# Detection in HPLC- Implementation

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

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

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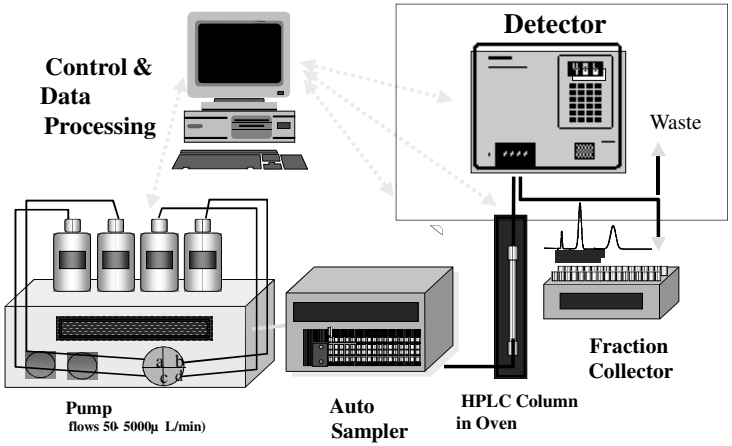
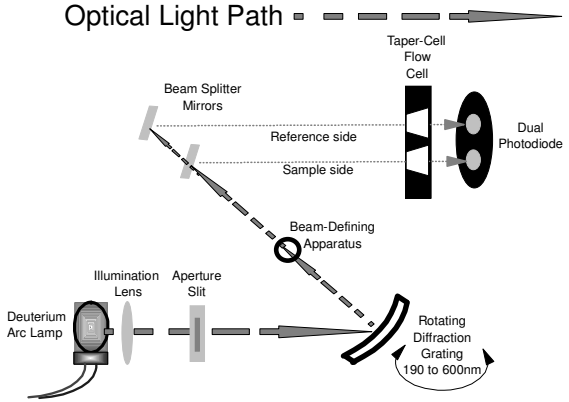
## The Detector is the "Eye" of the HPLC System




## Detectors

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

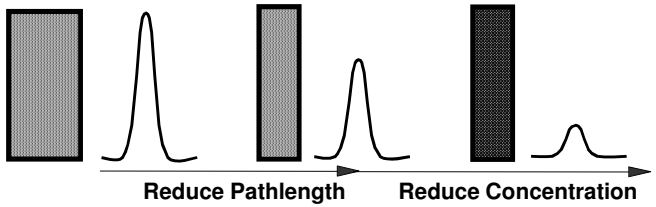
## Optical Bench of UV-VIS Detector



# Detection in HPLC- Implementation

## Beer's Law

Absorbance = Extinction Coefficient x Pathlength x Concentration




## UV Chromophores

| Chromophore | Chemical Configuration | λmax (nm) | ∈max (L/m/cm) | λmax (nm) | ∈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 |

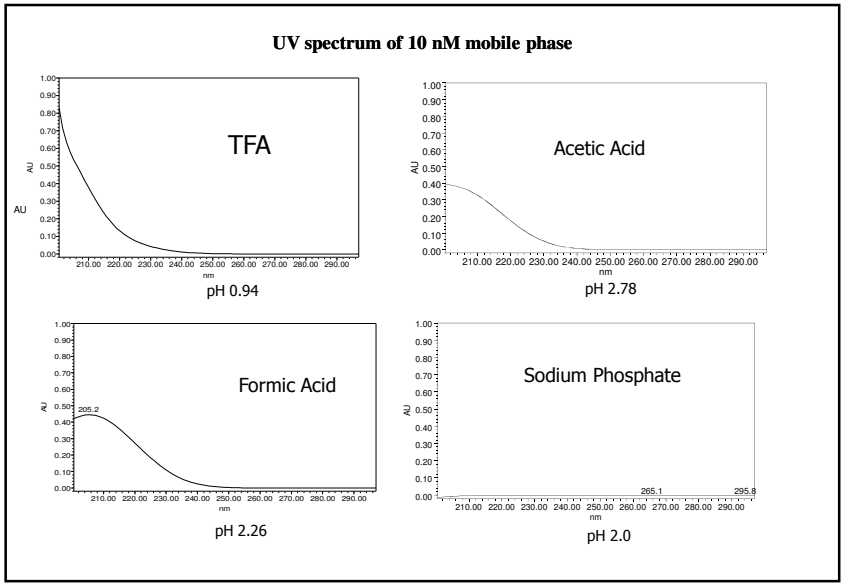
## UV Chromophores

| Chromophore | Chemical Configuration | λmax (nm) | ∈max (L/m/cm) | λmax (nm) | ∈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

| | λmax | Em x $10^{-3}$ @ λmax |
|---|---|---|
| **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- Implementation

