

# Detection in HPLC

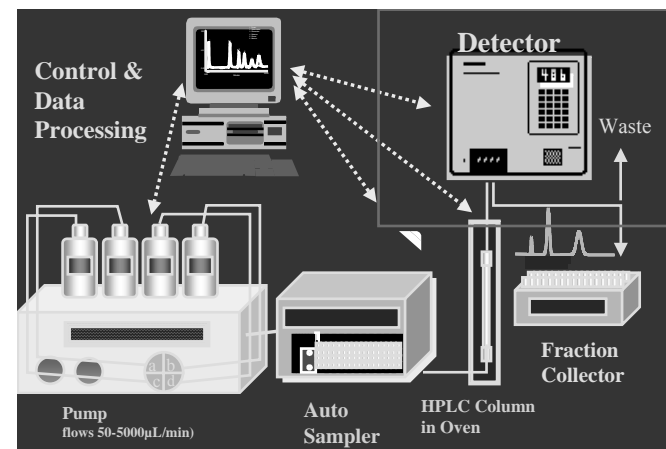


## Selecting the Right Detector: Types of Detectors in HPLC

- 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

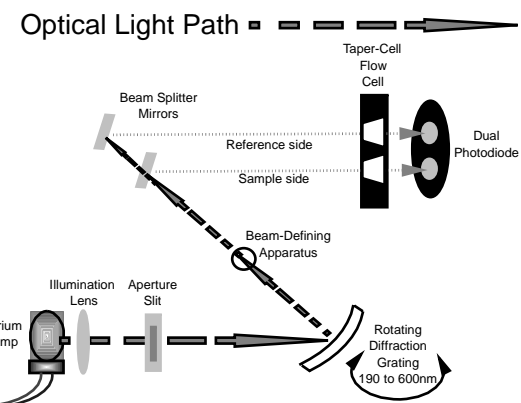
Electrochemical

Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

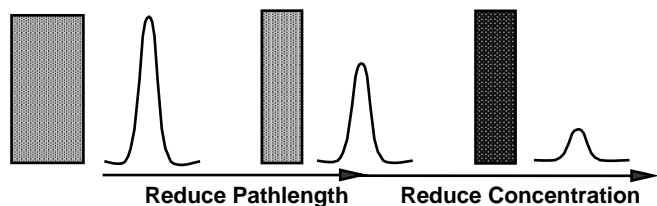
## Optical Bench of UV-VIS Detector



# Detection in HPLC

## Beer's Law

Absorbance = Extinction Coefficient x Pathlength x Concentration



## UV Chromophores

Chromophore	Chemical Configuration	$\lambda_{\text{max}}$ (nm)	$\epsilon_{\text{max}}$ (L/m/cm)	$\lambda_{\text{max}}$ (nm)	$\epsilon_{\text{max}}$ (L/m/cm)
Ether	—O—	185	1000		
Thioether	—S—	194	4600	215	1600
Amine	—NH <sub>2</sub>	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 <sub>2</sub> —	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 <sub>2</sub>	210	strong		
Nitrile	—ONO	220-230	1000-2000	300-400	10

## UV Chromophores

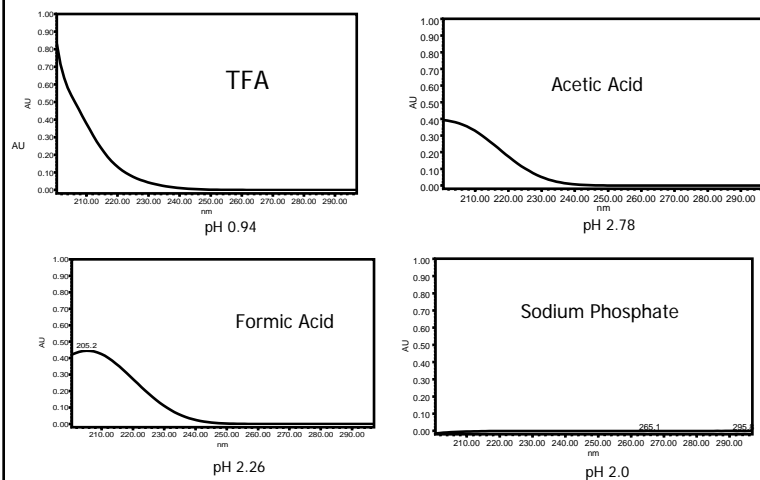
Chromophore	Chemical Configuration	$\lambda_{\text{max}}$ (nm)	$\epsilon_{\text{max}}$ (L/m/cm)	$\lambda_{\text{max}}$ (nm)	$\epsilon_{\text{max}}$ (L/m/cm)
Azo	—N=N—	285-400	3-25		
Nitroso	—N=O	302	100		
Nitrate	—ONO <sub>2</sub>	270 (shoulder)	12		
Allene	—(C=C) <sub>2</sub> — (acyclic)	210-230	21,000		
Allene	—(C=C) <sub>3</sub> —	260	35,000		
Allene	—(C=C) <sub>4</sub> —	300	52,000		
Allene	—(C=C) <sub>5</sub> —	330	118,000		
Allene	—(C=C) <sub>2</sub> — (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 <sub>2</sub>	229	9,500		

## UV-Vis chromophores

	$\lambda_{\text{max}}$	$\epsilon_{\text{m}} \times 10^{-3} @ \lambda_{\text{max}}$
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



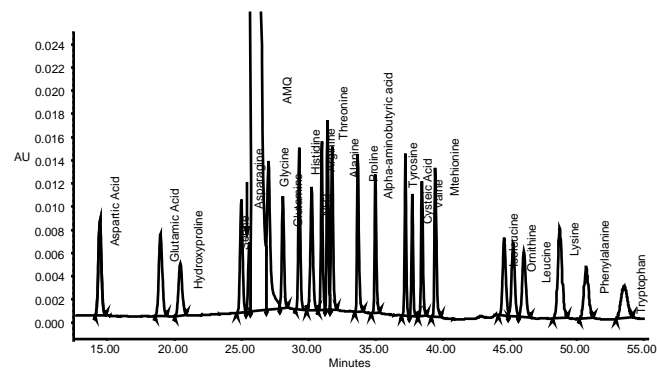
## U.V. Cut-offs for some Common Solvents

*Remember Solvents chosen can affect detection!!*

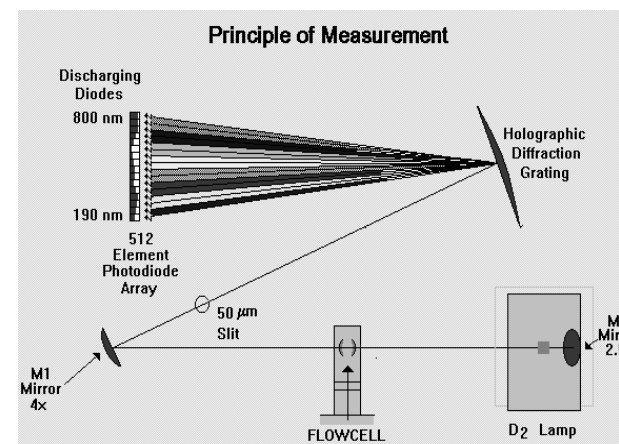
<u>Solvent</u>	<u>UV Cutoff</u>	<u>Solvent</u>	<u>UV Cutoff</u>
Water	180	N-Heptane	197
Methanol	205	Cyclohexane	200
N-Propanol	205	Carbon tetrachloride	265
Acetonitrile	190	Chloroform	245
THF	225	Benzene	280
Acetone	330	Toluene	285
Methyl acetate	260	Methylene chloride	232
Ethyl Acetate	260	Tetrachloroethylene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

