

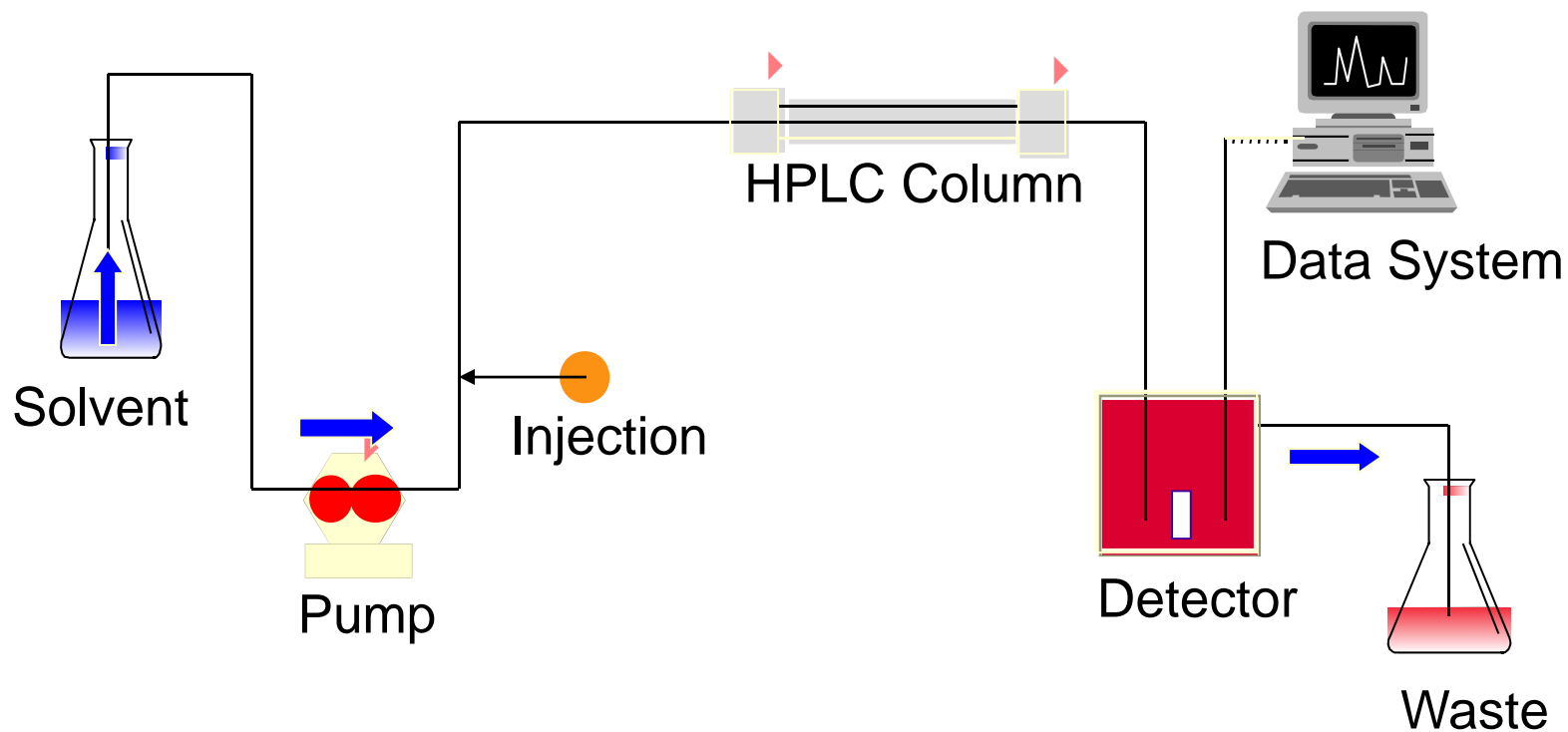
HPLC instrumentation and Trends

Content

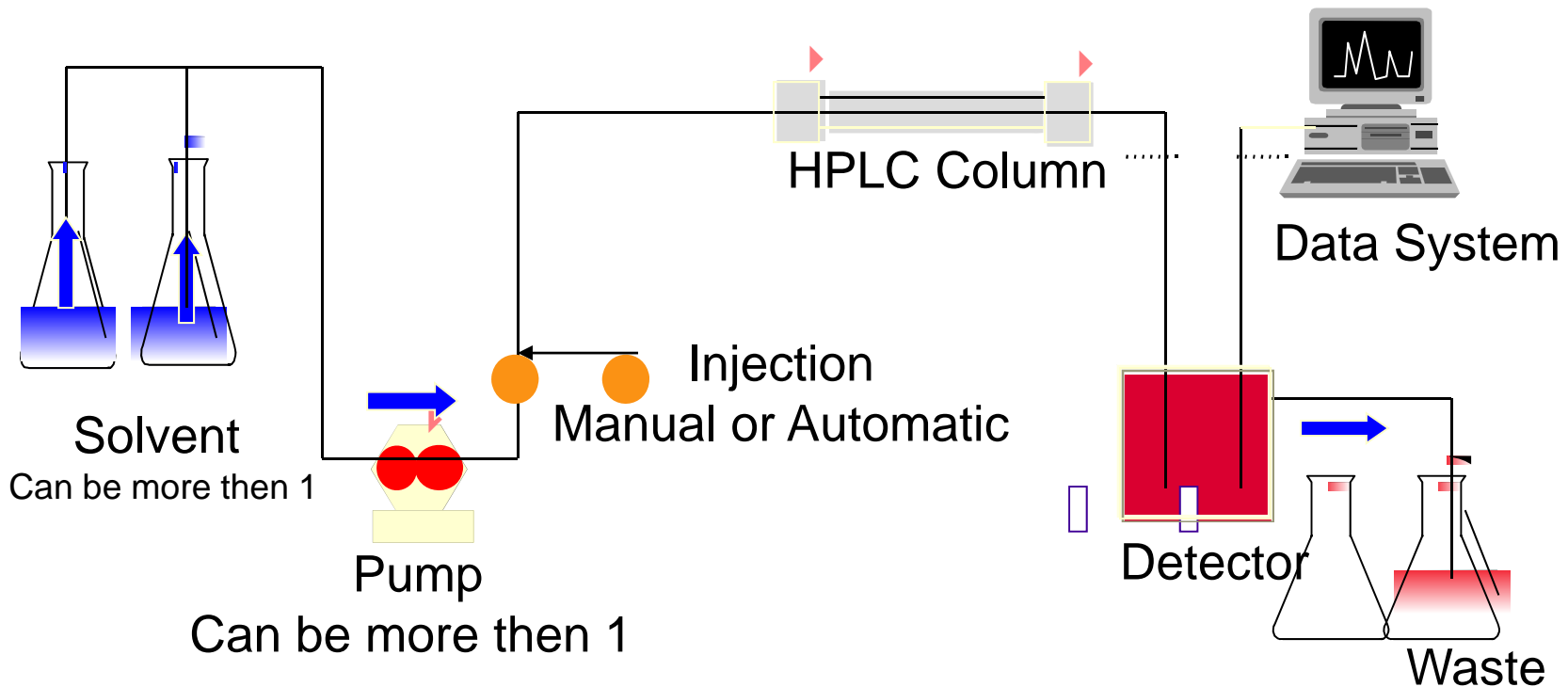
- Composition of the HPLC system
- Pumps
- Injectors
- Detectors
- Data handling system

The Chromatographic System

Basic



The Chromatographic System Advanced



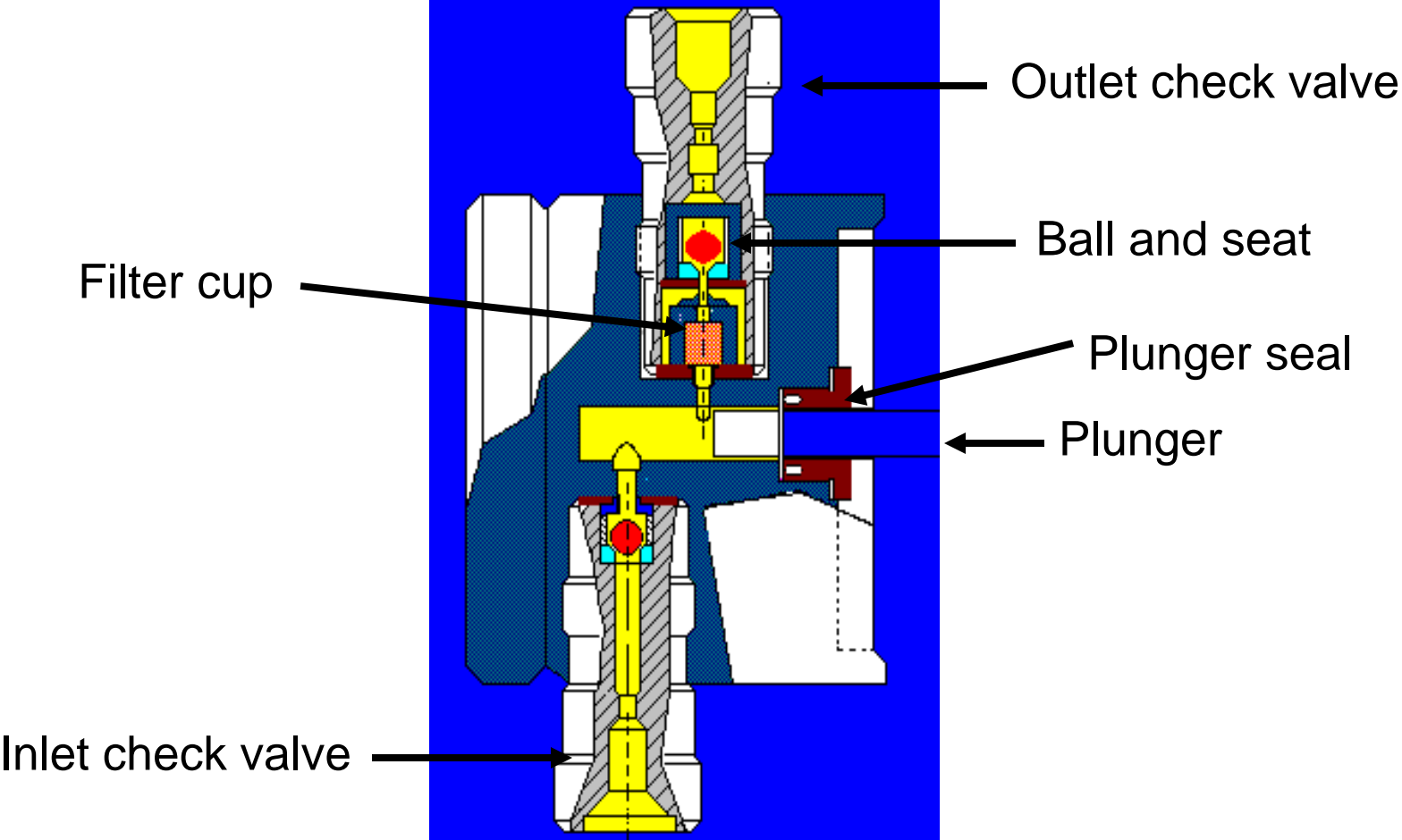
Criteria for HPLC Solvent Delivery System (Pumps)

