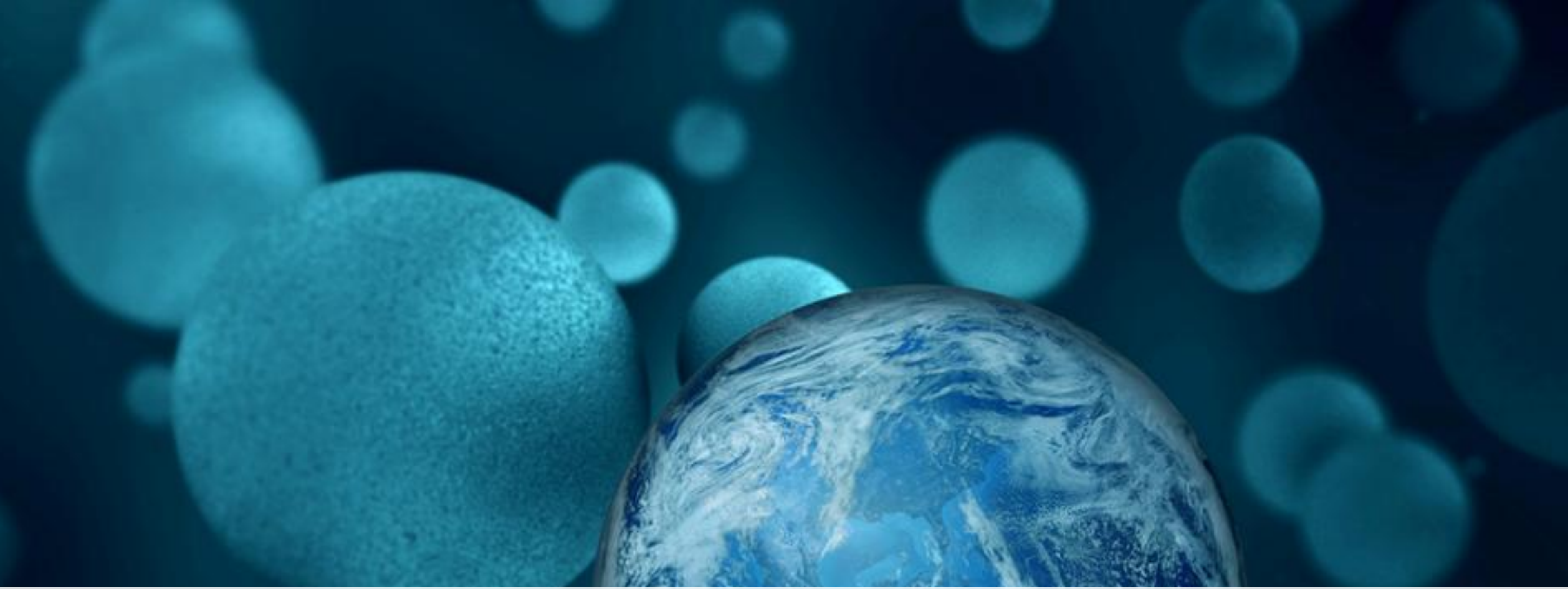

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Introduction to UV-based Detection

Jan Pettersson
Nordic HPLC & Chromeleon CDS Support Specialist
Thermo Fisher Scientific

UV Vis Detectors

- The ideal detector ?

- Why do we get a signal?

- Optics
  - Lamps
  - Flow cell
  - Band and slit width
  - Data collection rate and time constant
  - Reference
  - Stray light, refractive index effects & noise

Thermo Scientific™ Vanquish™ HPLC

UV Vis Detectors – The Ideal Detector?

- A workhorse for detection and quantification of organic compounds

VWD

MWD

DAD

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- The ideal detector ?

