

Detection in HPLC

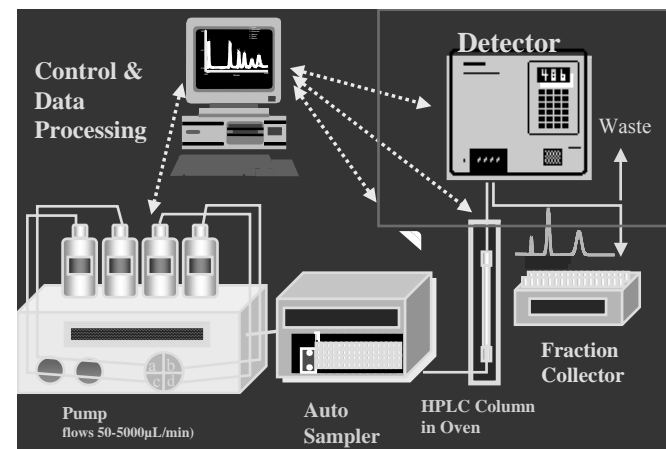


Selecting the Right Detector: Types of Detectors in HPLC

- UV/VIS
- Refractive index
- Fluorescence
- Electrochemical
- Conductivity
- Mass-spectrometric (LC/MS)
- Evaporative light scattering
- Appendix:
- Cutoff of solvents UV
- Troubleshooting of RI detector as an example

Shulamit Levin

The Detector is the “Eye” of the HPLC System



Detectors

UV/VIS

Refractive index

Fluorescence

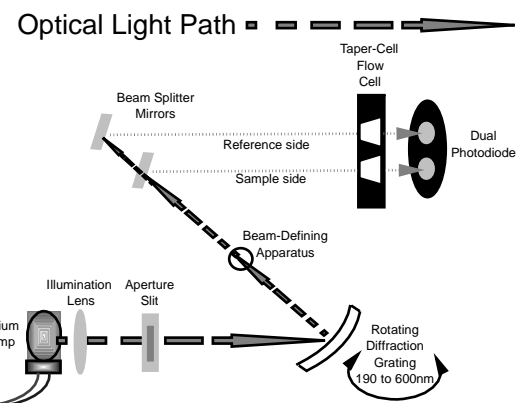
Electrochemical

Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

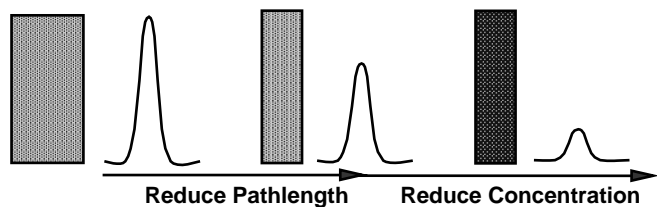
Optical Bench of UV-VIS Detector



Detection in HPLC

Beer's Law

Absorbance = Extinction Coefficient x Pathlength x Concentration



UV Chromophores

Chromophore	Chemical Configuration	λ_{max} (nm)	ϵ_{max} (L/m/cm)	λ_{max} (nm)	ϵ_{max} (L/m/cm)
Ether	—O—	185	1000		
Thioether	—S—	194	4600	215	1600
Amine	—NH ₂	195	2800		
Thiol	—SH	195	1400		
Disulfide	—S—S—	194	5500	255	400
Bromide	—Br	208	300		
Iodide	—I	260	400		
Nitrile	—C≡N	160	—		
Acetylide	—C≡C—	175-180	6000		
Sulfone	—SO ₂ —	180	—		
Oxime	—NOH	190	5000		
Azido	>C=N—	190	5000		
Ethylene	—C=C—	190	8000		
Ketone	>C=O	195	1000	270-285	18-30
Thioketone	>C=S	205	strong		
Esters	—COOR	205	50		
Aldehyde	—CHO	210	strong	280-300	11-18
Carboxyl	—COOH	200-210	50-70		
Sulfoxide	>S=O	210	1500		
Nitro	—NO ₂	210	strong		
Nitrile	—ONO	220-230	1000-2000	300-400	10

UV Chromophores

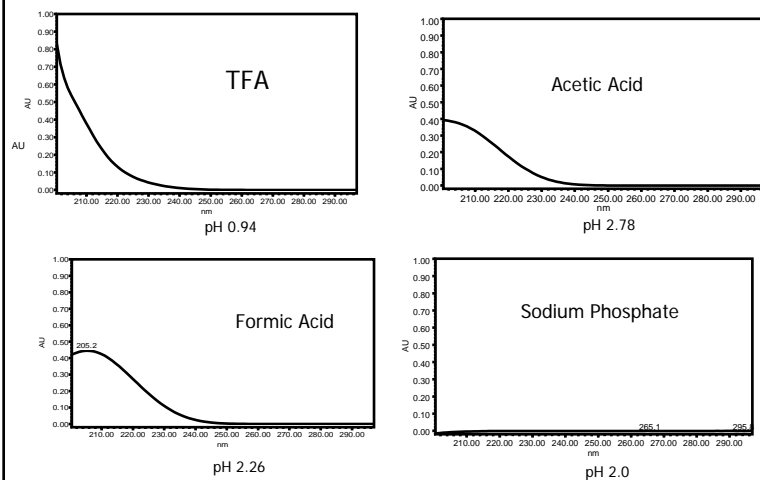
Chromophore	Chemical Configuration	λ_{max} (nm)	ϵ_{max} (L/m/cm)	λ_{max} (nm)	ϵ_{max} (L/m/cm)
Azo	—N=N—	285-400	3-25		
Nitroso	—N=O	302	100		
Nitrate	—ONO ₂	270 (shoulder)	12		
Allene	—(C=C) ₂ — (acyclic)	210-230	21,000		
Allene	—(C=C) ₃ —	260	35,000		
Allene	—(C=C) ₄ —	300	52,000		
Allene	—(C=C) ₅ —	330	118,000		
Allene	—(C=C) ₂ — (alicyclic)	230-260	3000-8000		
Ethylenic/Acetylenic	C=C—C≡C	219	6,500		
Ethylenic/Amido	C=C—C=N	220	23,000		
Ethylenic/Carbonyl	C=C—C=O	210-250	10,000-20,000		
Ethylenic/Nitro	C=C—NO ₂	229	9,500		

UV-Vis chromophores

	λ_{max}	$\epsilon_{\text{m}} \times 10^{-3} @ \lambda_{\text{max}}$
Adenine	260.5	E = 13.4
Guanine	275	E = 8.1
Cytosine	267	E = 6.1
Thymine	264.5	E = 7.9
Uracil	259.5	E = 8.2
NADH	340	E = 6.23
NAD	260	E = 18

Detection in HPLC

UV spectrum of 10 nM mobile phase



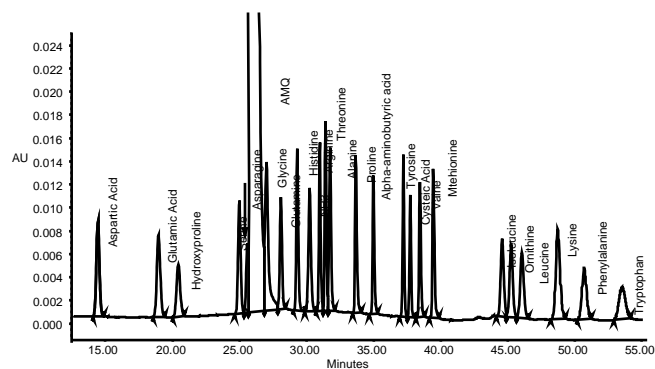
U.V. Cut-offs for some Common Solvents

Remember Solvents chosen can affect detection!!