- Constructed of materials that are chemically resistant to the mobile phase
- Provide precise and pulse-free delivery of solvents at typical flow rates
- Range for flow rate 0.1–10 mL/min and pressure up to 6,000 psi (42MPa)
- Flow reproducibility \ll 1% R.S.D
- Desirable to have small holdup volume for rapid solvent changes/gradient elution
- Compatible with common organic solvents, buffers, and salts
- Reliable operation with long pump seal life

Pressure Units and Conversion

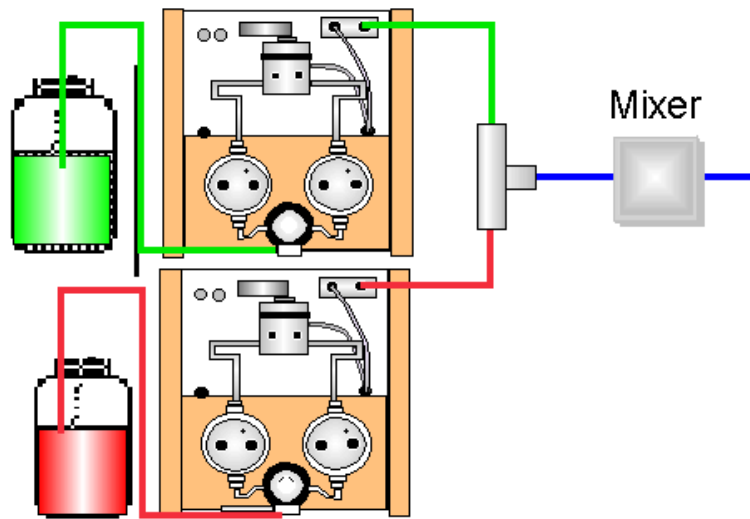
- Bar
- Pounds per Square Inch (PSI)
- Pascal
- Atm
 - ◆ 1 Bar = 14.5 Psi
 - ◆ 1 Bar = 0.9869 Atm
 - ◆ 1 Bar = 100000 Pascal

Pump Head

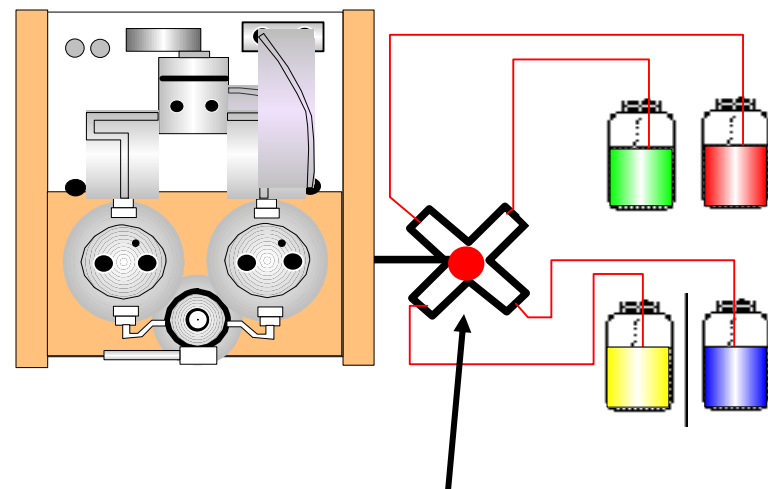


Pumps in Gradient System

2 pump gradient system
Mixing on high pressure side



1 pump gradient system
Mixing on low pressure side



Gradient proportioning valve

Gradient Systems

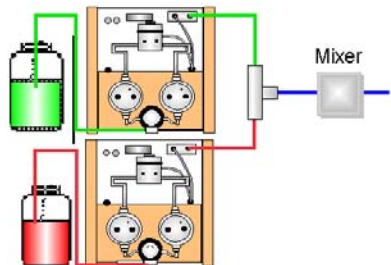
“High-pressure mixing”

- Advantages

- ◆ Usually lower system volume
- ◆ Degassing not as critical

- Disadvantages

- ◆ One pump per solvent
- ◆ Only practical with up to 3 solvents (usually only 2)



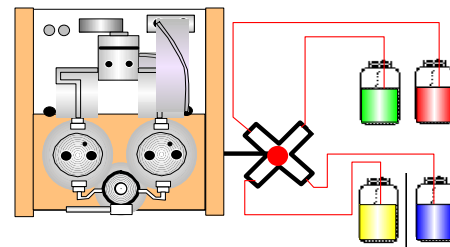
“Low-pressure mixing”

- Advantages

- ◆ Only one pump
- ◆ Usually more solvents (normally 4)

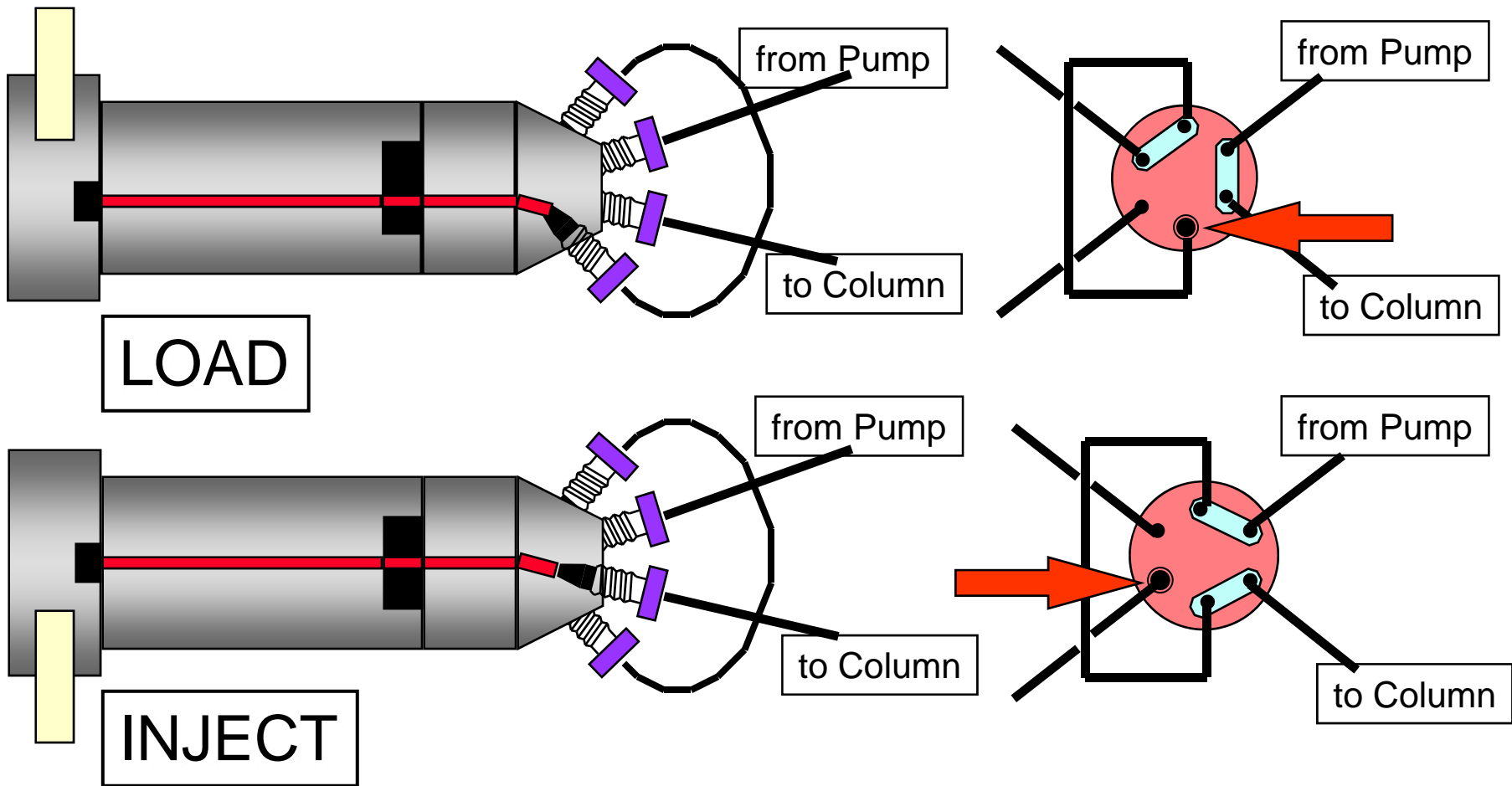
- Disadvantages

- ◆ Usually higher system volume
- ◆ Degassing more critical



INJECTORS

An HPLC injector is used to introduce the sample to the column under high pressure. A common injector is the Rheodyne model 7125 or 7725 injector,



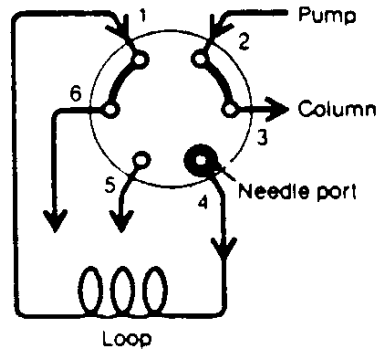
Loading of sample loop

The sample loop can be loaded in two ways :

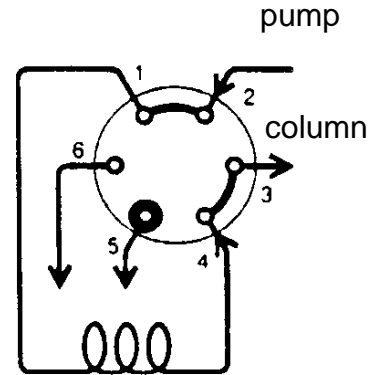
- Complete Filling : The loop is flushed with an excess amount of sample. For best results the sample volume should be more than twice of the volume of loop. The maximum injection volume can be changed by replacing the sample volume.
- Partial filling : The loop can be partially filled with a specially calibrated syringe or pump. Any volume between 0.1 μL and the volume of the loop itself can be injected.

