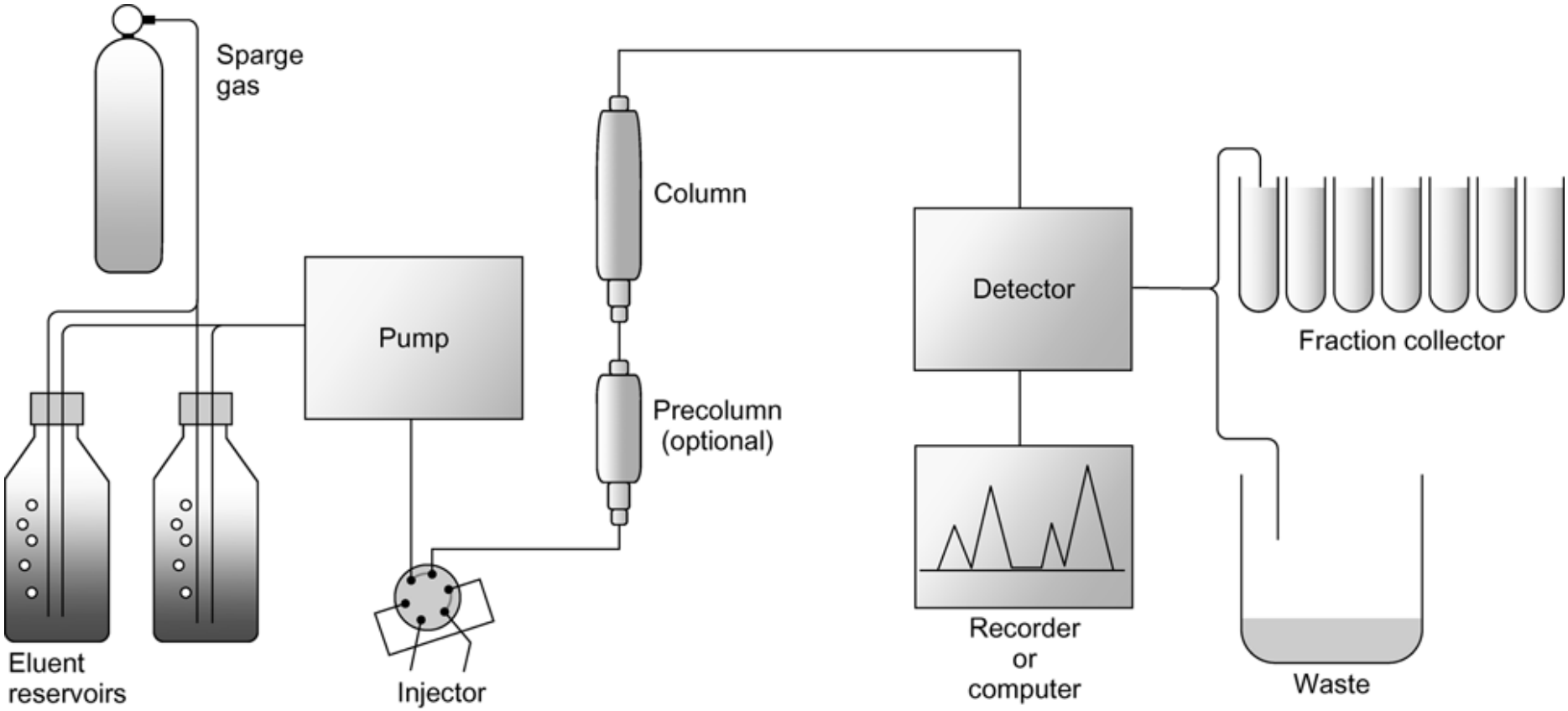
High performance liquid chromatography with UV & fluorescence detection

HPLC system

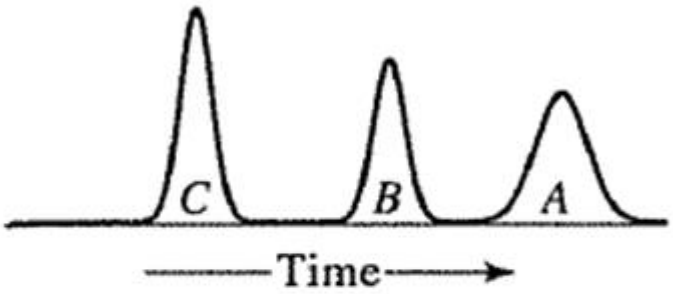

Sparge gas
Pump
Column
Precolumn (optional)
Detector
Fraction collector
Eluent reservoirs
Injector
Recorder or computer
Waste

Normal phase

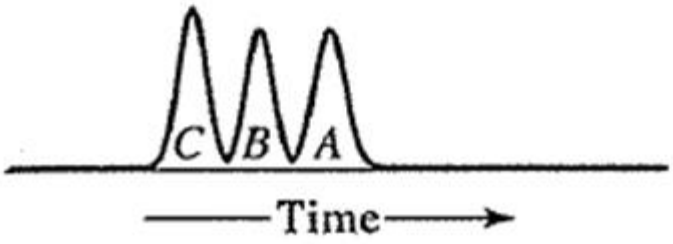
Solute polarities: $A > B > C$

Normal-phase chromatography

Low-polarity mobile phase



Medium-polarity mobile phase

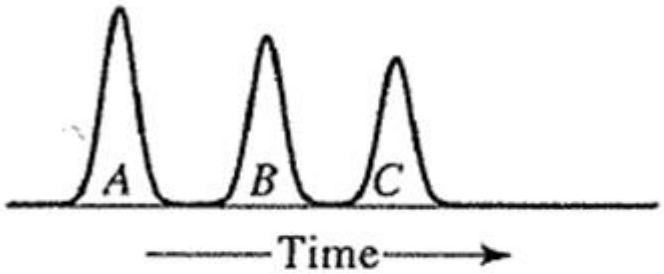


Reversed phase

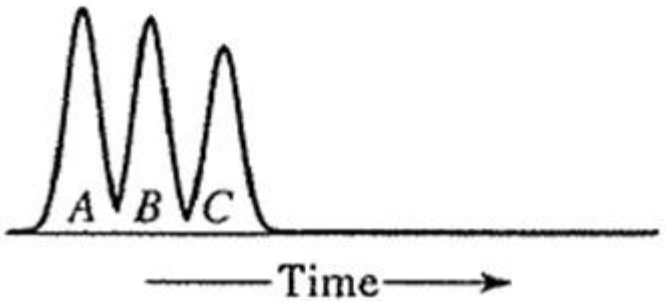
Solute polarities: $A > B > C$

Reversed-phase chromatography

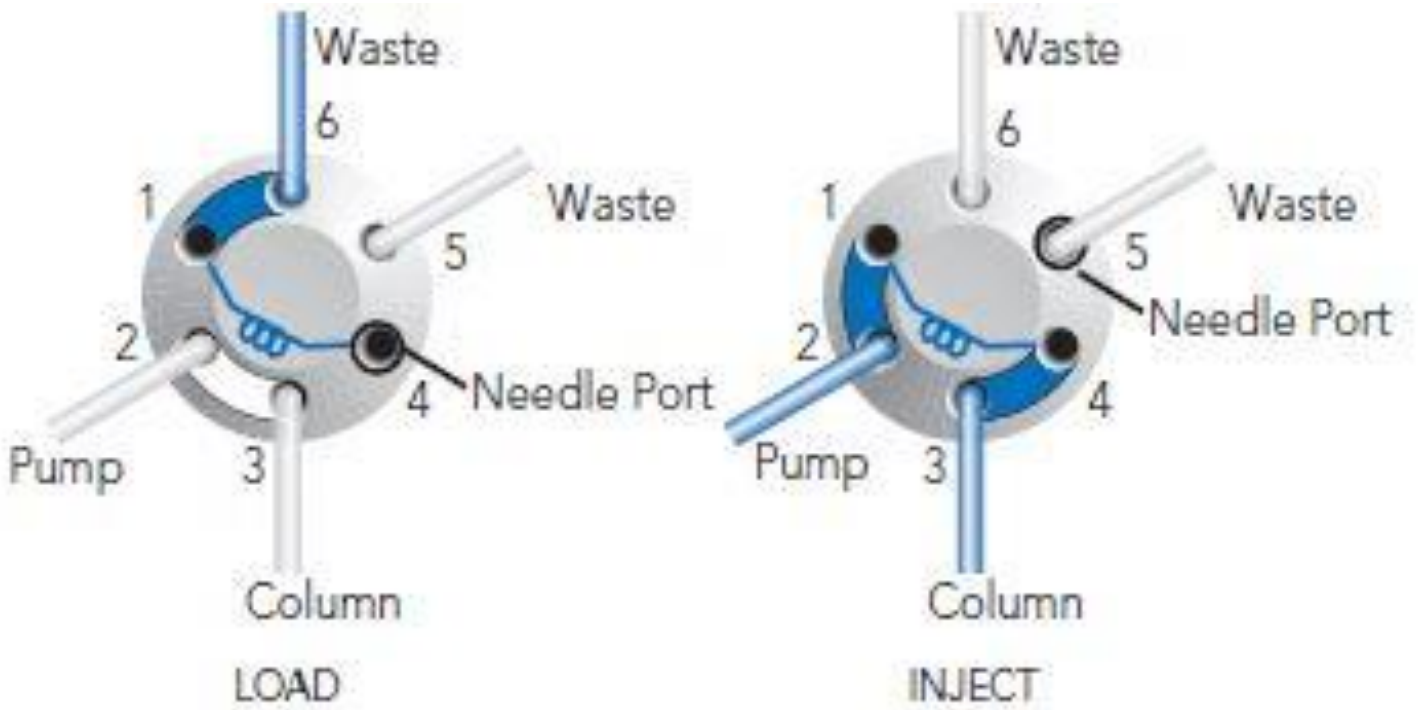
High-polarity mobile phase



Medium-polarity mobile phase



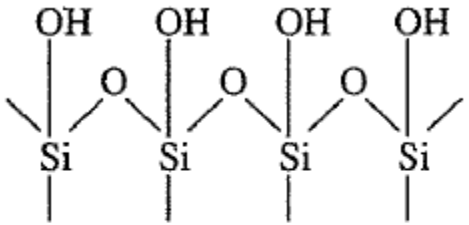
The injector: Rheodyne™

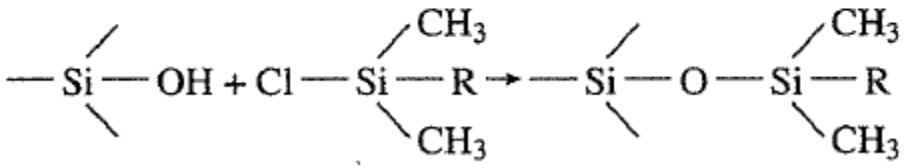


Flow path for the typical dual mode injector

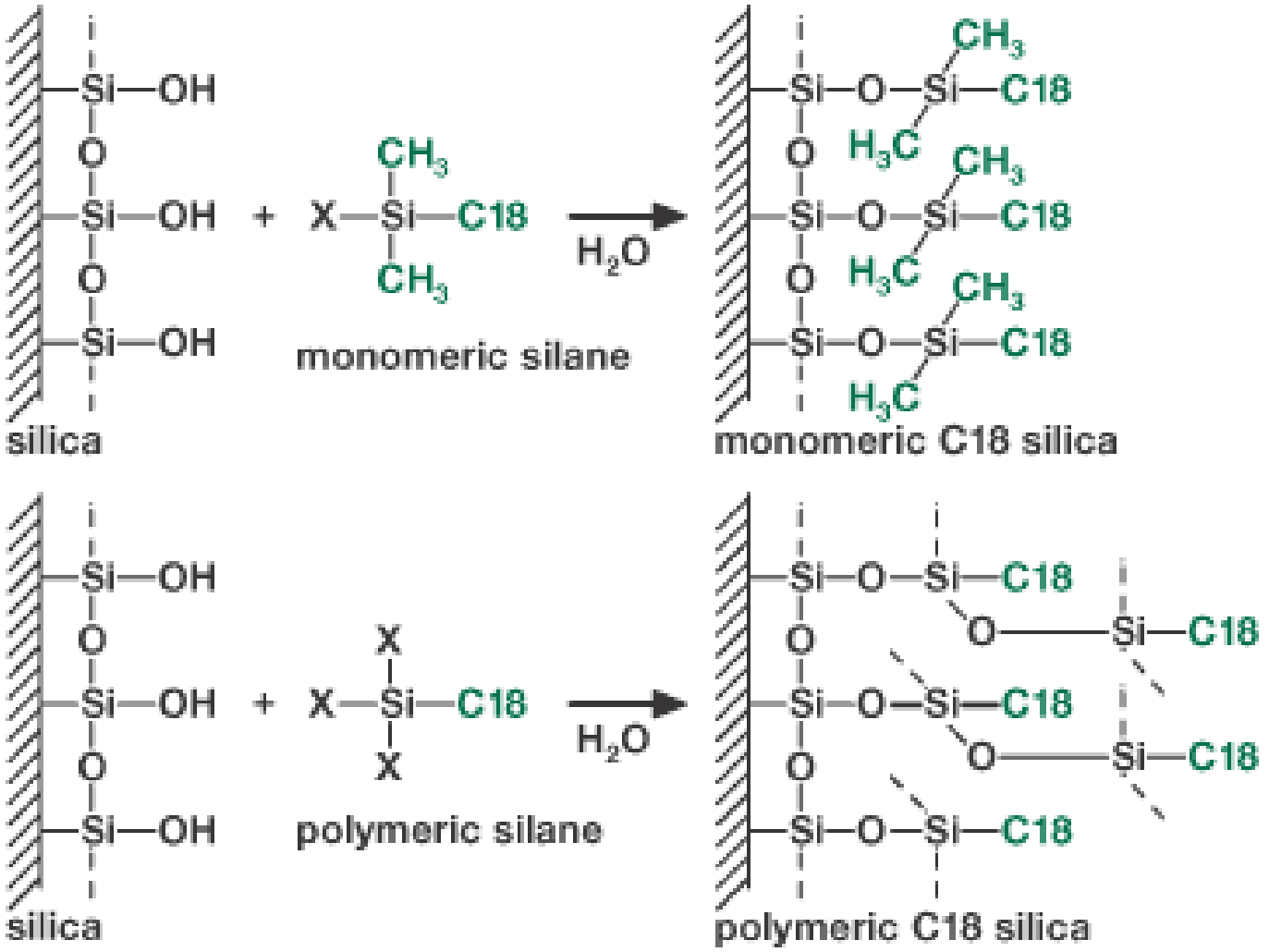
http://kinesis.co.uk/knowledgebase/rheodyne-dual-mode-sample-injectors/