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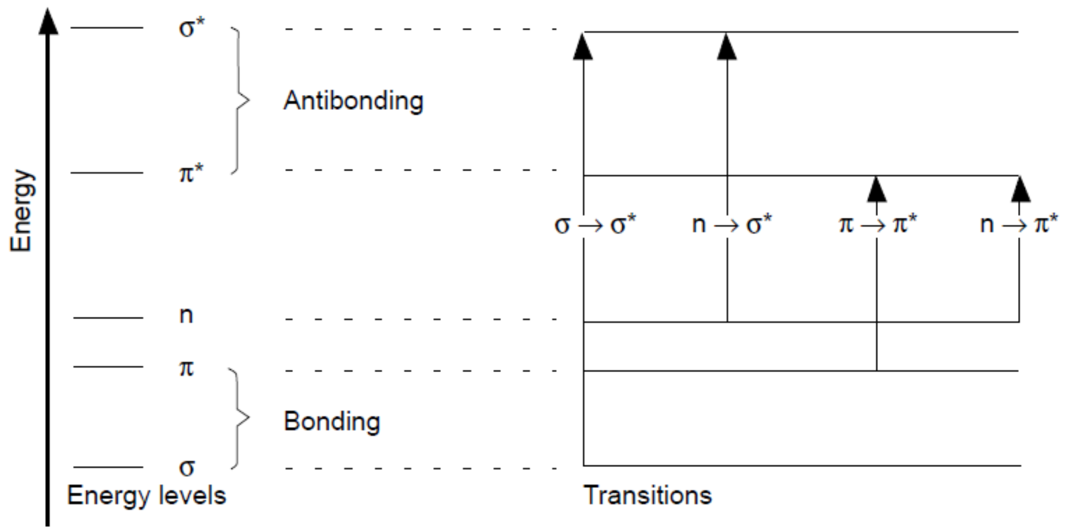
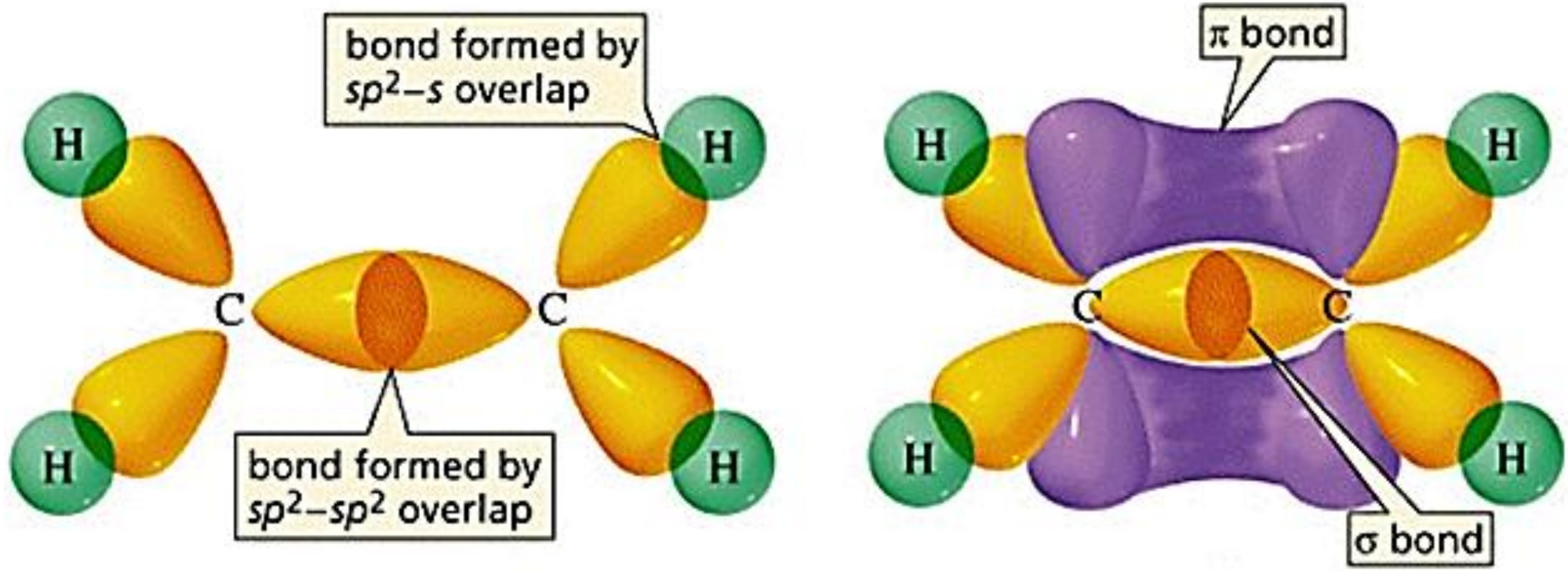


Why Do We Get a Signal?

Very simplified:

- The light from the lamp excites the electrons in the sample to a higher state of energy.
- Different molecules absorbs light at different frequencies.
- The shorter the wavelength the higher the energy

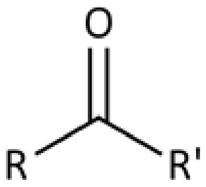
Why Do We Get a Signal?