Fixed loop Injection Valve

Position 1 (load)

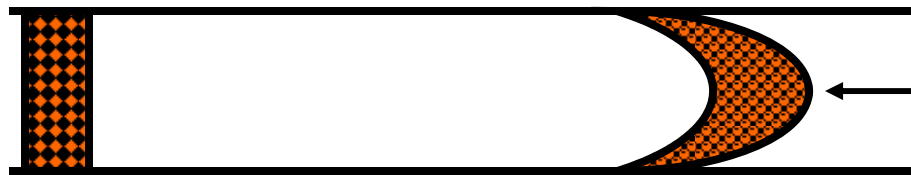


Position 2 (inject)



Sample loop

Tube wall



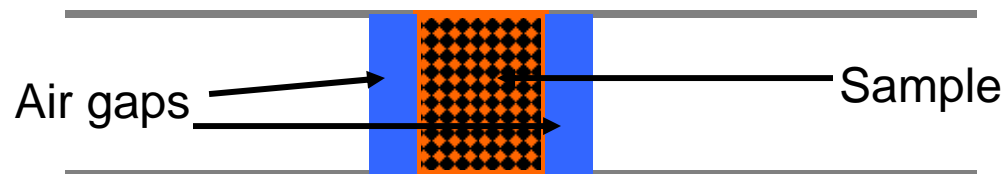
Laminar flow

Initial fluid element

Fluid element after flow

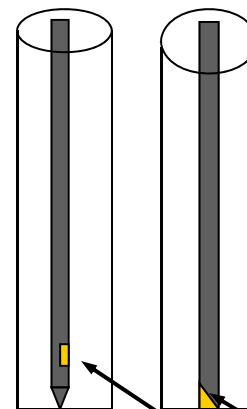
Characteristics of a Fixed Loop Injector

- As a consequence of laminar flow each 1ul loaded in the loop occupies 2ul.
- Full loop injections means that 2-3 times the loop size should be loaded.
- Partial loop injections this means that only up to 50% of loop size should be loaded.
- Using pre- and post- sample air gaps (2-3ul) the laminar flow can be eliminated



Auto samplers, Sample Formats, Vials

- Crimp cap
 - ◆ Cheapest solution
 - ◆ Needs a crimping tool
- Snap cap
 - ◆ Easy to use - no tools
 - ◆ Traditional or New **LectraBond™** versions
- Screw cap
 - ◆ The universal version
 - ◆ Traditional or New **LectraBond™** versions
- Limited volume vials
 - ◆ Inserts
 - ◆ With internal taper



Needle opening



Auto samplers, Sample Formats, Plates

- Plate formats
 - ◆ For traditional vials
 - ◆ 96- or 384-well plates



HPLC Detectors - Criteria

An HPLC detector measures the concentration (or mass) of eluting analytes by monitoring one of their inherent properties, such as UV absorbance. A detector can be “universal” to all analytes or “specific” to particular classes of analytes.

- Operates by registering an output in response to sample detection.
- A linear relationship is expected between response of the detector and concentration of the sample, and calibration techniques are designed to promote this relationship.
- Not all detectors are linear and effects from other components of the HPLC system may cause the detector to deviate from a linear relationship between response and concentration.
- High sensitivity
- Negligible baseline noise
- Large linear dynamic range
- Response independent of variations in operating parameters (pressures, temperature, flow-rate...etc.)
- Response independent of mobile phase

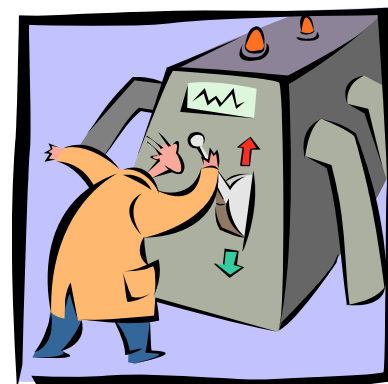
HPLC Detectors - Types

- UV/Visible
 - ◆ Variable wavelength
 - ◆ Photo diode array (PDA/DAD)
- Fluorescence
- Refractive Index
- Conductivity
- Electrochemical
- Mass Spectrometry
- Evaporative Light Scattering
- Nuclear Magnetic Resonance (NMR)

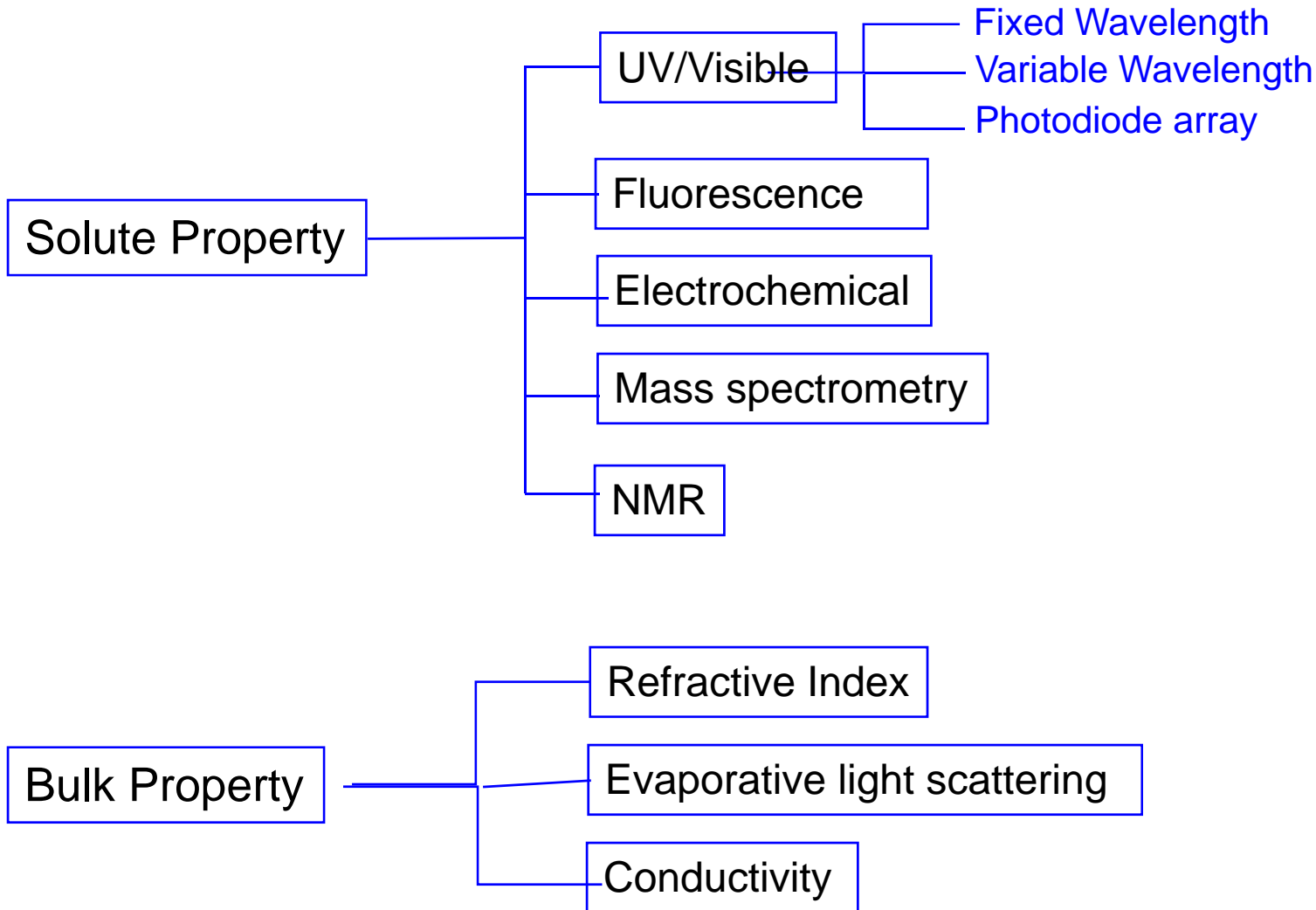
HPLC Detectors

There is no single detector that can be employed for all HPLC separations “the magic box”!

- HPLC detectors can be classified as:
 - ◆ Solute property detectors -respond to physical or chemical properties of the solute that are generally not exhibited by the mobile phase.
 - ◆ Bulk property detectors -respond to an overall change in the physical property of mobile phase with and without solute.



Classification of Different Detector Types



UV/Visible Detectors

The UV/Vis absorbance detector monitors the absorption of UV or visible light in the HPLC eluent.

- Most frequently used detector in HPLC analysis
- Compounds must contain a UV absorbing chromophore
- Must work in the linear range of Beer's Law

A diagram illustrating the Beer's Law equation $A = \epsilon b c$. The equation is centered at the top. Three arrows point from labels below to the variables in the equation: 'Absorbance' points to 'A', 'Molar extinction coefficient' points to ' ϵ ', and 'Sample concentration (moles/l)' points to 'c'. A fourth arrow points from 'cell path length (cm)' to 'b'.