*All wavelengths reported in nm.*

UV Detection of AccQ-Tag Amino Acid Derivatives

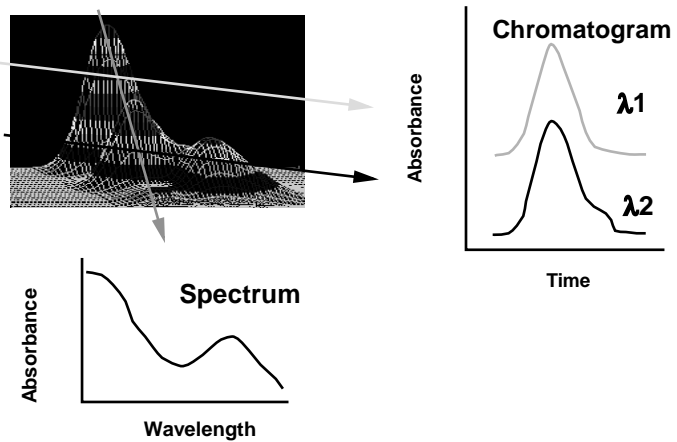


## Diode Array Detector



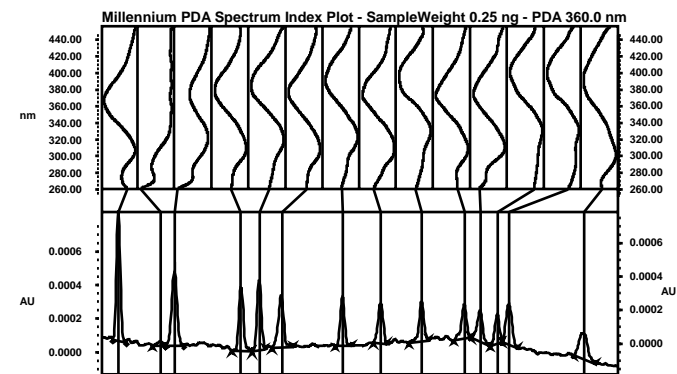
# Detection in HPLC

## Extraction of 3D Data

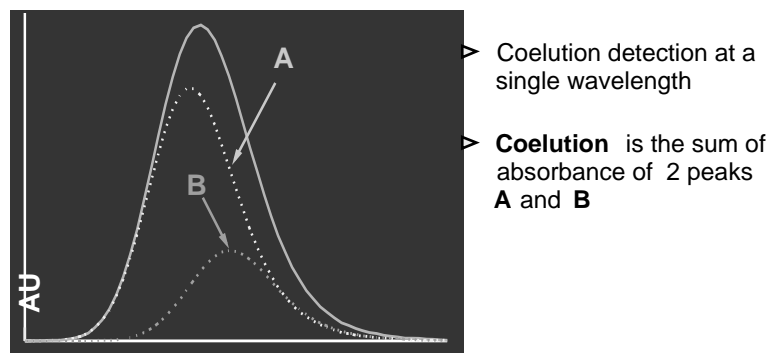


## PDA Spectrum Index Plot

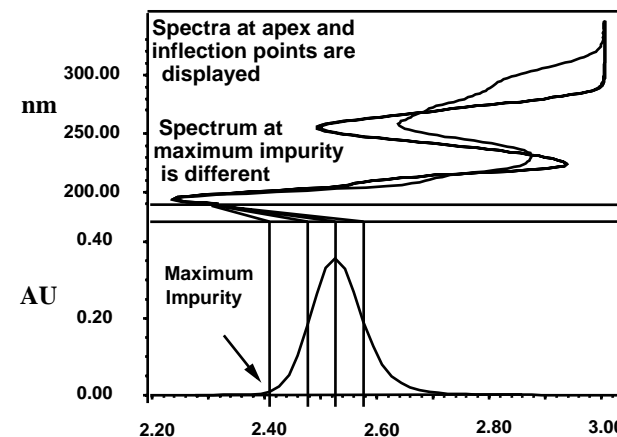
DNPH Derivatives 0.25 ng Each Peak



## Coelution of 2 Peaks

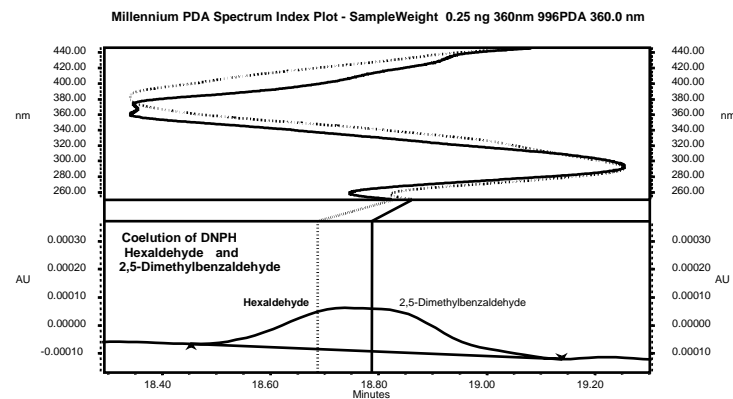


## Peak Purity Measurement



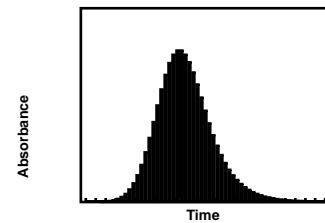
# Detection in HPLC

## Maximum Impurity Detection



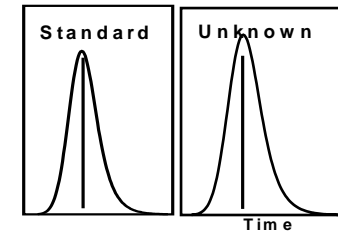
## Determination of Peak Purity

### Peak Purity



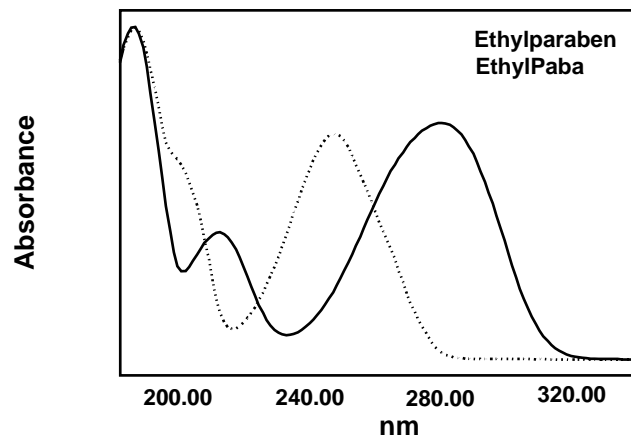
Peak Purity analyzes all spectra (minimum 15) within a peak against the apex spectrum of the peak itself.

### Spectral Matching

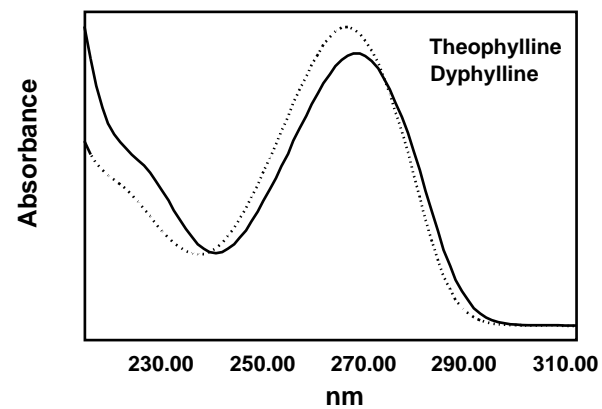


Spectral match of apex spectrum of the unknown against the apex spectrum of a standard, stored in a user's library.