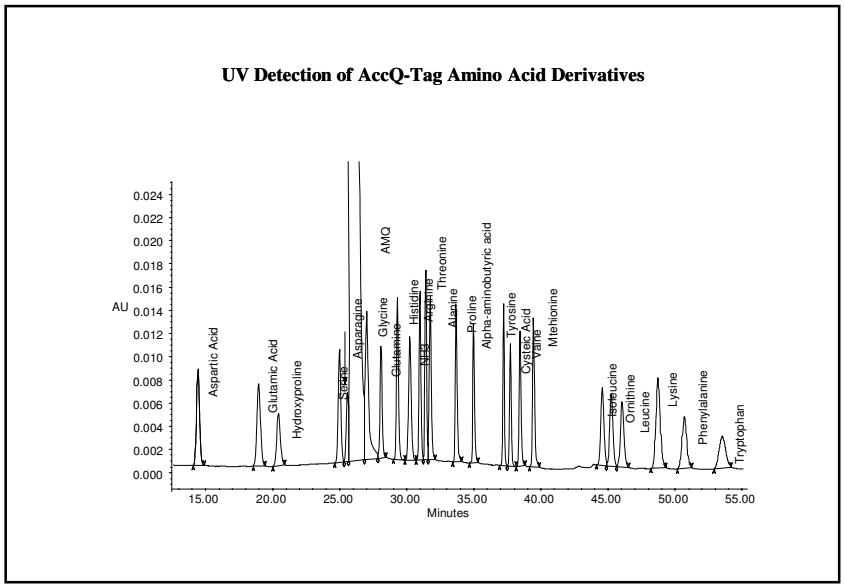



## U.V. Cut-offs for some Common Solvents

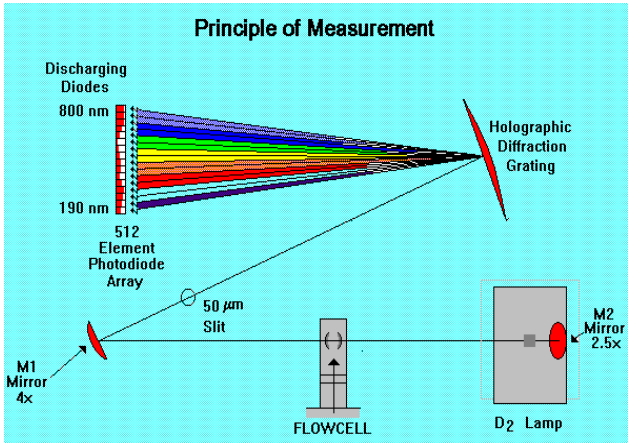
*Remember Solvents chosen can affect detection!!*

| Solvent | UV Cutoff | Solvent | UV Cutoff |
|---|---|---|---|
| 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.*

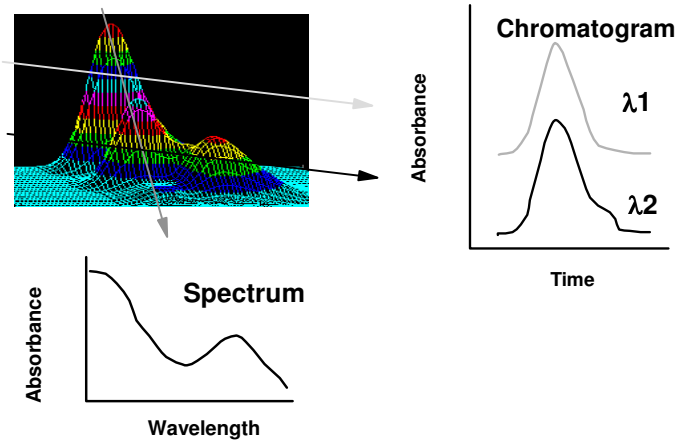



## Diode Array Detector



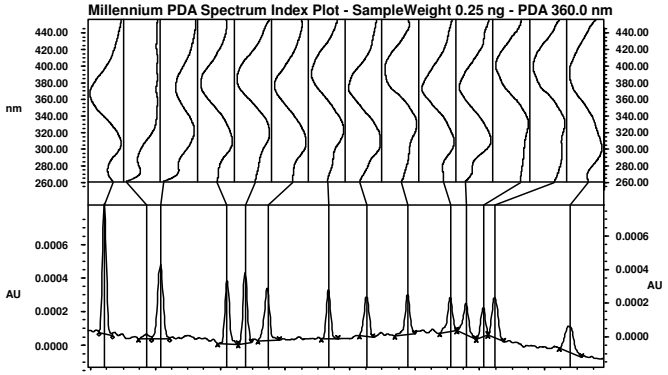
# Detection in HPLC- Implementation

## Extraction of 3D Data

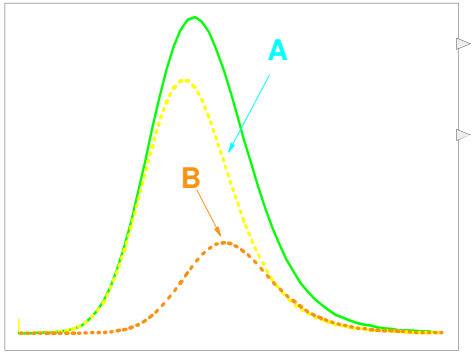



## PDA Spectrum Index Plot

DNPH Derivatives 0.25 ng Each Peak

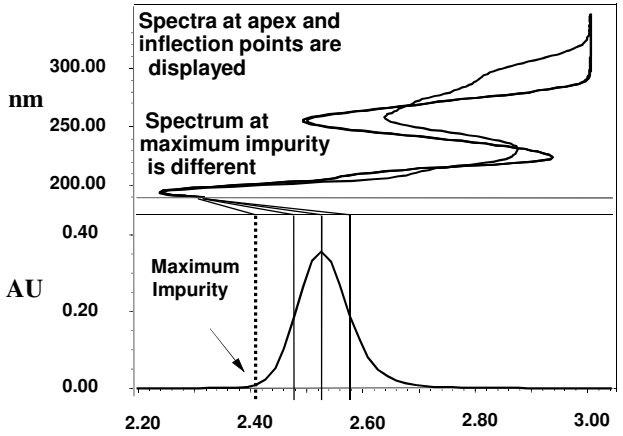



## Coelution of 2 Peaks



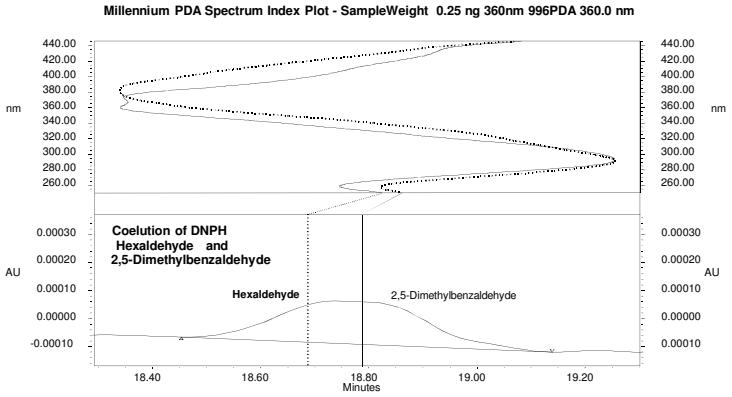

- Coelution detection at a single wavelength
- **Coelution** is the sum of absorbance of 2 peaks **A** and **B**