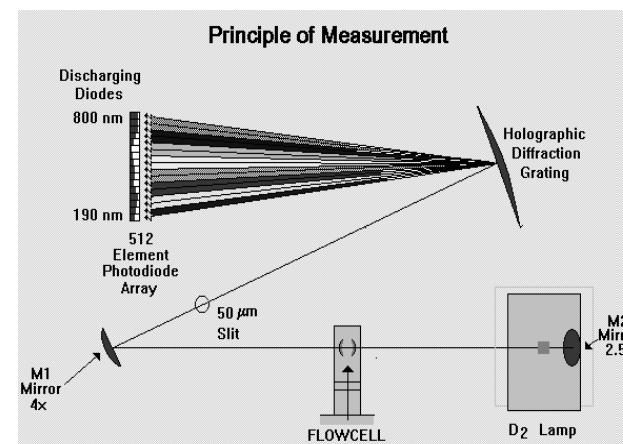
<u>Solvent</u>	<u>UV Cutoff</u>	<u>Solvent</u>	<u>UV Cutoff</u>
Water	180	N-Heptane	197
Methanol	205	Cyclohexane	200
N-Propanol	205	Carbon tetrachloride	265
Acetonitrile	190	Chloroform	245
THF	225	Benzene	280
Acetone	330	Toluene	285
Methyl acetate	260	Methylene chloride	232
Ethyl Acetate	260	Tetrachloroethylene	280
Nitromethane	380	1,2-Dichloroethane	225

All wavelengths reported in nm.

UV Detection of AccQ-Tag Amino Acid Derivatives

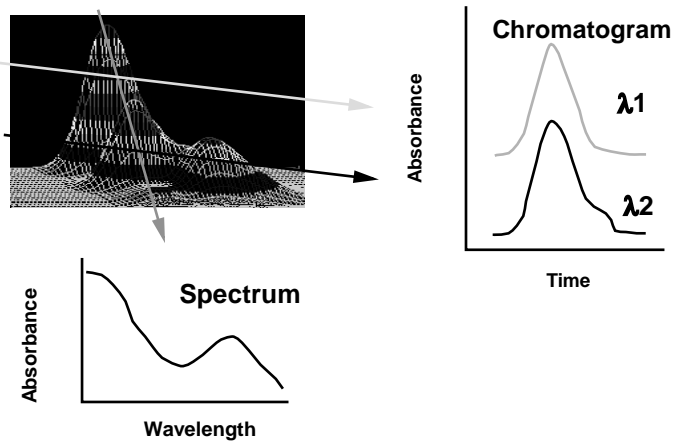


Diode Array Detector



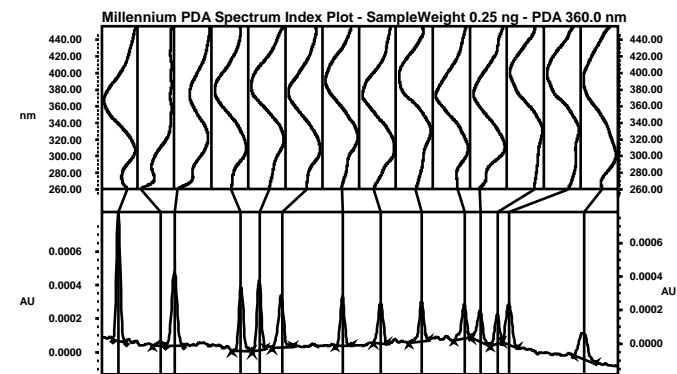
Detection in HPLC

Extraction of 3D Data

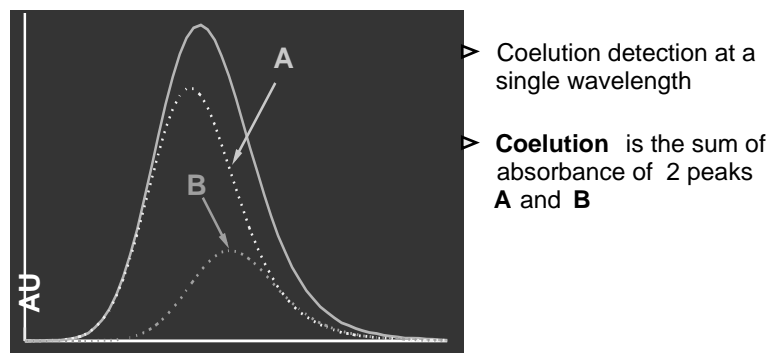


PDA Spectrum Index Plot

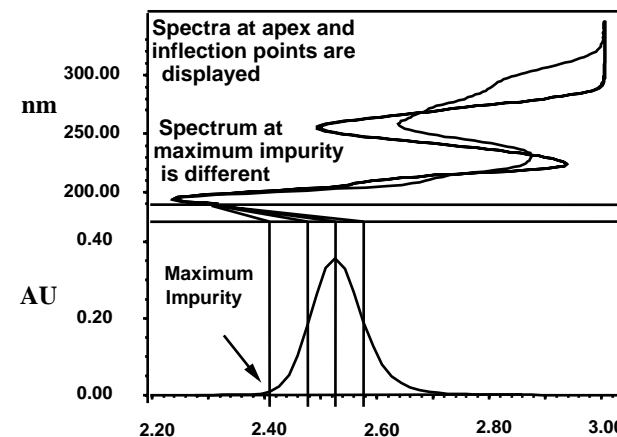
DNPH Derivatives 0.25 ng Each Peak



Coelution of 2 Peaks

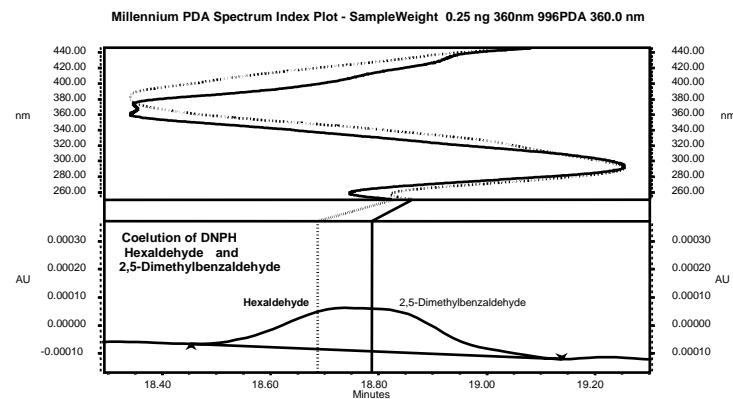


Peak Purity Measurement



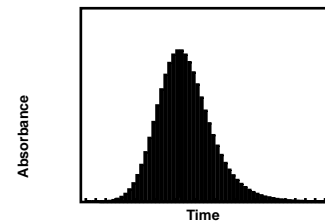
Detection in HPLC

Maximum Impurity Detection



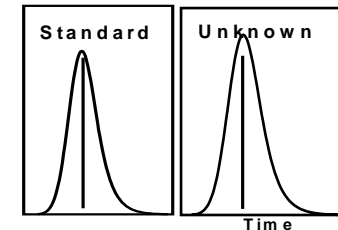
Determination of Peak Purity

Peak Purity



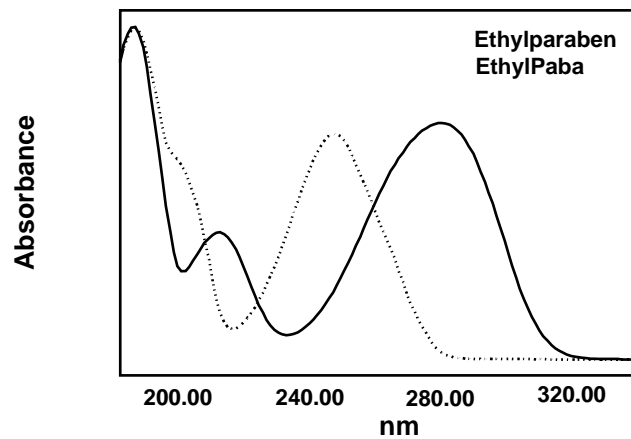
Peak Purity analyzes all spectra (minimum 15) within a peak against the apex spectrum of the peak itself.