## Different Spectra – 53 deg



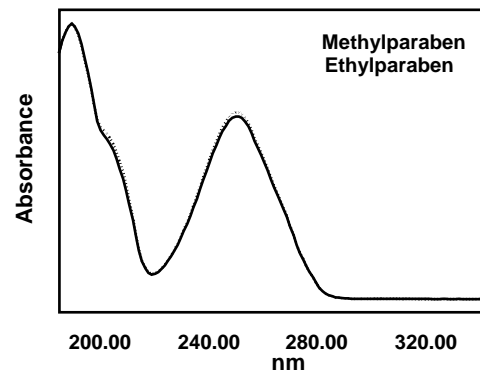
## 10 deg of Spectral Contrast



Similar spectra for structurally related compounds

# Detection in HPLC

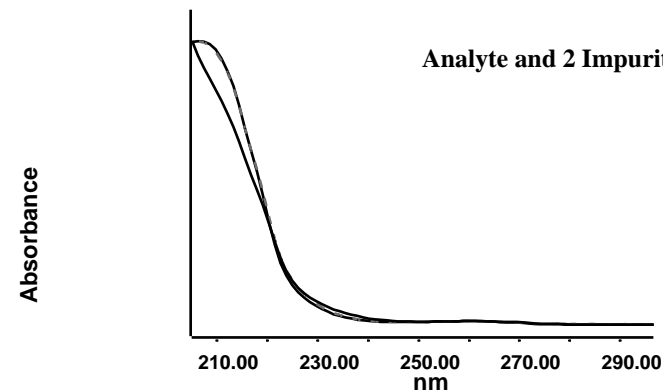
## Spectral Contrast 0.5 Degrees



Very similar spectra,  
CH<sub>2</sub> 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

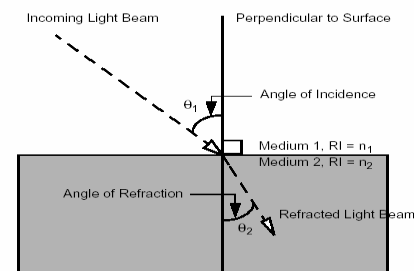


Figure 1-3 Refraction of Light

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:

θ<sub>1</sub> = Angle of incidence

θ<sub>2</sub> = Angle of refraction

n<sub>1</sub> = RI of medium 1

n<sub>2</sub> = RI of medium 2

# Detection in HPLC

## Refractive Index Detector

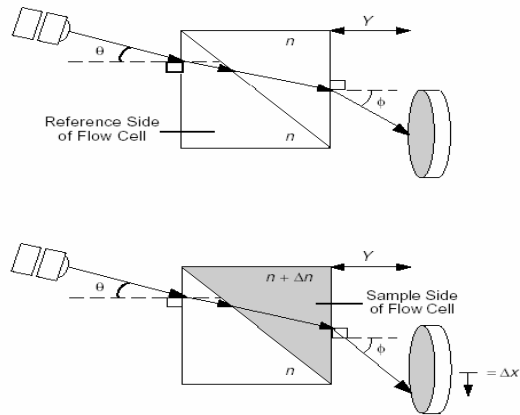
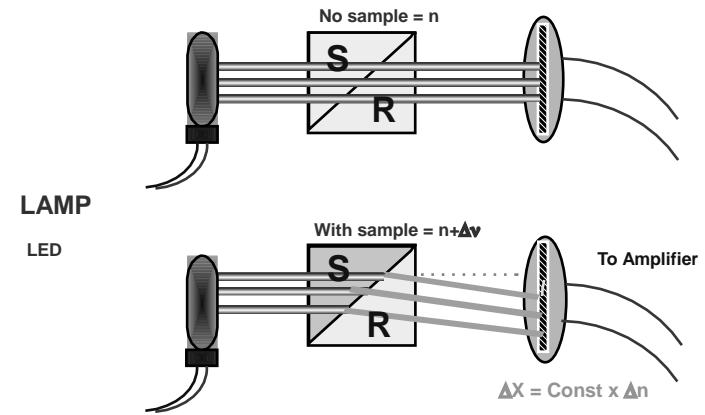
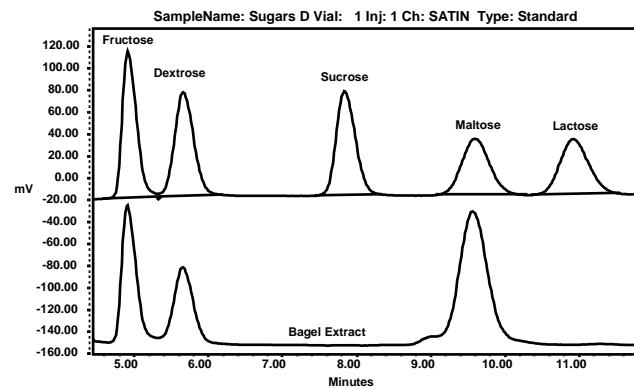


Figure 1-5 How Refraction Changes the External Angle of Deflection

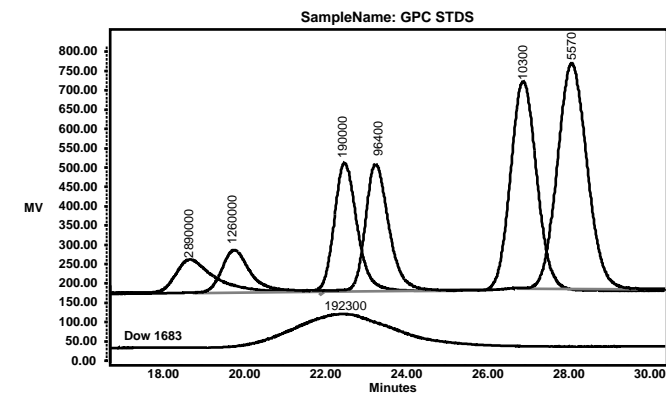
## Differential Refractive Index Detector



