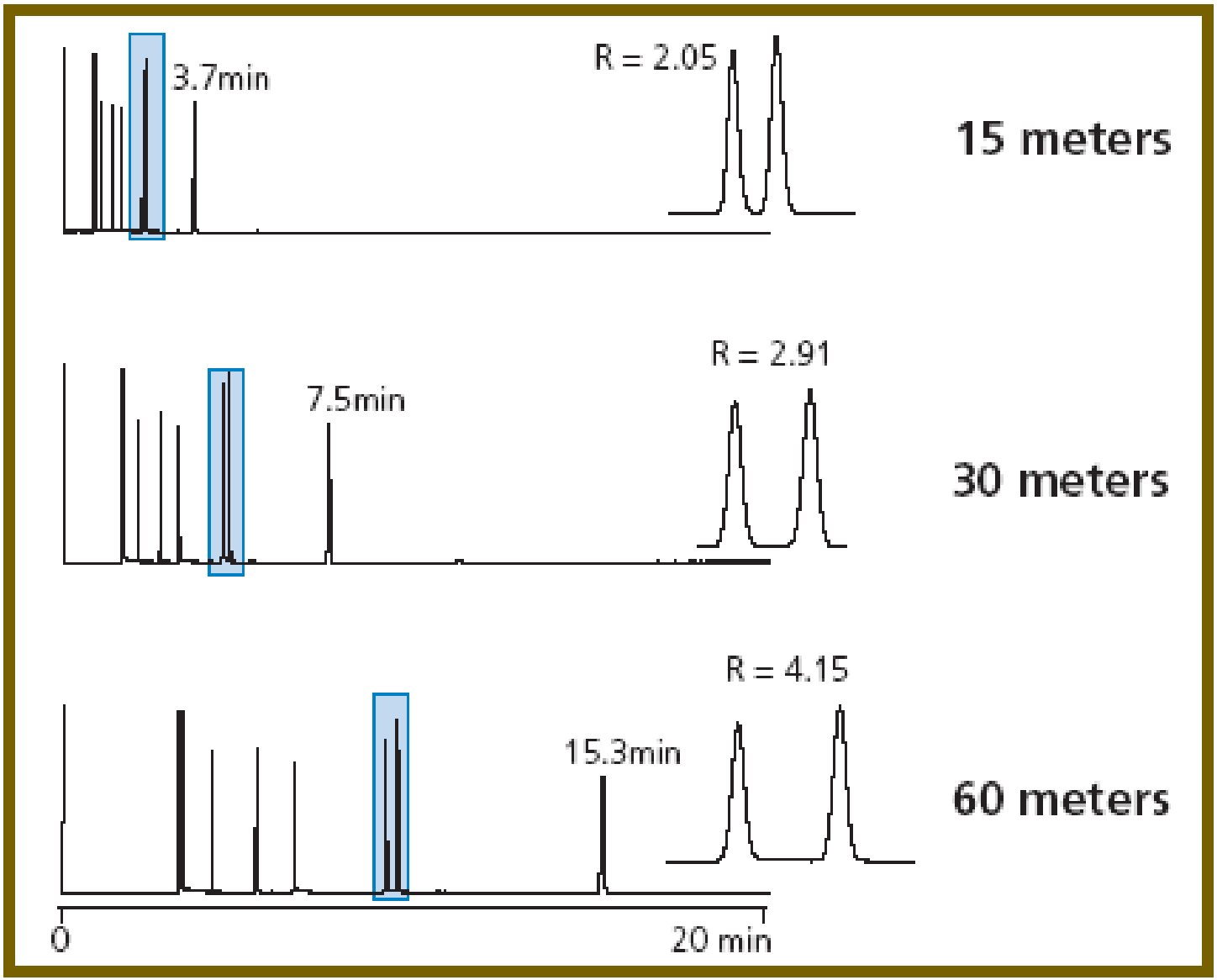
- The smaller the diameter, the faster the analysis.
- 0.25mm ID columns are suitable for most applications, especially GC-MS.



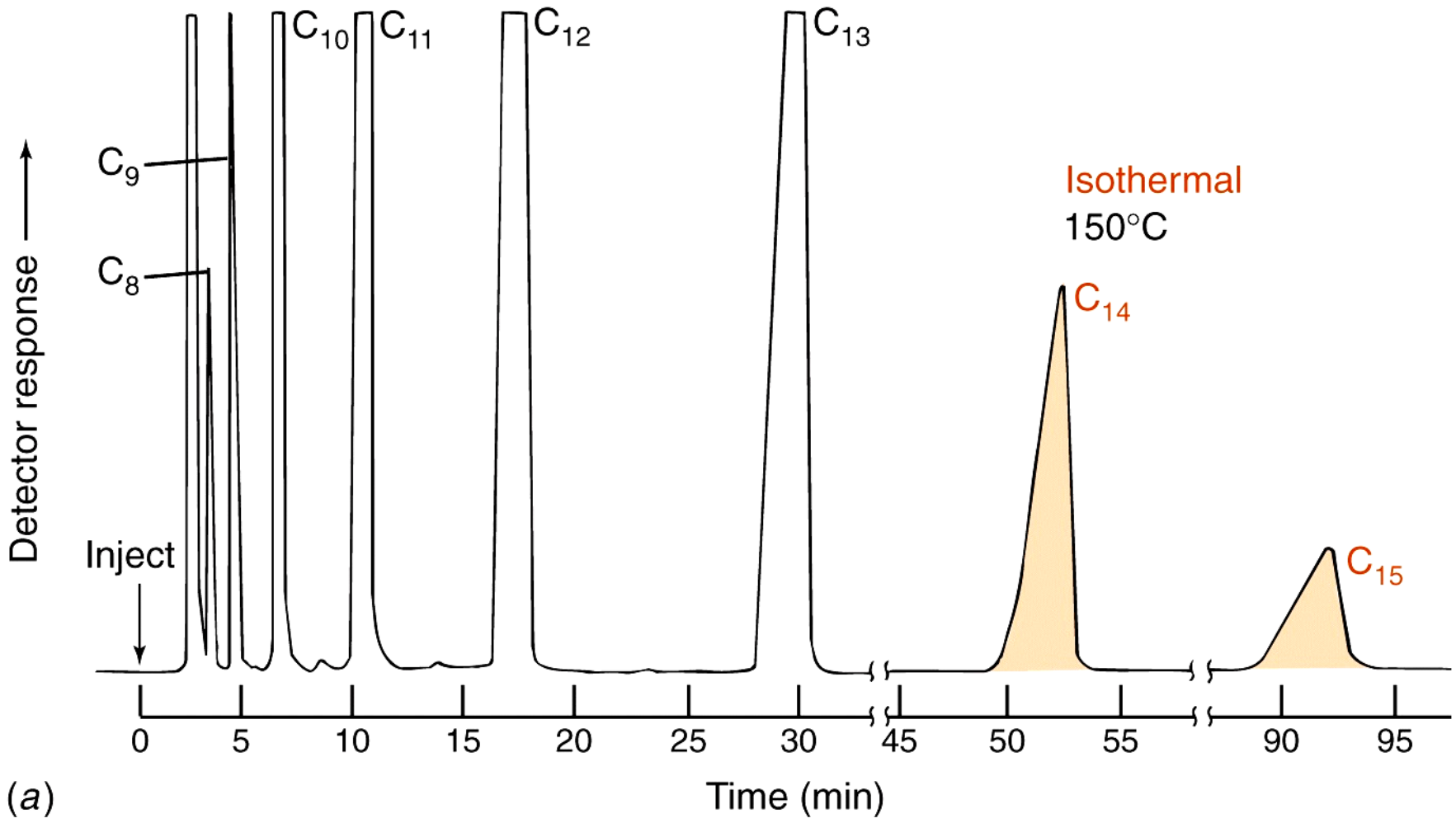
- 0.25um films are standard.
- Use >= 0.5um films for volatile compounds.
- Thicker film columns will result in longer run times but better resolution