Spectral Matching

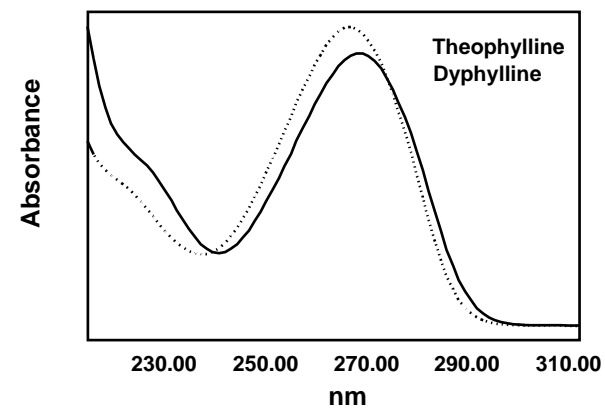


Spectral match of apex spectrum of the unknown against the apex spectrum of a standard, stored in a user's library.

Different Spectra – 53 deg



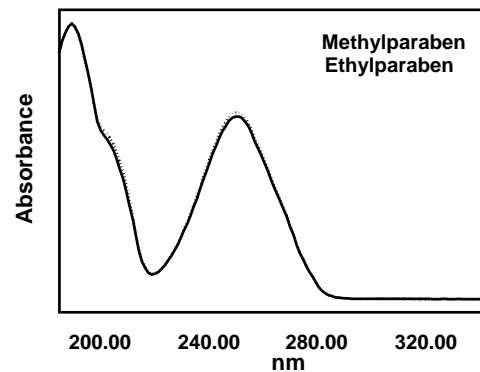
10 deg of Spectral Contrast



Similar spectra for structurally related compounds

Detection in HPLC

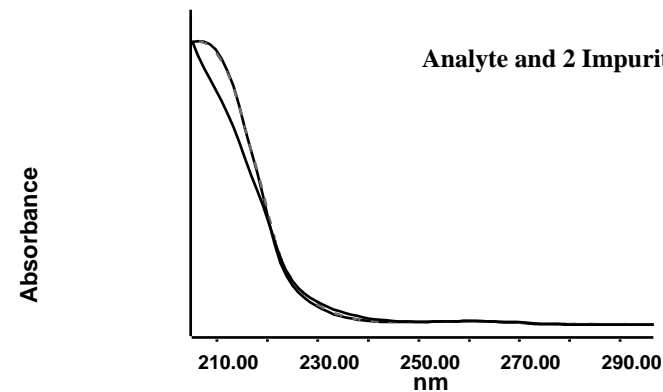
Spectral Contrast 0.5 Degrees



Very similar spectra,
CH₂ difference

Spectral Contrast can
differentiate these
spectra

Spectra of non-UV Active Compounds



Analyte and 2 Impurities

Detectors

UV/VIS

Refractive index

Fluorescence

Electrochemical

Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

Refractive Index Detector

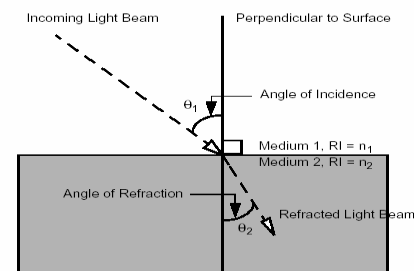


Figure 1-3 Refraction of Light

The relationship between the refractive indices of the two media and the angles of incidence and refraction is described by Snell's Law:

$$n_1(\sin \theta_1) = n_2(\sin \theta_2)$$

where:

θ₁ = Angle of incidence

θ₂ = Angle of refraction

n₁ = RI of medium 1

n₂ = RI of medium 2

Detection in HPLC

Refractive Index Detector

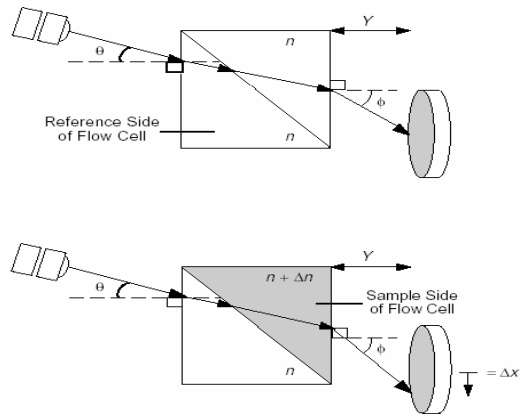
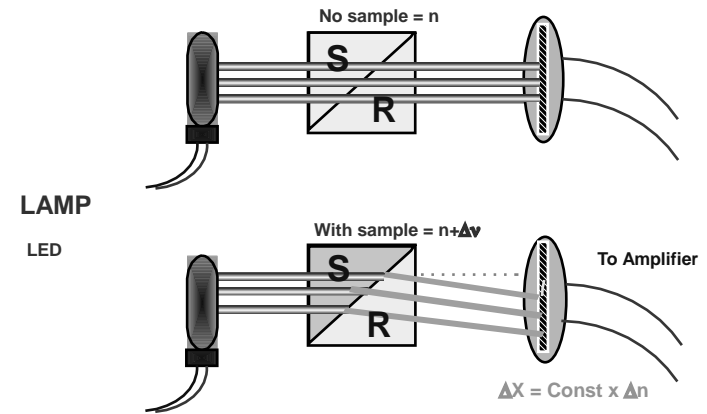
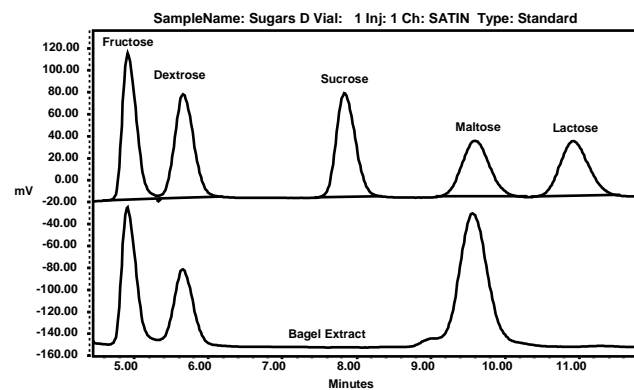


Figure 1-5 How Refraction Changes the External Angle of Deflection

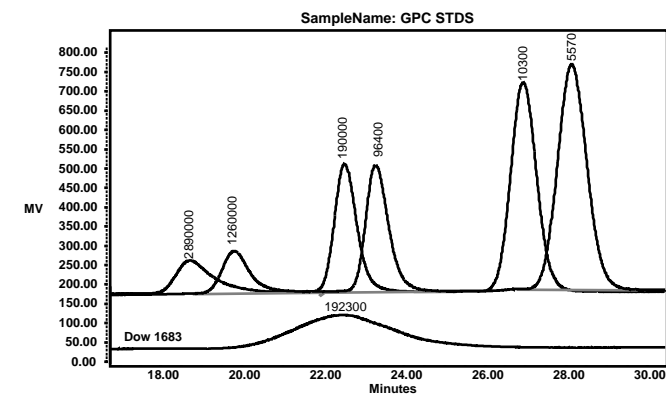
Differential Refractive Index Detector



Sugar Analysis

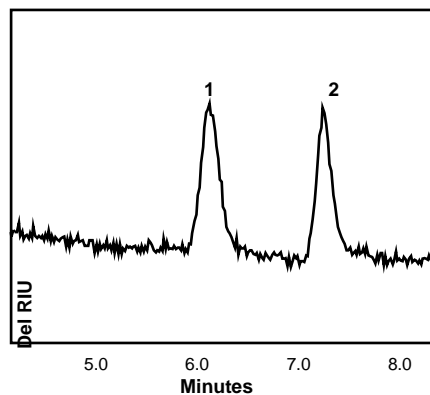


Polymer Analysis



Detection in HPLC

Lipids



► 250 ng on column
1=Tristearin
2=Myristic acid

► Styragel HR 0.5,
4.6 x 300 mm,
35°C, 0.35 mL/min

► dRI sensitivity =
32X, 32°C

Detectors

UV/VIS

Refractive index

Fluorescence

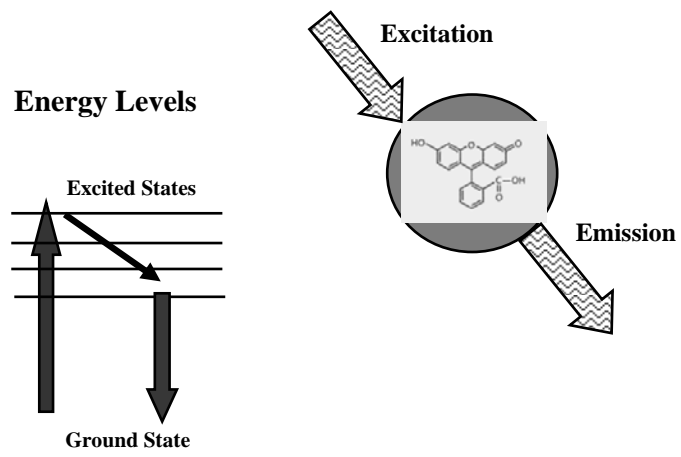
Electrochemical

Conductivity

Mass-spectrometric (LC/MS)