The column

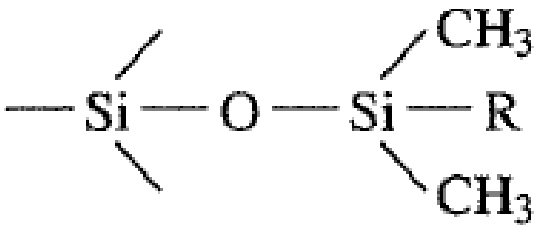




Monomeric vs. polymeric C18 bonding. Use of trifunctional bonding reagent results in a more complex multilayered C18 bonded phase.



http://www.seaviewsci.com/vydac/vydacpubs/NL01SPR/NL01SPR.html



Peak identification
1. Uracil
2. Phenol
3. Acetophenone
4. Nitrobenzene
5. Methyl benzoate
6. Toluene

R = CH_3

R = C_8H_{17}

R = $C_{18}H_{37}$

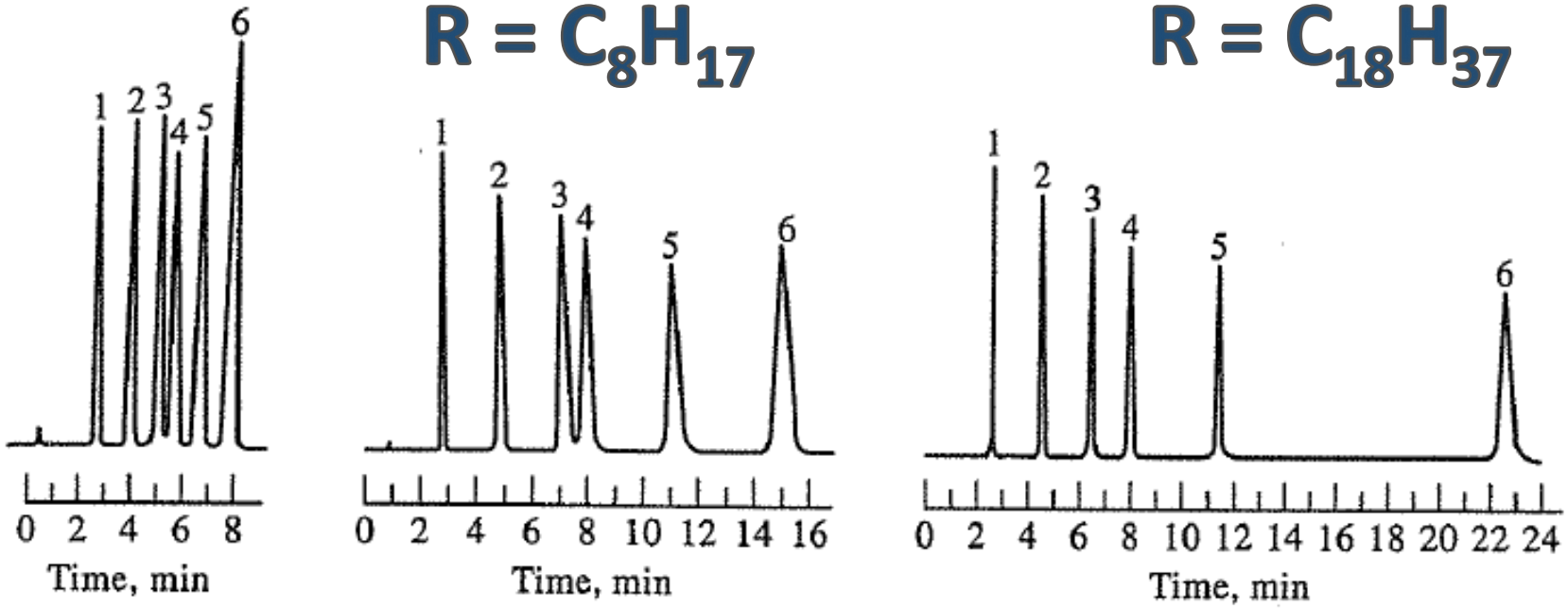


FIGURE 28-15 Effect of chain length on performance of reversed-phase siloxane columns packed with 5-μm particles. Mobile phase: 50:50 methanol-water. Flow rate: 1.0 ml/min.

Effect of particle size

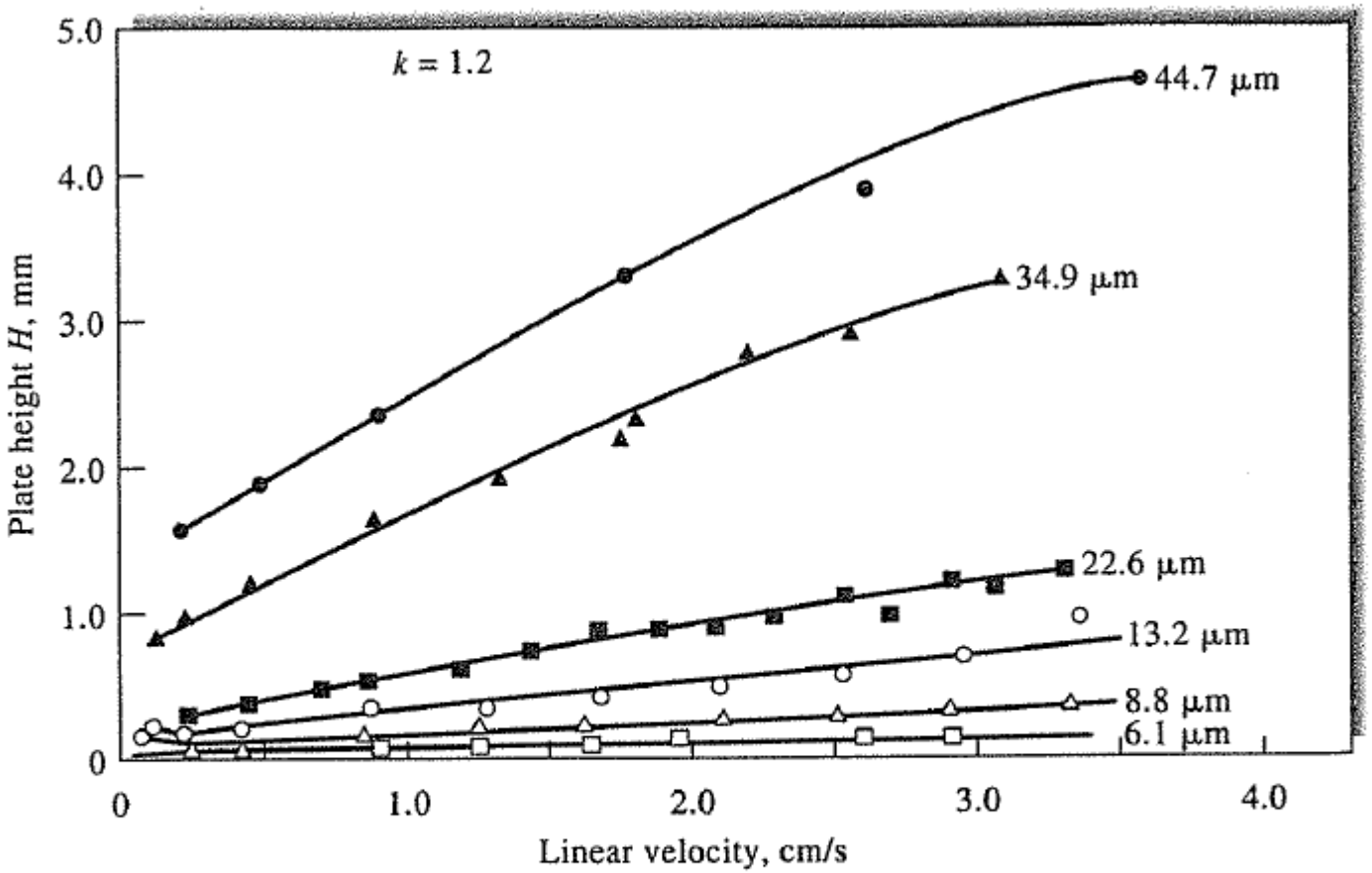


FIGURE 28-2 Effect of particle size of packing and flow rate on plate height H in LC. Column dimensions: 30 cm × 2.4 mm. Solute: *N,N'*-diethyl-*p*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. (From R. E. Majors, *J. Chromatogr. Sci.*, **1973**, *11*, 88. With permission.)

Effect of solvent gradient

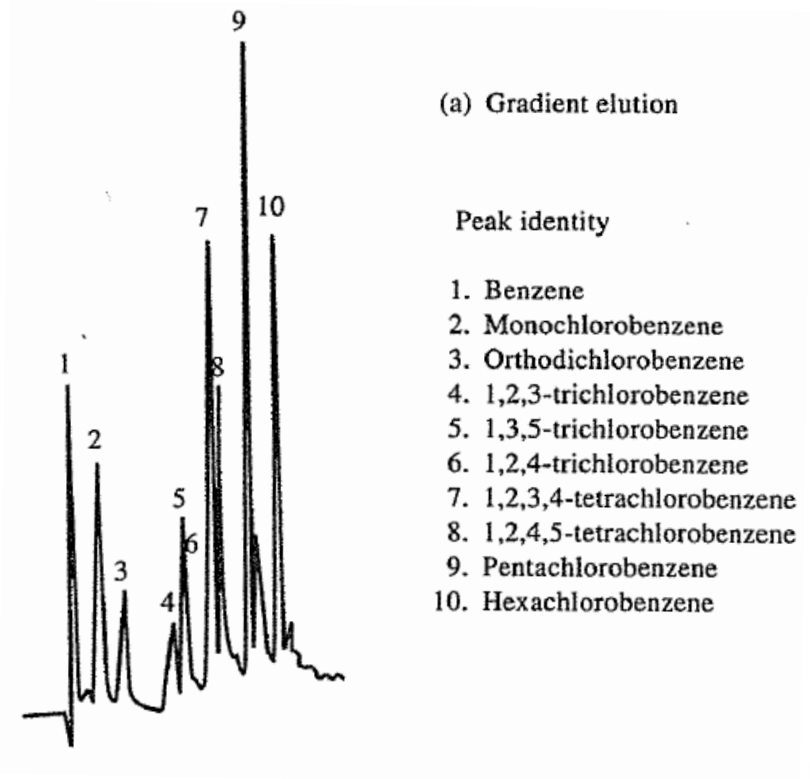
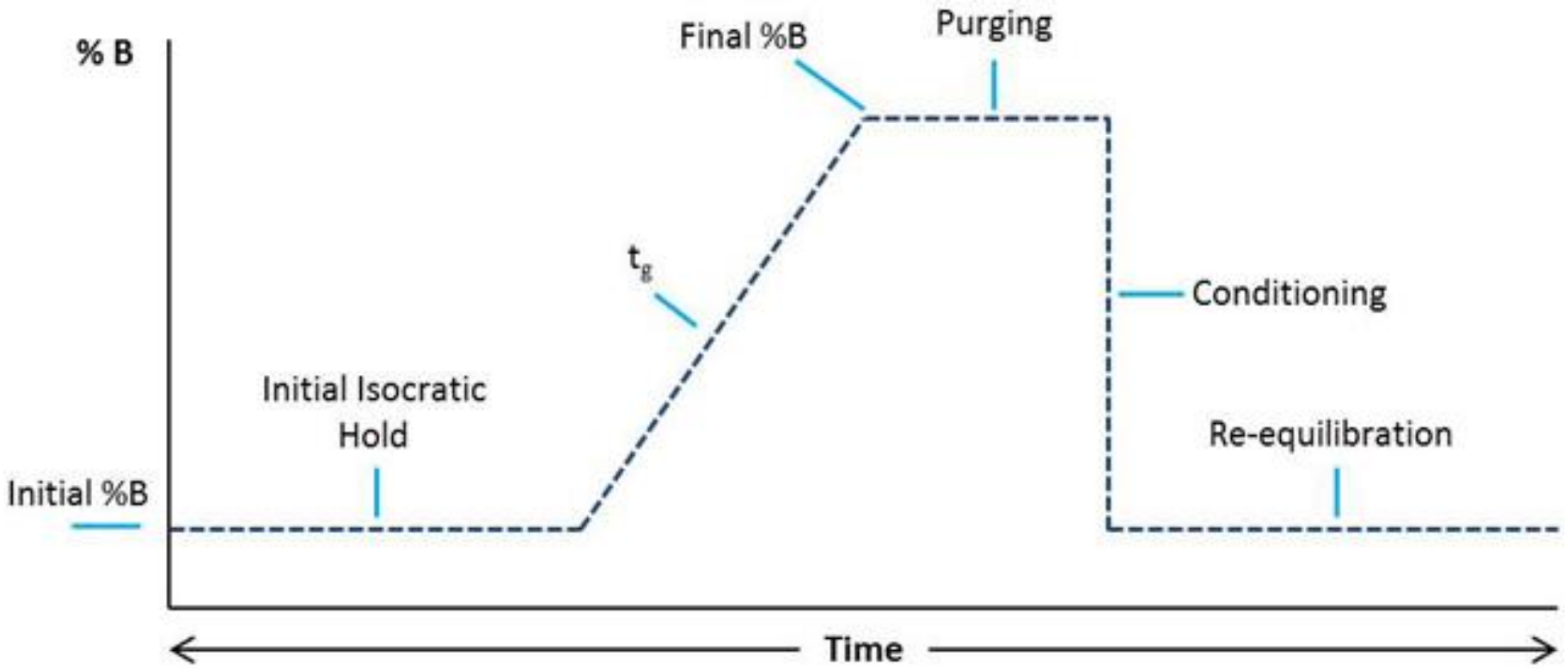
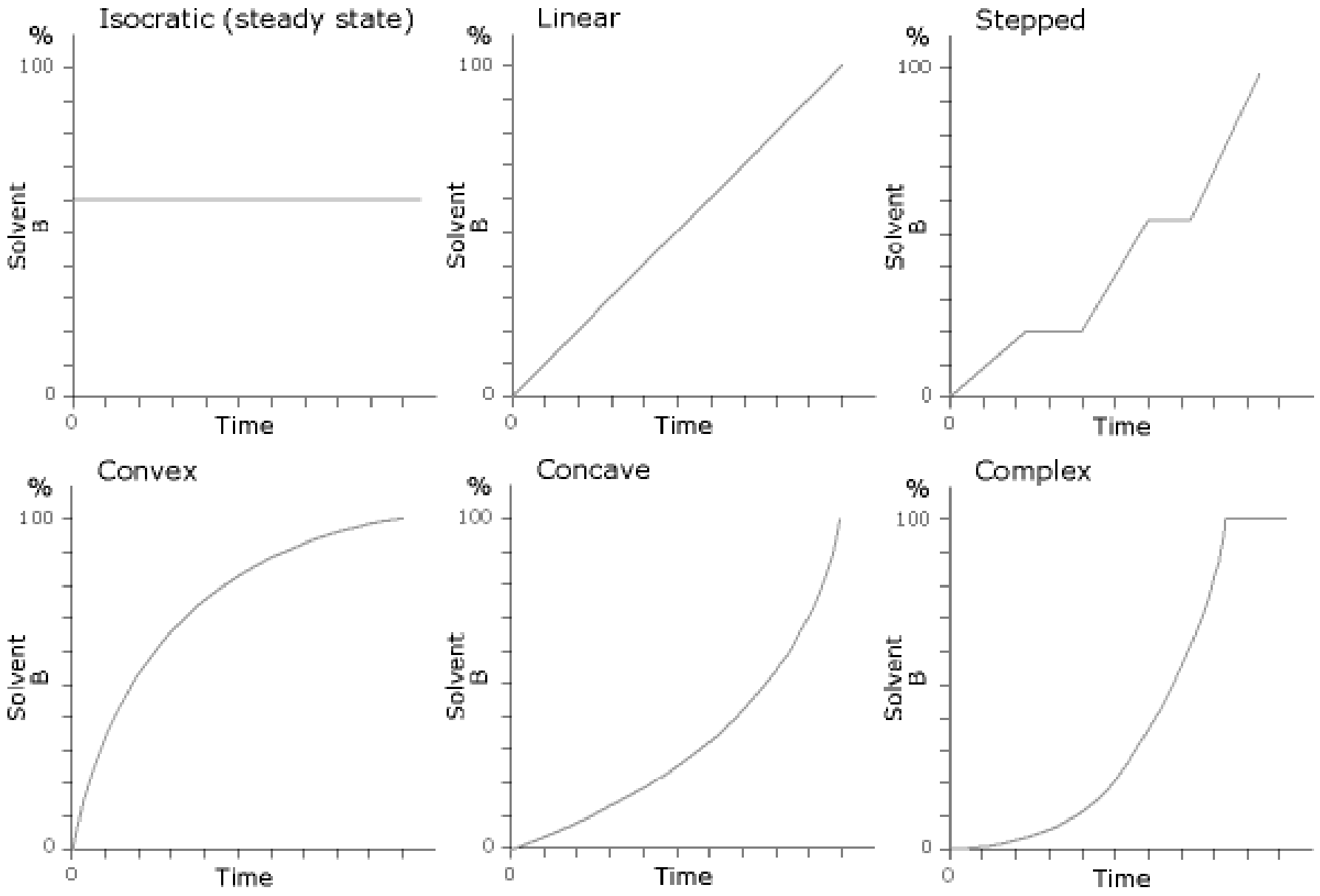