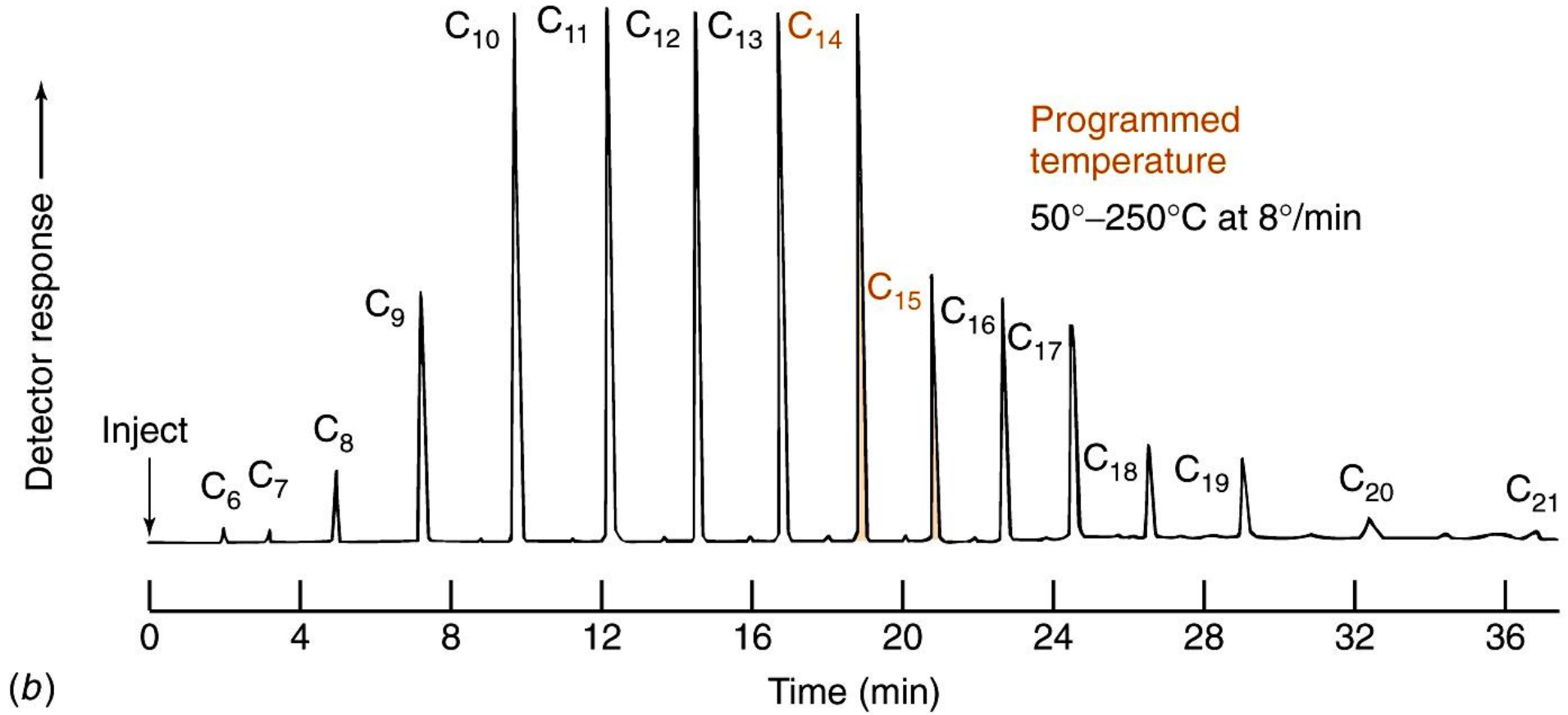
- Doubling the column length improves resolution by 40%.
- 30 meter columns are standard.
- Choose short columns for active compounds.



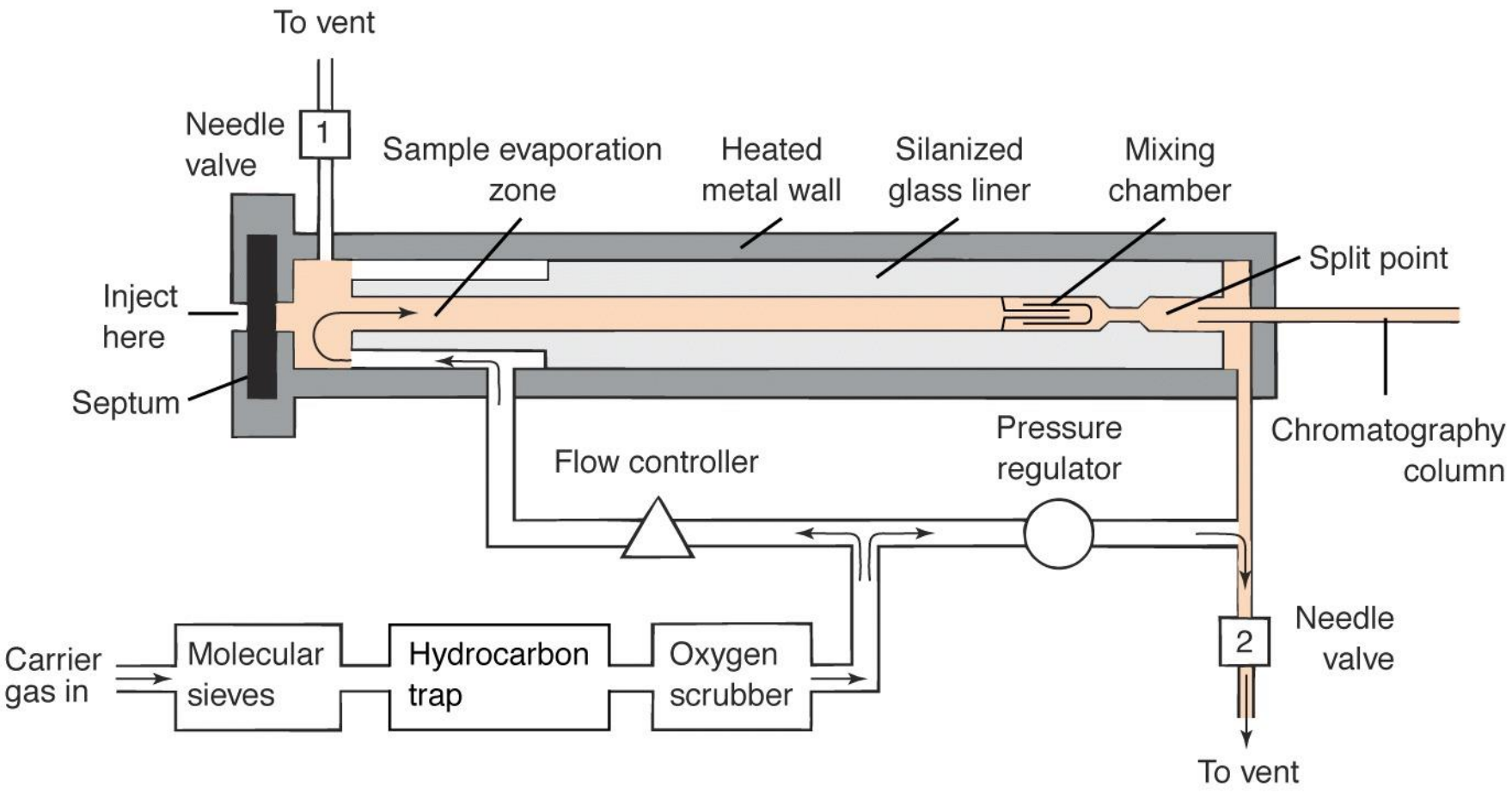
Column Temperature (Isothermal)