Absorbance

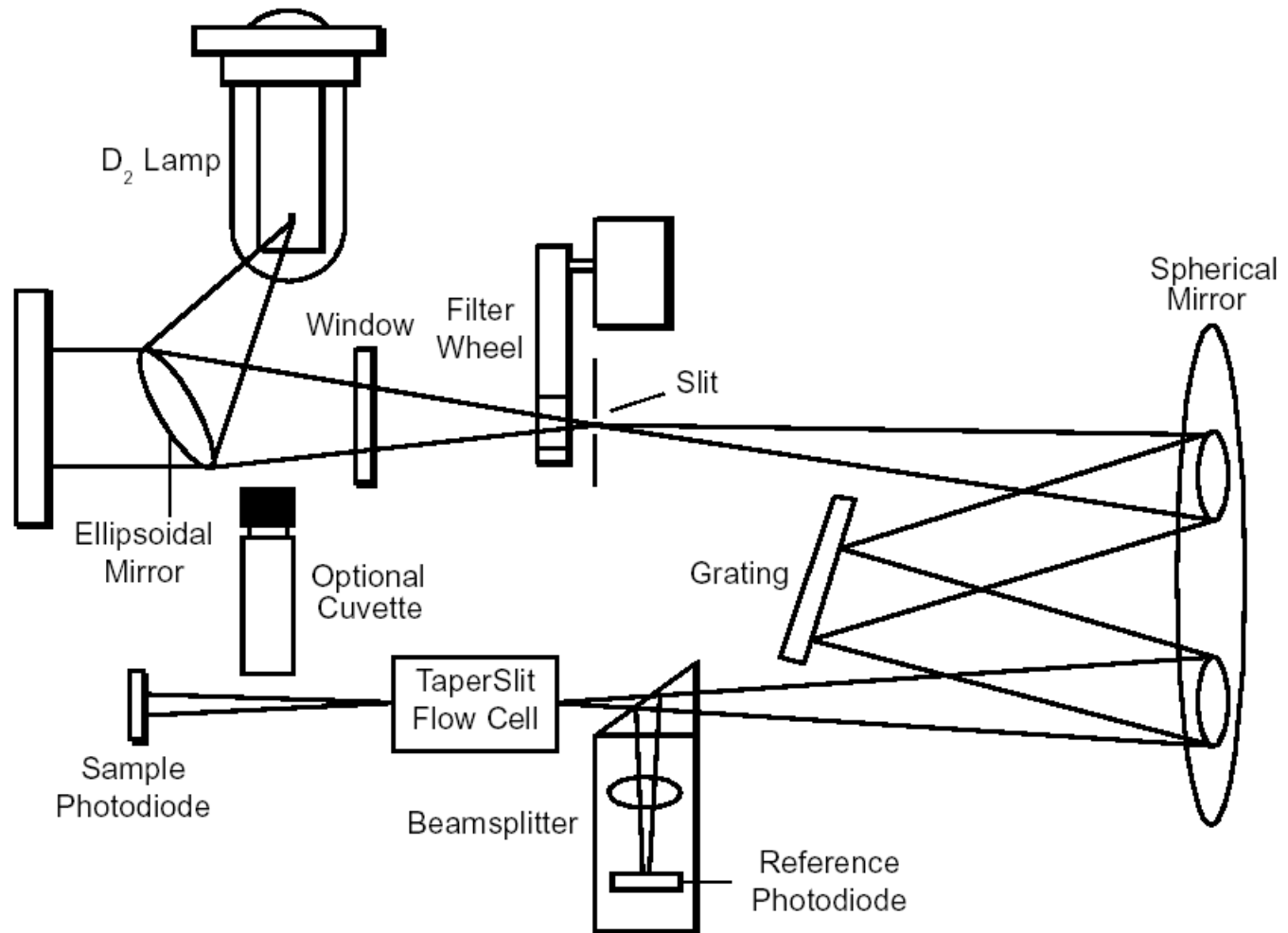
Molar extinction coefficient

Sample concentration (moles/l)

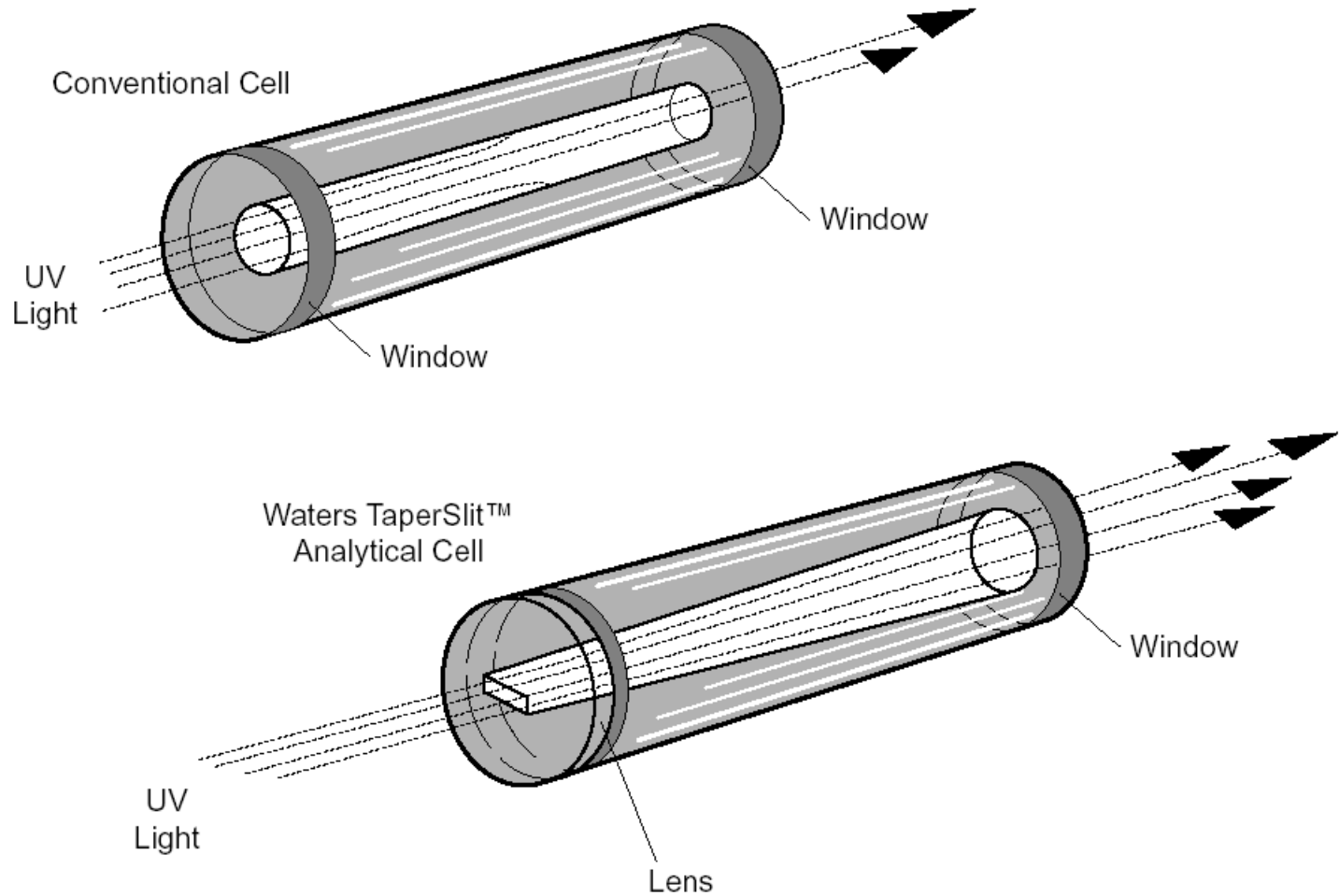
cell path length (cm)

$$A = \epsilon b c$$

Schematics of a Variable Wavelength UV/Vis Detector



Flow Cell Design

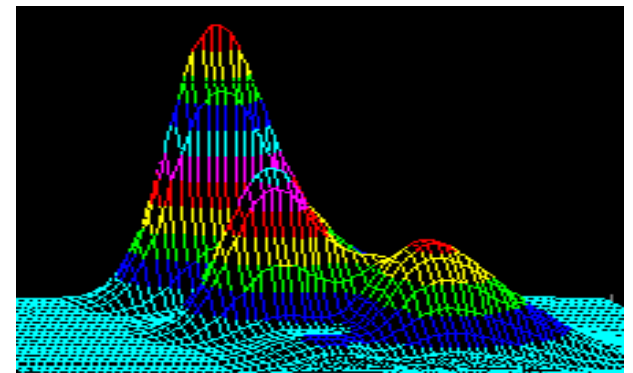


The Photodiode Array (PDA) Detector

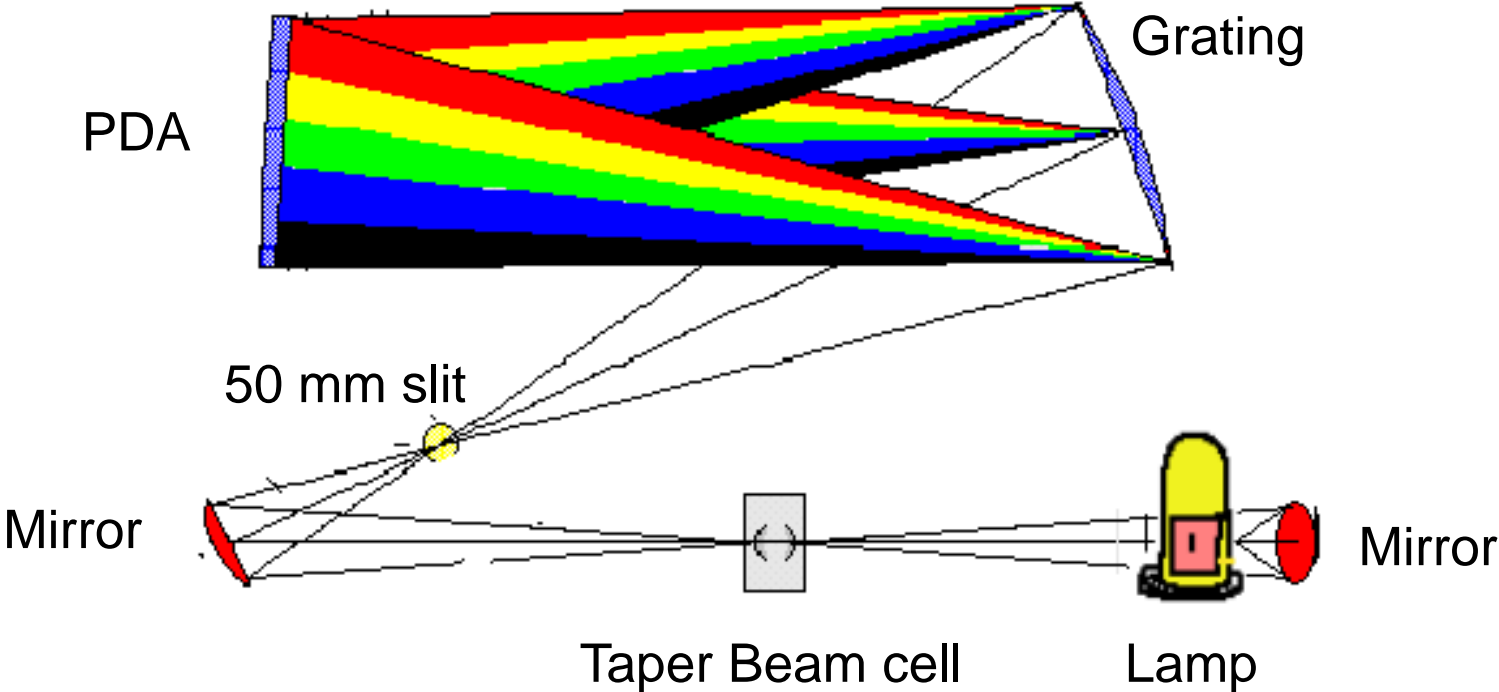
A photodiode array detector (PDA), also known as a diode array detector (DAD), provides UV spectra of eluting peaks while functioning as a multi wavelength UV/Vis absorbance detector. It facilitates peak identification and is the preferred detector for method development.