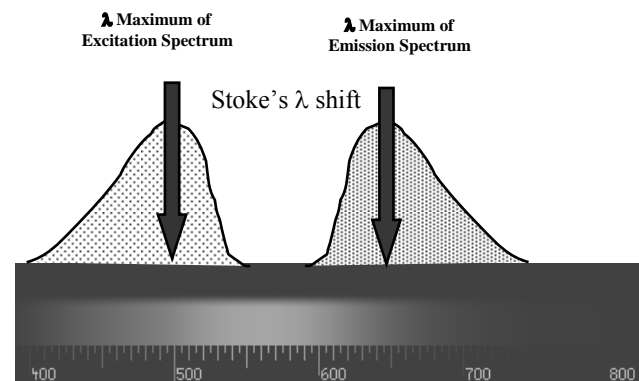
Evaporative light scattering

Fluorescence Process



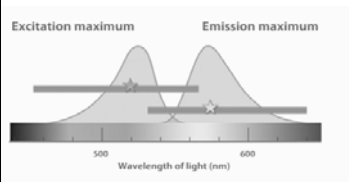
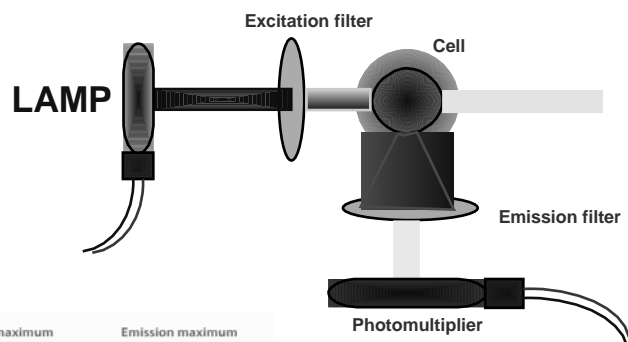
Excitation-Emission Spectra

Lifetime= $10^{-9} - 10^{-15}$ sec

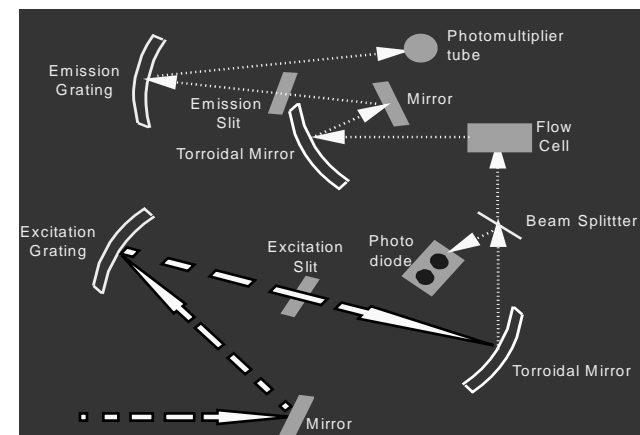


Detection in HPLC

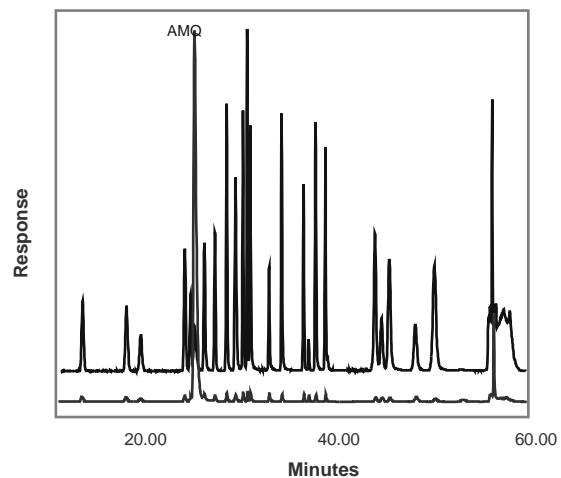
Fluorescence Detectors



Fluorescence Detector Optical Bench



UV vs Fluorescence Sensitivity

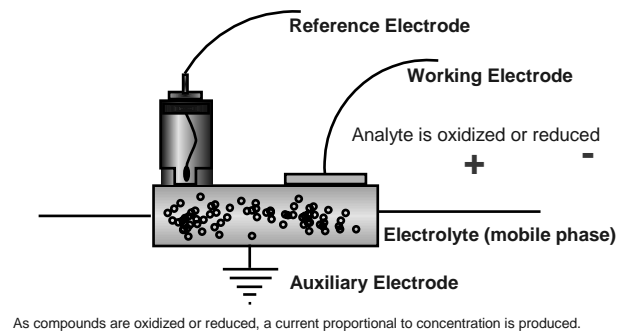


Detectors

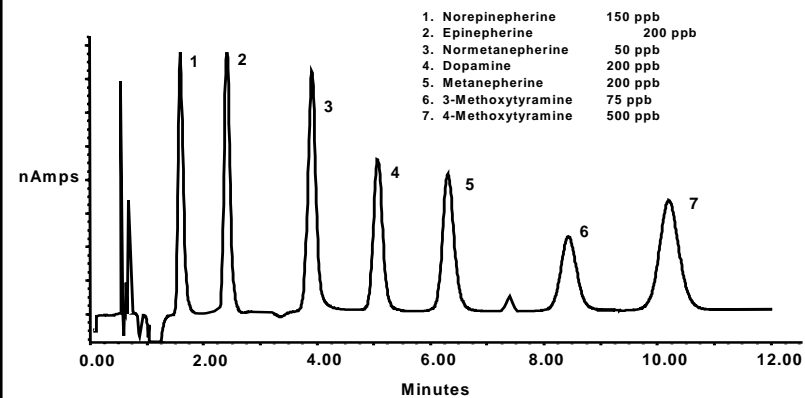
UV/VIS
 Refractive index
 Fluorescence
 Electrochemical
 Conductivity
 Mass-spectrometric (LC/MS)
 Evaporative light scattering

Detection in HPLC

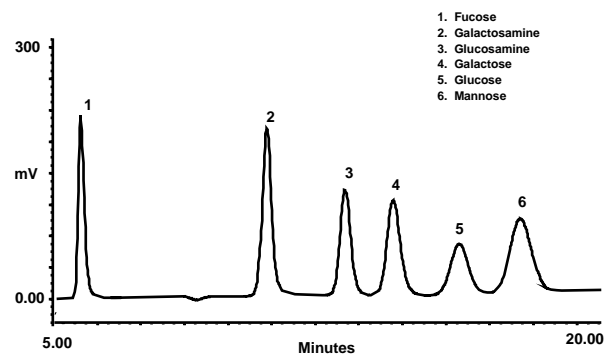
Electrochemical Detector



Electrochemical Detection of Catecholamines & Related Compounds



Pulsed Amperometric Detection of Monosaccharides



Detectors

UV/VIS

Refractive index

Fluorescence

Electrochemical

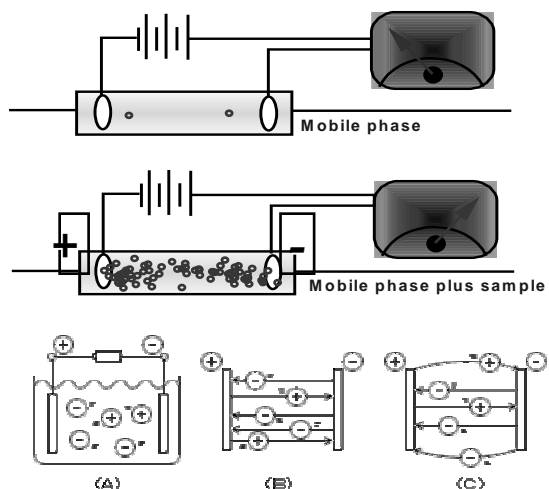
Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