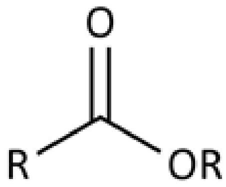
UV Chromophores

Ethylene
190 nm

Amine
195 nm



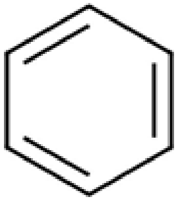
Ketone
195 nm



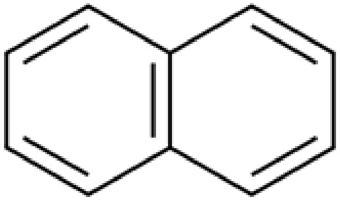
Ester
205 nm

Nitro
310 nm

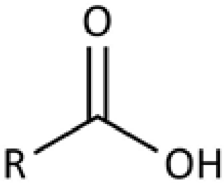
Methanol
205 nm



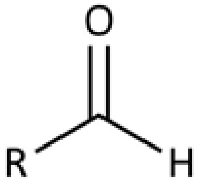
Phenyl
202, 255 nm



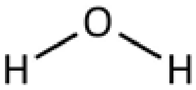
Naphthyl
220, 275 nm



Carboxyl
200-210 nm



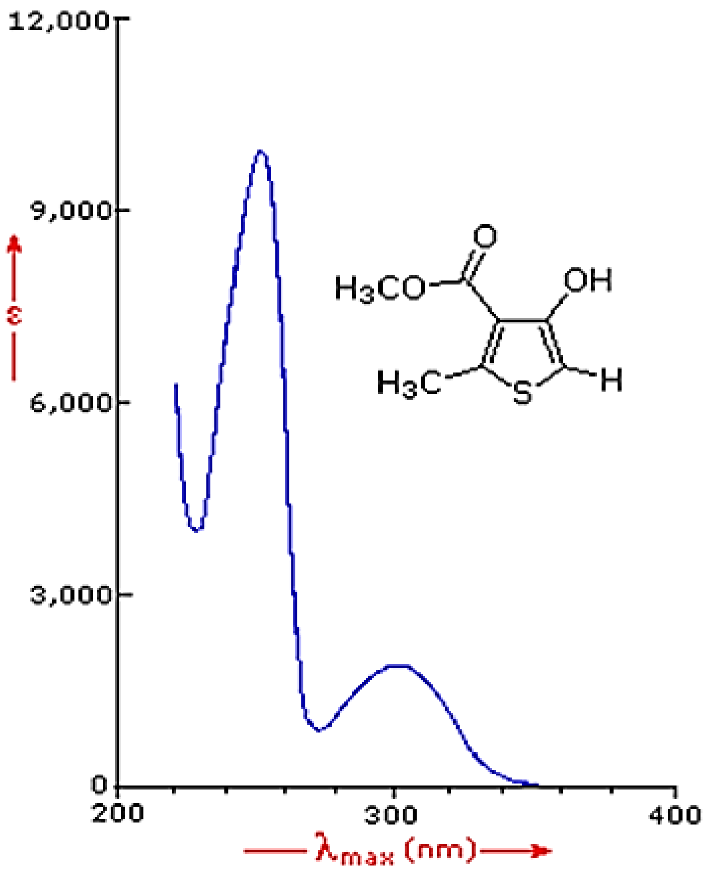
Aldehyde
210 nm

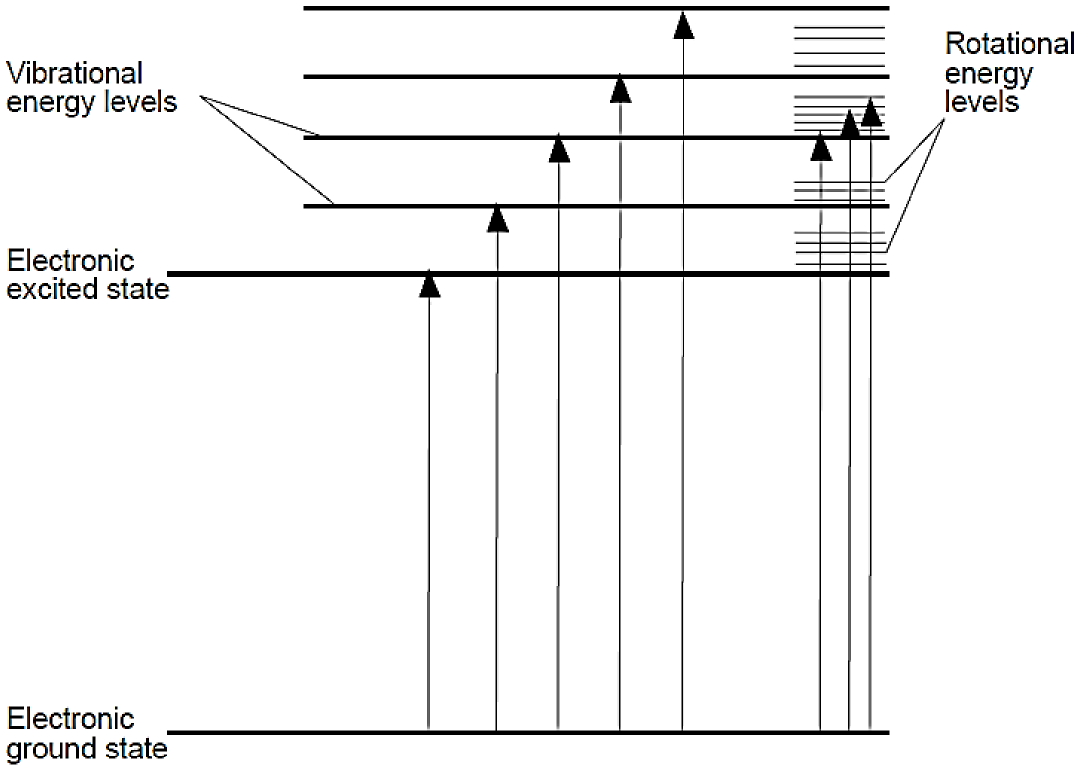