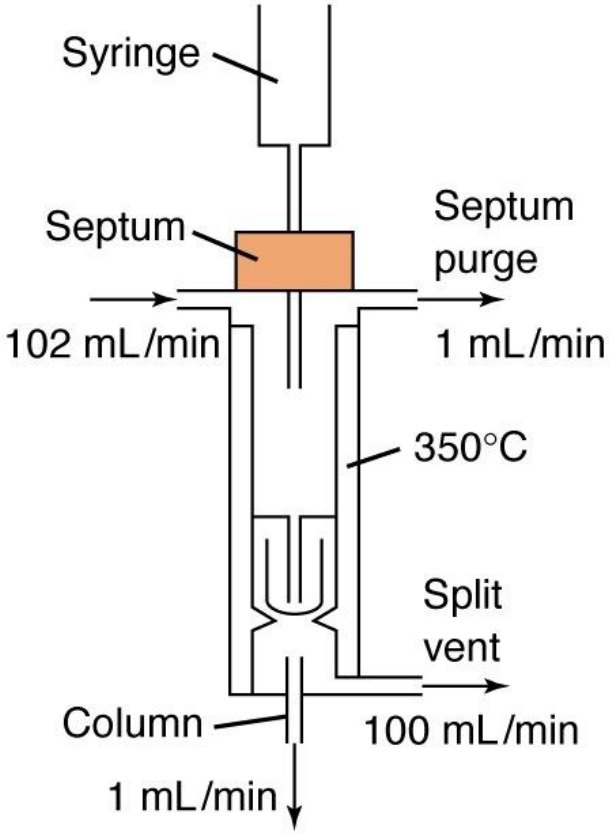


Column Temperature (Programmed)

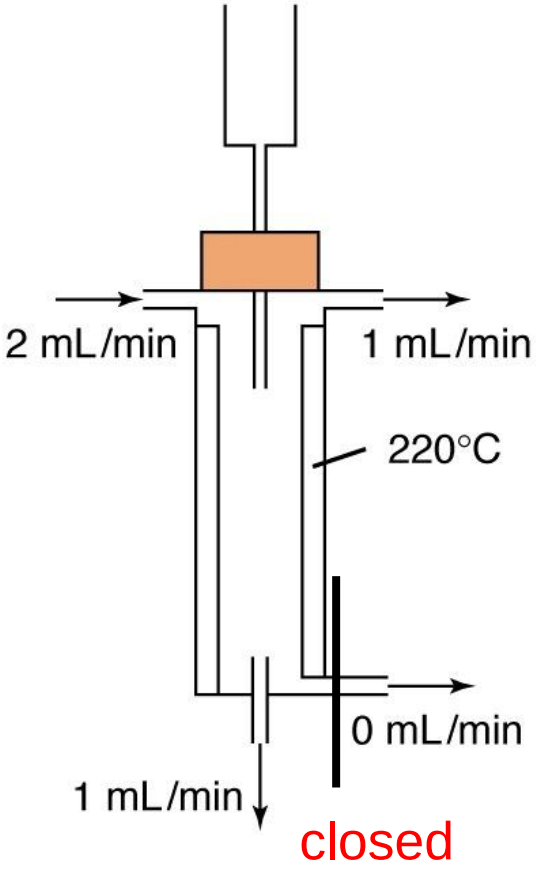


Injector Port

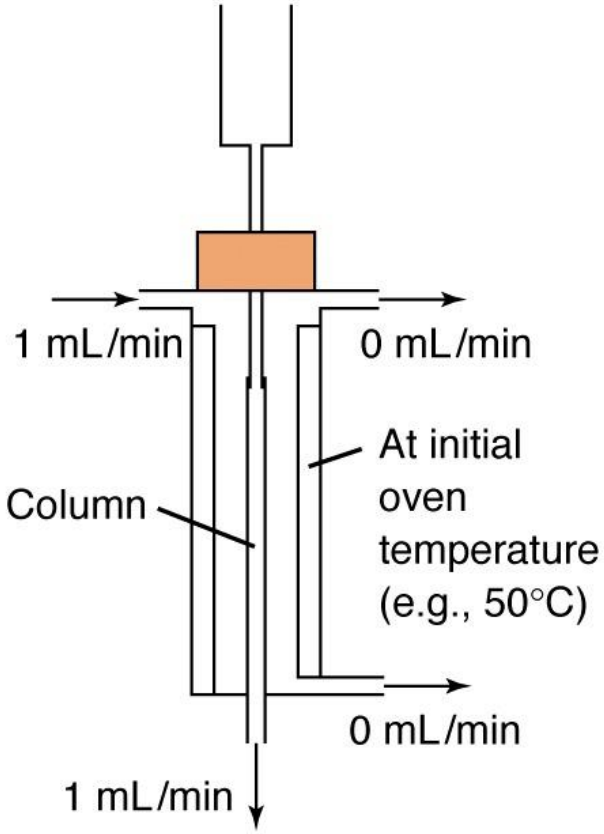




Split injection



Splitless injection

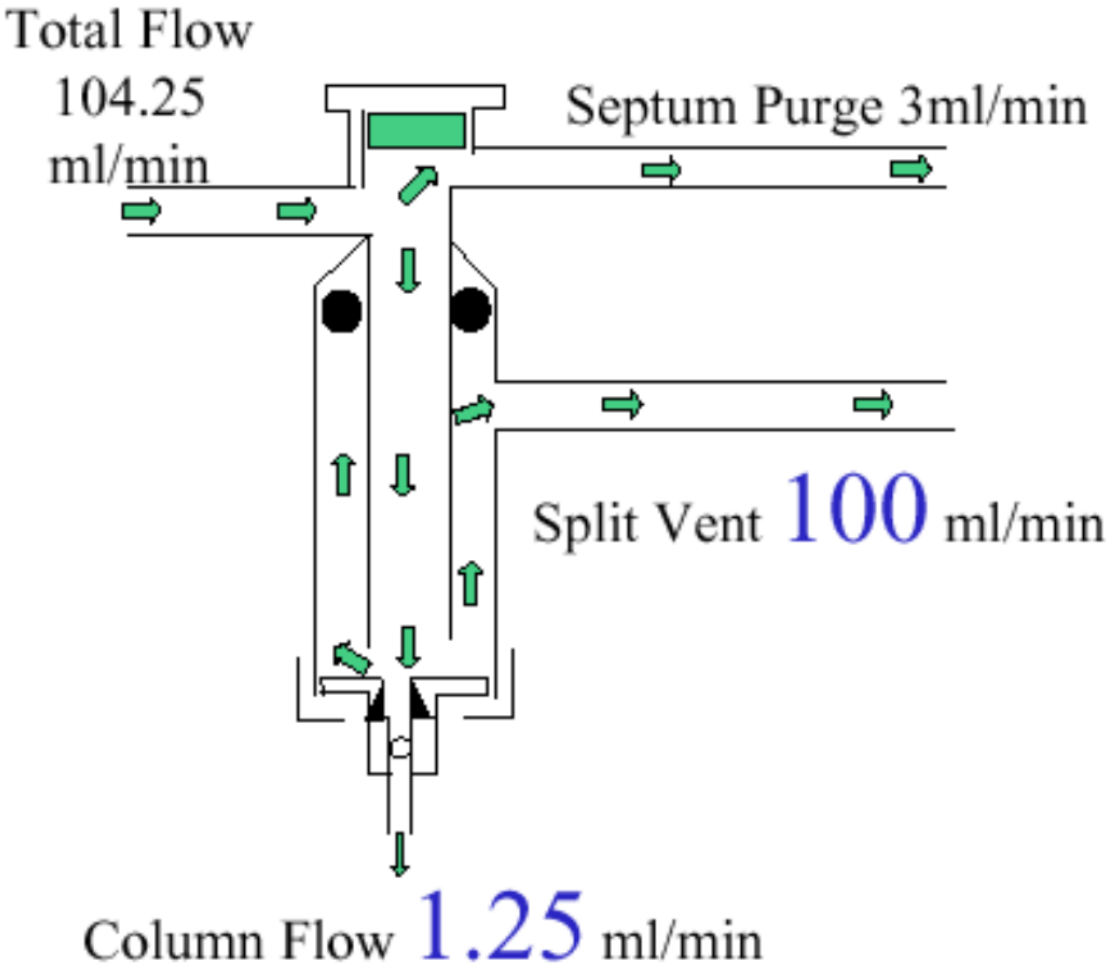


On-column injection

Split Ratio

You are here:

Split Ratio

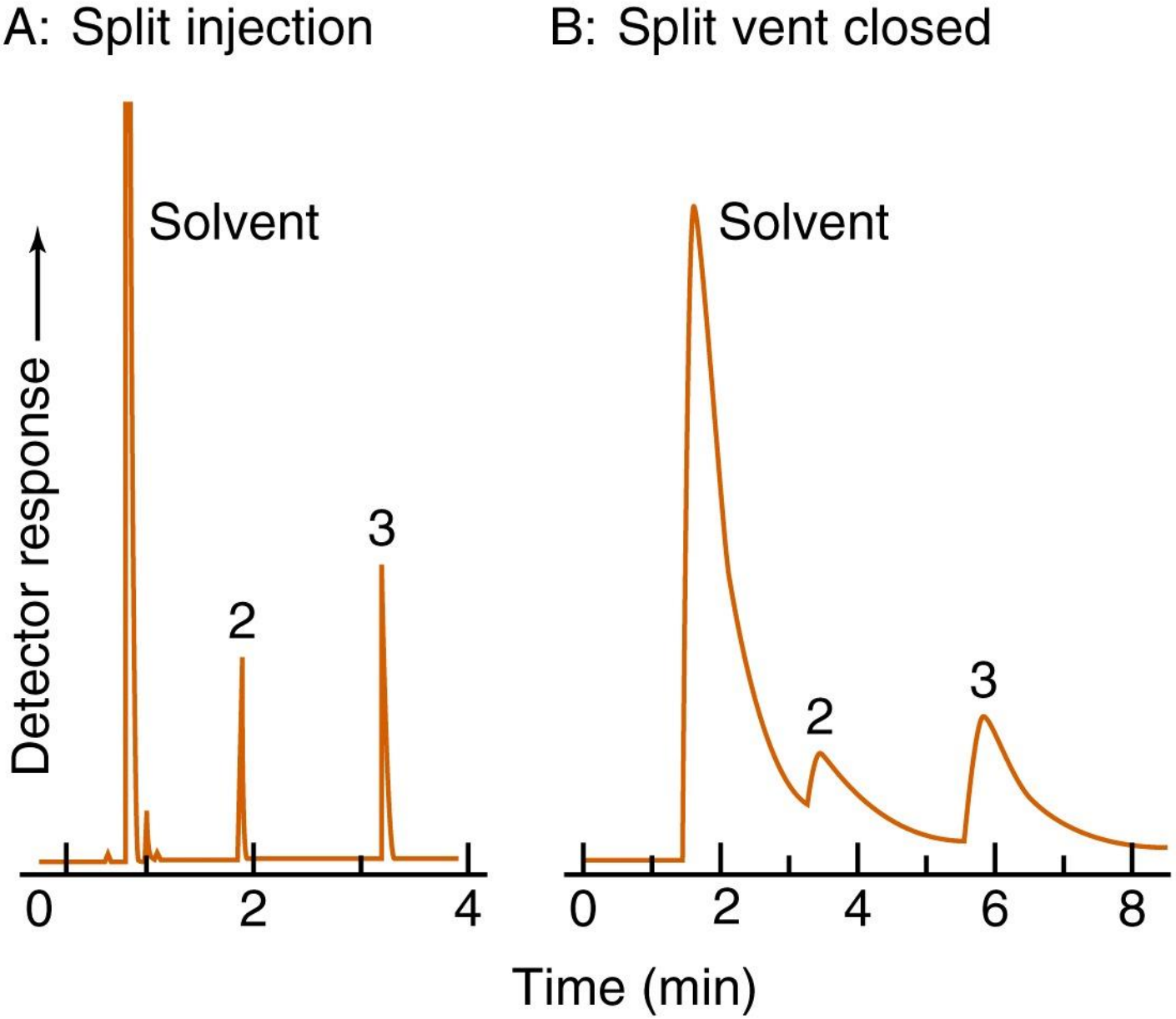


Split Ratio Calculation

$$= \frac{\text{Split Vent Flow}}{\text{Column Flow}}$$

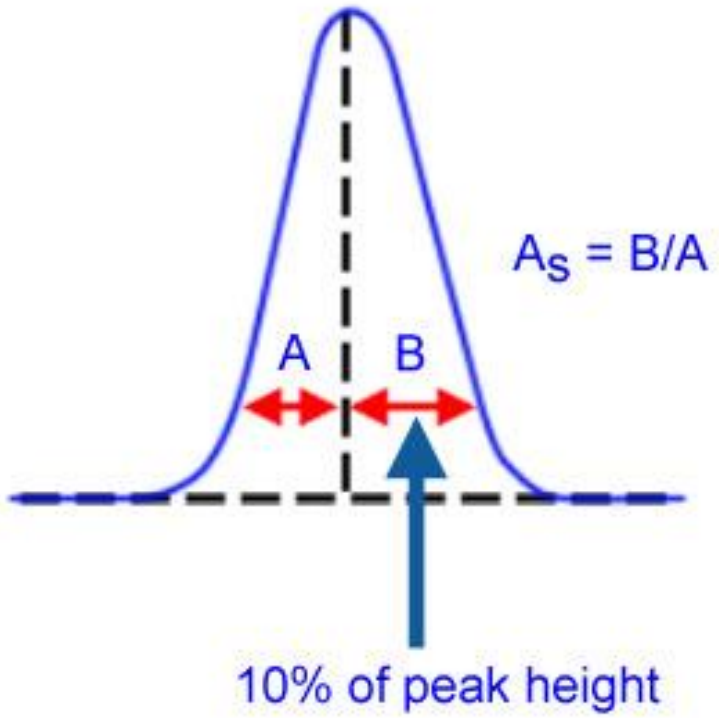
$$= \frac{100}{1.25}$$

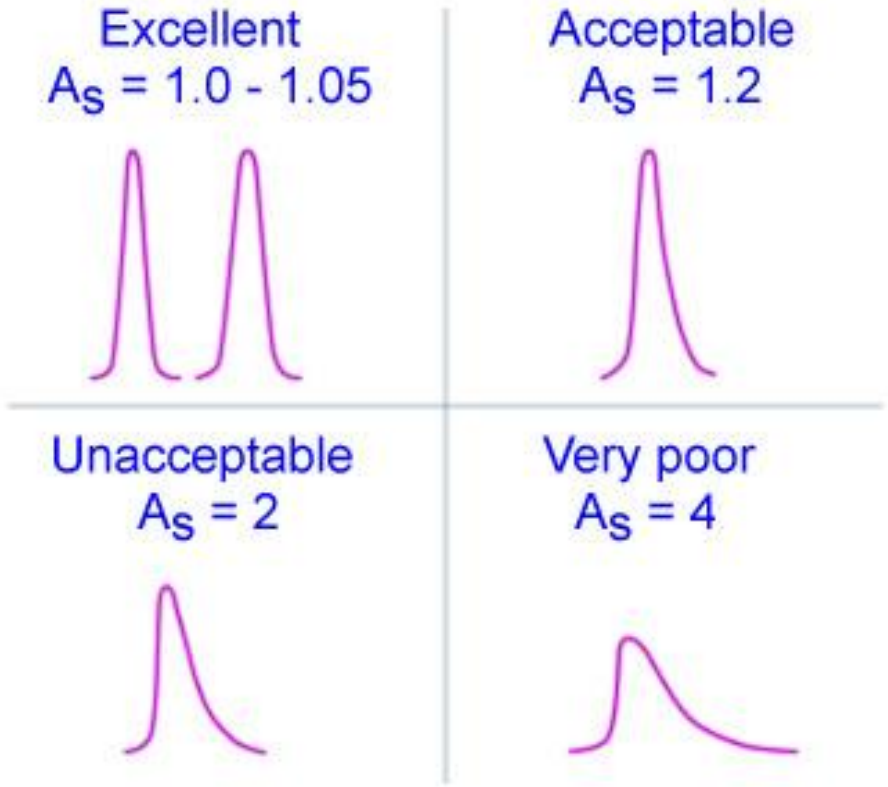
$$= 80 \text{ to } 1$$


A: Split injection
B: Split vent closed
Solvent
Solvent
2
3
2
3
Detector response
0
2
4
0
2
4
6
8
Time (min)