## Sugar Analysis

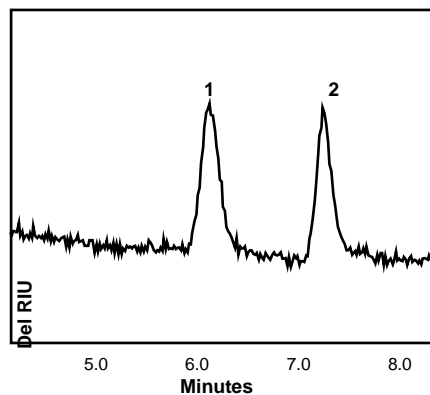


## Polymer Analysis



# 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

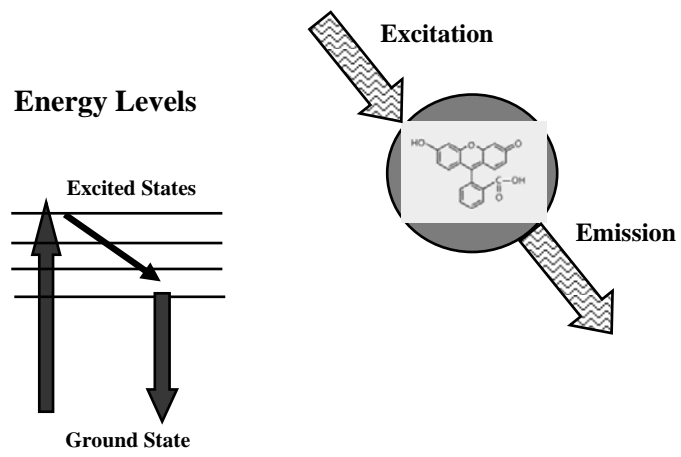
Electrochemical

Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

## Fluorescence Process



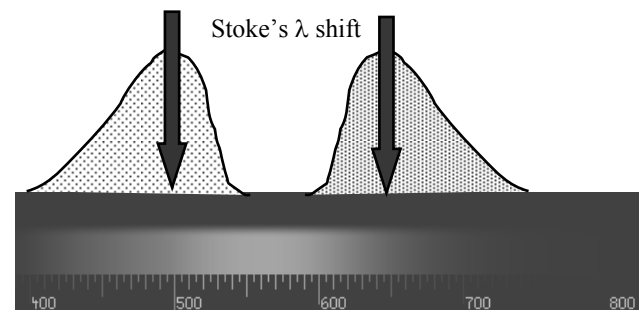
## Excitation-Emission Spectra

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

λ Maximum of  
Excitation Spectrum

λ Maximum of  
Emission Spectrum

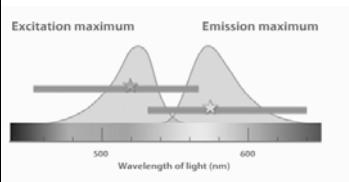
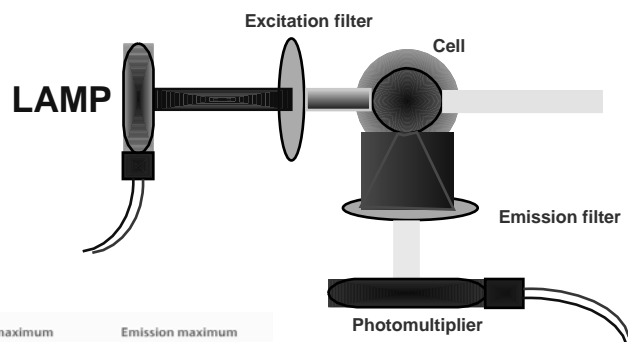
Stoke's λ shift



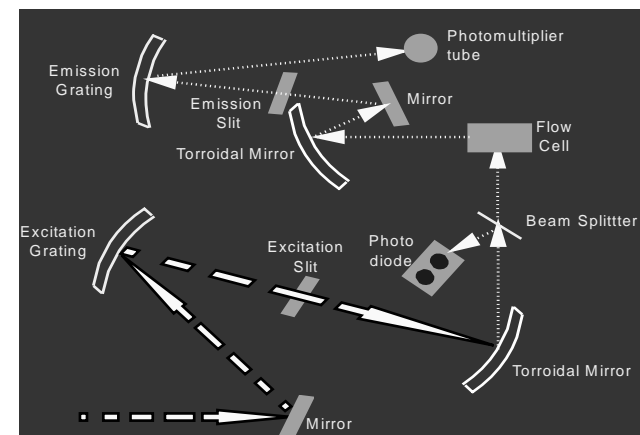


# Detection in HPLC

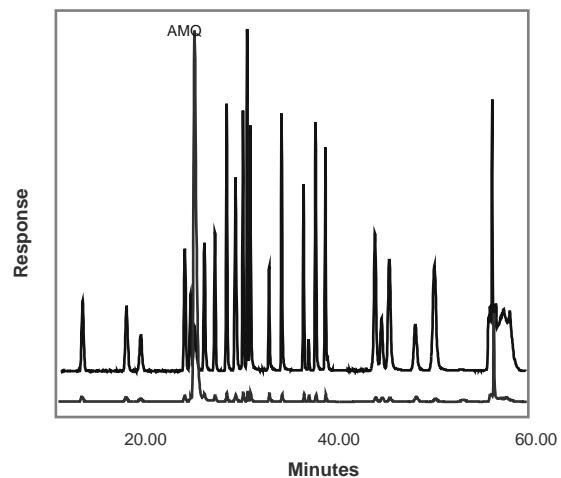
## Fluorescence Detectors



## Fluorescence Detector Optical Bench



## UV vs Fluorescence Sensitivity

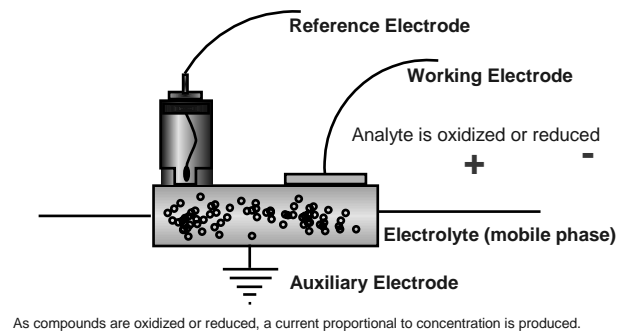


## Detectors

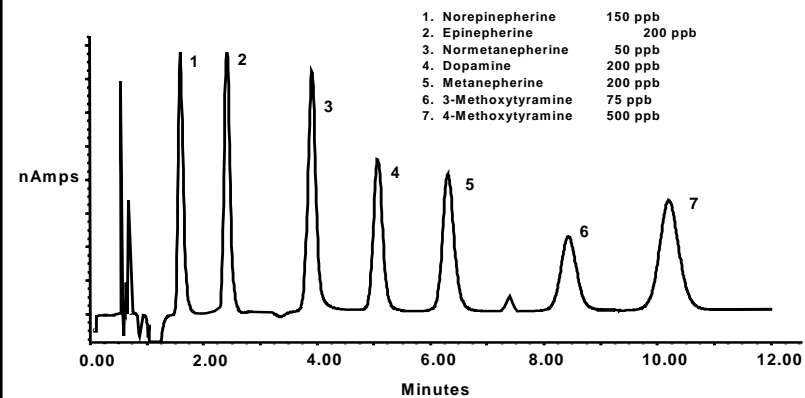
UV/VIS  
 Refractive index  
 Fluorescence  
 Electrochemical  
 Conductivity  
 Mass-spectrometric (LC/MS)  
 Evaporative light scattering

# Detection in HPLC

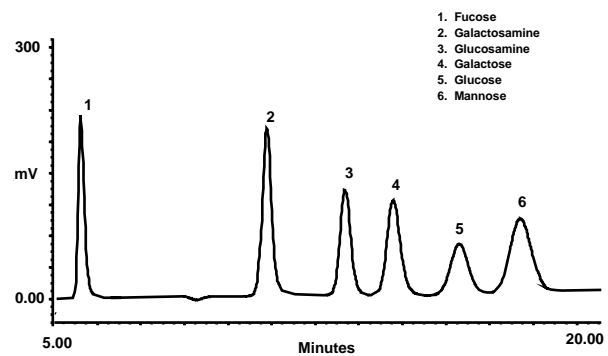
## Electrochemical Detector



## Electrochemical Detection of Catecholamines & Related Compounds



## Pulsed Amperometric Detection of Monosaccharides



## Detectors

UV/VIS

Refractive index

Fluorescence

Electrochemical

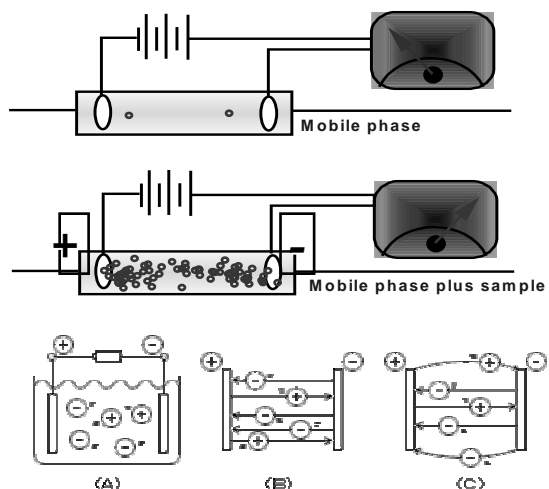
Conductivity

Mass-spectrometric (LC/MS)