Detection in HPLC

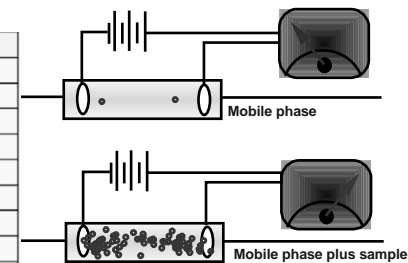
Conductivity Detector



Conductivity Detector

Limiting Equivalent Conductance of Ions in Water at 25 °C

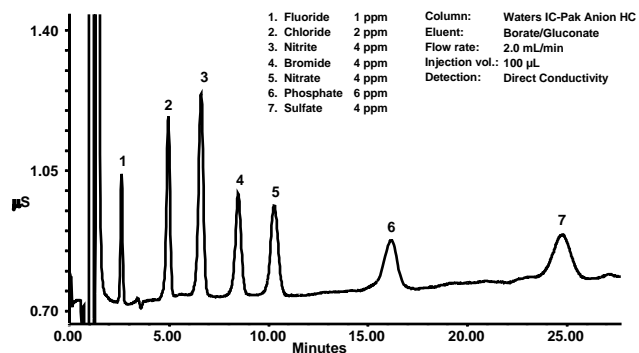
Cations	λ_{+}	Anions	λ_{-}
H ⁺	349.8	OH ⁻	198.6
Li ⁺	38.6	F ⁻	55.4
Na ⁺	50.1	Cl ⁻	76.4
K ⁺	73.5	Br ⁻	78.1
Rb ⁺	77.8	I ⁻	76.8
Ag ⁺	61.9	NO ₃ ⁻	71.5
NH ₄ ⁺	73.3	ClO ₄ ⁻	64.6
(CH ₃) ₄ NH ⁺	51.8	ClO ₃ ⁻	67.4
Hg ₂ ²⁺	53.0	IO ₃ ⁻	54.5
Mg ²⁺	53.1	Formate	54.6
Ca ²⁺	59.5	Acetate	40.9
Ba ²⁺	63.6	Benzoate	32.4
Cu ²⁺	53.6	SO ₄ ²⁻	80.0
Zn ²⁺	52.8	CO ₃ ²⁻	69.3
La ³⁺	69.7	Fe(CN) ₆ ⁴⁻	111.0
Ce ³⁺	69.8		



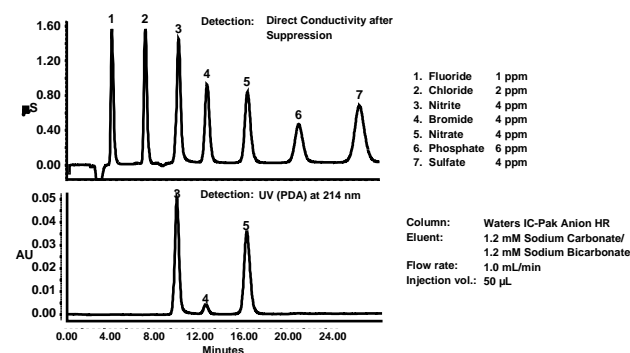
$$G = \frac{\lambda C}{10^{-3}}$$

G = measured conductance of the solution, in Siemens (1 S = ohm⁻¹)
 C = concentration in equivalents per 1000 cm³
 K = length/area of cell (the cell constant)
 λ = equivalent conductance in S cm² equiv⁻¹

Anion Analysis by IC



Anion analysis by IC



Detection in HPLC

Applications

- Sensitivities for compounds such as phenol, catecholamines, nitrosamines, and organic acids are in the picomole (nanogram) range.

The mobile phase must be made electrically conductive, usually by the addition of a suitable salt:

Ion Exchange

Reversed Phase and Ion-Pair RP

No normal phase separations

Detectors

UV/VIS

Refractive index

Fluorescence

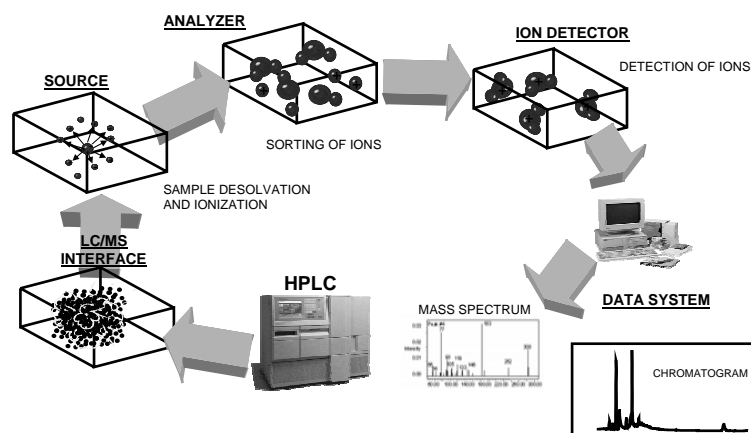
Electrochemical

Conductivity

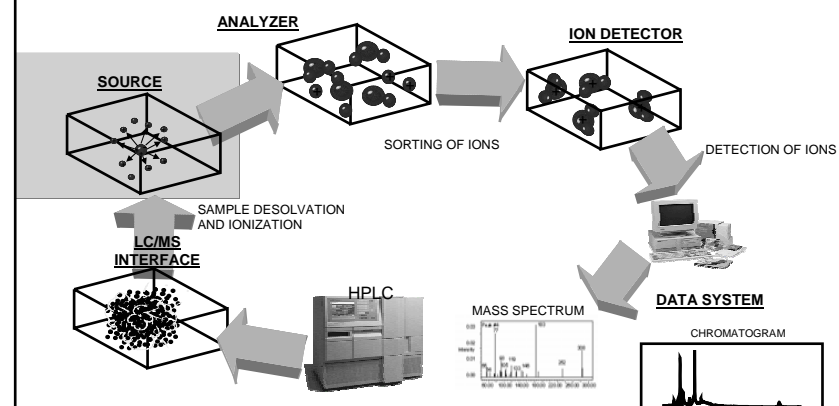
Mass-spectrometric (LC/MS)

Evaporative light scattering

Typical LC/MS System Progression



Typical LC/MS System Progression

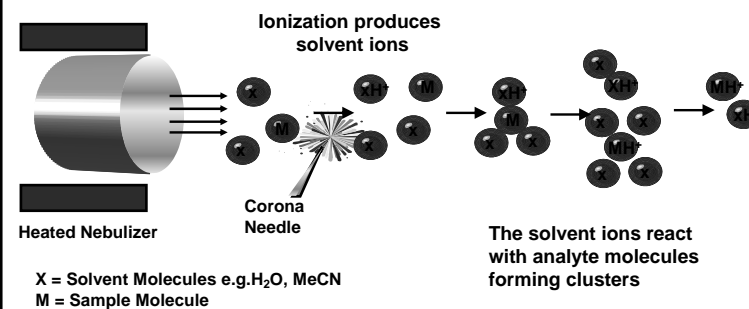


Detection in HPLC

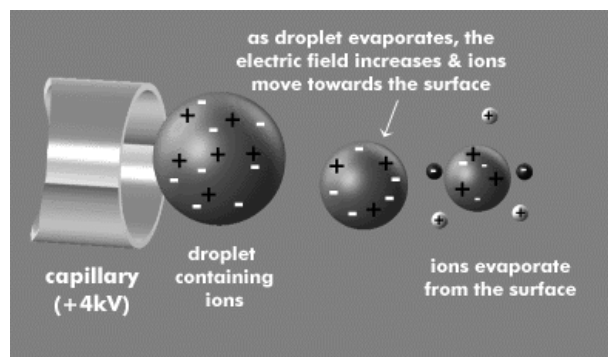
Transition from LC to MS

- State of Matter: **Liquid to Gas**
- Charge State: “**Neutral**” to **Ion**
- Pressure: **760 torr** to **10^{-5} to 10^{-8} torr**

APCI Mechanism



Electrospray Ionization



Positive or Negative?