- Advantages

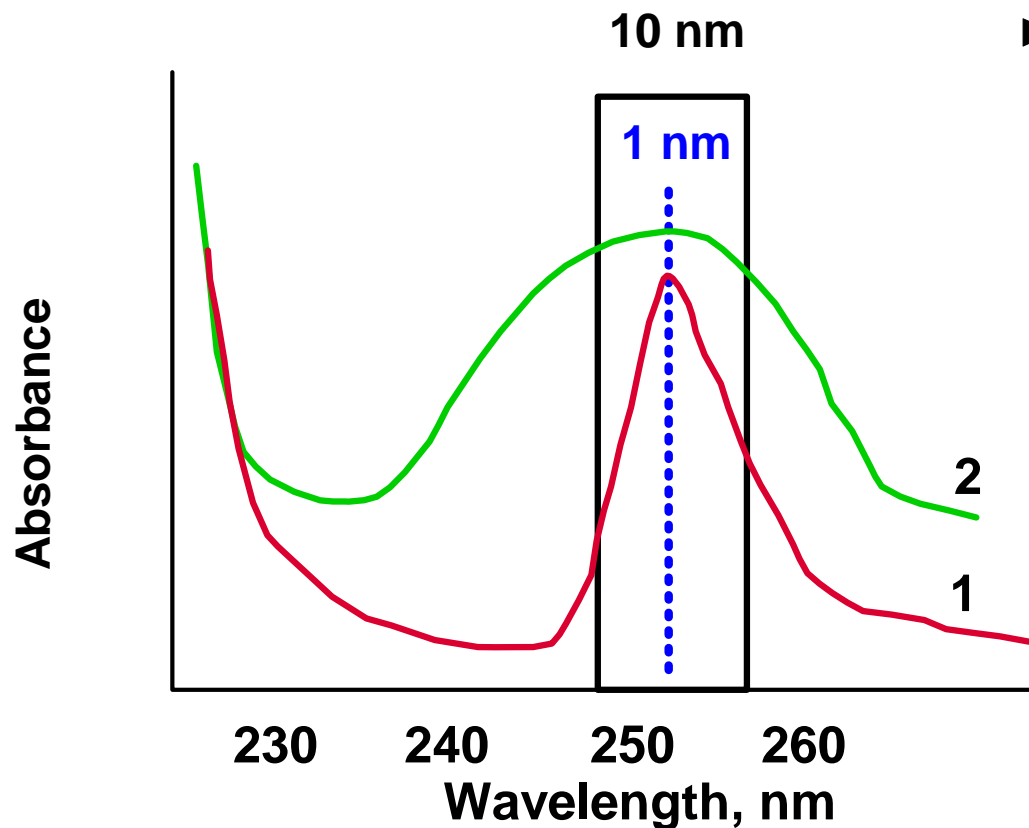
- ◆ Can provide UV spectra across peaks
- ◆ Can be used to assess peak “purity”/ spectral homogeneity
- ◆ Can be used to track peaks via their UV spectra (library search)



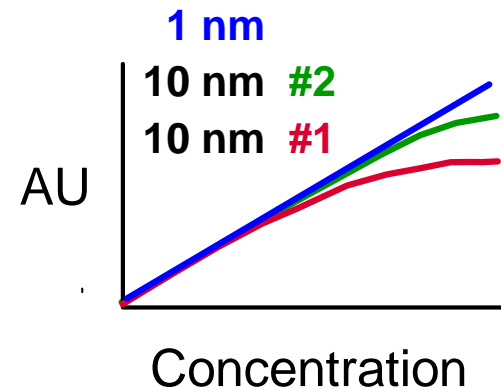
Schematic of a PDA Detector



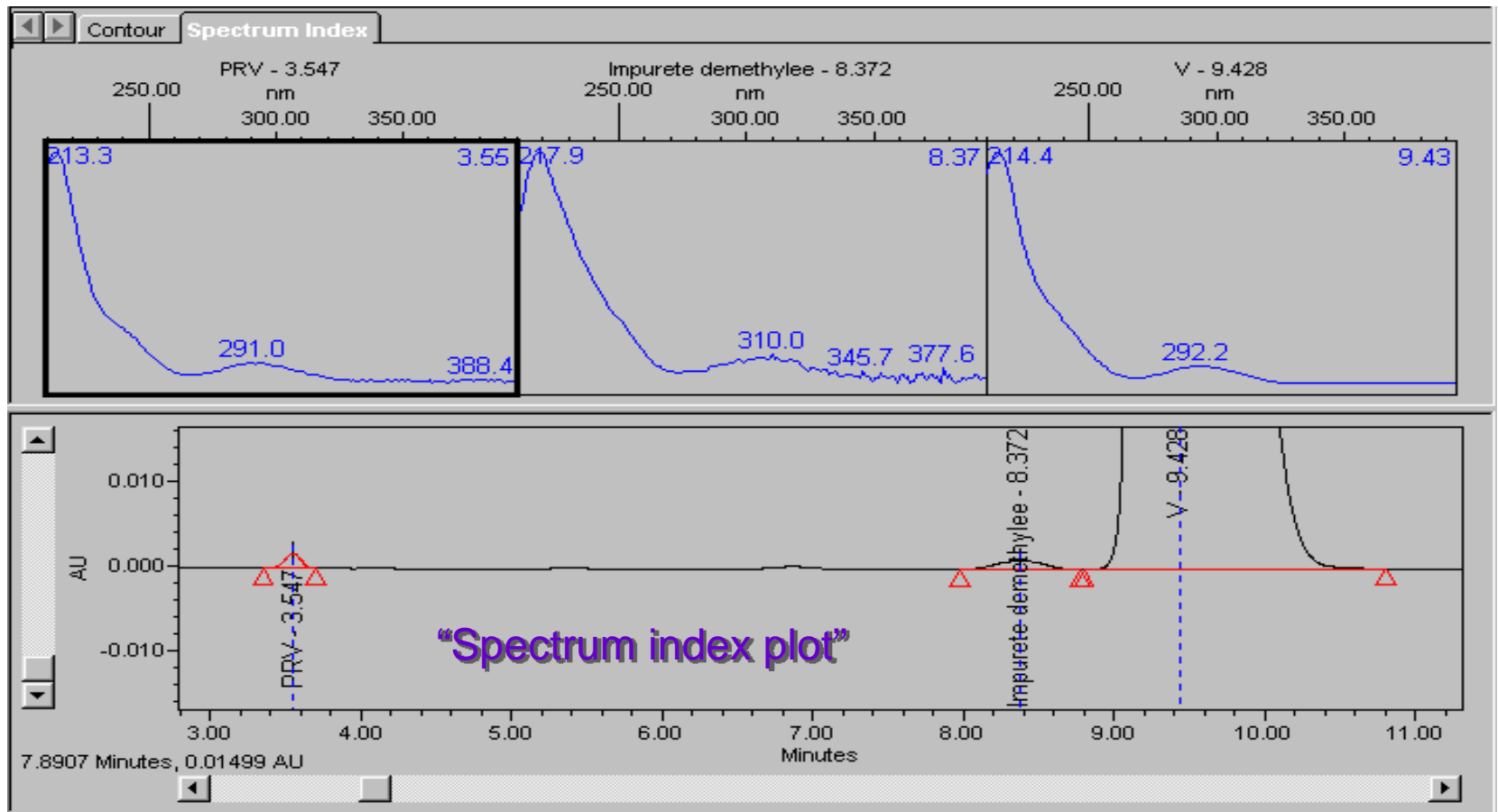
Optical Resolution Influence on Linearity



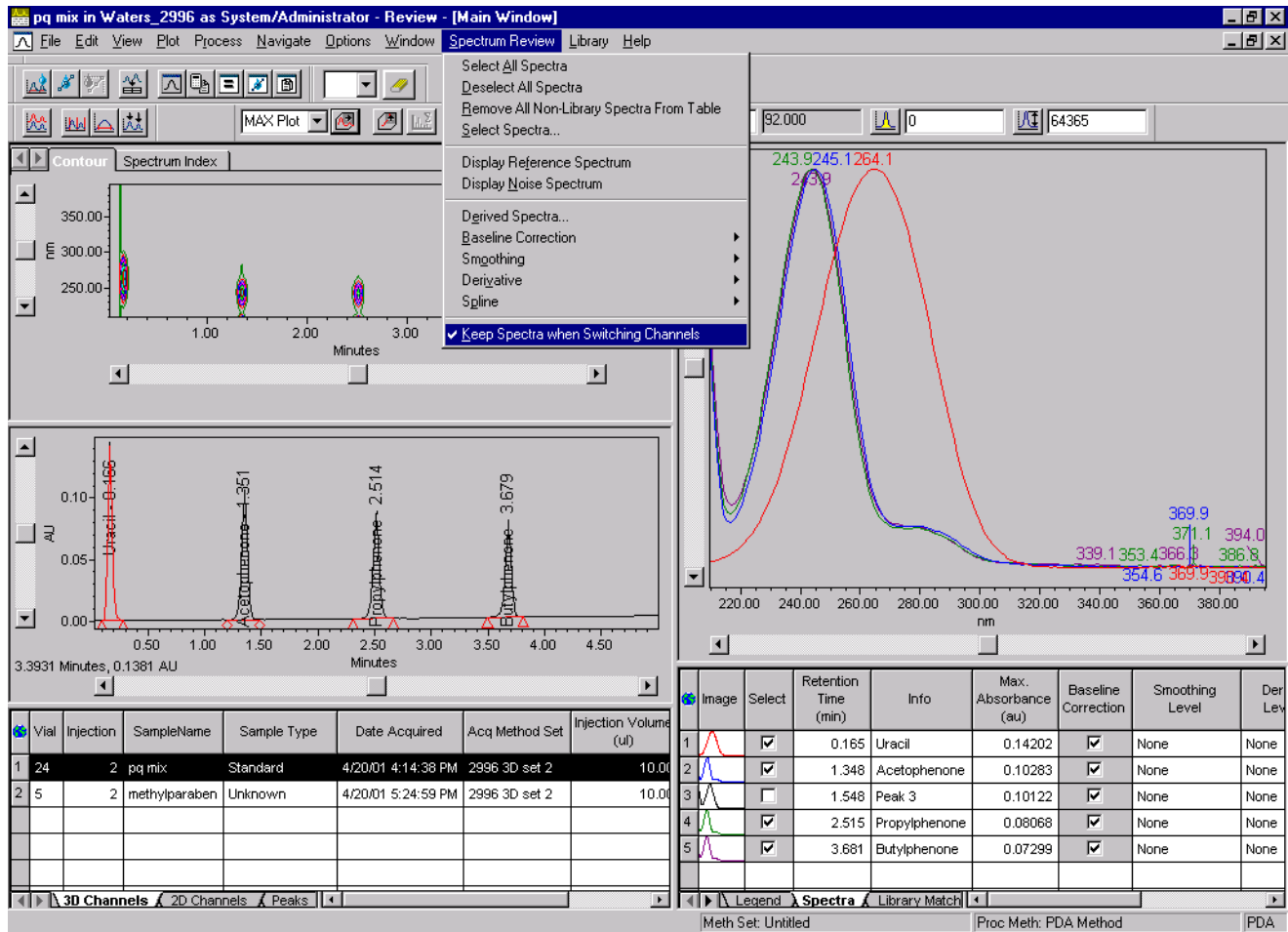
- ▶ Narrow spectral peak #1 with a wide bandpass (10 nm) is non-linear



Example of UV Spectra



Example of Library Search



U.V. Cutoffs for Some Common Solvents

Remember that 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

UV cutoff = Wavelength at which solvent absorb 1 AU

All wavelengths reported in nm.