Evaporative light scattering

# Detection in HPLC

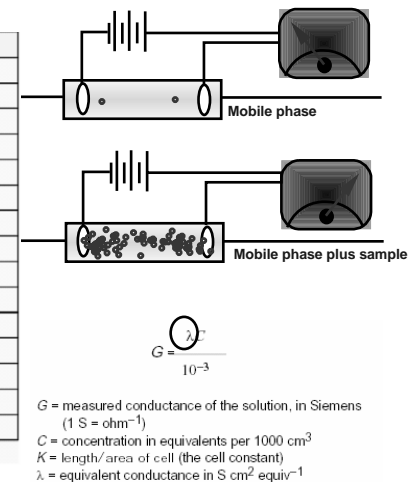
## Conductivity Detector



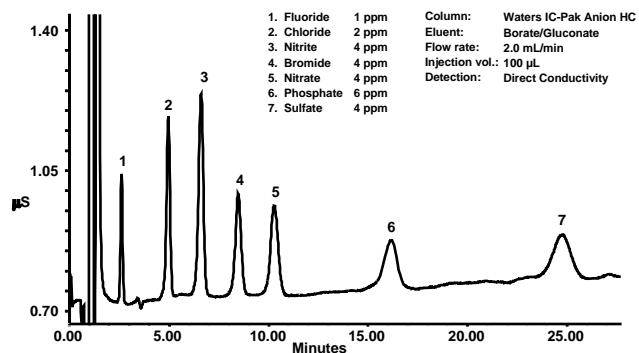
## Conductivity Detector

Limiting Equivalent Conductance of Ions in Water at 25 °C

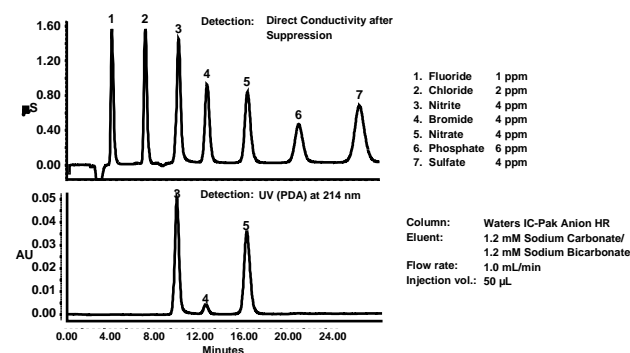
Cations	$\lambda_{+}$	Anions	$\lambda_{-}$
H <sup>+</sup>	349.8	OH <sup>-</sup>	198.6
Li <sup>+</sup>	38.6	F <sup>-</sup>	55.4
Na <sup>+</sup>	50.1	Cl <sup>-</sup>	76.4
K <sup>+</sup>	73.5	Br <sup>-</sup>	78.1
Rb <sup>+</sup>	77.8	I <sup>-</sup>	76.8
Ag <sup>+</sup>	61.9	NO <sub>3</sub> <sup>-</sup>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



## 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

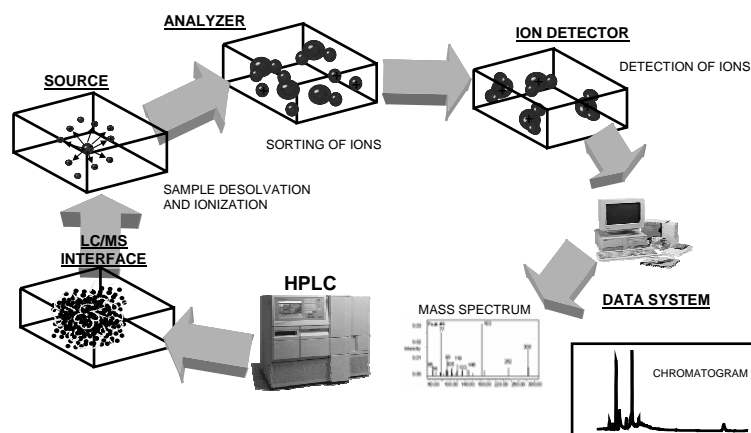
Electrochemical

Conductivity

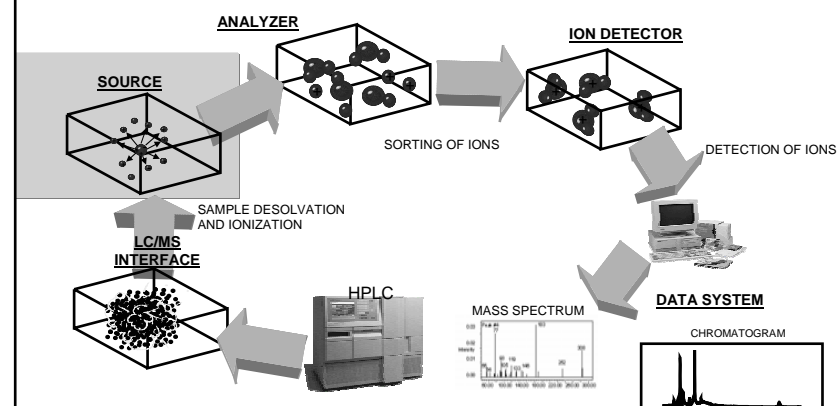
Mass-spectrometric (LC/MS)

Evaporative light scattering

## Typical LC/MS System Progression



## Typical LC/MS System Progression

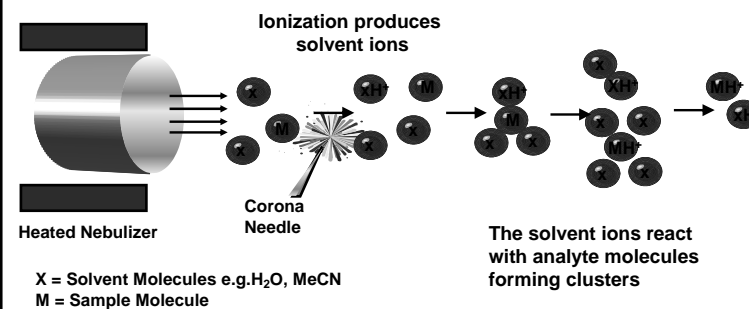


# Detection in HPLC

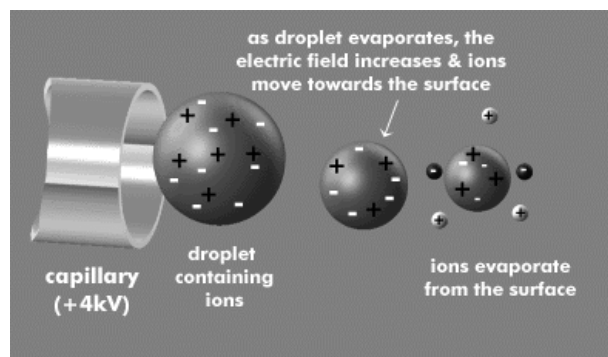
## Transition from LC to MS

- 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<sub>2</sub>)** **(M+H)<sup>+</sup>**