Basic Compounds ($-NH_2$) **$(M+H)^+$**

Acidic Compounds ($-CO_2H$, $-OH$) **$(M-H)^-$**

Detection in HPLC

Recognizing Multiply Charged Ions

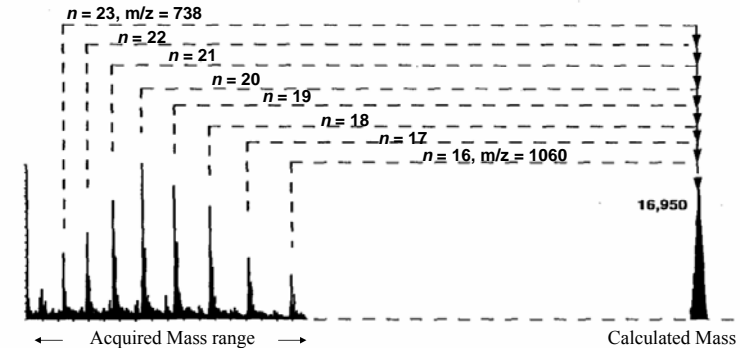
Mass spectrometers operate on the basis of mass-to-charge ratio (m/z). Mass assignments are normally made assuming a single charge per ion (i.e. $m/z = m$)

Single charge	Mass = (M+H)
Double charge	Mass = $1/2 (M+2H)$
n charge	Mass = $1/n (M+nH)$

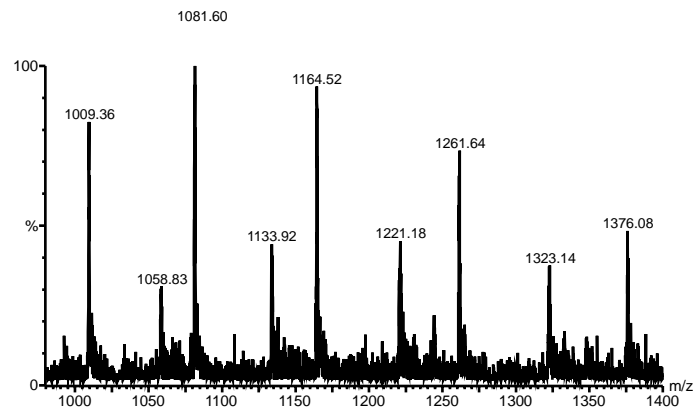
Isotopes of doubly charged ions are separated by 0.5 Da _____

Mass Range Multiply Charged Molecules

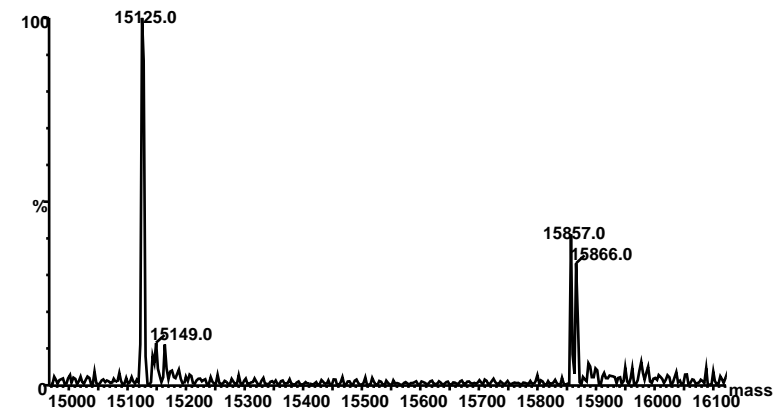
Horse Heart Myoglobin



Hemoglobin Spectrum Presence of More Than One Charged Envelope

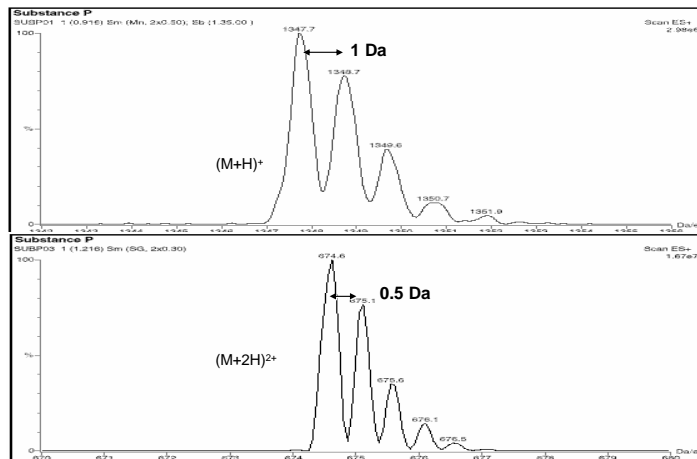


Deconvolution by MaxEnt Hemoglobin

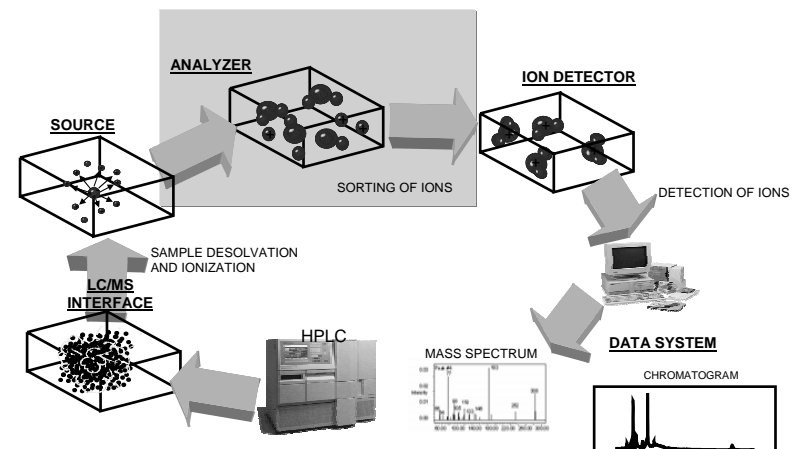


Detection in HPLC

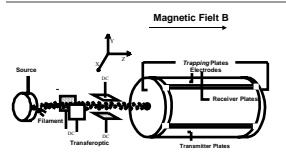
Multiply Charged Ions – How Many Charges?



Typical LC/MS System Progression

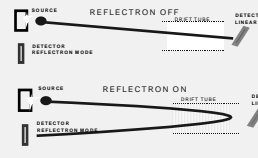


FT-ICR-Spectrometer

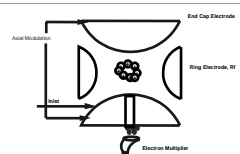


Mass Spectrometer's Analyzers

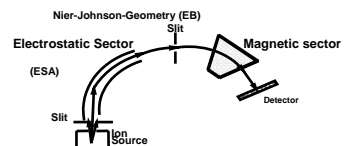
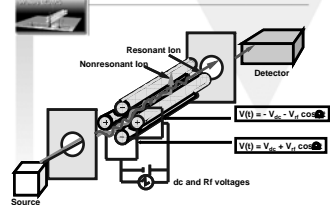
Time Of Flight Mass Analyzers