Fluorescence

A fluorescence detector monitors the emitted fluorescent light of the HPLC eluent in the flow cell

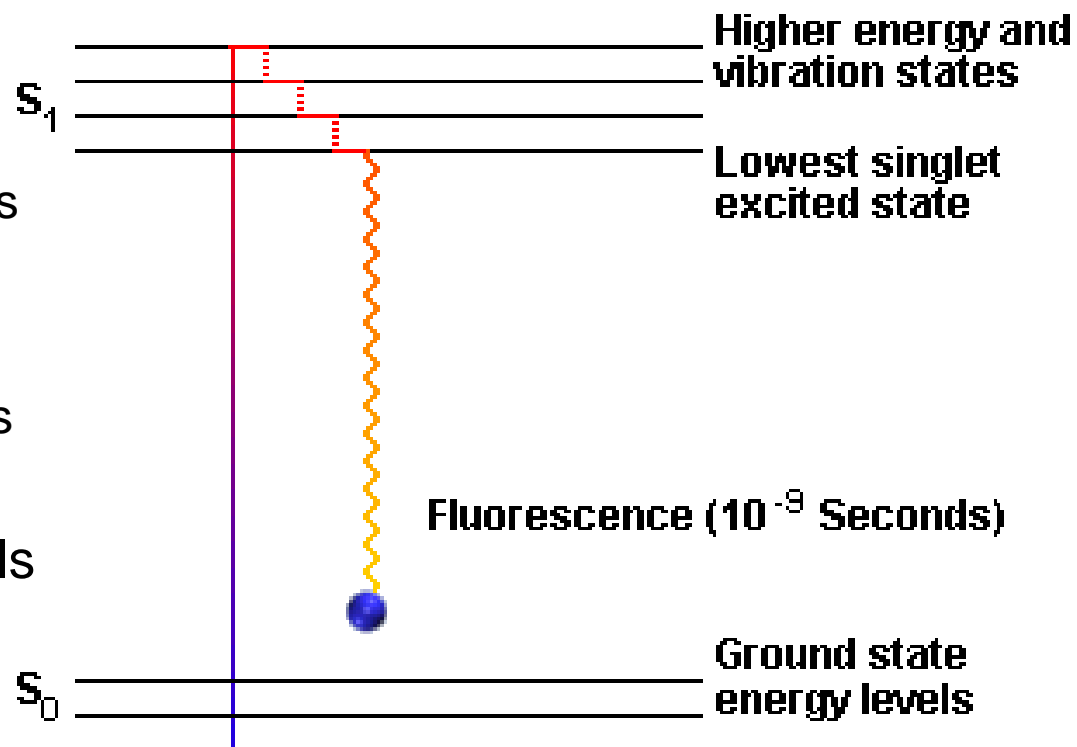
- Increased selectivity
- Analytes not only absorb UV/Vis radiation but also release the energy in the form of light of longer wavelength.
- This property is typically associated with non-ionic molecules which are strongly conjugated and have rigid structures.
- Sensitivity is in the femto gram region
- Detector is sensitive to the presence of dissolved gasses and other “quencher’s”

Fundamentals of Fluorescence

Conjugated and aromatic systems are most likely to exhibit fluorescence

Some Applications

- Environmental
 - ◆ Polycyclic Aromatic Hydrocarbons
 - ◆ Phenols, carbamates
- Food and Beverage
 - ◆ Aflatoxins in Food Products
 - ◆ Dyes
- Biotech and Pharmaceuticals
 - ◆ Derivatized amino acids (AccQ-Tag or OPA)

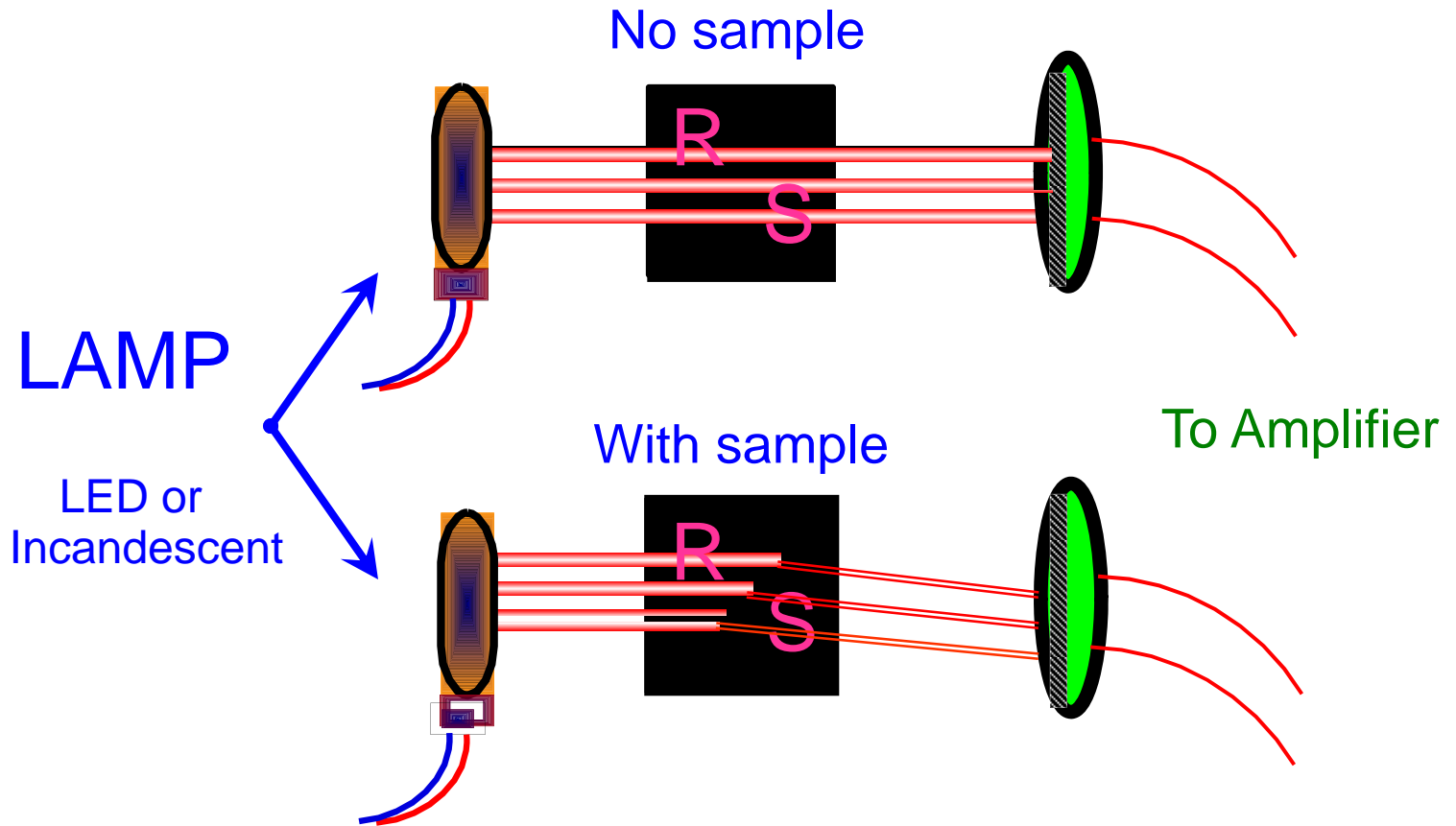


Refractive Index Detectors

A refractive index detector measures the refractive index change between the sample cell containing the eluting analyte and the reference cell purged with pure eluent

- First HPLC detector developed
- Typically referred to as Universal detectors
 - ◆ Detects all dissolved solutes- “non-specific”
 - ◆ RI response depends on the difference in RI between mobile phase and solute(s).
 - ◆ Sensitivity reaches maximum when RI differences are greatest.
- Utilizes Snell’s Law Principles
 - ◆ μg sensitivity but only for isocratic runs
 - ◆ Commonly used for:
Sugars, Polymers and Fatty Acids