## Peak Purity Measurement

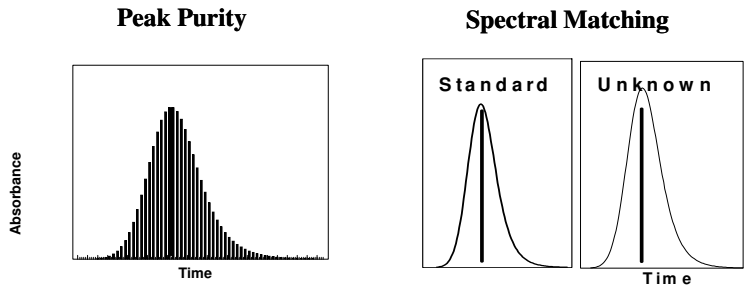


# Detection in HPLC- Implementation

## Maximum Impurity Detection



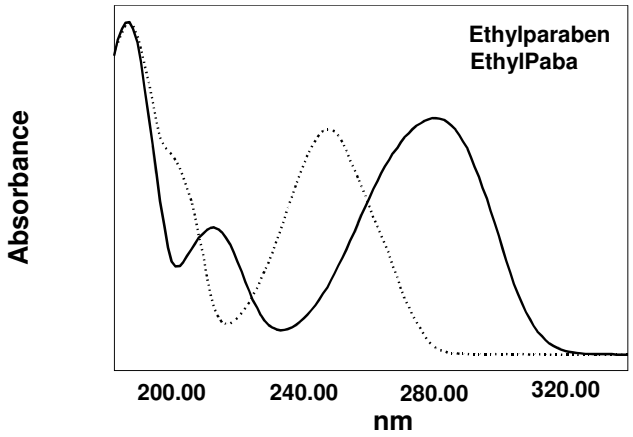

## Determination of Peak Purity



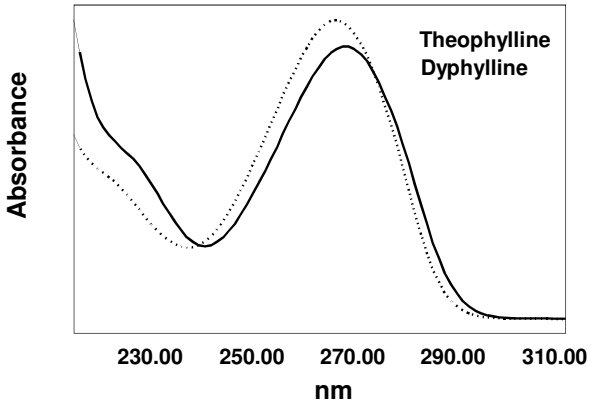

**Peak Purity analyzes all spectra (minimum 15) within a peak against the apex spectrum of the peak itself.**

**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



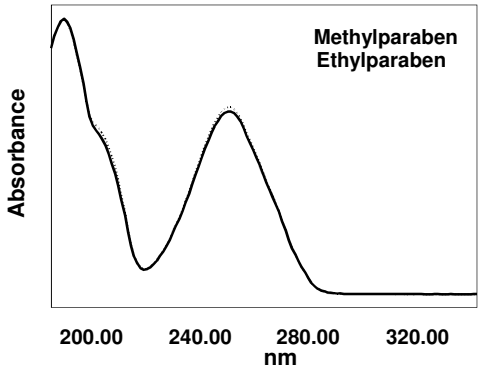

## 10 deg of Spectral Contrast




Similar spectra for structurally related compounds

# Detection in HPLC- Implementation

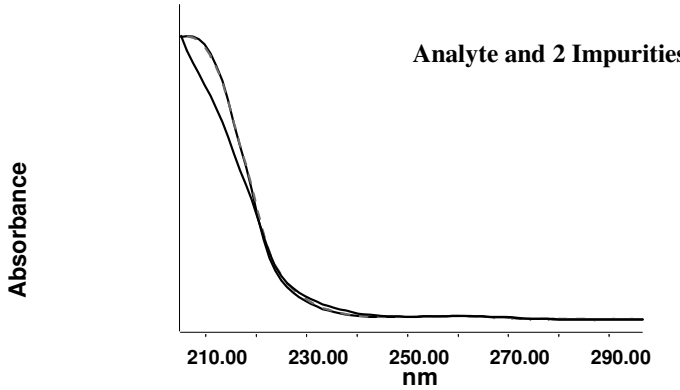
## Spectral Contrast 0.5 Degrees




Very similar spectra, CH2 difference

Spectral Contrast can differentiate these spectra

## Spectra of non-UV Active Compounds




## Detectors

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

## Refractive Index Detector

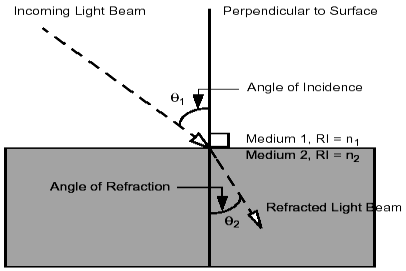



Figure 1-3 Refraction of Light

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:

$\theta_1$ = Angle of incidence
$\theta_2$ = Angle of refraction
$n_1$ = RI of medium 1
$n_2$ = RI of medium 2