FIGURE 28-4 Improvement in separation effectiveness by gradient elution. Column: 1 m × 2.1 mm inside-diameter, precision-bore stainless steel; packing: 1% Permaphase® ODS (C_{18}). Sample: 5 µL of chlorinated benzenes in isopropanol. Detector: UV photometer (254 nm). Conditions: temperature, 60°C, pressure, 1200 psi. (From J. J. Kirkland, *Modern Practice of Liquid Chromatography*, p. 88, New York: Interscience, 1971. Reprinted by permission of John Wiley & Sons, Inc.)

Gradient programming



http://www.chromacademy.com/chromatography-HPLC-Gradient-Elution-Baseline-Drift.html



http://simulab.ltt.com.au/5/Laboratory/StudyNotes/snAboutHPLC.htm

HPLC detectors

TABLE 28-1 Performance of HPLC Detectors

HPLC Detector	Commercially Available	Mass LOD* (typical)	Linear Range† (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

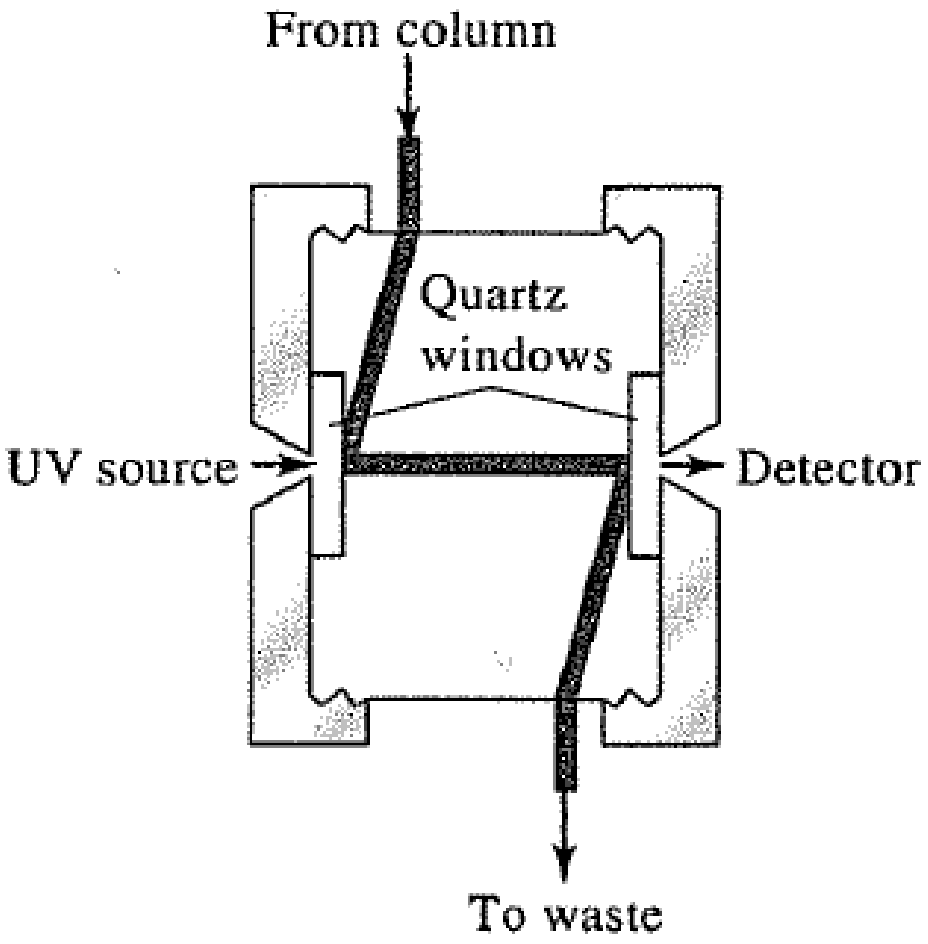
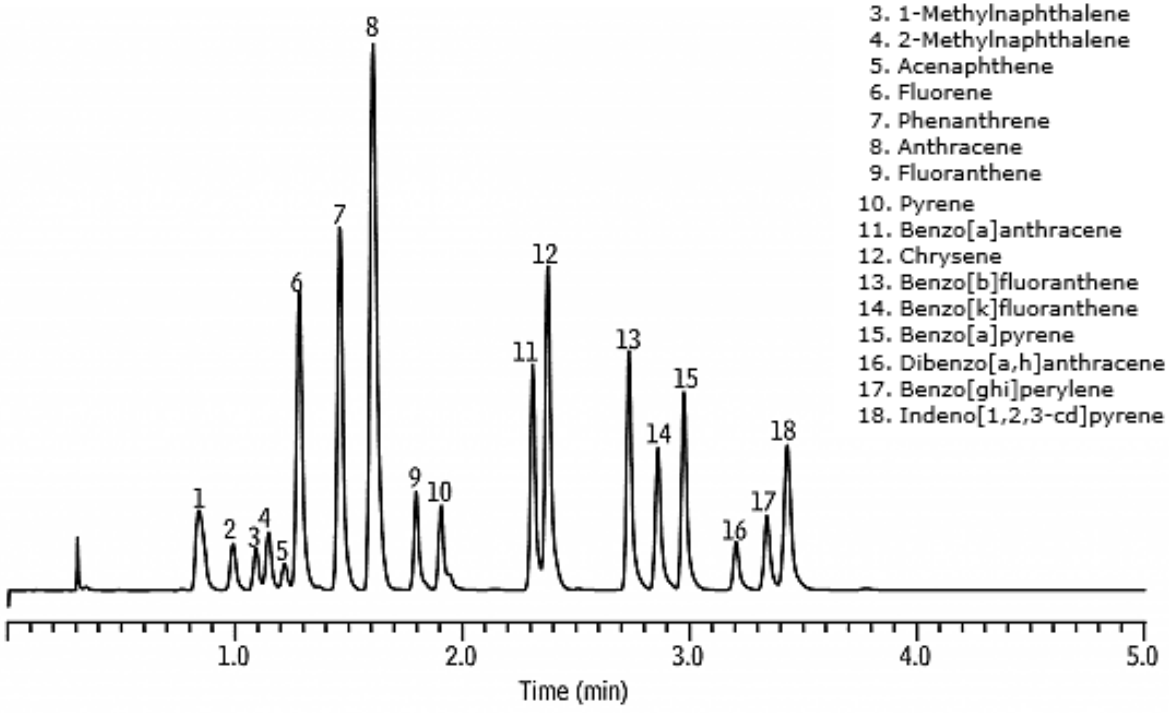


FIGURE 28-8 A UV-visible absorption cell for HPLC.

Example of UV detection: PACs on reversed phase

λ = 254 nm



Peaks

1. Naphthalene
2. Acenaphthylene
3. 1-Methylnaphthalene
4. 2-Methylnaphthalene
5. Acenaphthene
6. Fluorene
7. Phenanthrene
8. Anthracene
9. Fluoranthene
10. Pyrene
11. Benzo[a]anthracene
12. Chrysene
13. Benzo[b]fluoranthene
14. Benzo[k]fluoranthene
15. Benzo[a]pyrene
16. Dibenzo[a,h]anthracene
17. Benzo[ghi]perylene
18. Indeno[1,2,3-cd]pyrene

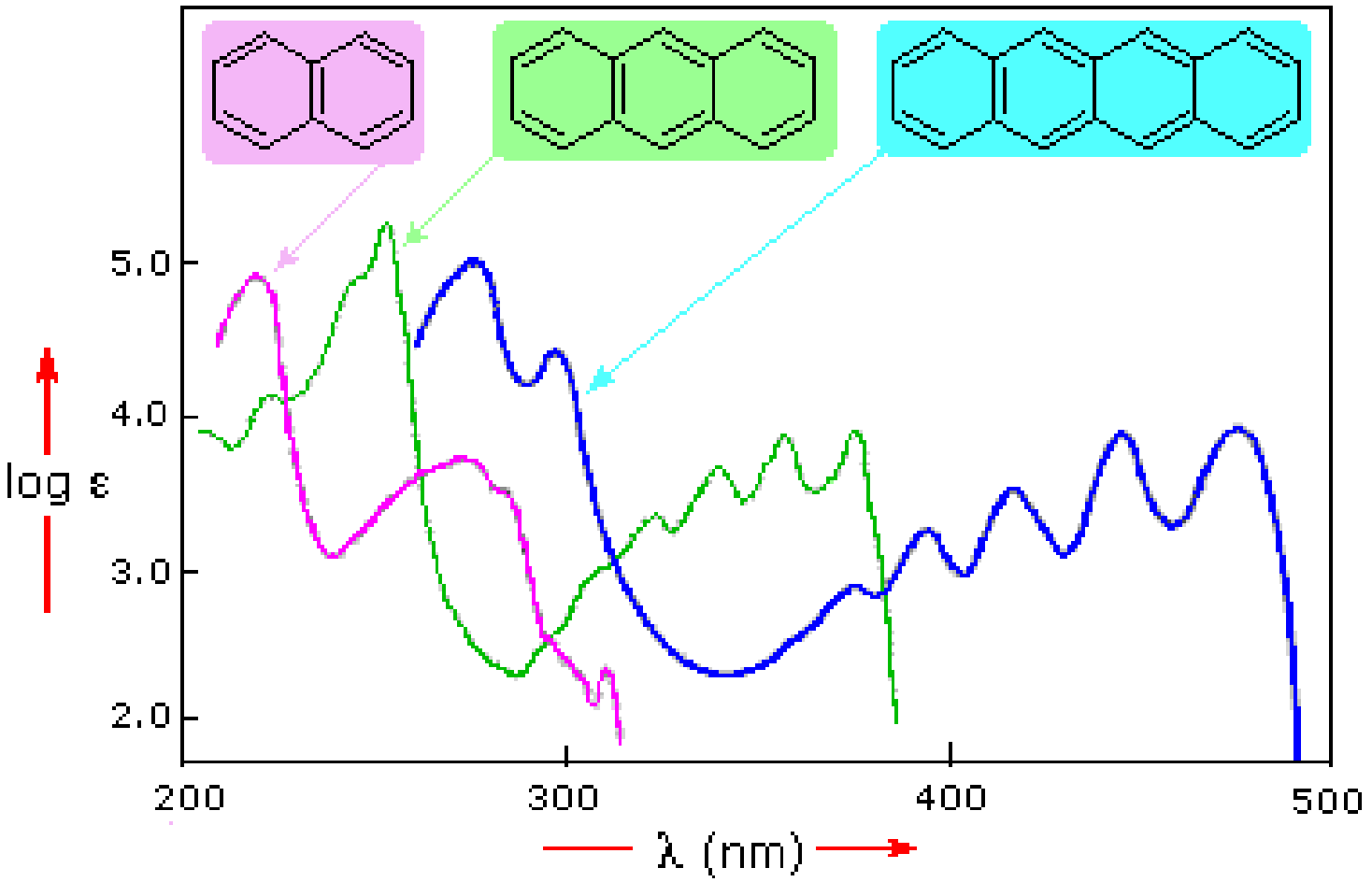
Column	Pinnacle® DB PAH (cat.# 9470252)
Dimensions:	50 mm x 2.1 mm ID
Particle Size:	1.9 µm
Pore Size:	140 Å
Temp.:	30 °C
Sample	
Diluent:	acetonitrile
Conc.:	20 µg/mL each component
Inj. Vol.:	2 µL
Mobile Phase	
A:	water
B:	acetonitrile

Time (min)	**%B**
0.00	50
1	60
3	100
5	100

Flow:	0.6 mL/min
Detector	UV/Vis @ 254 nm
Instrument	Jasco X-LC

http://www.restek.com/Technical-Resources/Technical-Library/Environmental/env_A027

UV absorption spectra of PACs



https://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/UV-Vis/spectrum.htm

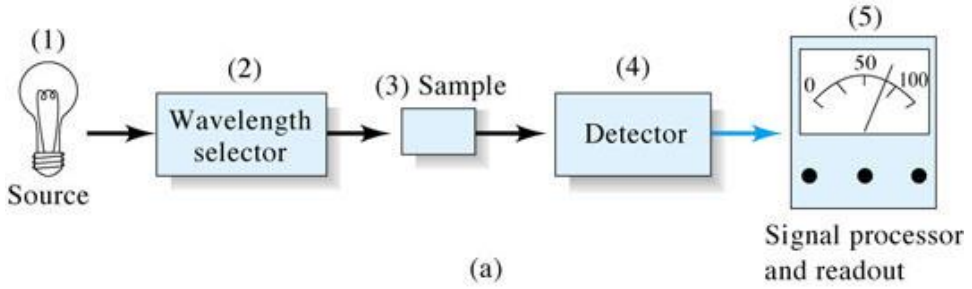
Fluorescence detection/spectrophotometry

- **Fluorescence:** radiation emitted from atoms or molecules rapidly ($\tau < 10^{-5}$ s) after the time of photo-excitation.