**Acidic Compounds (-CO<sub>2</sub>H, -OH)** **(M-H)<sup>-</sup>**

# Detection in HPLC

## Recognizing Multiply Charged Ions

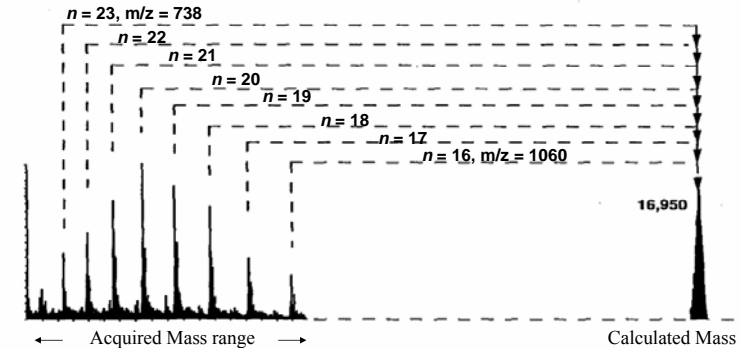
Mass spectrometers operate on the basis of mass-to-charge ratio ( $m/z$ ). Mass assignments are normally made assuming a single charge per ion (i.e.  $m/z = m$ )

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

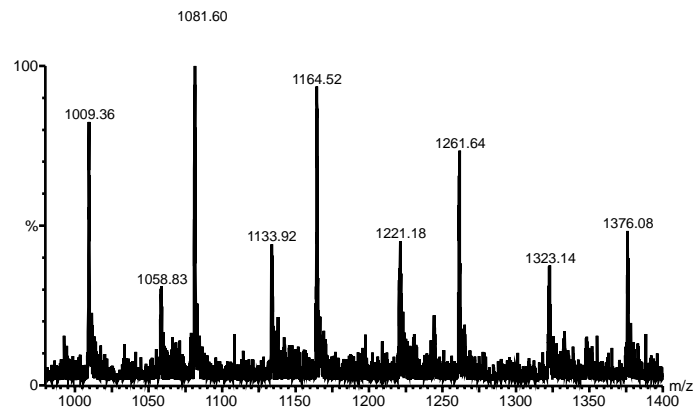
Isotopes of doubly charged ions are separated by 0.5 Da \_\_\_\_\_

## Mass Range Multiply Charged Molecules

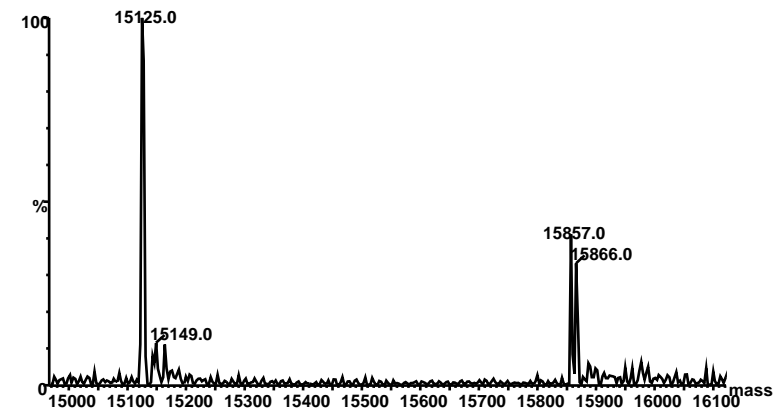
### Horse Heart Myoglobin



## Hemoglobin Spectrum Presence of More Than One Charged Envelope

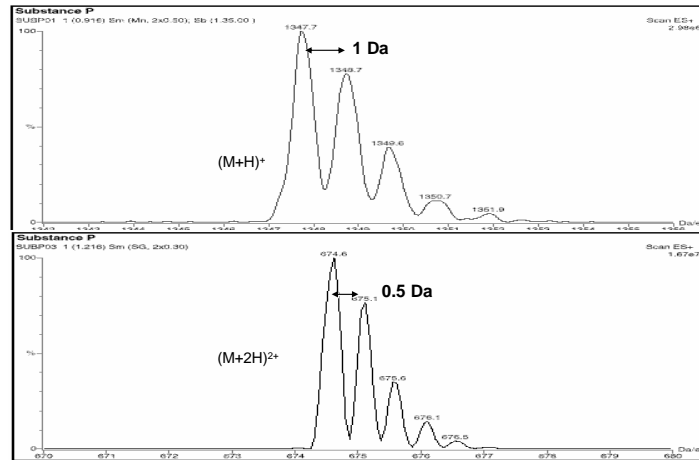


## Deconvolution by MaxEnt Hemoglobin

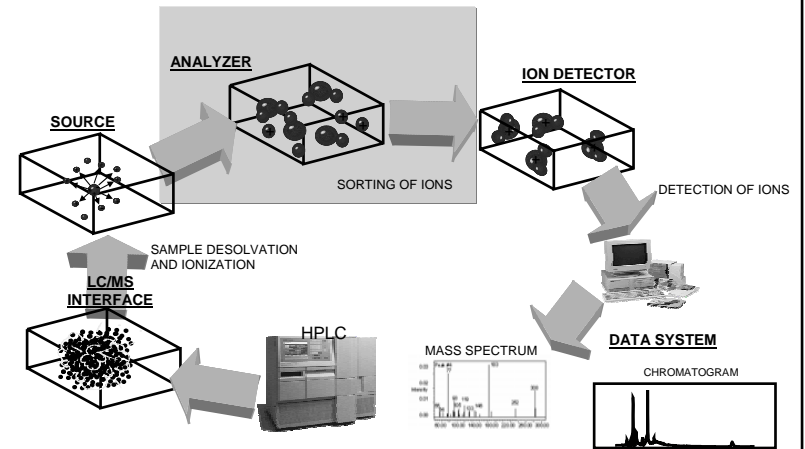


# Detection in HPLC

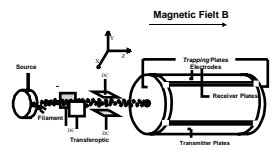
## Multiply Charged Ions – How Many Charges?



## Typical LC/MS System Progression

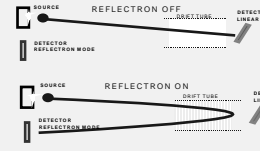


## FT-ICR-Spectrometer

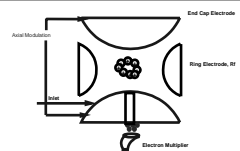


## Mass Spectrometer's Analyzers

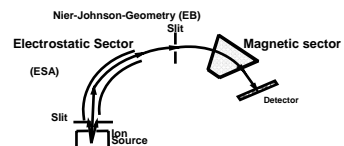
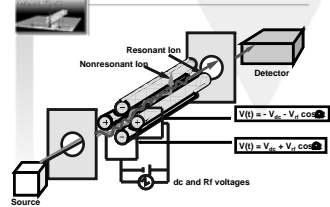
### Time Of Flight Mass Analyzers