# Detection in HPLC- Implementation

## Refractive Index Detector

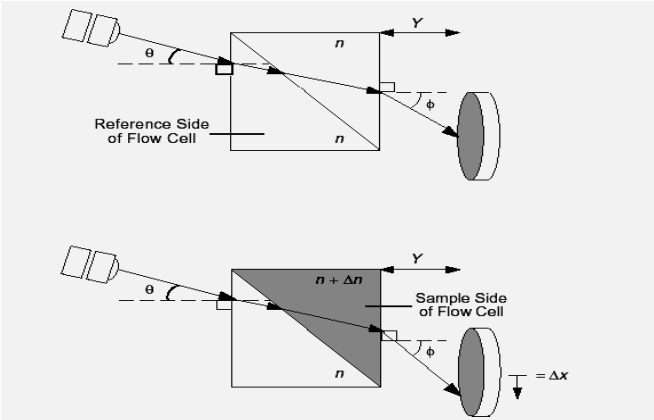
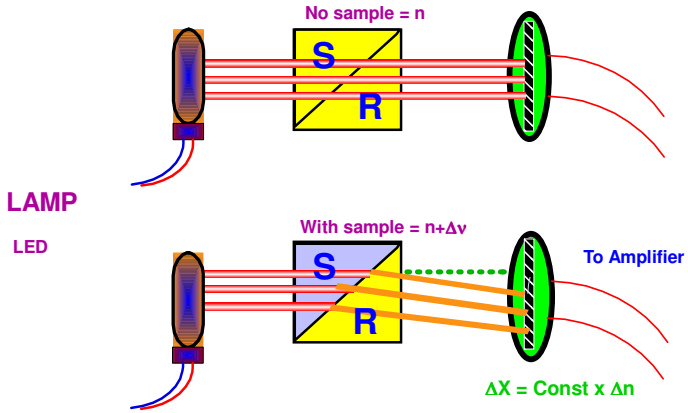



Figure 1-5 How Refraction Changes the External Angle of Deflection

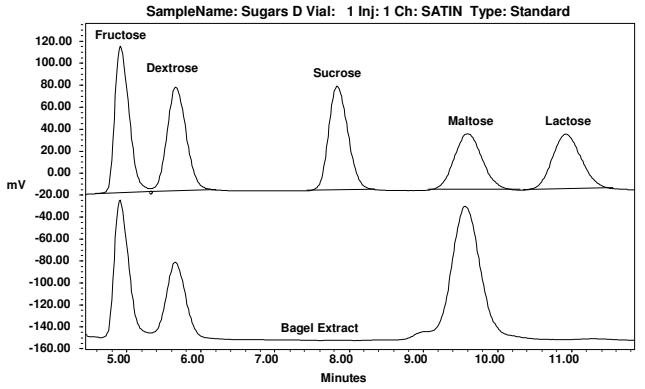
## Differential Refractive Index Detector




## What affects RI?

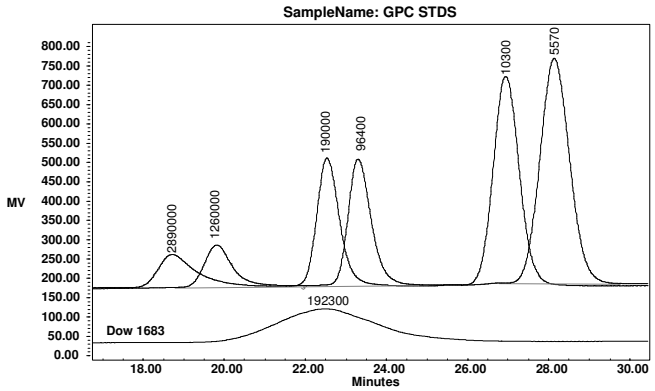
- Temperature
  - Temperature must be stable
  - Pressure free of pulses from solvent delivery
  - Changes in atmospheric pressure
- Composition
  - Vacuum Degassing, Cleanliness from Particulates
  - Thermal Stability & Warm-up is system dependent based on variables such as column heater type & de-gasser
  - Qualifying Conditions will vary between LC pumps!

## Sugar Analysis

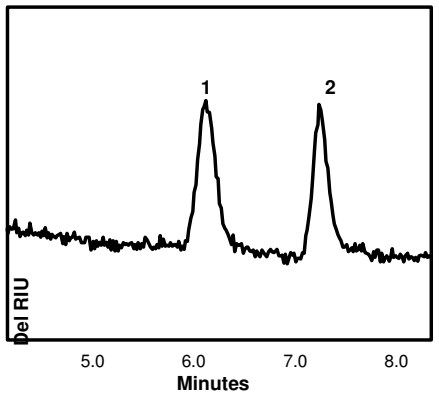


# Detection in HPLC- Implementation

## Polymer Analysis




## Lipids

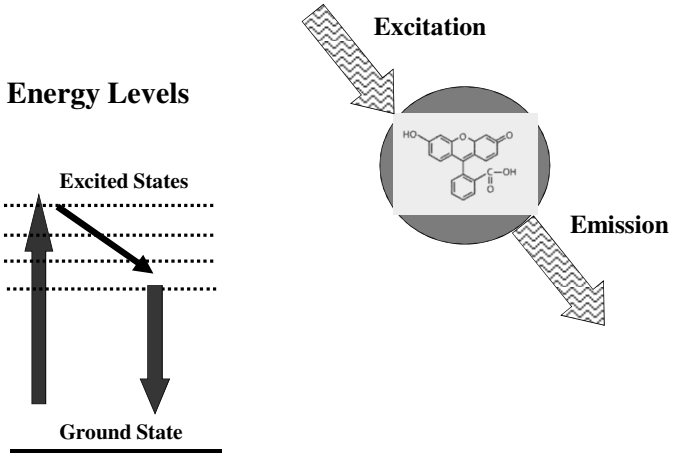



- 250 ng on column
  1=Tristearin
  2=Myristic acid
- Styragel HR 0.5, 4.6 x 300 mm, 35°C, 0.35 mL/min
- dRI sensitivity = 32X, 32°C