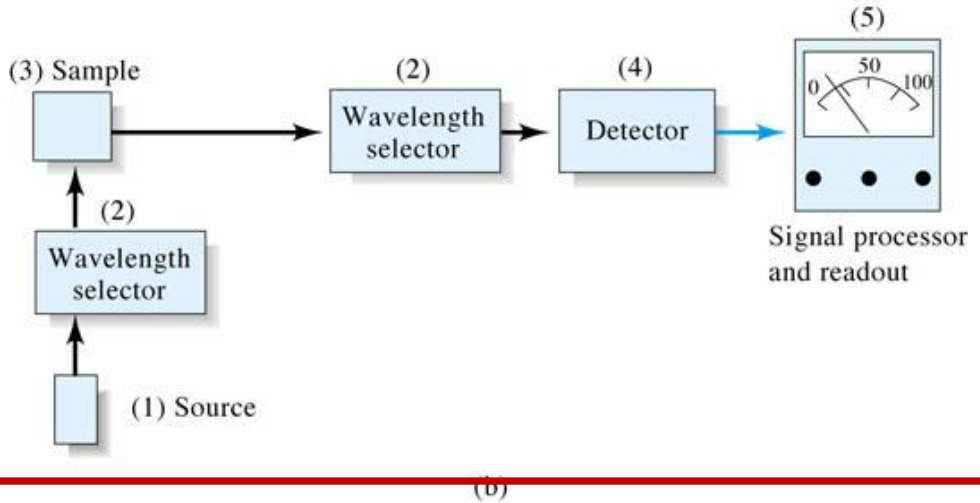
Resonance fluorescence: when $\lambda_e = \lambda_a$

Nonresonance fluorescence: when $\lambda_e > \lambda_a$

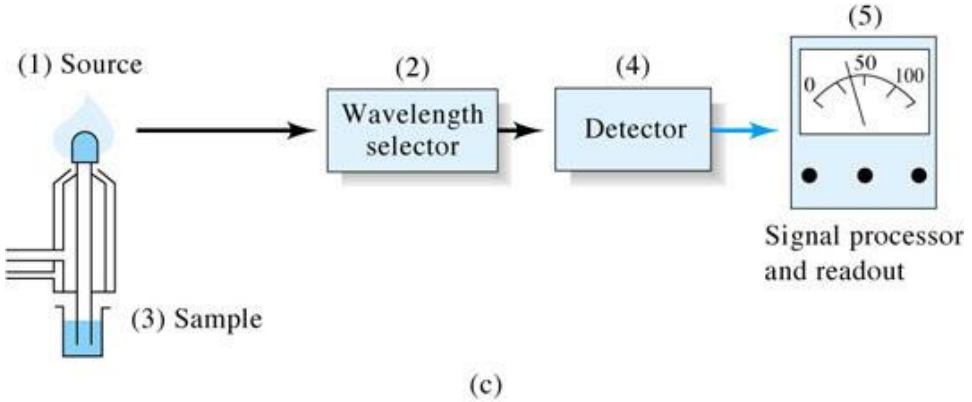
Absorption Spectroscopy



Fluorescence Spectroscopy



Emission Spectroscopy



Skoog et al., 2007

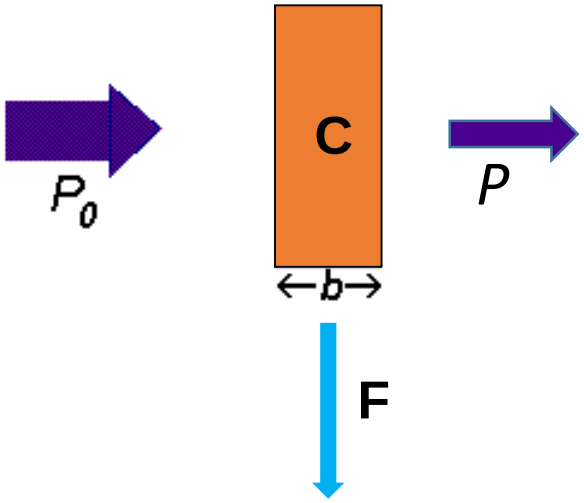
UV-Vis Fluorescence of Molecules

Quantum yield (ϕ):

Ratio of the number of molecules that luminescence to the total number of excited molecules. Determined by the relative rate constants (k_x) of deactivation processes:

$$\phi = \frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d}$$

f: fluorescence
i: intersystem crossing
ec: external conversion
ic: internal conversion
pd: predissociation
d: dissociation



$$A = \log \frac{P_0}{P} = \varepsilon b C$$

$$F = K'(P_0 - P)$$

$$F = \phi k' \varepsilon b P_0 C$$

Both absorbance and fluorescence are dependent on light path (b) and molar absorptivity (ε)

Fluorescence depends on quantum yield (ϕ) and incident light P_0

k' = constant depending on geometry and instrument used.

Some variables affecting fluorescence

- **Excitation wavelength**

  $\lambda > 250$ nm

- **Transition types (fluor.)**

  $\pi^* \rightarrow \pi$ transition $> \pi^* \rightarrow$ n transition $> \sigma^* \rightarrow \sigma$ transition

- **Molecular structure**

  - Usually aromatic compounds
  - ϕ increases with number of rings and degree of condensation
  - Increase in rigid structures
  - Increase for chelating agents when bound to metal.

- **Temperature:** increased fluor. at lower T.

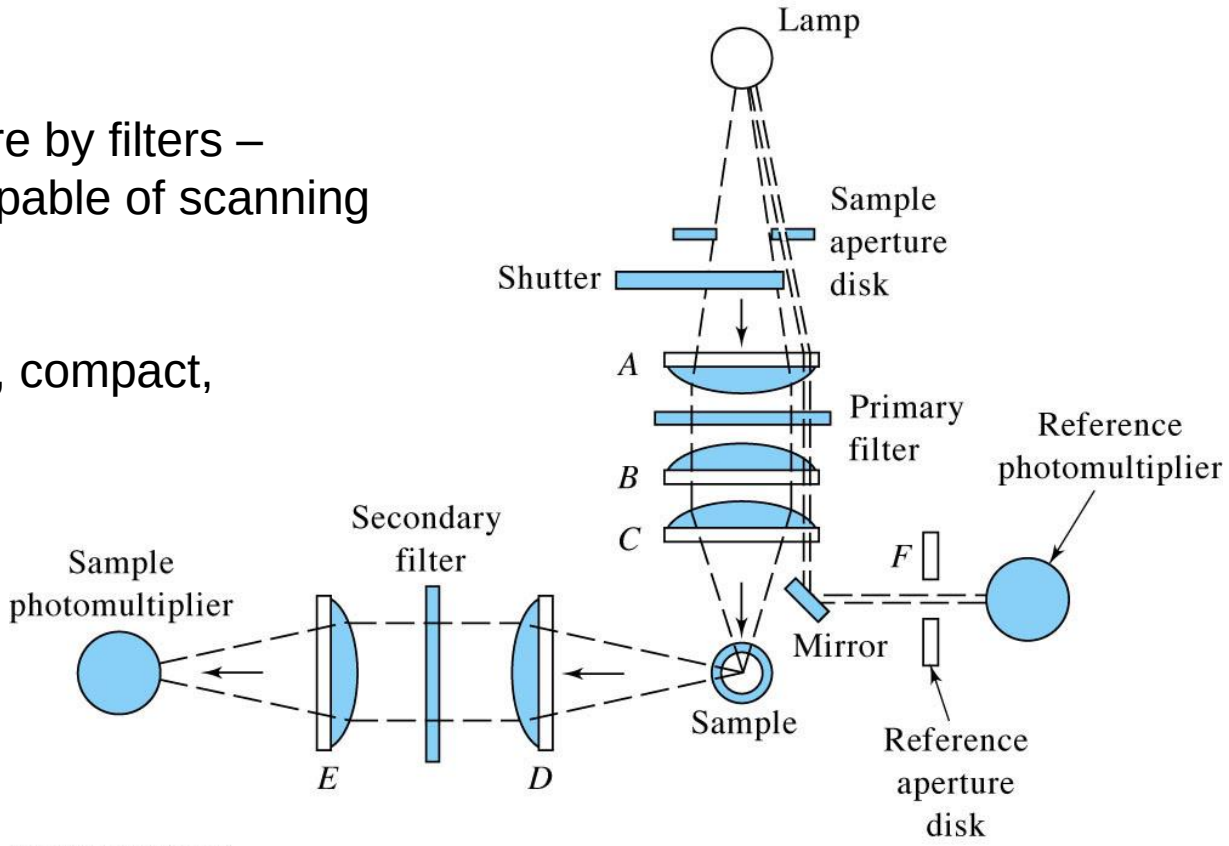
- **pH:** pH dependent for compounds with acid-base dissociations

UV-Vis Fluorescence Spectrophotometers

Components similar to UV/Vis absorption spectrophotometer, but the detector is located with a 90º angle from the incident light beam

Newer portable fluorimeters

- Source beam split into reference and sample beams, allowing to correct for source fluctuations.
- Wavelength selections are by filters – limited range analysis; incapable of scanning measurement.
- Simple, rugged, low cost, compact, portable



UV-Vis fluorescence spectra from grating instruments

➢ Excitation Spectrum

measure fluorescence at a fixed λ while varying the excitation wavelength.

➢ Emission Spectrum

measure fluorescence over a range of wavelengths using a fixed excitation λ.

➢ Total fluorescence Spectrum

measure fluorescence over a range of emission and excitation wavelengths

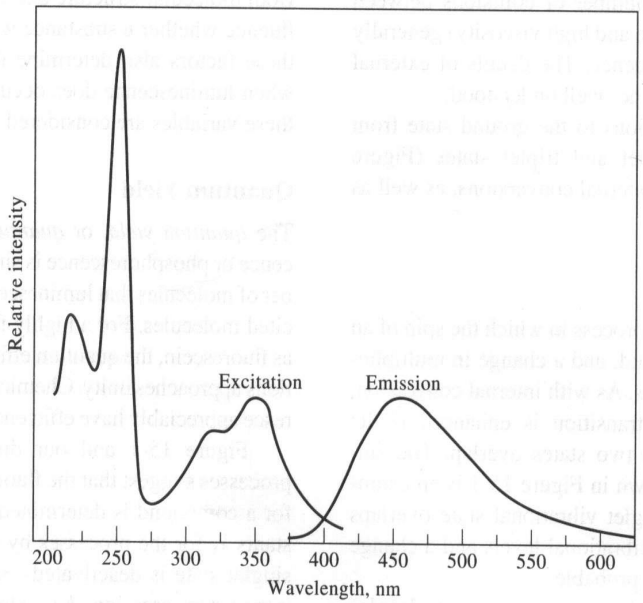
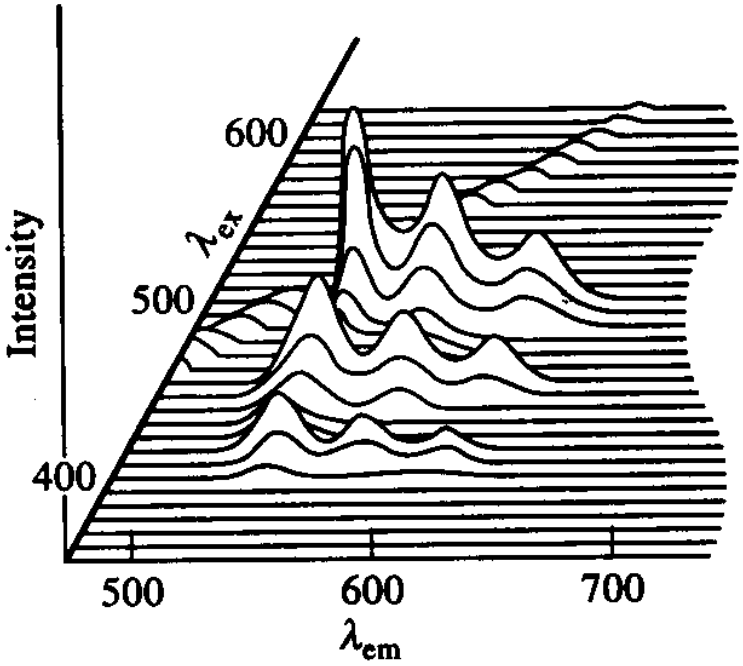