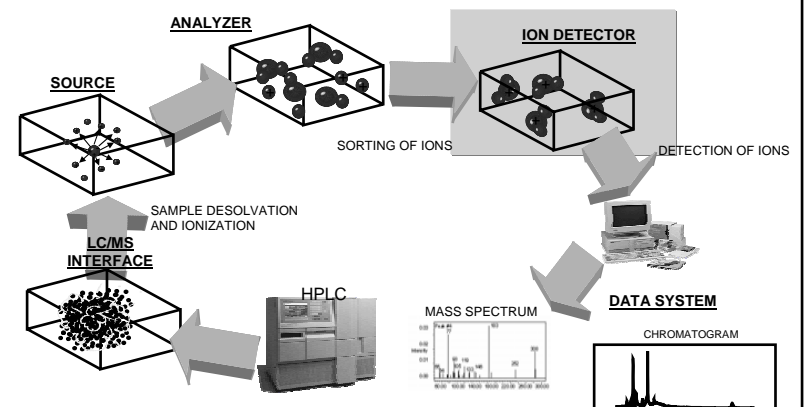
### Ion Traps



### Starting with the quadrupole

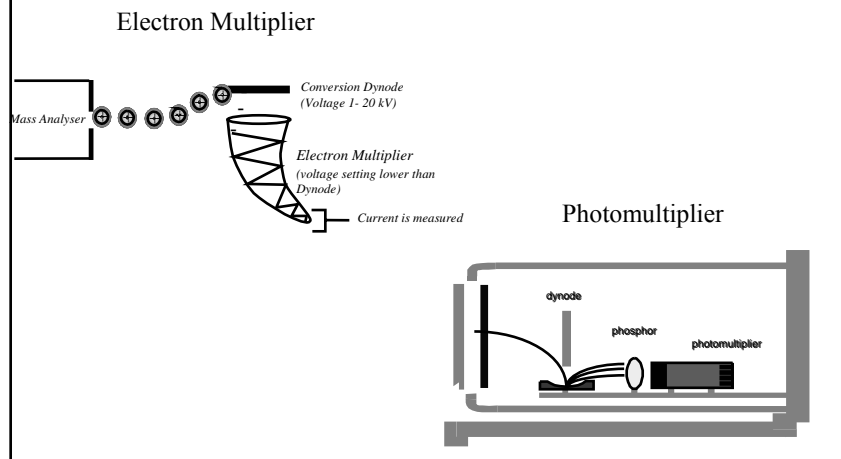


## Typical LC/MS System Progression

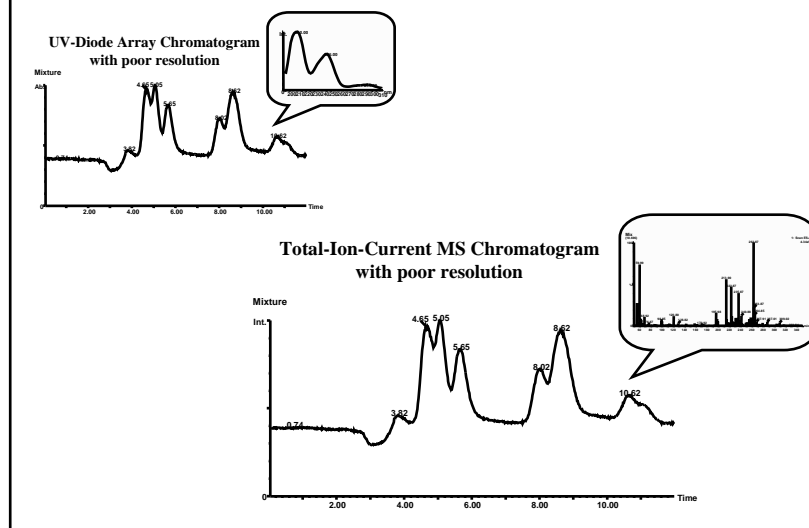


# Detection in HPLC

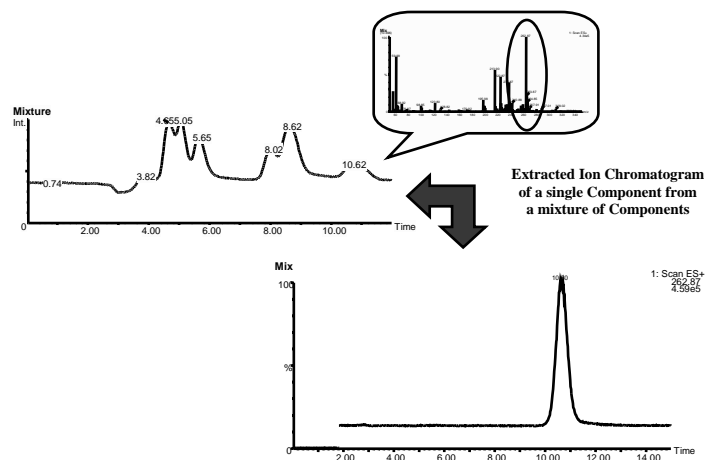
## MS Detectors



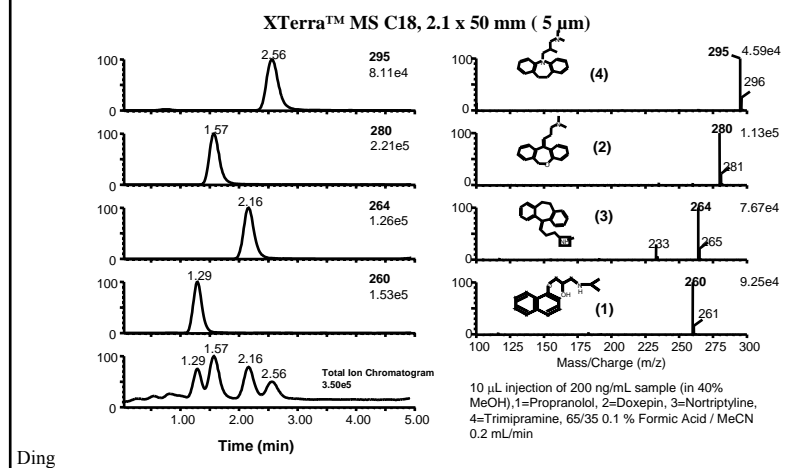
## Mass Spectrometer 3D Run



## Selectivity of Mass Spectrometer Detector



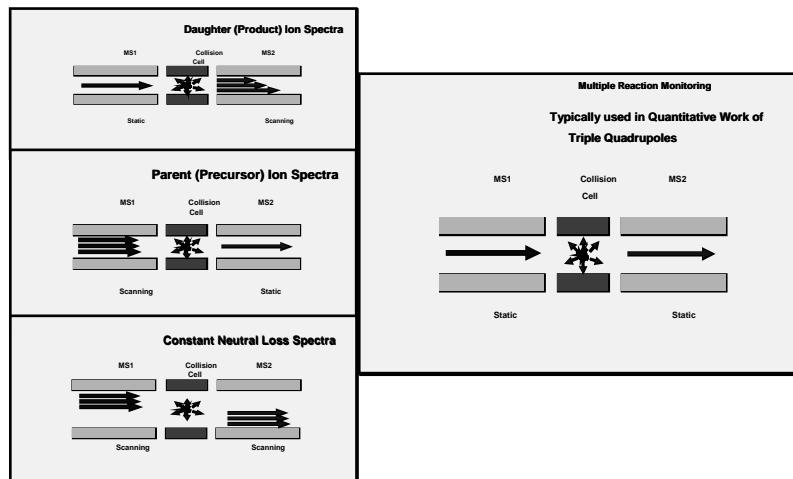
## LC-MS Analysis



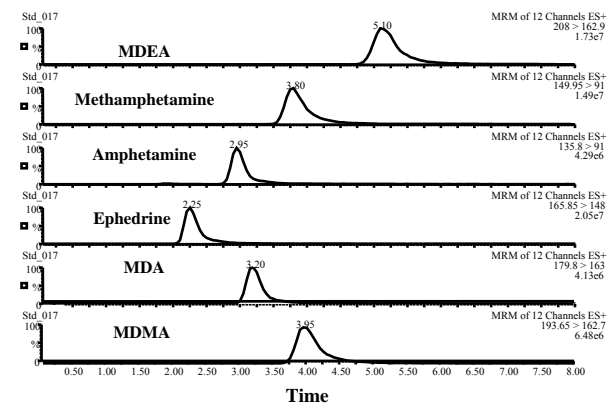


# Detection in HPLC

## Triple Quadrupoles: MS-MS Modes



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

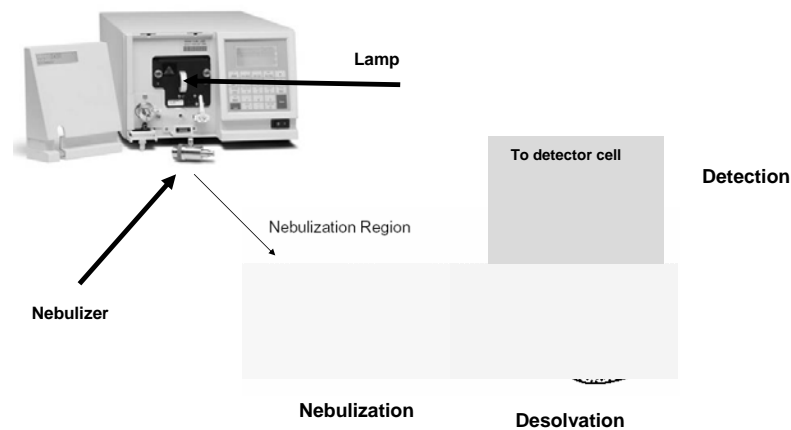


Highly specific and sensitive chromatograms

## Detectors

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

## Evaporative Light Scattering - ELS



# Detection in HPLC

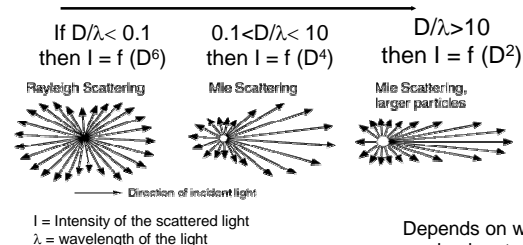
## Rayleigh Scattering – Why the Sky is blue

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