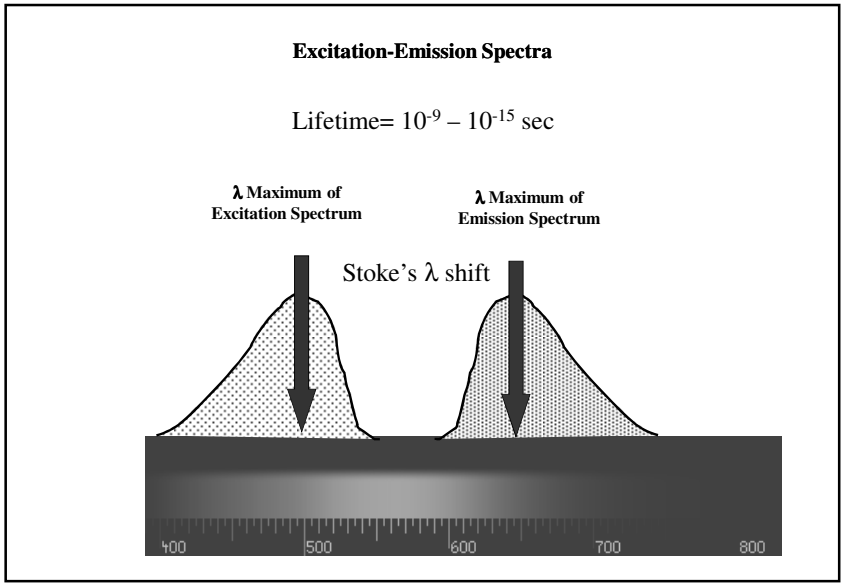
## Detectors

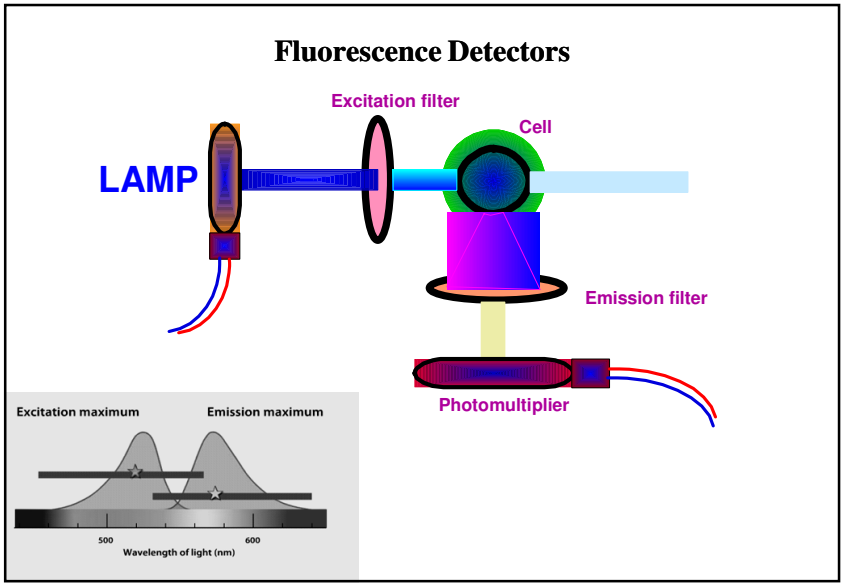
- UV/VIS
- Refractive index
- **Fluorescence**
- Electrochemical
- Conductivity
- Mass-spectrometric (LC/MS)
- Evaporative light scattering