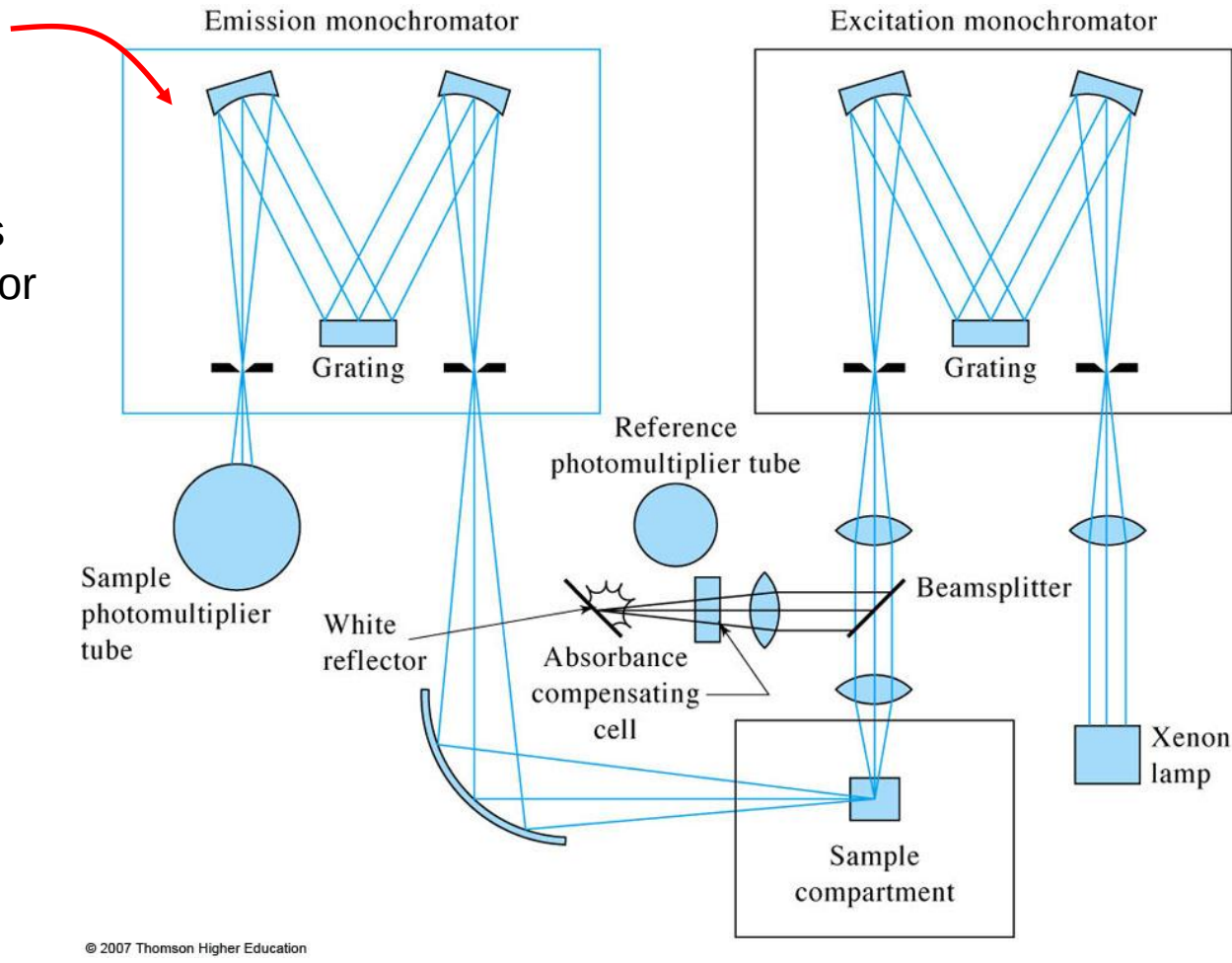
Figure 15-2 Fluorescence excitation and emission spectra for a solution of quinine.



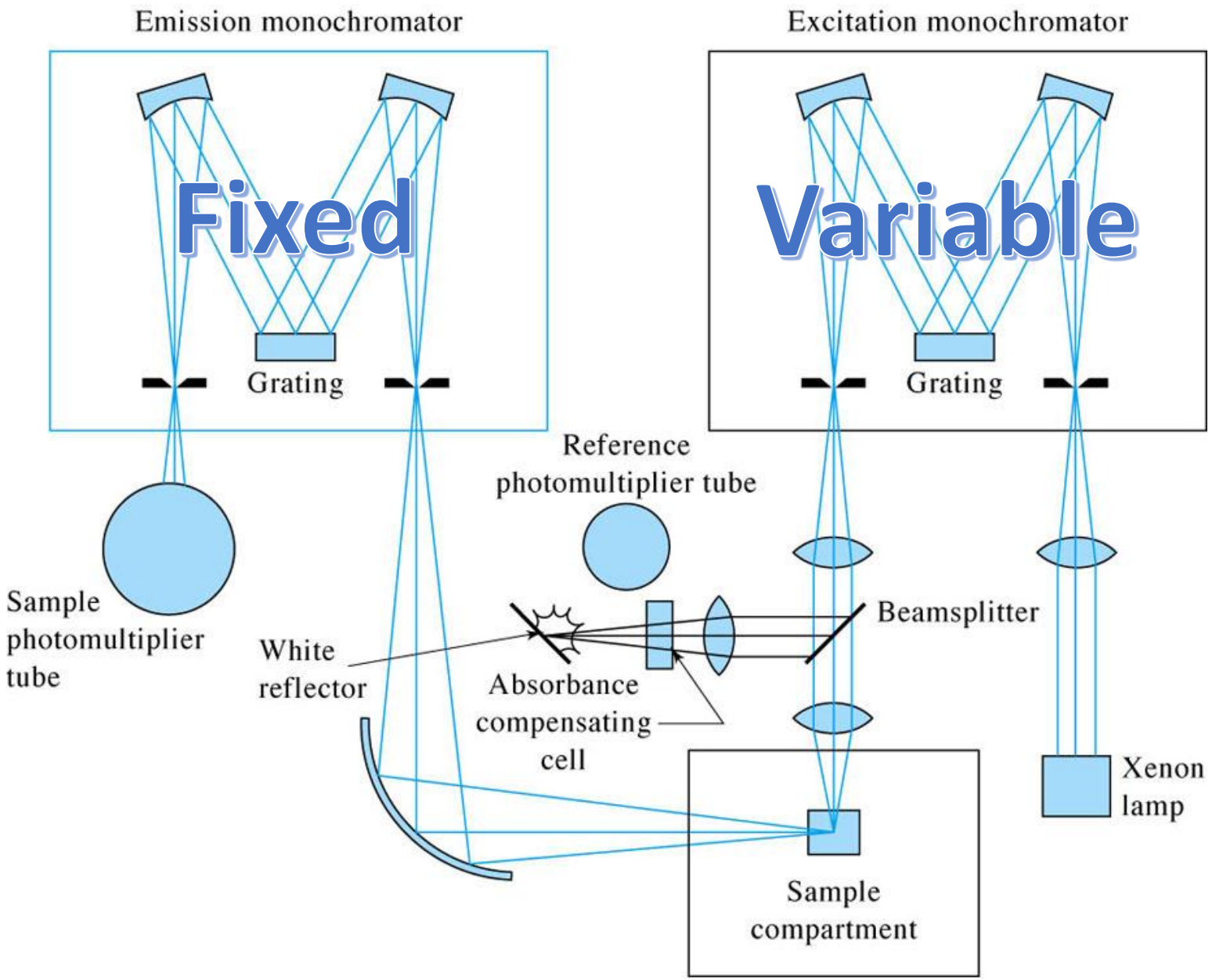
“Grating” Spectrofluorimeter

- Two grating monochromators (excitation, emission) allowing for obtaining both types of spectra

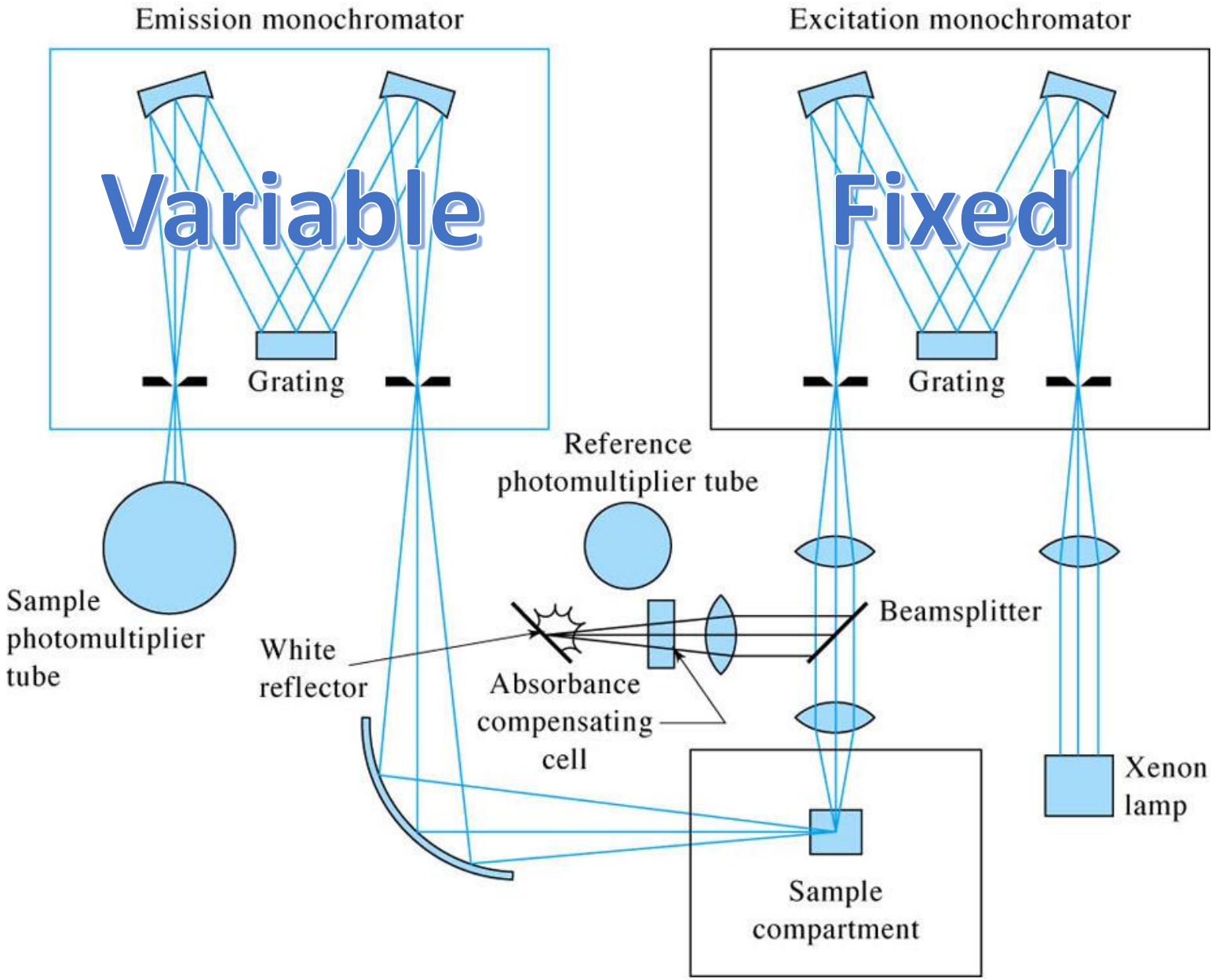
- The emission spectra are often instrument dependent (radiation source, transducer, monochromators) and thus not necessarily comparable.