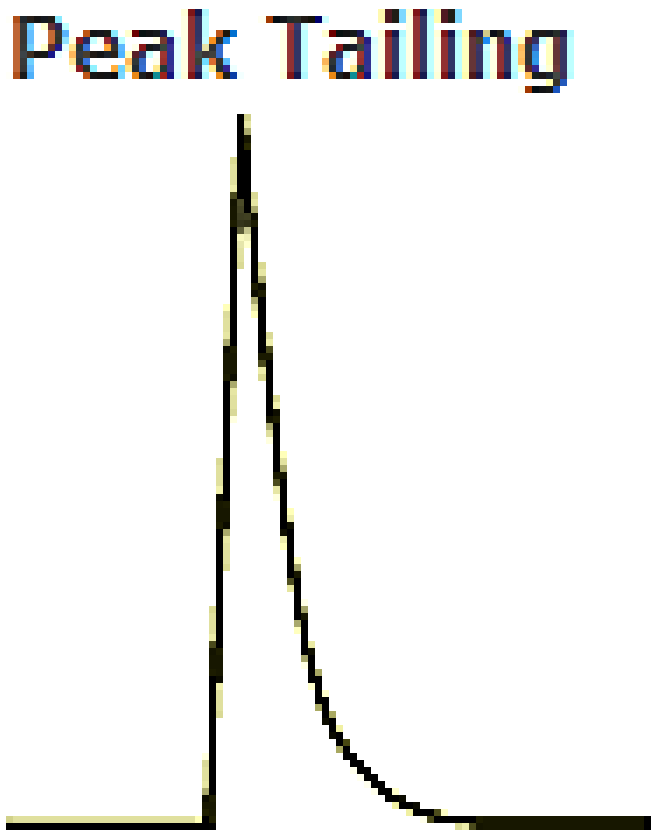
Ideal Peak Shapes

A_s = asymmetric factor





Peak Tailing



What are the Possible Causes for Peak Tailing? - General GC

- Column Contamination
- Column activity
- Solvent-phase polarity mismatch
- Solvent effect violation for splitless or on-column injections
- Too low of a split ratio
- Poor column installation
- Some active compounds always tail

Possible Solutions

1. Column Contamination:

-Trim the column (Remove 1/2-1 meter from the front of the column)

-Solvent rinse the columns (Only for bonded and cross-linked phases)

2.Column activity: This is irreversible & it only affects active compounds

3. Solvent-phase polarity mismatch:

-More tailing for the early eluting peaks or those closest to solvent front to resolve this change the sample solvent.

-Install a 3-5 meter retention gap

Possible Solutions

4. Solvent effect violation for splitless or on-column injections:

-Decrease the initial column temperature

5. Too low of a split ratio:

-Increase the split ratio

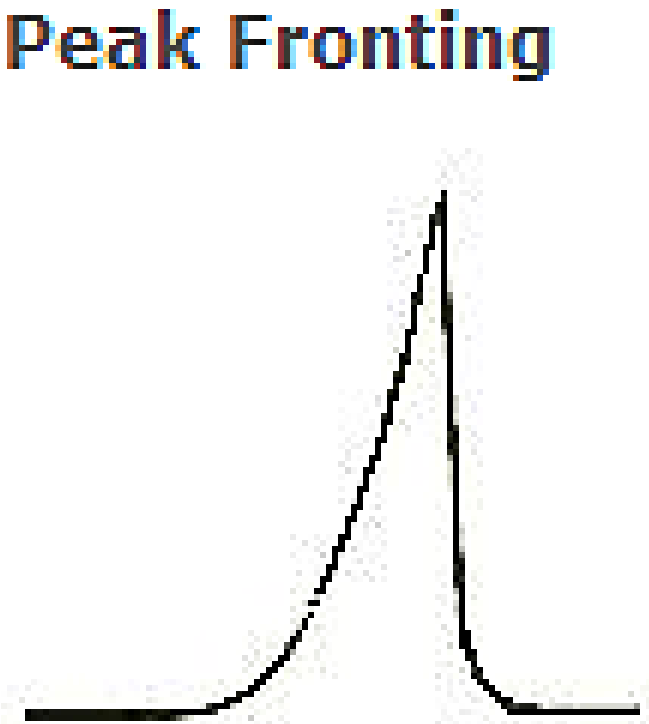
6. Poor column installation:

It results in more tailing for the early eluting peaks, reinstall the column.

7. Some active compounds always tail:

Most common for amines and carboxylic acids always tail

Peak Fronting



What are the Possible Causes for Peak Fronting? - General GC

- Column overload
- Reduce injection volume
- Reduce sample concentrations
- Use a column with larger i.d.