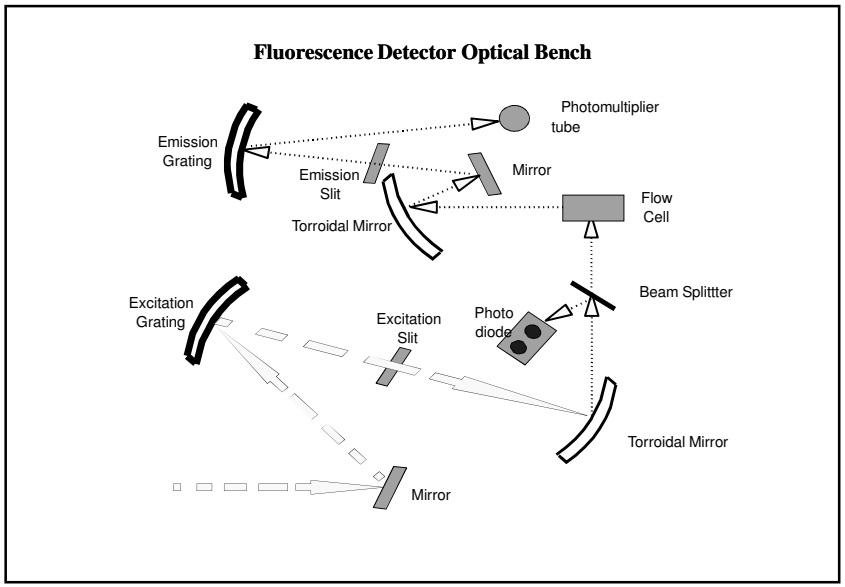
## Fluorescence Process

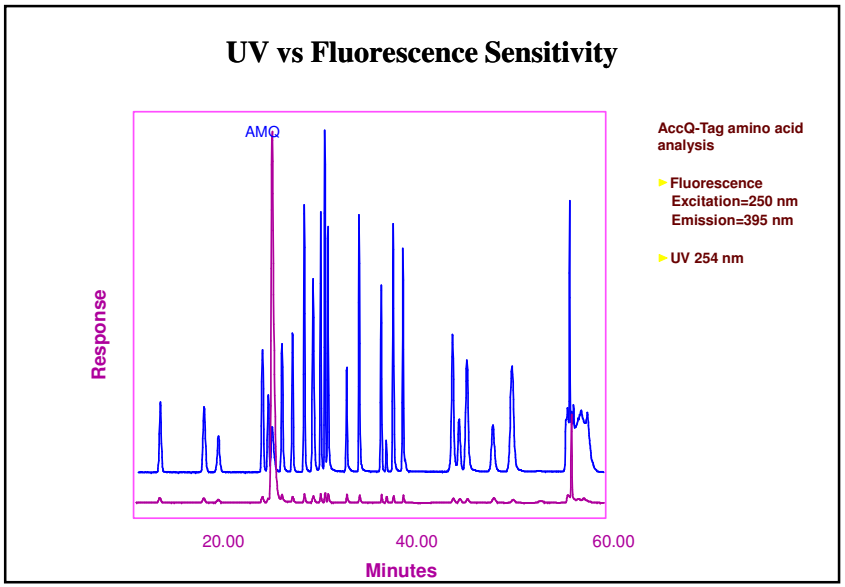


# Detection in HPLC- Implementation



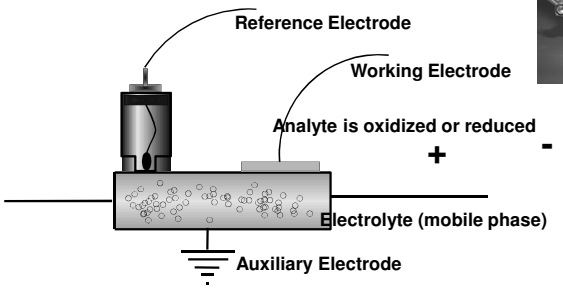



# Detection in HPLC- Implementation

## Detectors

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

## Electrochemical Detector

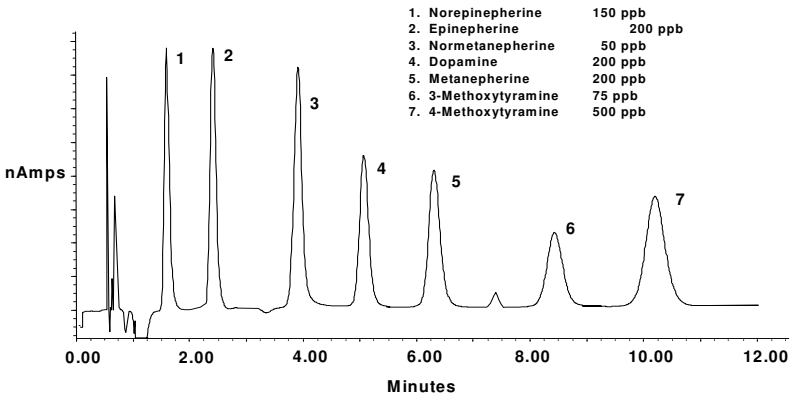

As compounds are oxidized or reduced,
a current proportional to concentration is produced.

## Electrochemical Detector (ECD)

- "Destructive"-detection mode
- Sample electrochemically modified in the cell
  - generation of current detected as response.
- Sensitivity
  - picogram ($10^{-12}$ grams) to high femtogram ($10^{-15}$ grams)
- Selection of buffer, electrode and voltage important

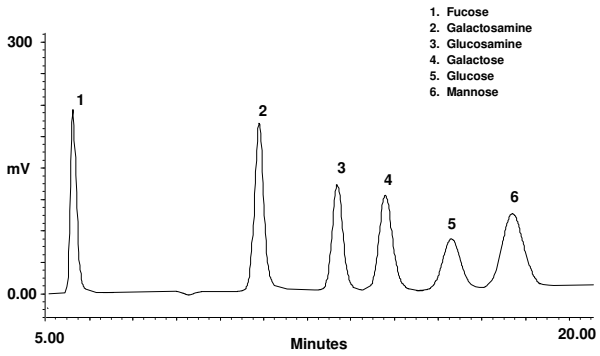
| Oxidation | Reduction |
|---|---|
| Phenolic | Ketones |
| Oximes | Aldehydes |
| Dihydroxy | Oximes |
| Mercaptans | Conjugated acids |
| Peroxides | Conjugated esters |
| Hydroperoxides | Conjugated unsaturation |
| Aromatic Amines, diamines | Activated halogens |
| Purines | Aromatic halogens |
| Heterocyclic Rings | Nitro compounds |
| | Heterocyclic rings |

## Electrochemical Detection of Catecholamines & Related Compounds



# Detection in HPLC- Implementation

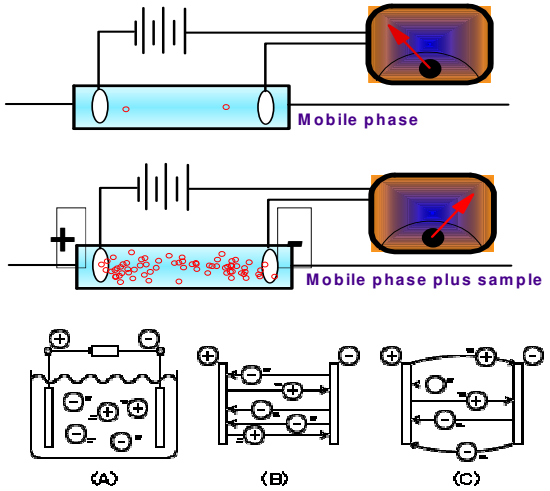
## Pulsed Amperometric Detection of Monosaccharides




## Detectors

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

## Conductivity Detector




## Conductivity Equations

Ohm's Law

$$\text{Electric current } (\mathbf{I}) = \frac{\text{Voltage } (\mathbf{E})}{\text{Resistance } (\mathbf{R})}$$

Unit: **I : A (Ampere)**
**E : V (Volt)**
**R : Ω (Ohm)**

$$\text{Conductivity}(\mathbf{k}) = \frac{\text{Electric current } (\mathbf{I})}{\text{Voltage } (\mathbf{E})} \times \frac{\text{length } (\mathbf{L})}{\text{Area } (\mathbf{S})}$$

$$\text{Cell constant } (\mathbf{K}) = \frac{\text{length } (\mathbf{L})}{\text{Area } (\mathbf{S})}$$

$$\therefore \text{Conductivity}(\mathbf{k}) = \frac{\text{Cell constant } (\mathbf{K})}{\text{Resistance } (\mathbf{R})}$$