To collect excitation spectrum



To collect fluorescence spectrum



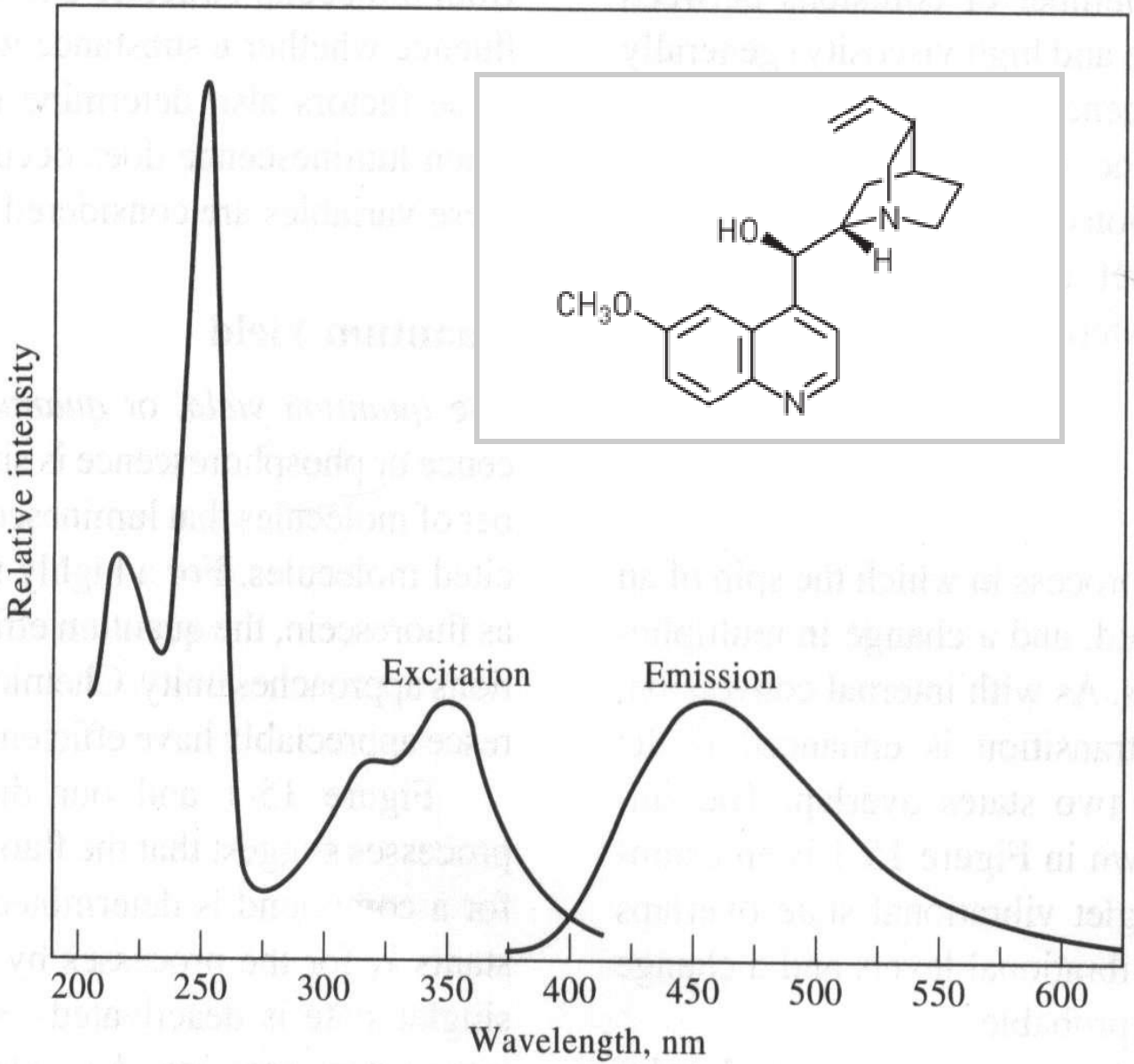
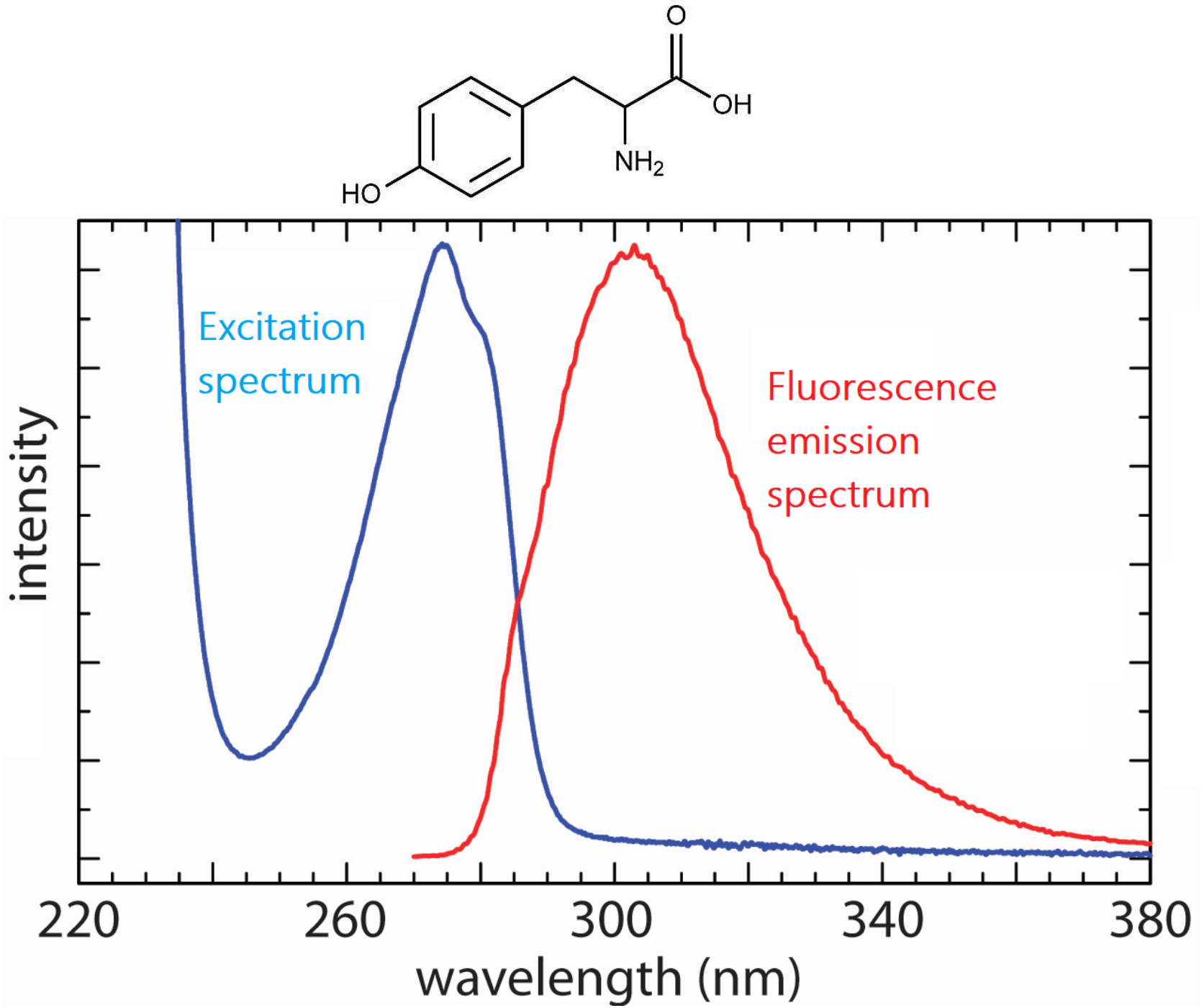


Figure 15-2 Fluorescence excitation and emission spectra for a solution of quinine.


O
OH
NH2
HO
Excitation
spectrum
Fluorescence
emission
spectrum
intensity
220
260
300
340
380
wavelength (nm)

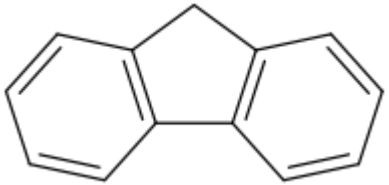
Method	Mass detection limit (moles)	Concentration detection limit (molar)	Advantages
Absorption	10^{-13} to 10^{-16}	10^{-5} to 10^{-8}	Universal
fluorescence	10^{-15} to 10^{-17}	10^{-7} to 10^{-9}	Sensitive

HPLC-Fluorescence

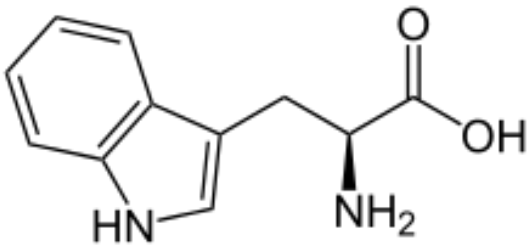
- Roughly about 15% of all compounds produce natural fluorescence.

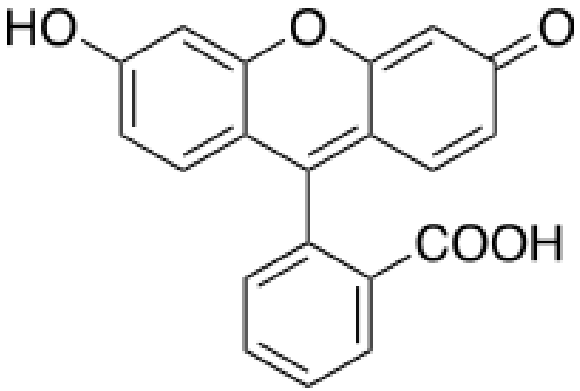
Molecules that fluoresce naturally

fluorene

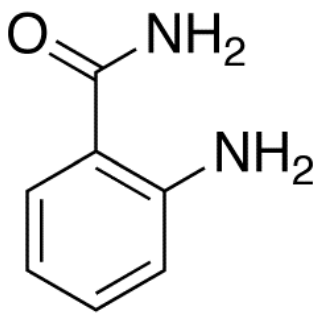


tryptophan





fluoresceine

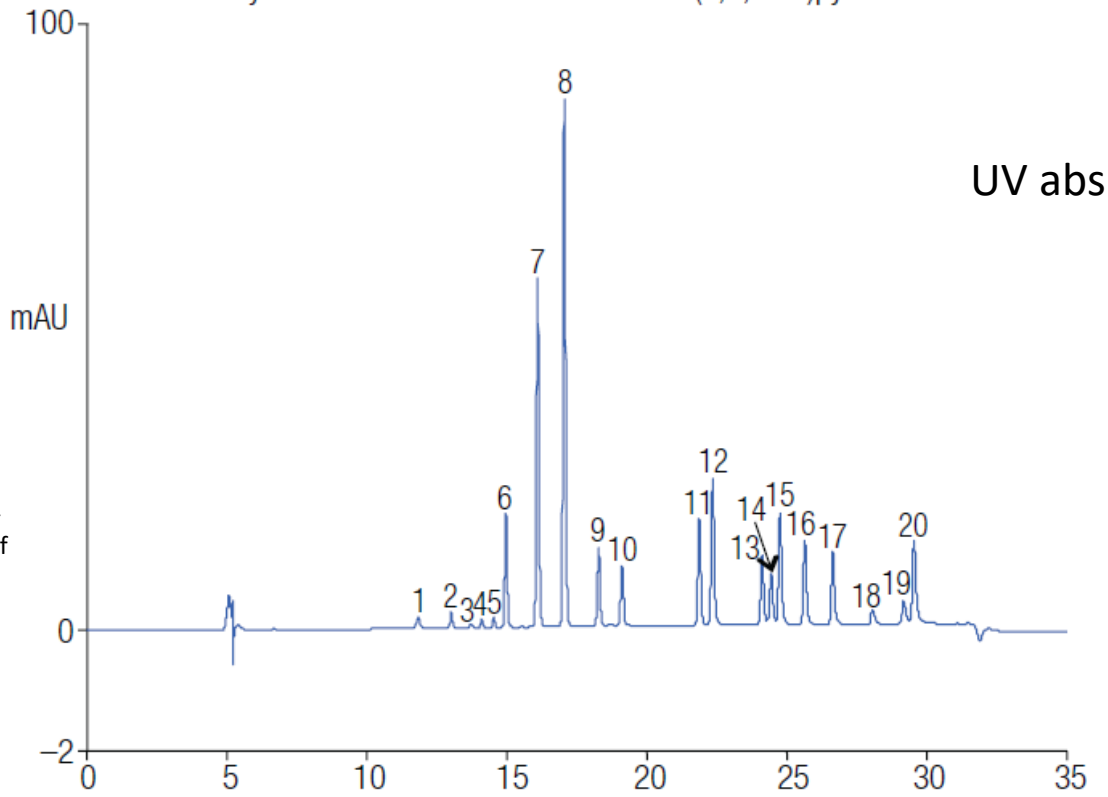


2-aminobenzamide

Polycyclic aromatics: UV-vis abs.

Peaks:

1. Naphthalene
2. Acenaphthylene
3. 1-Methylnaphthalene
4. 2-Methylnaphthalene
5. Acenaphthene
6. Fluorene
7. Phenanthrene
8. Anthracene
9. Fluoranthene
10. Pyrene
11. Benzo(a)anthracene
12. Chrysene
13. Benzo(j)fluoranthene
14. Benzo(e)pyrene
15. Benzo(b)fluoranthene
16. Benzo(a)pyrene
17. Benzo(k)fluoranthene
18. Dibenz(a,h)anthracene
19. Benzo(ghi)perylene
20. Indeno(1,2,3-cd)pyrene



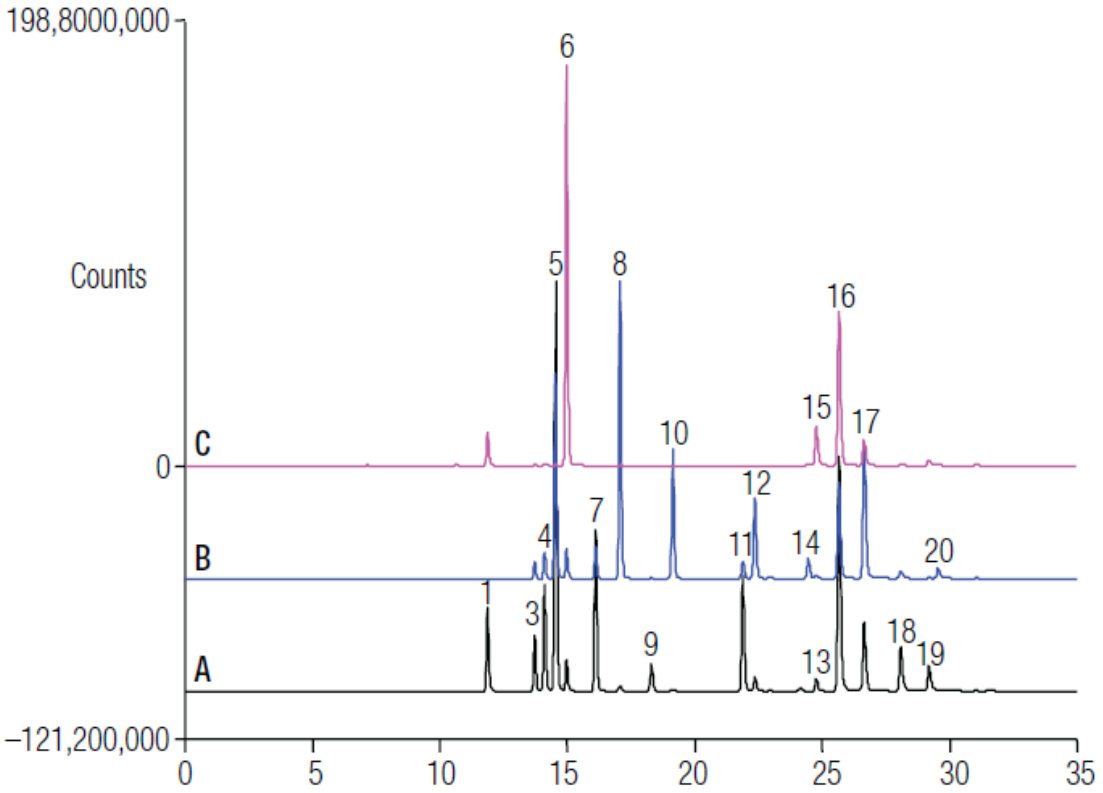
UV abs. 254 nm

http://www.dionex.com/en-us/webdocs/115260-AN-1085-HPLC-PAHs-Tap-Water-AN70923-EN.pdf

Polycyclic aromatics: Fluor.