Schematic of an RI Detector

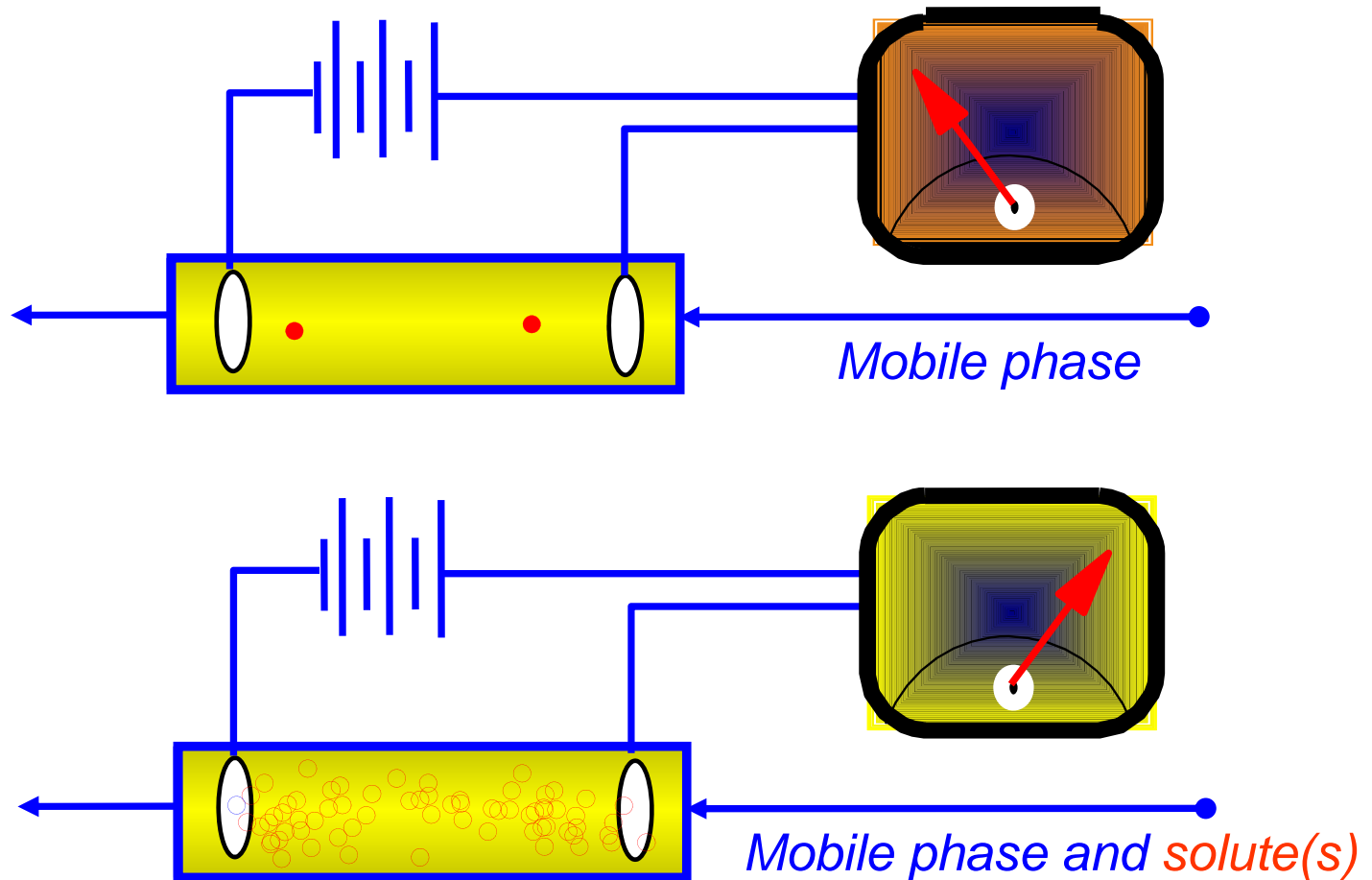


Conductivity Detectors

A conductivity detector measures the electrical conductivity of the HPLC eluent stream

- Generally used for ion Chromatography
- Detects the ability of analyte to carry a charge
 - ◆ solutes are ionic (that is, acid and bases)
 - ◆ Inorganic anions and cations
- Sensitivity is at the low ppb (ng/L) level
- Typically used with isocratic systems but can utilize isoconductive gradients

Schematics of Conductivity Detector

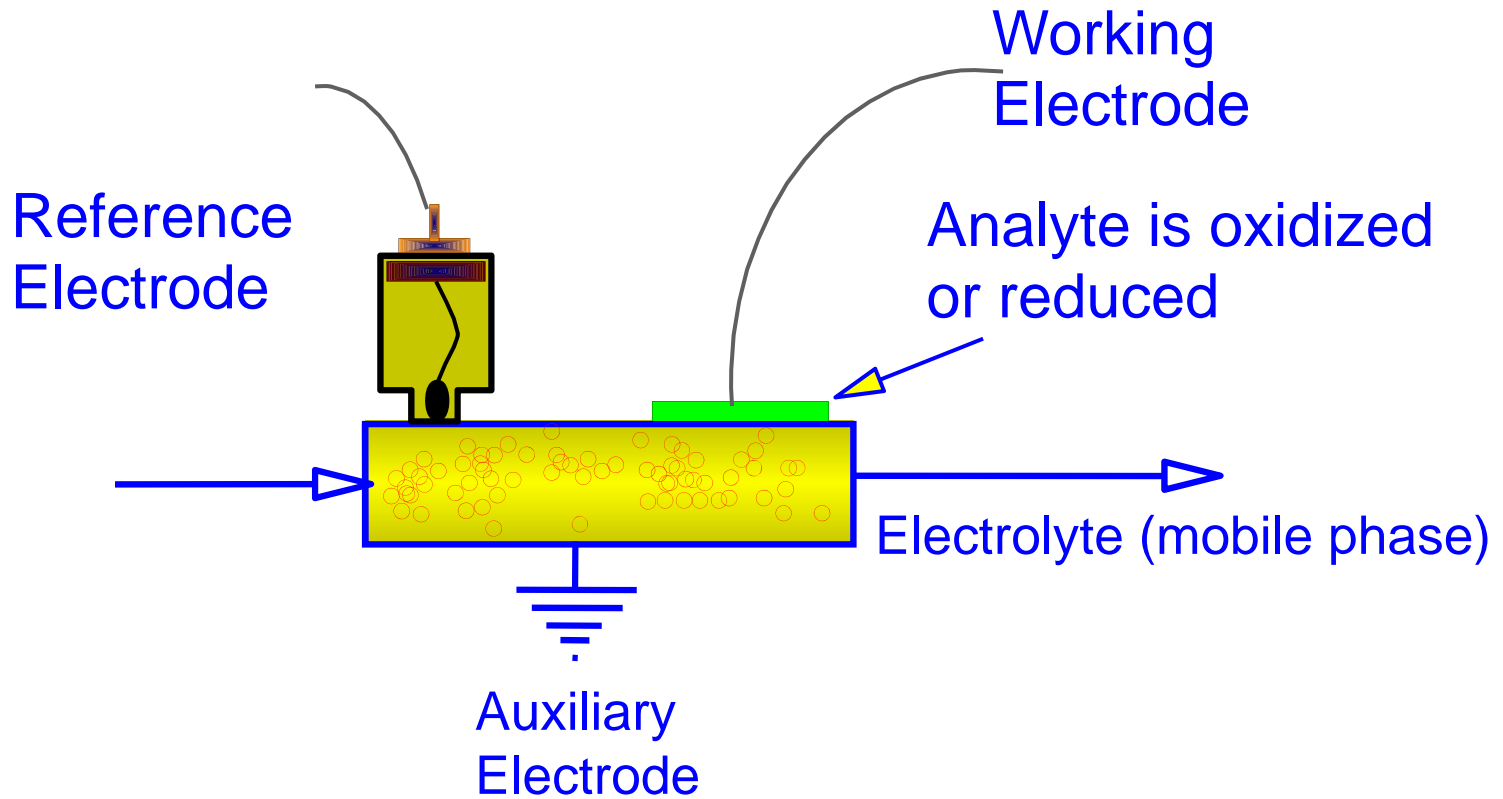


Electrochemical Detector (ECD)

An electrochemical detector (ECD) measures the electrical current generated by electroactive analytes in the HPLC eluent between electrodes in the flow cell

- “Destructive”-detection mode
- Sample is electrochemically modified in the cell with the concomitant generation of current detected as response.
- High sensitivity with picogram (10^{-12} grams) to high femtogram (10^{-15} grams) range.
- Proper selection of buffer, electrode and voltage is critical to success as well as conditioning the mobile phase.

Schematics of Electrochemical Cell



Some Compound Types Sensed by ECD

Oxidation

Phenolic

Oximes

Dihydroxy

Mercaptans

Peroxides

Hydroperoxides

Aromatic Amines, diamines

Purines

Heterocyclic Rings

Reduction

Ketones

Aldehydes

Oximes

Conjugated acids

Conjugated esters

Conjugated unsaturation

Activated halogens

Aromatic halogens

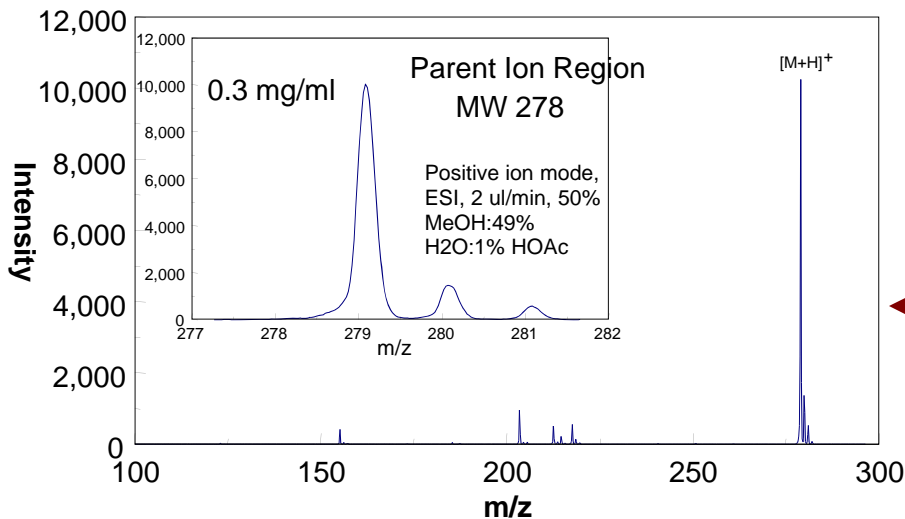
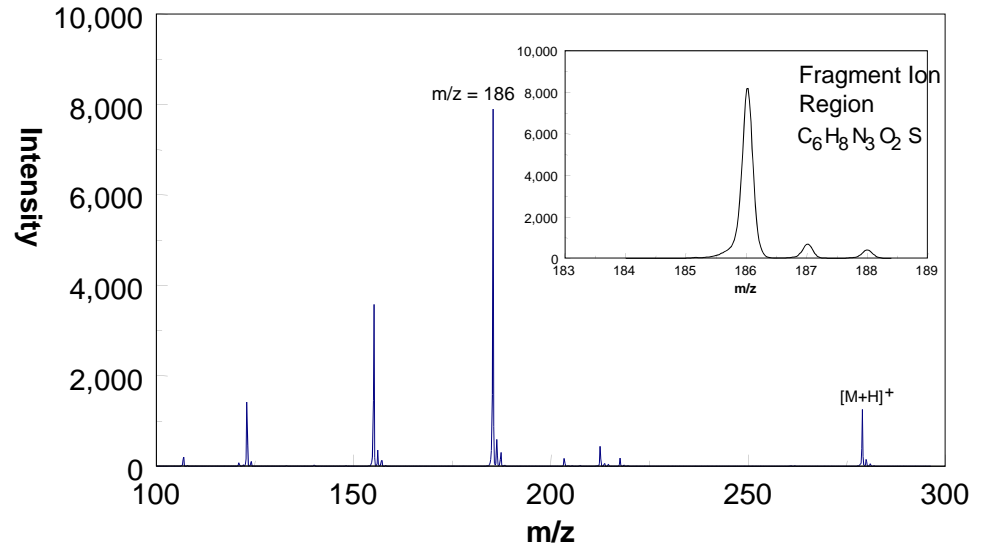
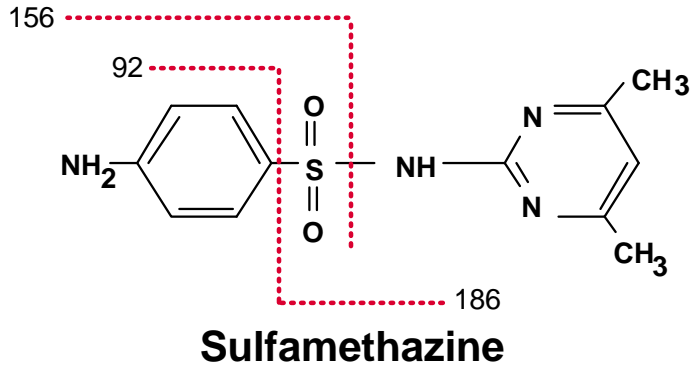
Nitro compounds