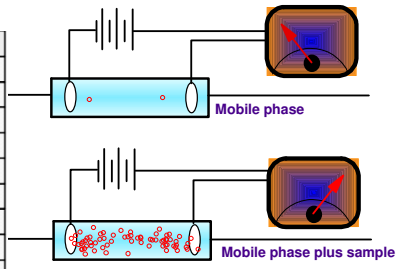
Unit $\mathbf{K}$ : $cm^{-1}$

# Detection in HPLC- Implementation

## Conductivity Detector

Limiting Equivalent Conductance of Ions in Water at 25 °C

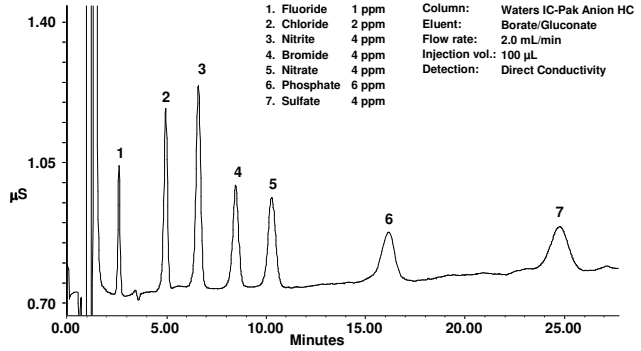
| Cations | $\lambda_+$ | Anions | $\lambda_-$ |
|---|---|---|---|
| $H^+$ | 349.8 | $OH^-$ | 198.6 |
| $Li^+$ | 38.6 | $F^-$ | 55.4 |
| $Na^+$ | 50.1 | $Cl^-$ | 76.4 |
| $K^+$ | 73.5 | $Br^-$ | 78.1 |
| $Rb^+$ | 77.8 | $I^-$ | 76.8 |
| $Ag^+$ | 61.9 | $NO_3^-$ | 71.5 |
| $NH_4^+$ | 73.3 | $ClO_3^-$ | 64.6 |
| $(CH_3)_2NH_2^+$ | 51.8 | $ClO_4^-$ | 67.4 |
| $Hg^{2+}$ | 53.0 | $IO_4^-$ | 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 | | |



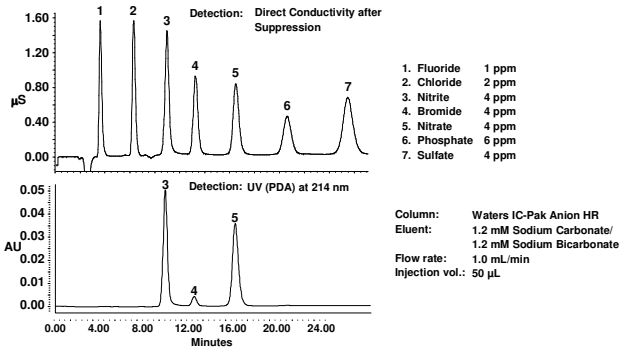

$$G = \frac{\lambda C}{10^{-3}}$$

$G$ = measured conductance of the solution, in Siemens ($1\ S = ohm^{-1}$)
$C$ = concentration in equivalents per 1000 $cm^3$
$K$ = length/area of cell (the cell constant)
$\lambda$ = equivalent conductance in S $cm^2$ $equiv^{-1}$

## Anion Analysis by IC




## Anion analysis by IC




1. Fluoride 1 ppm
2. Chloride 2 ppm
3. Nitrite 4 ppm
4. Bromide 4 ppm
5. Nitrate 4 ppm
6. Phosphate 6 ppm
7. Sulfate 4 ppm

Column: Waters IC-Pak Anion HR
Eluent: 1.2 mM Sodium Carbonate/ 1.2 mM Sodium Bicarbonate
Flow rate: 1.0 mL/min
Injection vol.: 50 µL

## 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

# Detection in HPLC- Implementation

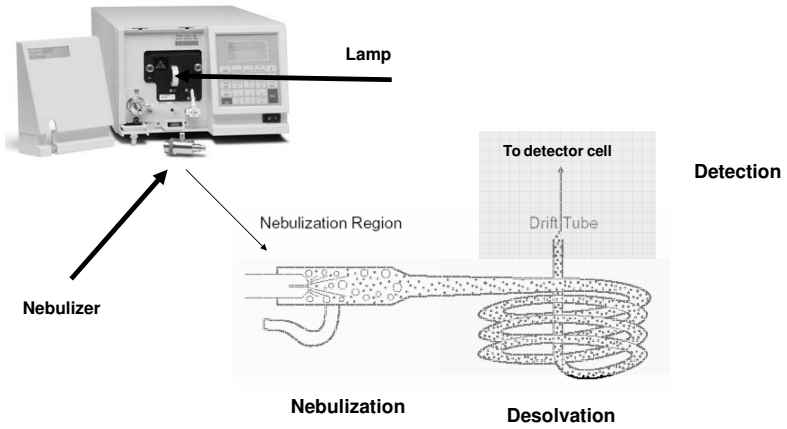
## Choosing a Detector for HPLC

| | RI | UV/VIS | Fluor. | ECD | Cond. |
|---|---|---|---|---|---|
| Response | Universal | Selective (Chromaphor) | Selective (Fluorophor) | Selective (Redox) | Selective (Ions) |
| Sensitivity | µgram | nanogram | picogram | picogram | picogram |
| Linear Range | $10^4$ | $10^5$ | $10^3$ | $10^6$ | $10^5$ |
| Flow Sensitivity | Yes | No | No | Yes | Yes |
| Temp. Sensitive | Yes | No | NO | Yes | Yes |