Peaks:

1. Naphthalene
2. Acenaphthylene
3. 1-Methylnaphthalene
4. 2-Methylnaphthalene
5. Acenaphthene
6. Fluorene
7. Phenanthrene
8. Anthracene
9. Fluoranthene
10. Pyrene
11. Benzo(a)anthracene
12. Chrysene
13. Benzo(j)fluoranthene
14. Benzo(e)pyrene
15. Benzo(b)fluoranthene
16. Benzo(a)pyrene
17. Benzo(k)fluoranthene
18. Dibenz(a,h)anthracene
19. Benzo(ghi)perylene
20. Indeno(1,2,3-cd)pyrene



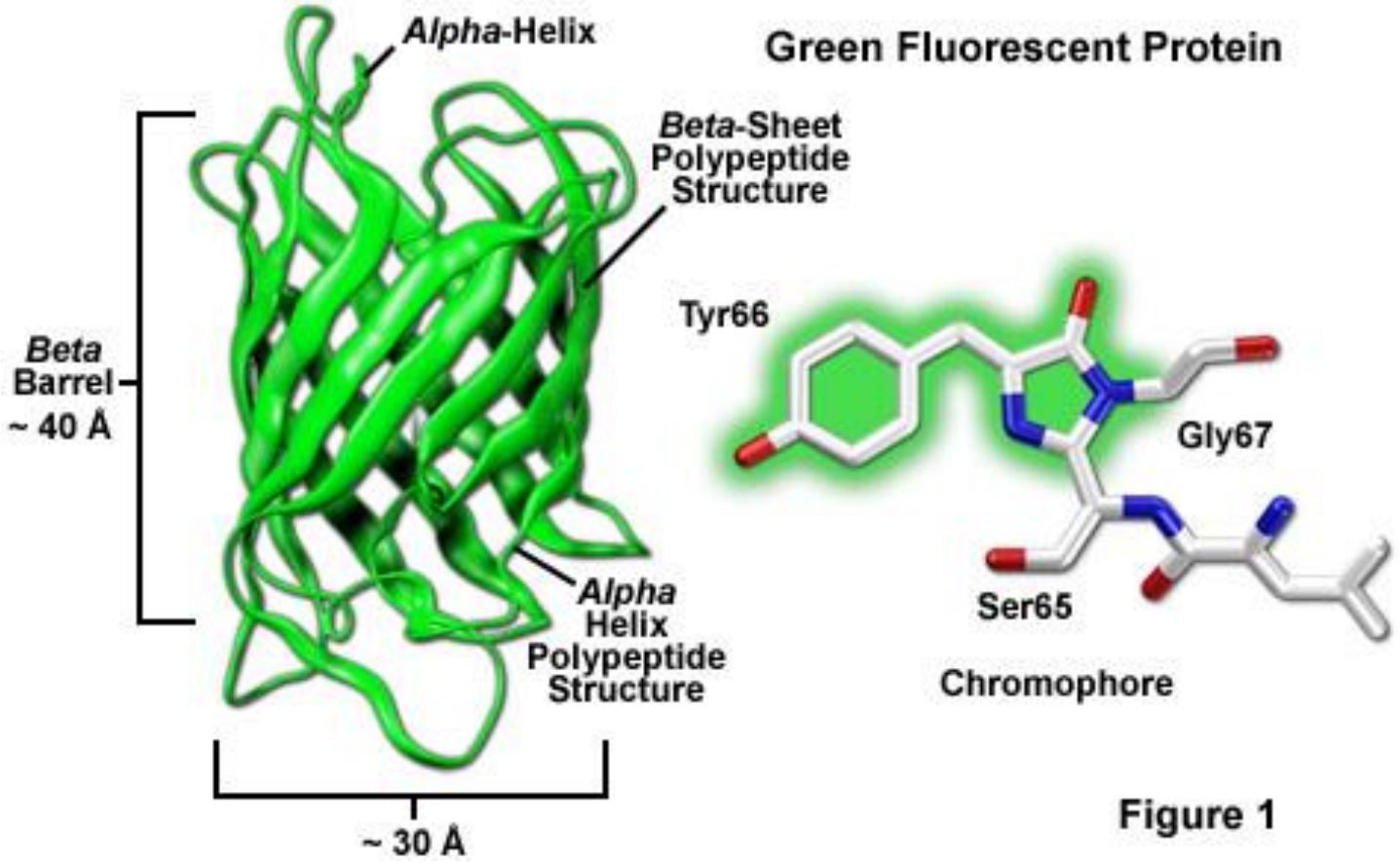
http://www.dionex.com/en-us/webdocs/115260-AN-1085-HPLC-PAHs-Tap-Water-AN70923-EN.pdf

Table 2. Ex/Em maximums for each PAH and programmed wavelength switching times.

Time (min)	Fluorescence Detection Channel	Ex/Em Wavelengths (nm)	PAH	Peak No.
0.0	Emission_1	219/330	Naphthalene	1
13.45	Emission_1	225/333	1-Methylnaphthalene	3
			2-Methylnaphthalene	4
	Emission_2	235/332	Acenaphthene	5
	Emission_3	263/310	Fluorene	6
15.50	Emission_1	247/364	Phenanthrene	7
	Emission_2	247/401	Anthracene	8
17.80	Emission_1	281/453	Fluoranthene	9
	Emission_2	236/389	Pyrene	10
20.50	Emission_1	281/391	Benzo(a)anthracene	11
	Emission_2	264/381	Chrysene	12
23.50	Emission_1	240/510	Benzo(j)fluranthene	13
	Emission_2	283/394	Benzo(e)pyrene	14
	Emission_3	249/443	Benzo(b)fluoranthene	15
25.40	Emission_1	243/412	Benzo(k)fluoranthene	16
	Emission_2	260/408	Benzo(a)pyrene	17
27.50	Emission_1	290/398	Dibenz(a,h)anthracene	18
28.70	Emission_1	292/415	Benzo(ghi)perylene	19
	Emission_2	246/503	Indeno(1,2,3-cd)pyrene	20

http://www.dionex.com/en-us/webdocs/115260-AN-1085-HPLC-PAHs-Tap-Water-AN70923-EN.pdf

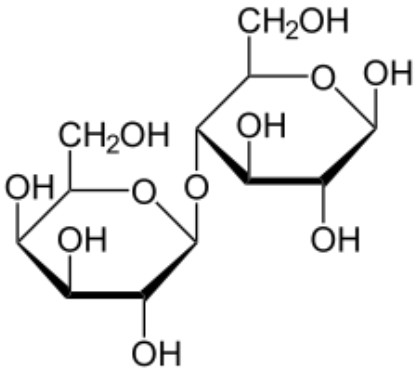
Molecules that fluoresce naturally



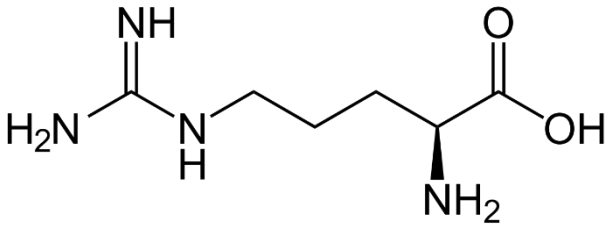
http://zeiss-campus.magnet.fsu.edu/articles/probes/fpintroduction.html

Molecules that need a fluorophore for HPLC

Carbohydrates



Amino acids

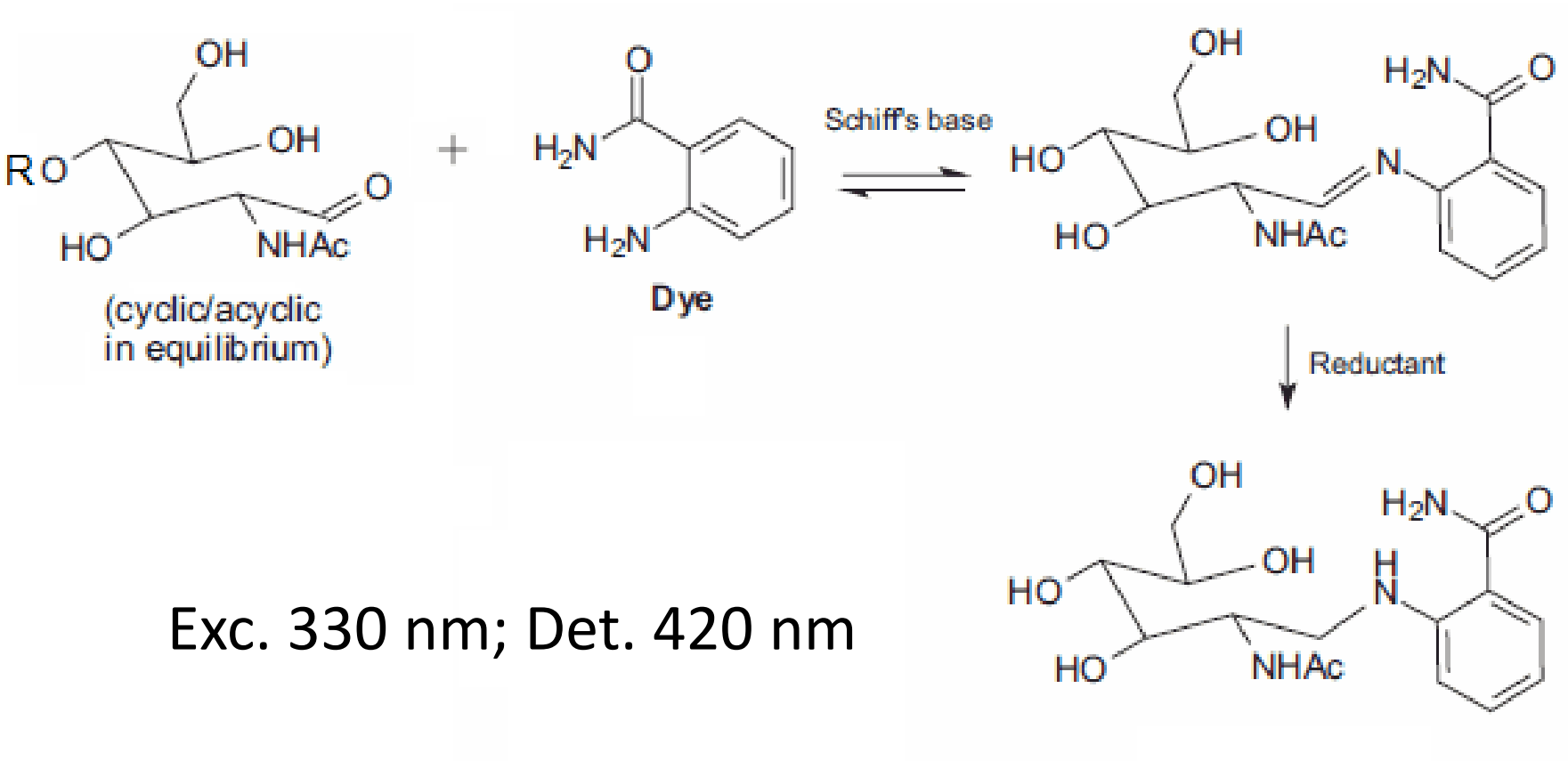


Metal ions Zn^{2+}

DNA

The fluorophore selectively binds to a specific region or functional group on the target molecule and can be attached chemically.

Carbohydrates: tagging with 2-aminobenzamide or 2-aminobenzoic acid



Exc. 330 nm; Det. 420 nm

Analysis of monosaccharides tagged with 2-aminobenzoic acid

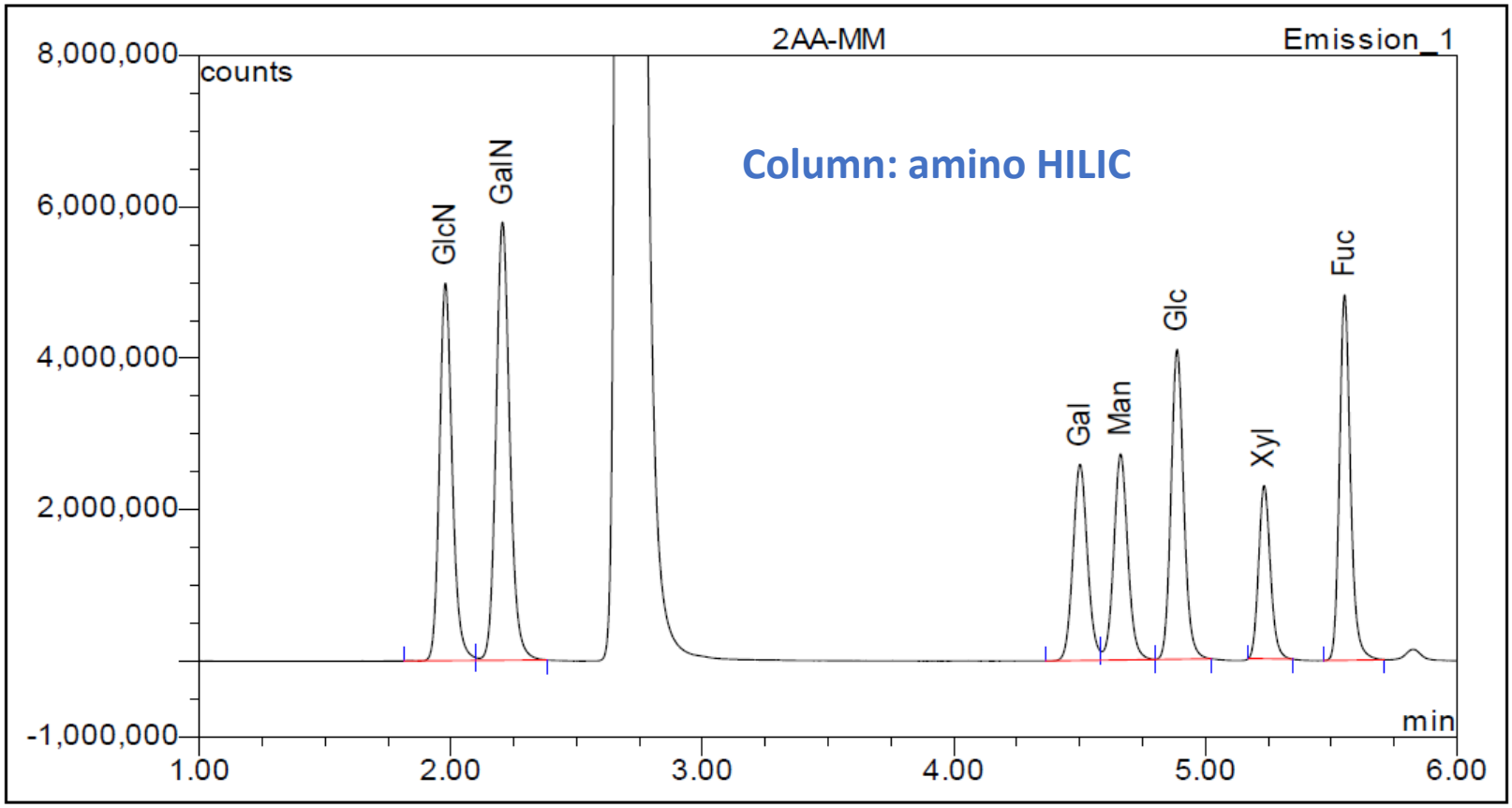
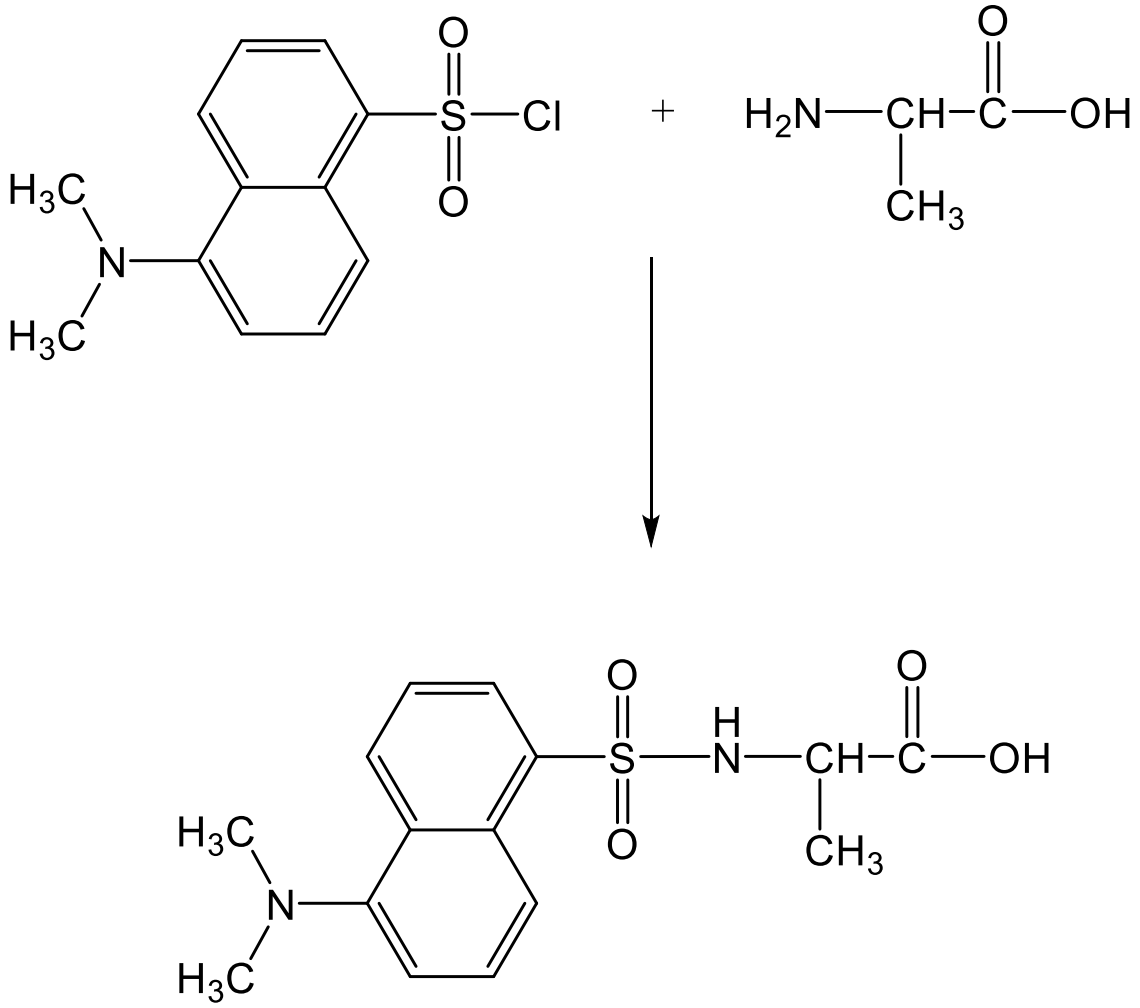