- Scattering is independent of the particle's chemical properties, where:
  - N = # of particles
  - $\alpha$  = 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



$D \propto C^{1/3}$   
(Often see solute density)

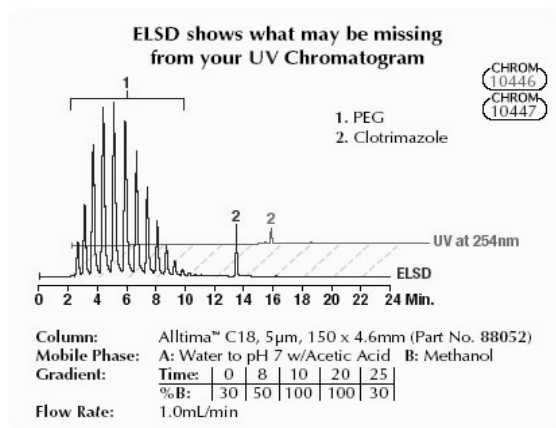
$I \propto (C^b)$   
With  $2 > b > 2/3$   
2 is the limiting value for Rayleigh symmetrical scattering

Depends on which type of scattering is predominant

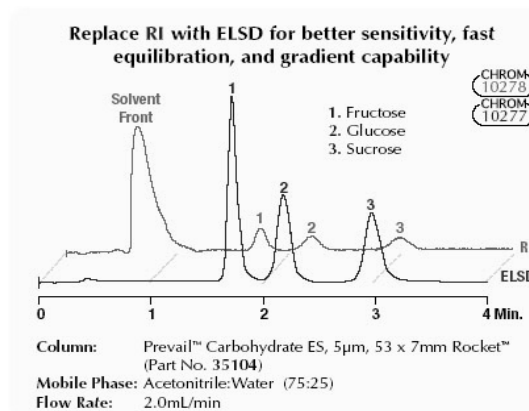
> **Non-linear mass detector**

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

## ELSD vs UV

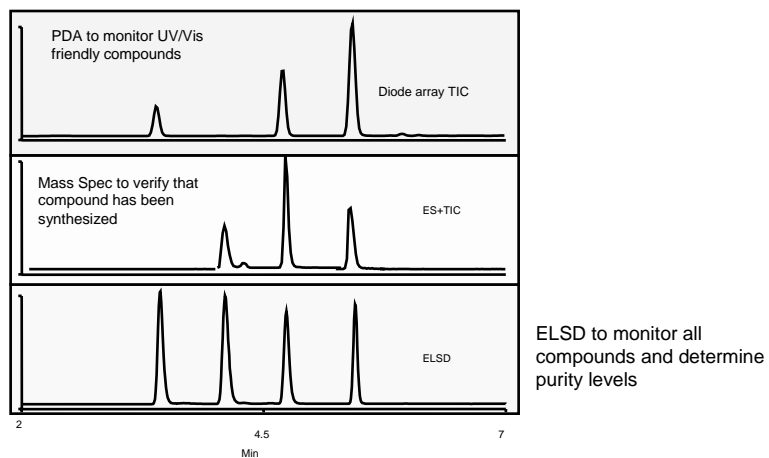


## ELSD vs RI

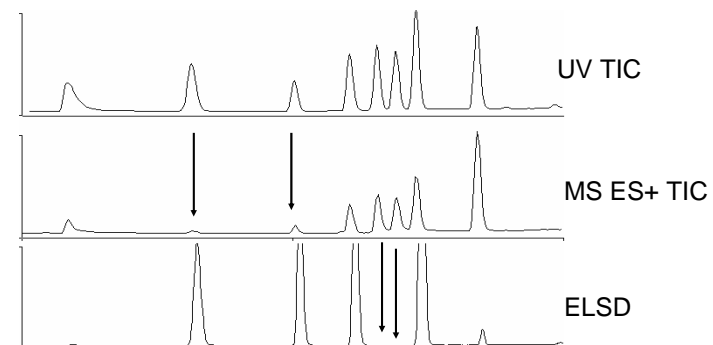


# Detection in HPLC

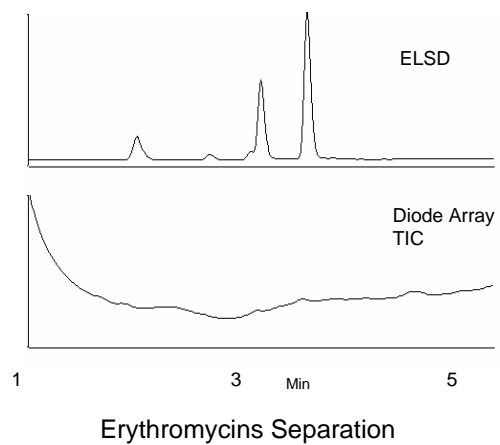
## ELSD Used with Other Detectors



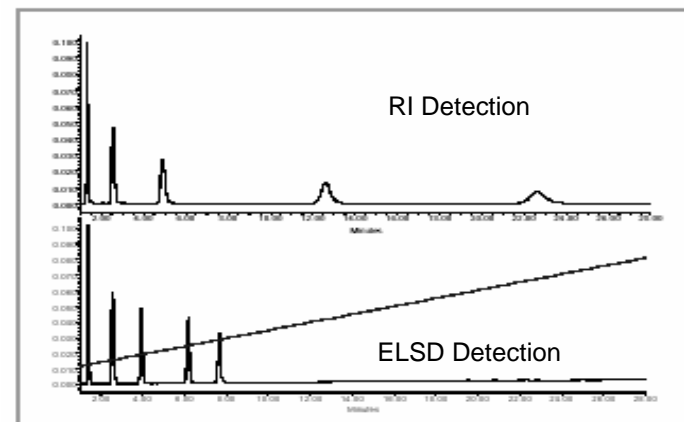
## Not a Universal Detector *Typically Used with Other Detectors*



## See Non-UV Absorbing Compounds



## See Your Peaks Faster *Use of Gradients Versus Isocratic*



## Detection in HPLC