## Detectors

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

## Evaporative Light Scattering - ELS




## 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

# Detection in HPLC- Implementation

## Scattering Models

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

If $D/\lambda < 0.1$ then $I = f(D^6)$ — Rayleigh Scattering

$0.1 < D/\lambda < 10$ then $I = f(D^4)$ — Mie Scattering

$D/\lambda > 10$ then $I = f(D^2)$ — Mie Scattering, larger particles

Direction of incident light

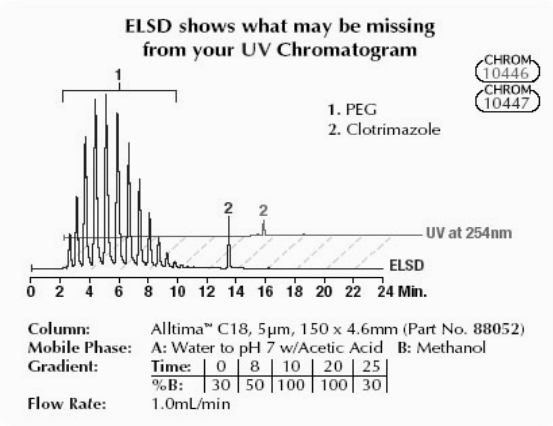
I = Intensity of the scattered light
λ = wavelength of the light

$D \alpha C^{1/3}$
(Often see solute density)
$I \alpha (C^b)$
With $2>b>2/3$
2 is the limiting value for Rayleigh symmetrical scattering

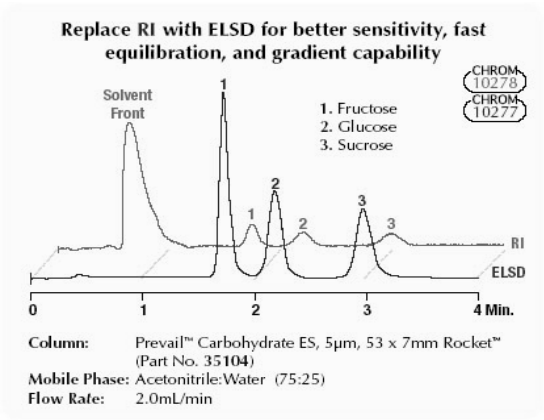
Depends on which type of scattering is predominant

- ***Non-linear mass detector***
  - ❖use chromatography data software quadratic curve or log/log curve to fit calibration curve

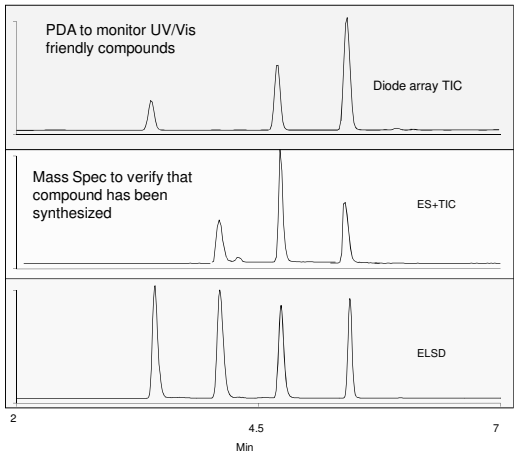
## ELSD vs UV




## ELSD vs RI




## ELSD Used with Other Detectors

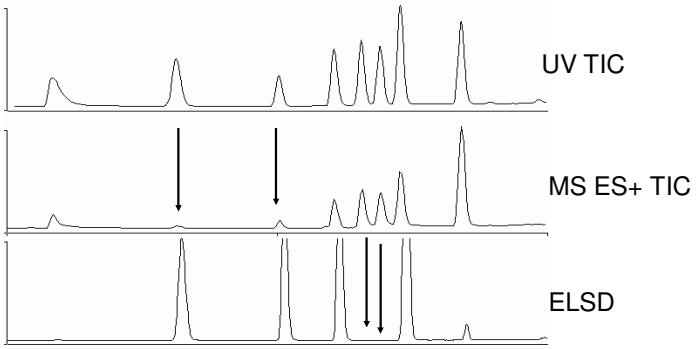



ELSD to monitor all compounds and determine purity levels

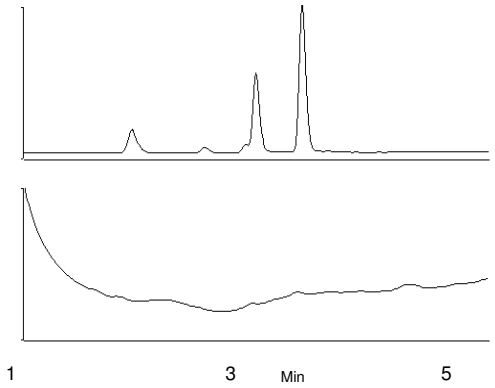
# Detection in HPLC- Implementation

## Not a Universal Detector

*Typically Used with Other Detectors*



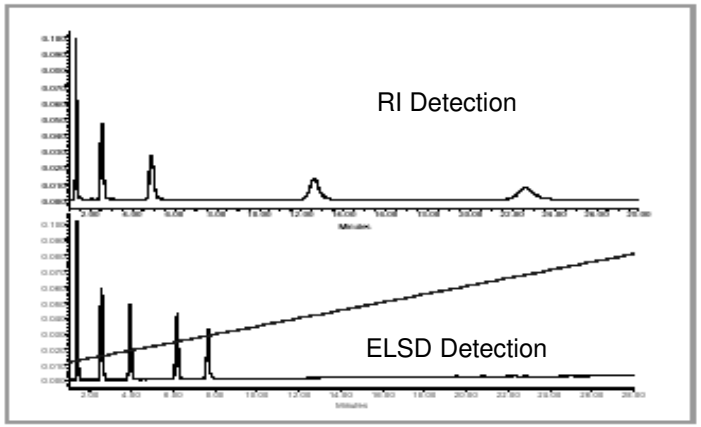

## See Non-UV Absorbing Compounds




Erythromycins Separation

## See Your Peaks Faster

*Use of Gradients Versus Isocratic*




## Evaporative Light-scattering Detector

Estrogen analogues and salt