Figure 1: 2AA-labeled monosaccharide standards profiled on a LudgerSep uR2 UHPLC column (Cat No. LS-UR2-2.1x50). Peaks for the following monosaccharides appear within 8 minutes; glucosamine (GlcN), galactosamine (GalN), galactose (Gal), mannose (Man), glucose (Glc), xylose (Xyl) and fucose (Fuc).

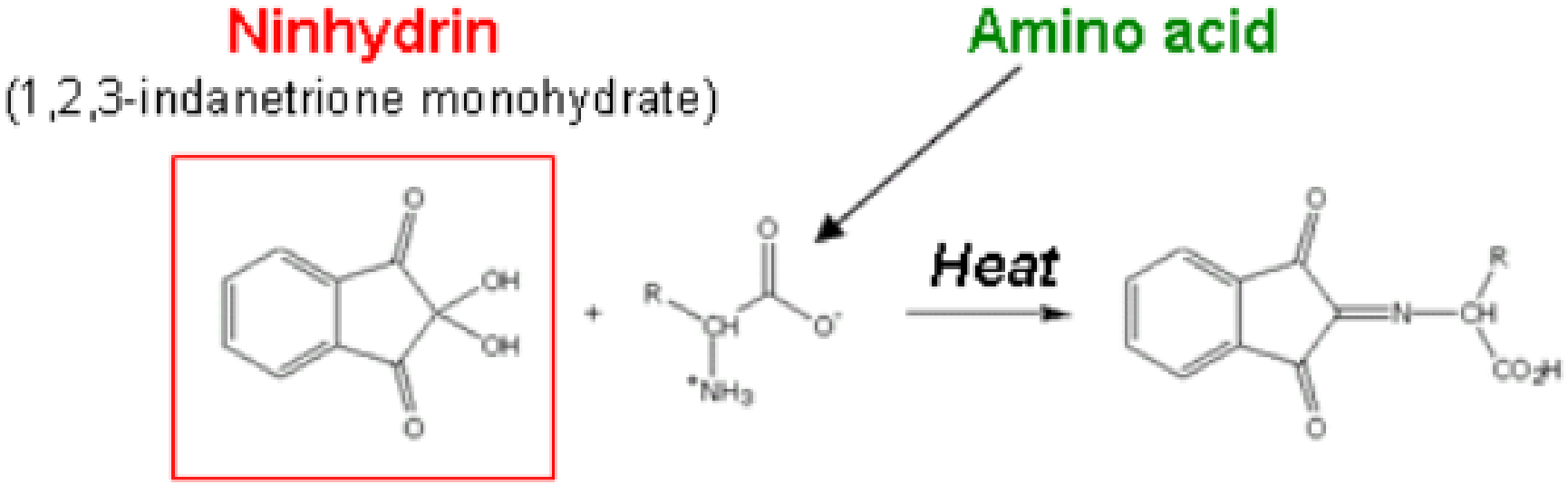
http://glycotools.qa-bio.com/Monosaccharide-Kit

Amino acids: tagging with dansyl chloride



Exc. 350 nm; Det. 520 nm

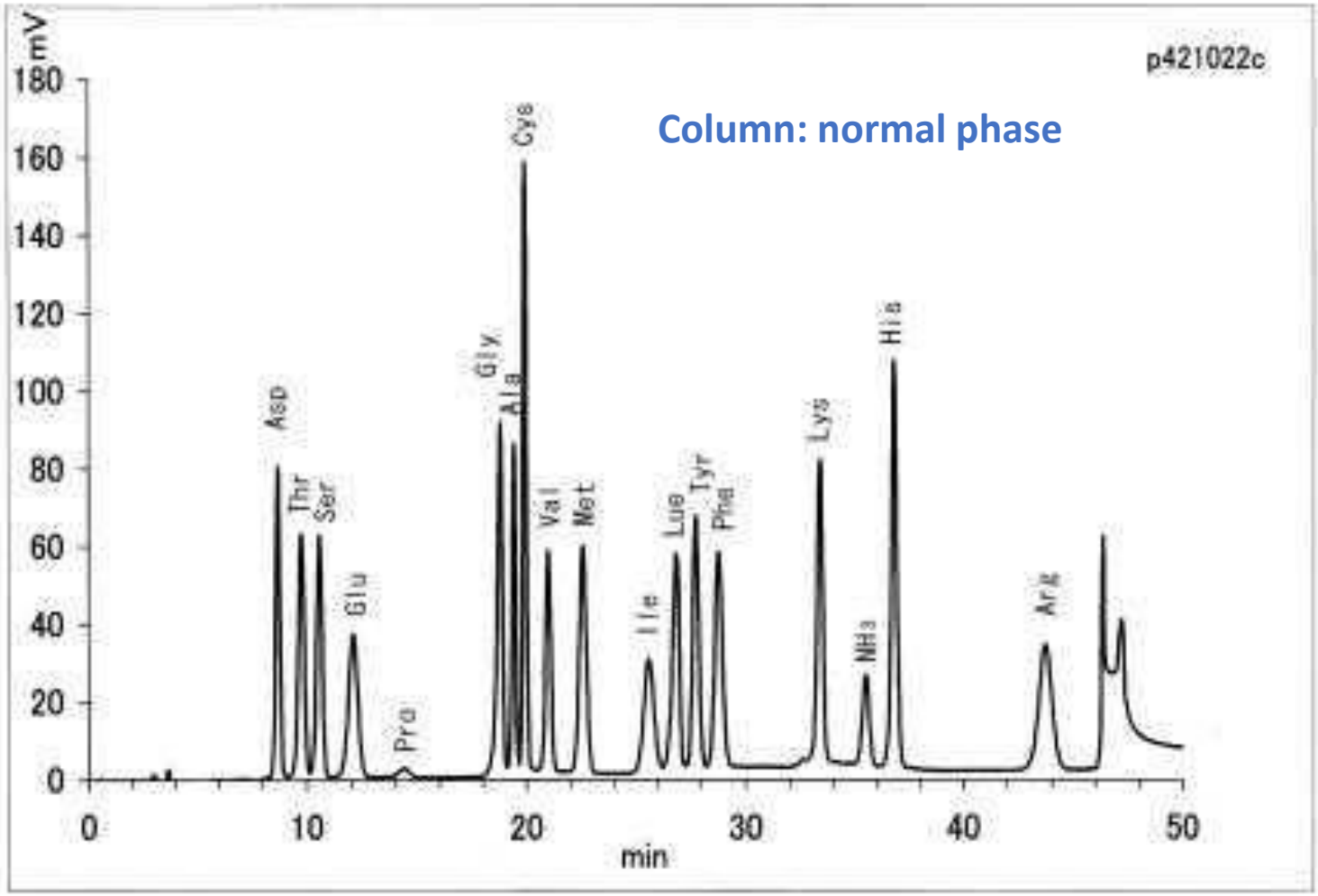
Amino acids: tagging with ninhydrin



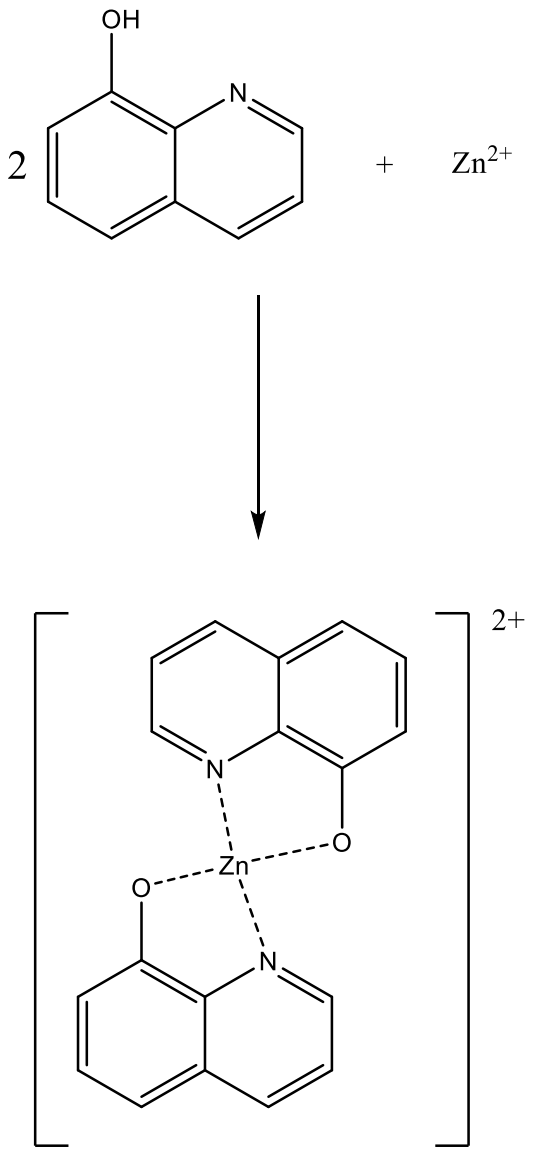
Exc: 300 nm Fluo: 570 nm

http://classroom.sdmesa.edu/eschmid/Lab4-Biol210.htm

Analysis of amino acids tagged with nihydrin



Zn^{2+} : chelating with 8-hydroxyquinoline



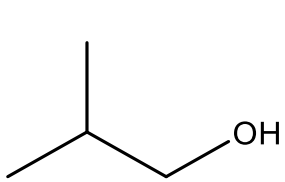
Exc. 320 nm; Det. 550 nm

Summary on HPLC with UV/Fluor.

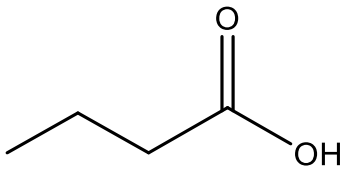
- For solid or liquid samples
- Different types of stationary phases available (normal and reversed phases)
- Fraction collection possible for sample purification
- UV and fluorescence detectors may be used separately or lined up
- UV: different compound have different ε so no direct quantitation
- Fluor: each compound has different combination of λ_{exc} and λ_{em}
- Non-absorbent and non-fluorescent compounds must be derivatized
- Fluor. more sensitive than UV-vis in general

Questions

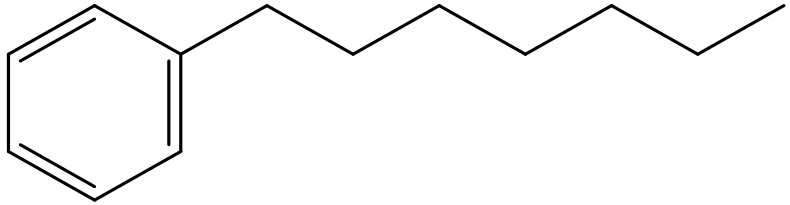
1. Given the three compounds shown here to be separated by reversed phase HPLC:



1



2



3

- What would be their elution order? Justify.

2. What are the excitation and fluorescence spectra of organic molecules and how are they obtained using a spectrofluorimeter?

3. Comment on the use of dansyl chloride reagent for high performance liquid chromatography.

a) What kind of compounds is it useful for and how does it enhance detection?

b) In terms of quantitative analysis by HPLC, what is the main advantage obtained from the use of dansyl chloride?

4. From the following chromatogram obtained with a 30 cm column, determine:

-The capacity constant for the compound eluting at 6 min.
-The resolution
-The number and height of theoretical plates

The first peak is from a compound that is not retained at all on the column.