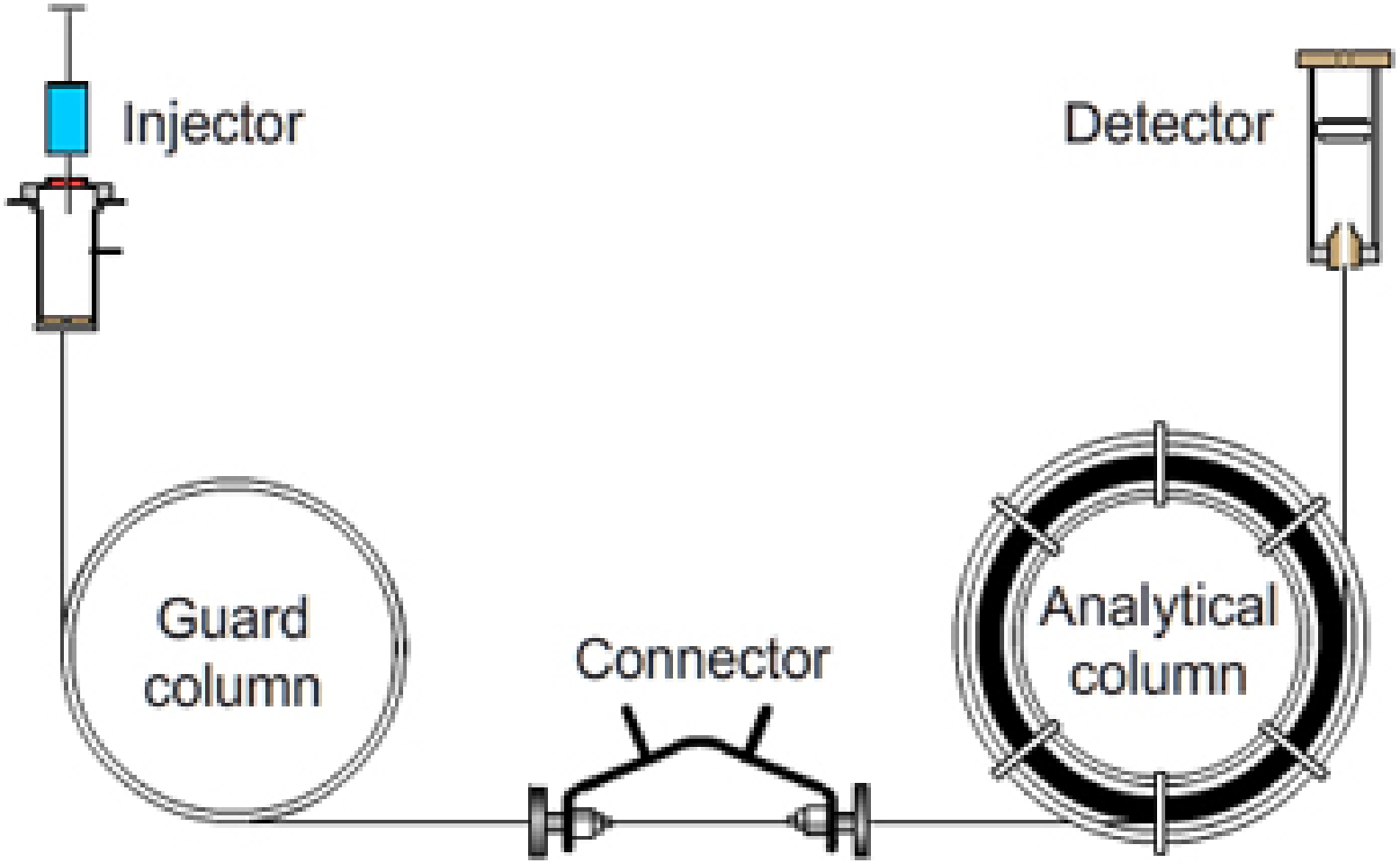
Guard Column

- installed between the injector and the analytical column.

- designed to increase the lifetime of an analytical column by protecting the analytical column from unwanted materials,

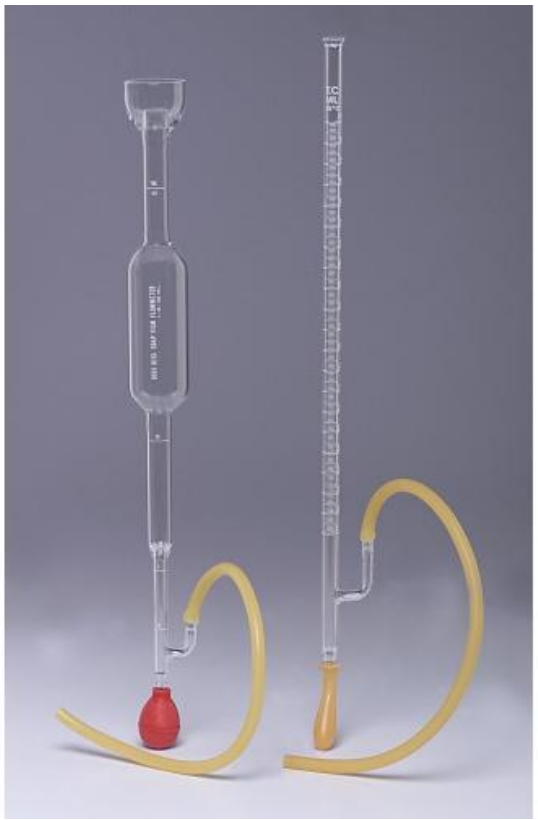
  – highly retained (very long retention time)
  – irreversibly retained compounds (stick forever inside the column)
  – particulate matter (clogging)

Guard Column



Flow Rate Measurement

- Manual flow meter (bubble)
- Automatic flow meter
- EPC (electronic pneumatic control)



$$Flow\ rate = \frac{volume\ (mL)\ between\ marks}{transverse\ time\ (min)}$$

Flow Rate Measurement

- Automatic flow meter
  - digital flowmeter (bubble)
  - Flowmeter

Detectors of GC

- Different detectors will give different types of selectivity.
- A *non-selective* detector responds to all compounds except the carrier gas.
- A *selective detector* responds to a range of compounds with a common physical or chemical property.
- A *specific detector* responds to a single chemical compound.

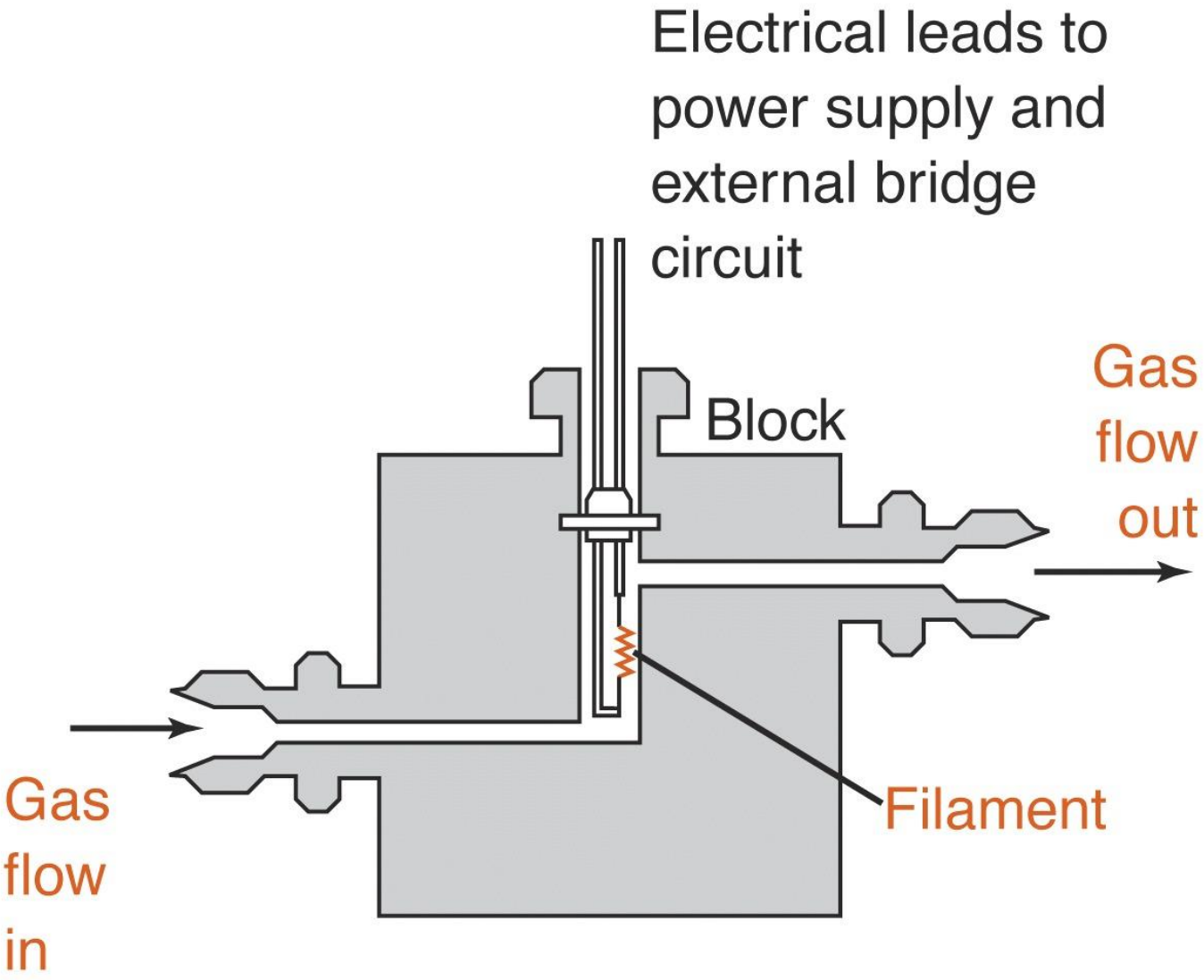
Detector	Type	Support gases	Selectivity	Detection limit	Dynamic range
Flame ionization	Mass flow	Hydrogen and air	Most organic cpds.	100 pg	10^7
Thermal conductivity	Conc.	Reference	Universal	1 ng	10^7
Electron capture	Conc.	Make-up	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics	50 fg	10^5
Nitrogen-phosphorus	Mass flow	Hydrogen and air	Nitrogen, phosphorus	10 pg	10^6
Flame photometric	Mass flow	Hydrogen and air possibly oxygen	Sulphur, phosphorus, tin, boron, arsenic, germanium, selenium, chromium	100 pg	10^3
Photo-ionization (PID)	Conc.	Make-up	Aliphatics, aromatics, ketones, esters, aldehydes, amines, heterocyclics, organosulphurs, some organometallics	2 pg	10^7
Hall electrolytic conductivity	Mass flow	Hydrogen, oxygen	Halide, nitrogen, nitrosamine, sulphur		