Water
190 nm

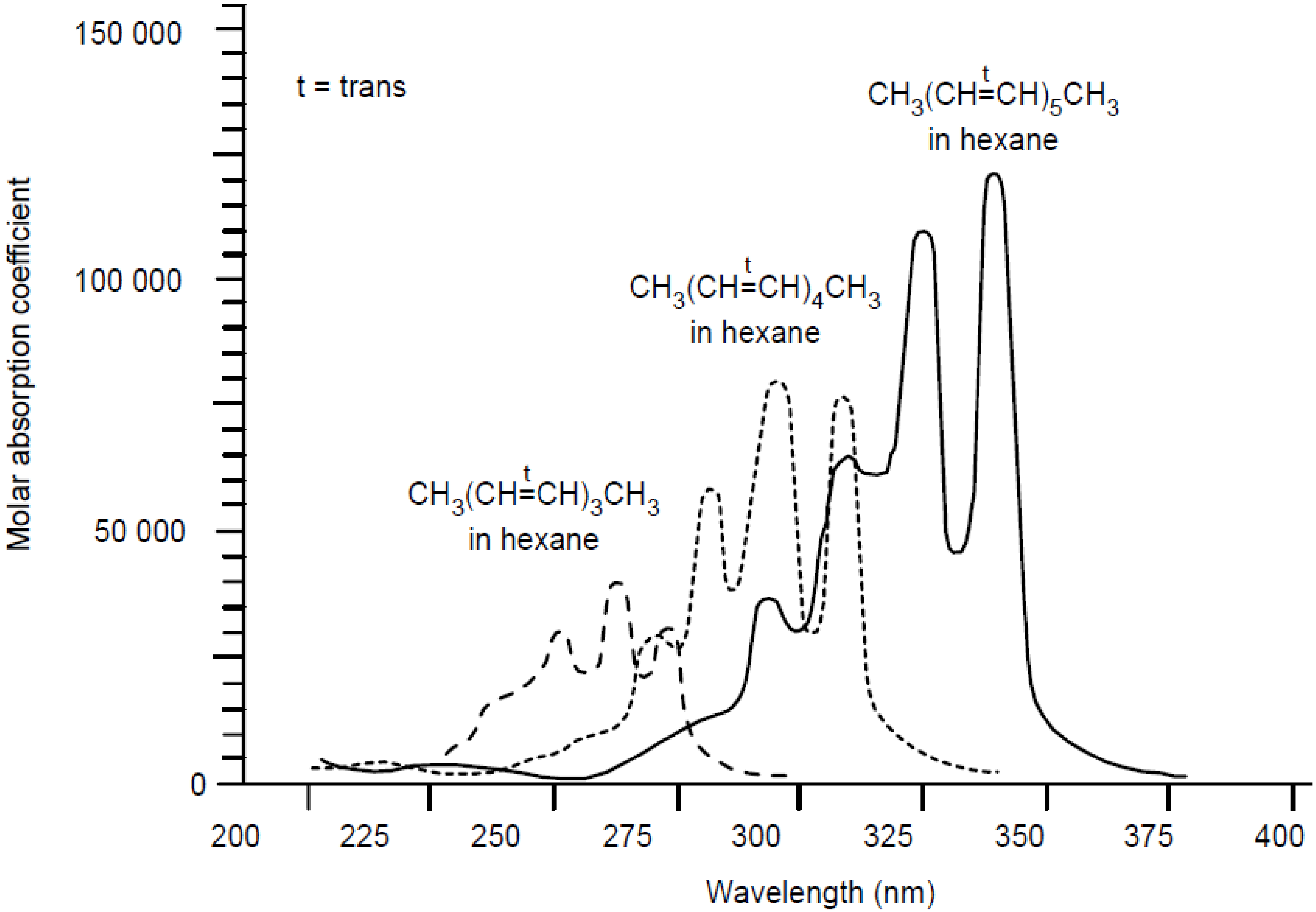
Acetonitrile
190 nm

UV Spectra


12,000
9,000
6,000
3,000
0
ε
200
300
400
λmax (nm)
O
H3CO
OH
H3C
S
H


Vibrational energy levels
Rotational energy levels
Electronic excited state
Electronic ground state