Heterocyclic rings

Benefits of MS Detection

- Additional information
 - ◆ Molecular weight
 - ◆ Structure
- Better Selectivity
 - ◆ Possible Quantification of “UV co-eluted compounds”
 - ◆ Shorter analysis
- Sensitivity
 - ◆ In Sir mode, MS detection can be much more sensitive than UV detection (100 times)
- Cross check UV and MS library search
 - ◆ Improved compounds characterization and differentiation
- Speed up method development
 - ◆ Improve impurities identification
 - ◆ Improve impurities characterization

Additional Information

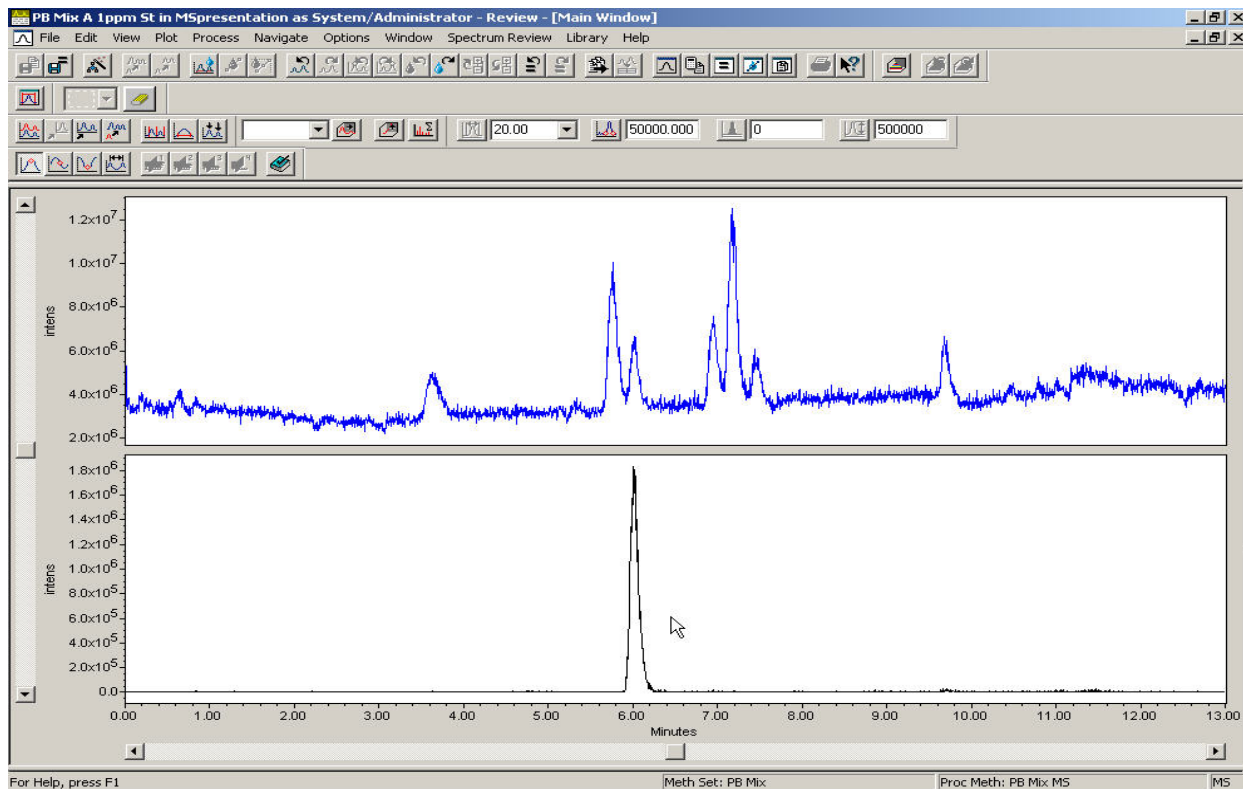


↑
**At High Cone Voltage
(fragmentation occurs)**

←
**At Low Cone Voltage
(typically a Protonated Molecule is
observed)**

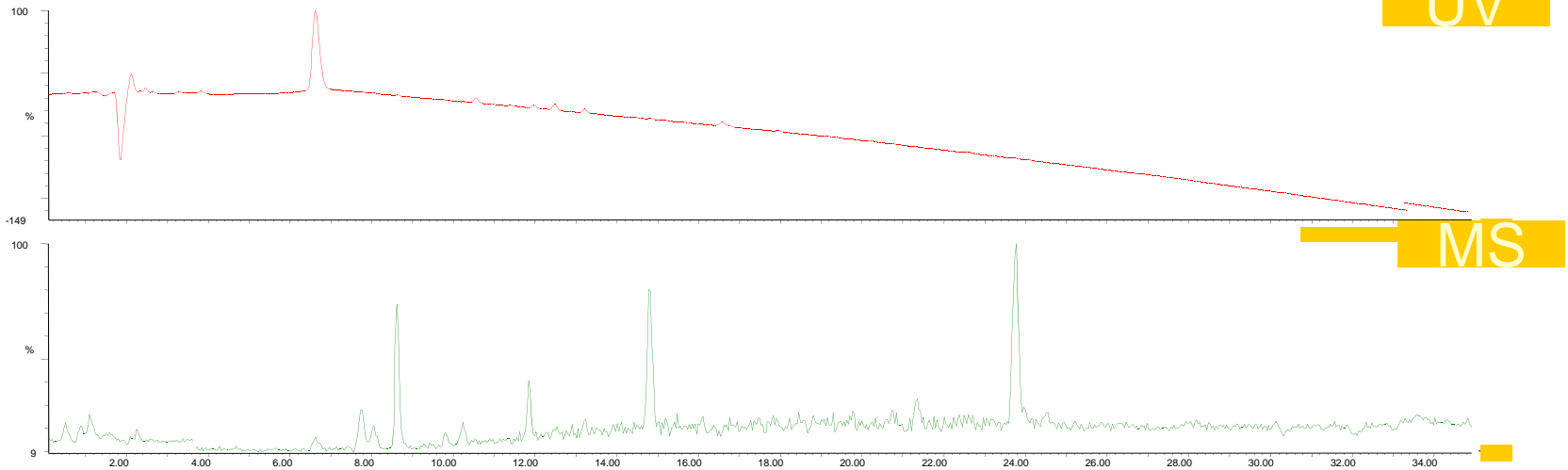
Better Selectivity

- Need for Selectivity.
 - ◆ Matrix interference
 - ◆ very similar structures, impossible separation



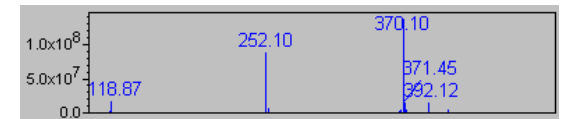
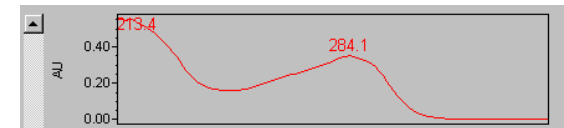
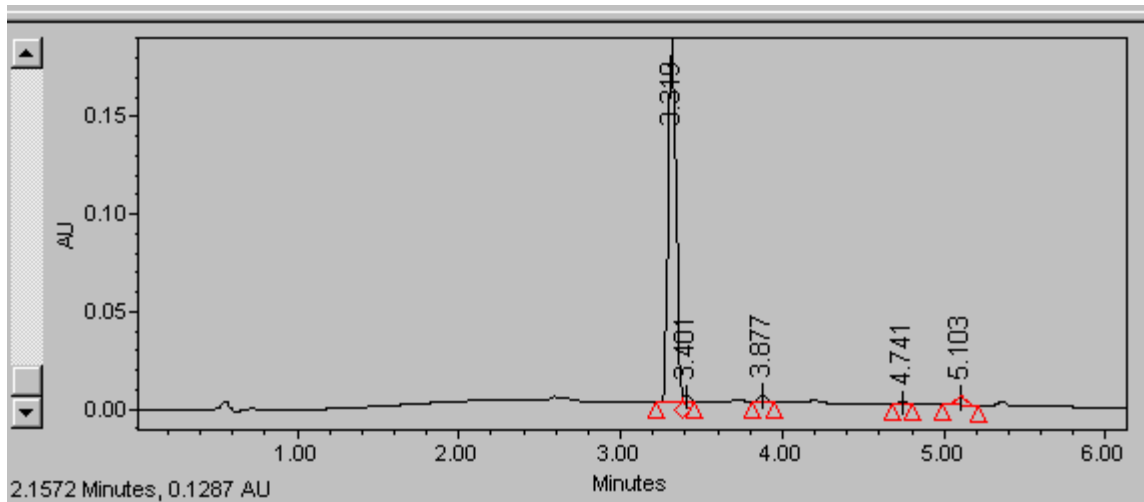
Sensitivity

- The molecules are difficult to detect.
 - ◆ Zero or poor UV absorbance or Fluorescence
 - ◆ Matrix interference



UV and MS Libraries

- Need for better Qualitative Information
 - ◆ Component Confirmation
 - ◆ Mass Spectrum (next to UV-Spectrum)
 - ◆ Library
 - ◆ Valuable information on Unknowns



Choosing a Detector for HPLC

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