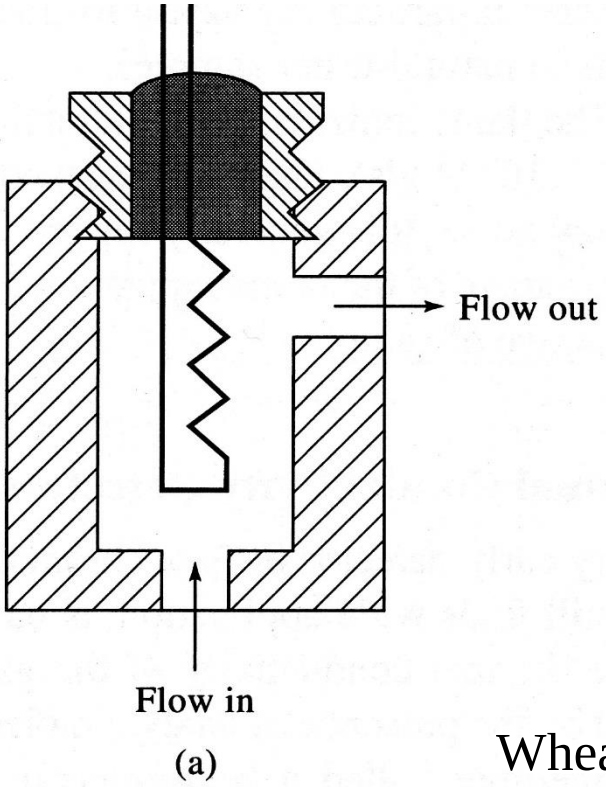
Thermal Conductivity Detector (TCD)

- A TCD detector consists of an electrically-heated wire or thermistor.
- The temperature of the sensing element depends on the thermal conductivity of the gas flowing around it.
- Changes in thermal conductivity, such as when organic molecules displace some of the carrier gas, cause a temperature rise in the element which is sensed as a change in resistance.
- The TCD is not as sensitive as other detectors but it is non-specific and non-destructive.

- Two pairs of TCDs are used in gas chromatographs.
- One pair is placed in the column effluent to detect the separated components as they leave the column, and another pair is placed before the injector or in a separate reference column.
- The resistances of the two sets of pairs are then arranged in a bridge circuit.

Thermal Conductivity Detector (TCD)





Wheatstone bridge

$R_{s1} \cdot R_{s2} = R_{r1} \cdot R_{r1}$

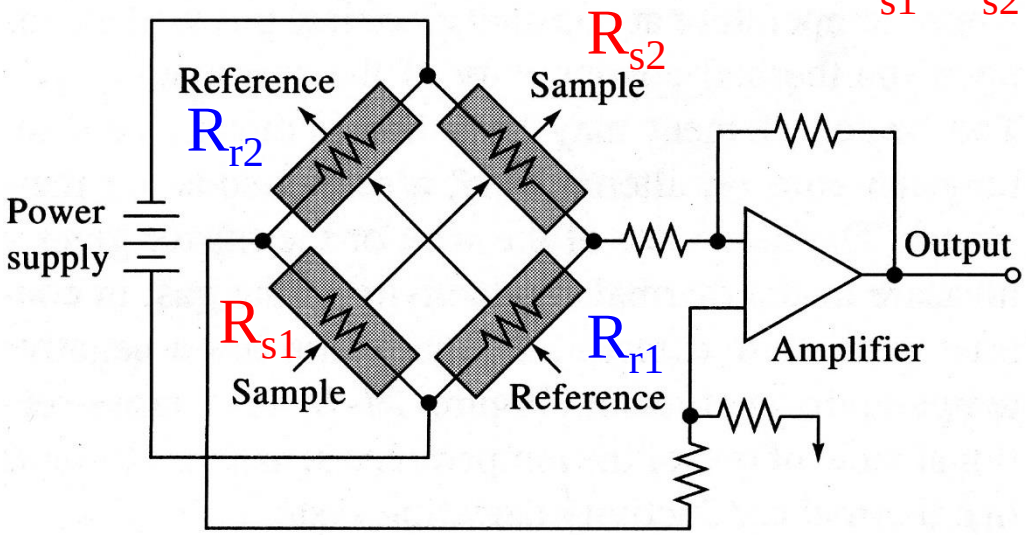


Table 24-4 Thermal conductivity at 273 K and 1 atm

Gas	Thermal conductivity J/(K · m · s)
H_2	0.170
He	0.141
NH_3	0.021 5
N_2	0.024 3
C_2H_4	0.017 0
O_2	0.024 6
Ar	0.016 2
C_3H_8	0.015 1
CO_2	0.014 4
Cl_2	0.007 6

The energy per unit area per unit time flowing from a hot region to a cold region is given by

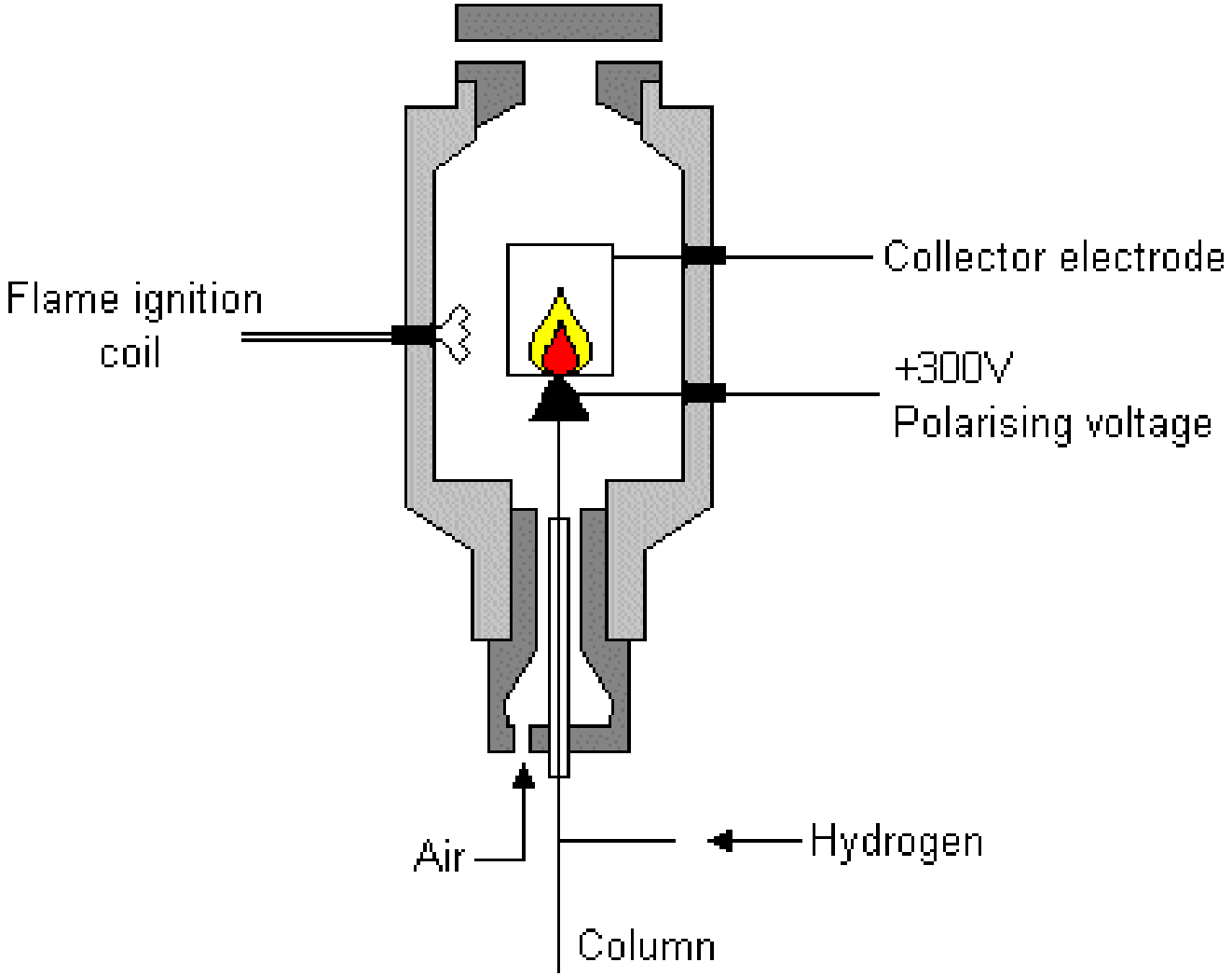
$$\text{Energy flux } (\text{J/m}^2 \cdot \text{s}) = -\mathtt{k}(dT/dx)$$