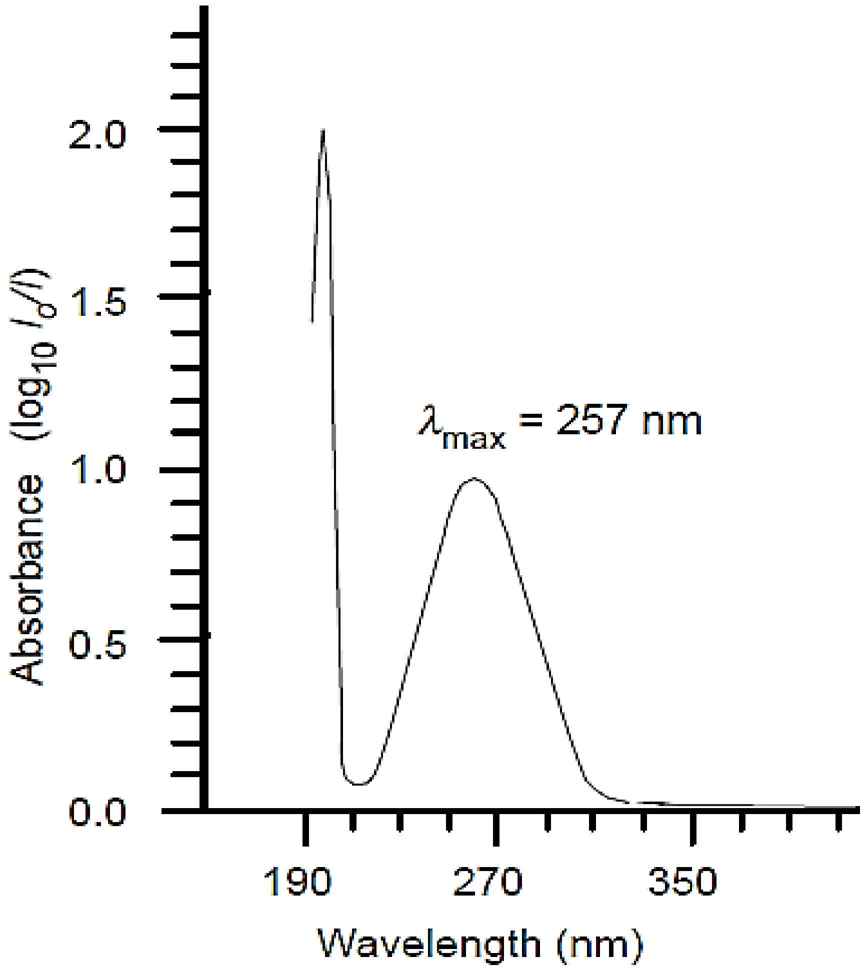
Conjugation Effects



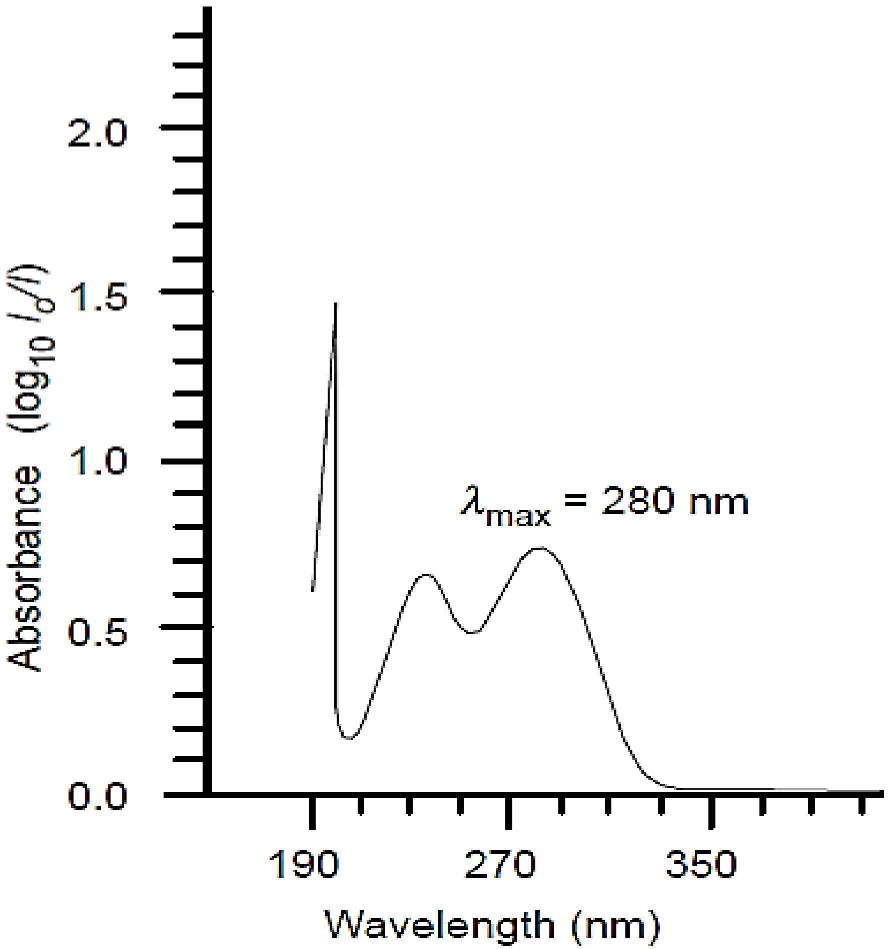
Solvent Effects – Why Water and ACN are Popular

Solvent (nm)	Minimum wavelength
Acetonitrile	190
Water	191
Cyclohexane	195
Hexane	201
Methanol	203
Ethanol	204
Ethoxyethane	215
Dichloromethane	220
Trichloromethane	237
Tetrachloromethane	257