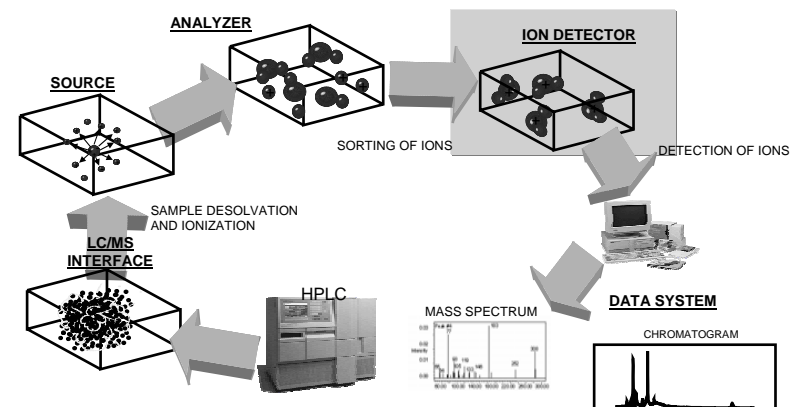
Ion Traps



Starting with the quadrupole

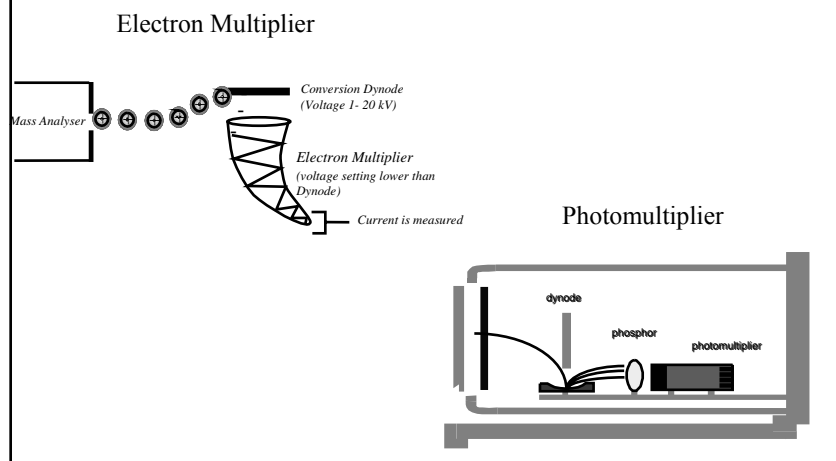


Typical LC/MS System Progression

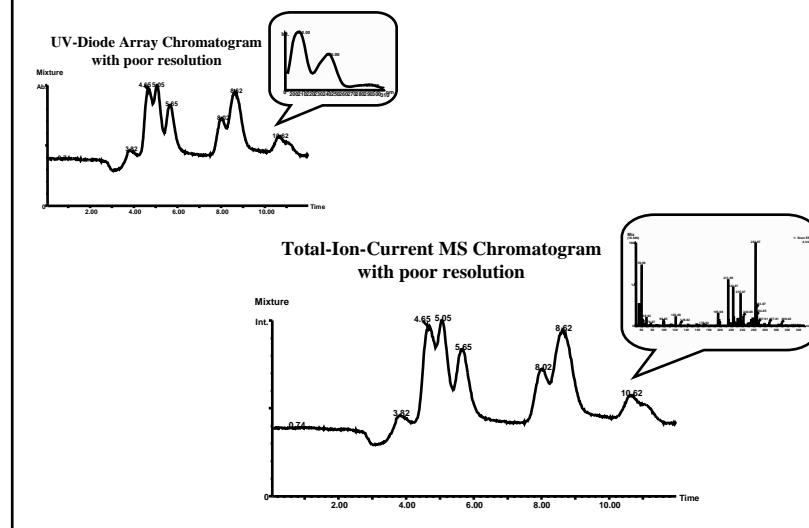


Detection in HPLC

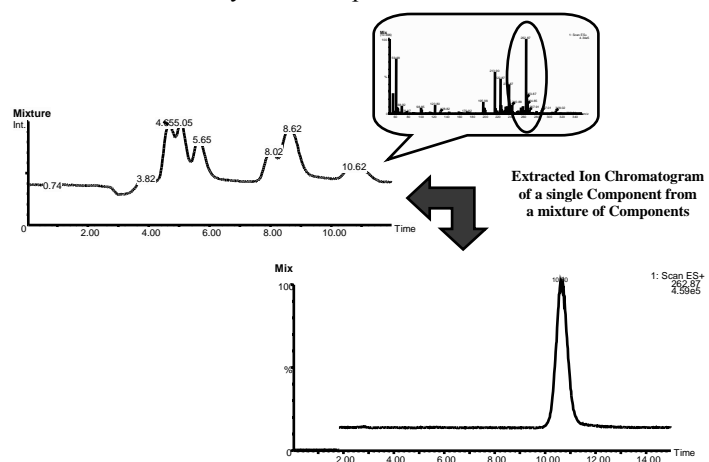
MS Detectors



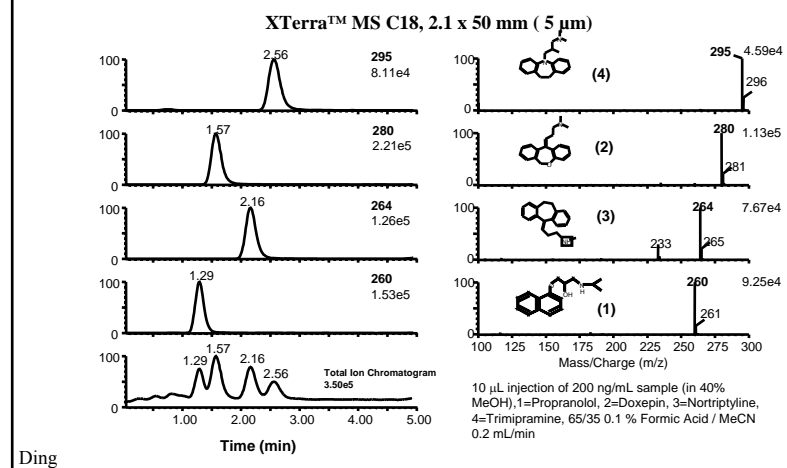
Mass Spectrometer 3D Run



Selectivity of Mass Spectrometer Detector



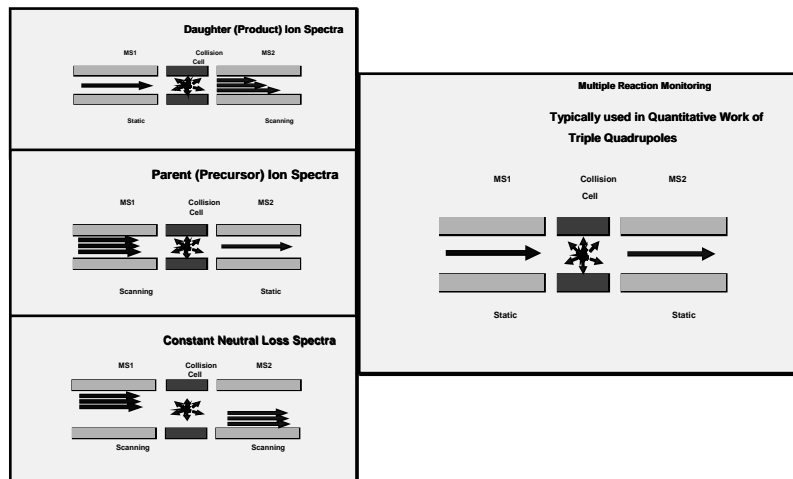
LC-MS Analysis



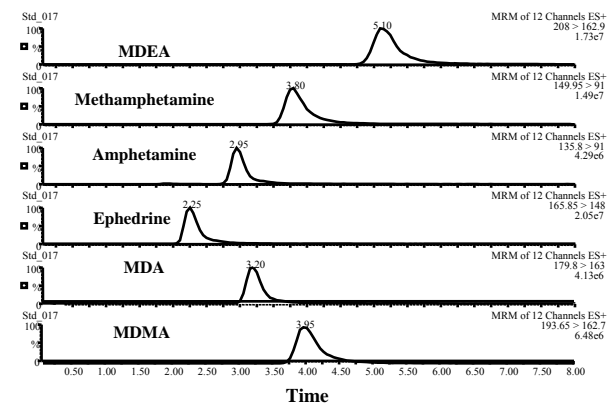
Ding

Detection in HPLC

Triple Quadrupoles: MS-MS Modes



Typical Quantitative Analysis Using Triple Quadrupoles: Simultaneous MRM analysis of 6 amphetamines