where $\mathtt{k}$ is the thermal conductivity [units = J/(K · m · s)] and dT/dx is the temperature gradient (K/m). Thermal conductivity is to energy flux as the diffusion coefficient is to mass flux.

Flame Ionization Detector (FID)

- The effluent from the column is mixed with hydrogen and air, and ignited.
- Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame.
- A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame.
- The current resulting from the pyrolysis of any organic compounds is measured.
- General detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise.
- It is also robust and easy to use, but unfortunately, it destroys the sample.

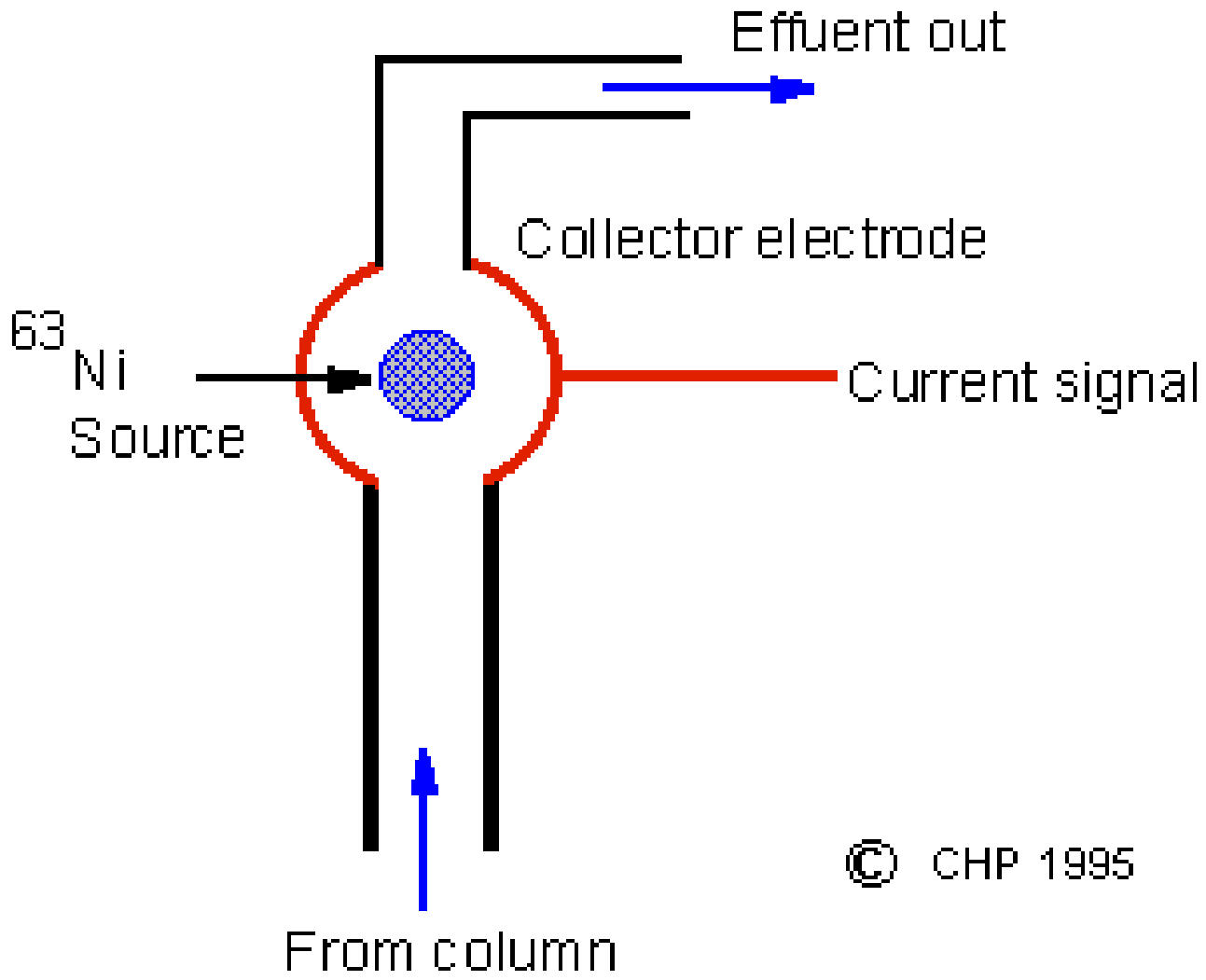
The Flame Ionisation Detector



Electron Capture Detector (ECD)

- The ECD uses a radioactive Beta emitter (electrons) to ionize some of the carrier gas and produce a current between a biased pair of electrodes.
- When organic molecules that contain electronegative functional groups, such as halogens, phosphorous, and nitro groups pass by the detector, they capture some of the electrons and reduce the current measured between the electrodes.
- The ECD is as sensitive as the FID but has a limited dynamic range and finds its greatest application in analysis of halogenated compounds.

Electron Capture Detector (ECD)



Nitrogen Phosphorus Detector (NPD)

- NPD is a highly sensitive and selective to organic compounds containing **nitrogen and/or phosphorus**.
- NPD is often used to detect pesticides, herbicides, drugs of abuse, and other trace compounds.
- NPD is similar in design to the FID, except it uses a thermionic NPD bead to generate ions in a hydrogen and air plasma.
- Like FID, NPD uses a stainless steel jet to deliver sample-laden carrier gas and hydrogen gas to the detector, and a positively charged collector electrode that also serves as the detector exhaust.

Nitrogen Phosphorus Detector (NPD)

- In a detector body, an electrically heated thermionic bead (NPD bead) is positioned between the jet orifice and the collector electrode.
- The bead is coated with an alkali metal (Cs or Rb) which promotes the ionization of compounds that contain nitrogen or phosphorus.
- Hydrogen and air flows create a hydrogen plasma around the hot NPD bead.
- When molecules containing nitrogen or phosphorus enter the plasma from the column and jet orifice, they undergo a catalytic surface chemistry reaction, producing thermionic electrons.
- The resulting ions are attracted to a positively charged collector electrode, then amplified and output to the data system.

Nitrogen Phosphorus Detector (NPD)