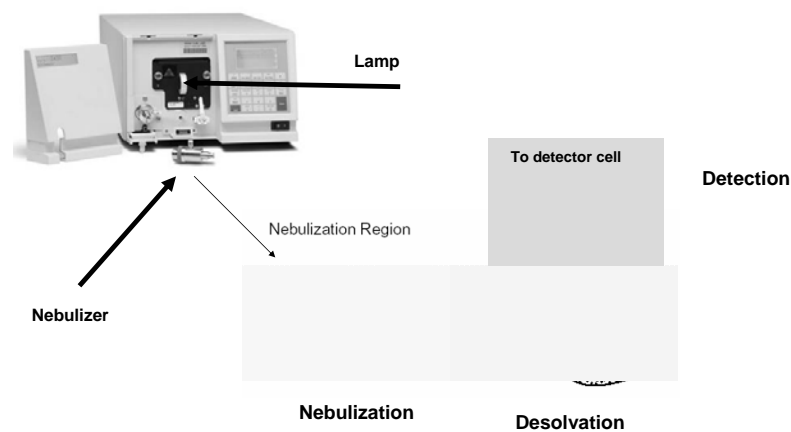


Highly specific and sensitive chromatograms

Detectors

- UV/VIS
- Refractive index
- Fluorescence
- Electrochemical
- Conductivity
- Mass-spectrometric (LC/MS)
- Evaporative light scattering

Evaporative Light Scattering - ELS



Detection in HPLC

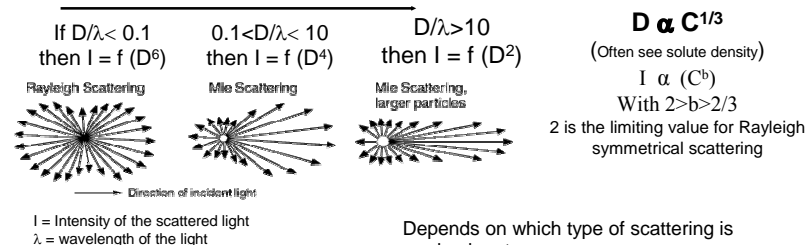
Rayleigh Scattering – Why the Sky is blue

$$I = I_0 \frac{8 \pi^4 N \alpha^2 (1 + \cos^2 \theta)}{\lambda^4 R^2}$$

- Scattering is independent of the particle's chemical properties, where:
 - N = # of particles
 - α = Polarizability i.e. the sum of the dipoles of all the molecules in the particle. For a homogeneous particle this is proportional to the particle volume.
 - R = Distance of observer from scatterer
 - Dependence on wavelength of incident light, shorter wavelengths produce greater scattering

Scattering Models

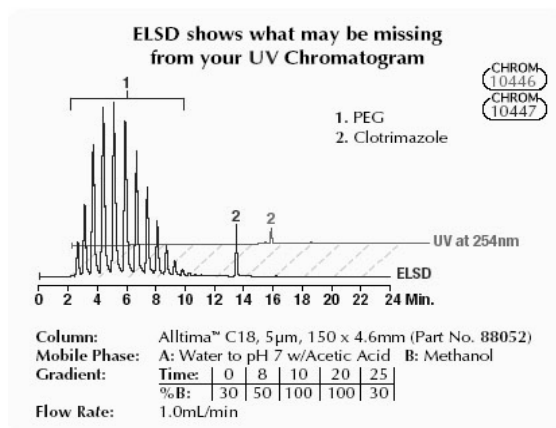
Scattering is dependent on particle size "D" Increasing particle size



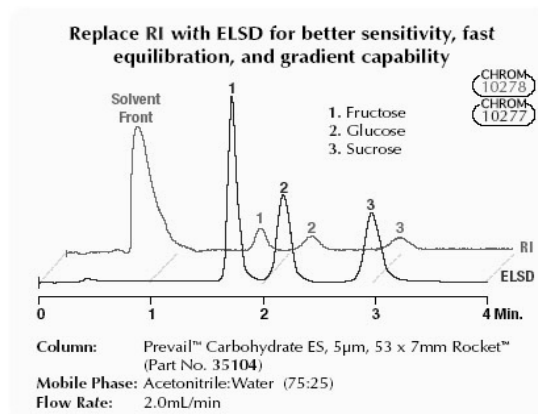
> Non-linear mass detector

♦ use chromatography data software
 quadratic curve or log/log curve to fit
 calibration curve

ELSD vs UV

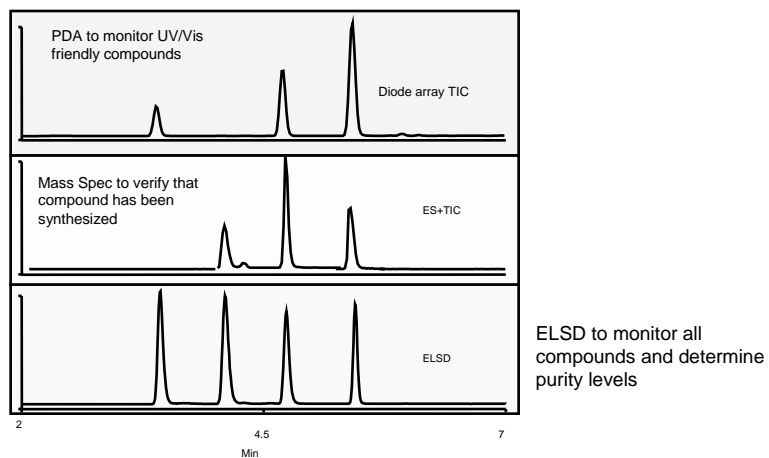


ELSD vs RI

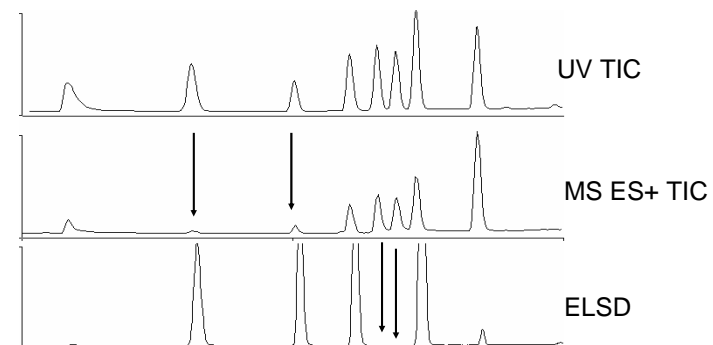


Detection in HPLC

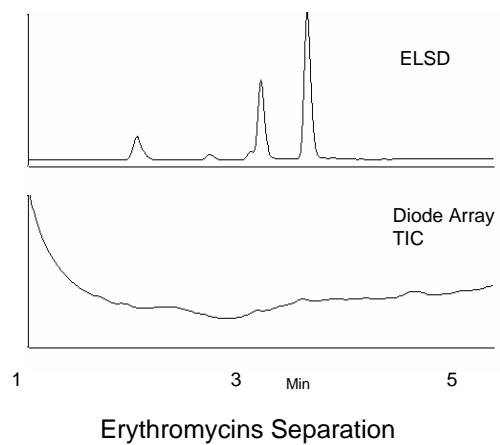
ELSD Used with Other Detectors



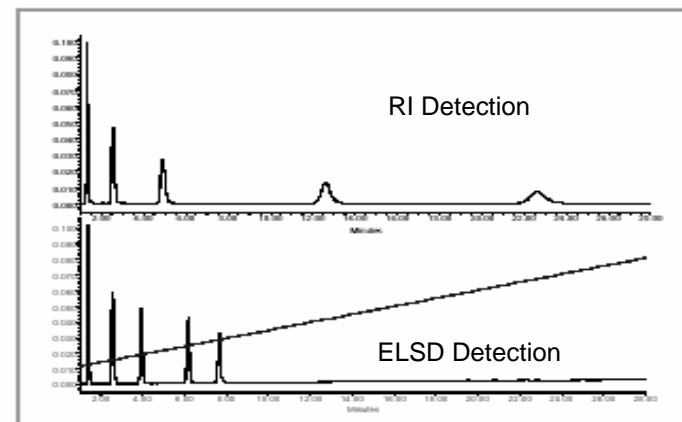
Not a Universal Detector *Typically Used with Other Detectors*



See Non-UV Absorbing Compounds



See Your Peaks Faster *Use of Gradients Versus Isocratic*



Detection in HPLC