Solvent Effects – Polarity

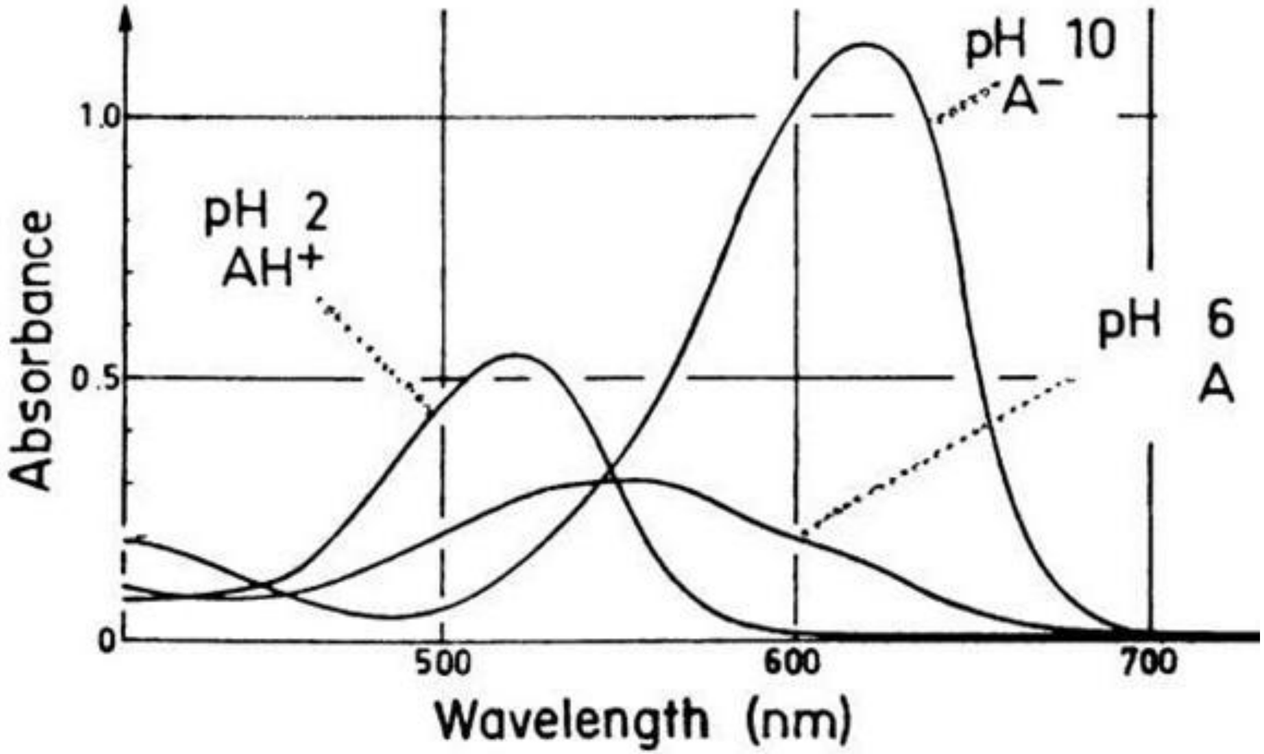


UV-Visible spectrum of acetone in water

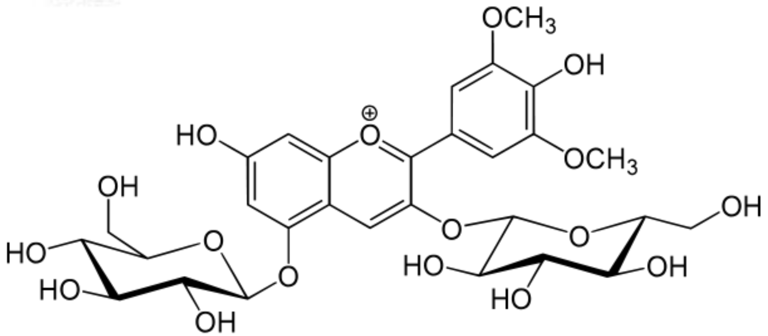


UV-Visible spectrum of acetone in hexane

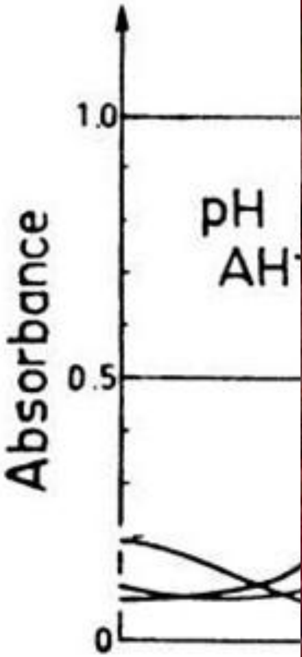
pH Effects



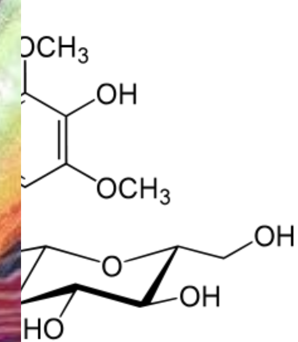
Anthocyanin pigment in buffers of varying pH



pH Effects



n pigment in
arying pH



Temperature Effects

- Expansion of the solvent may change absorbance
- Temperature may affect equilibria
- Changes in refractive index with temperature can be significant
- Convection currents cause different temperatures to occur in different parts of the cell

UV Vis Detectors

- The Ideal detector ?
- Why do we get a signal?
- Optics
  - Lamps
  - Flow cell
  - Band and slit width
  - Data collection rate and time constant
  - Reference
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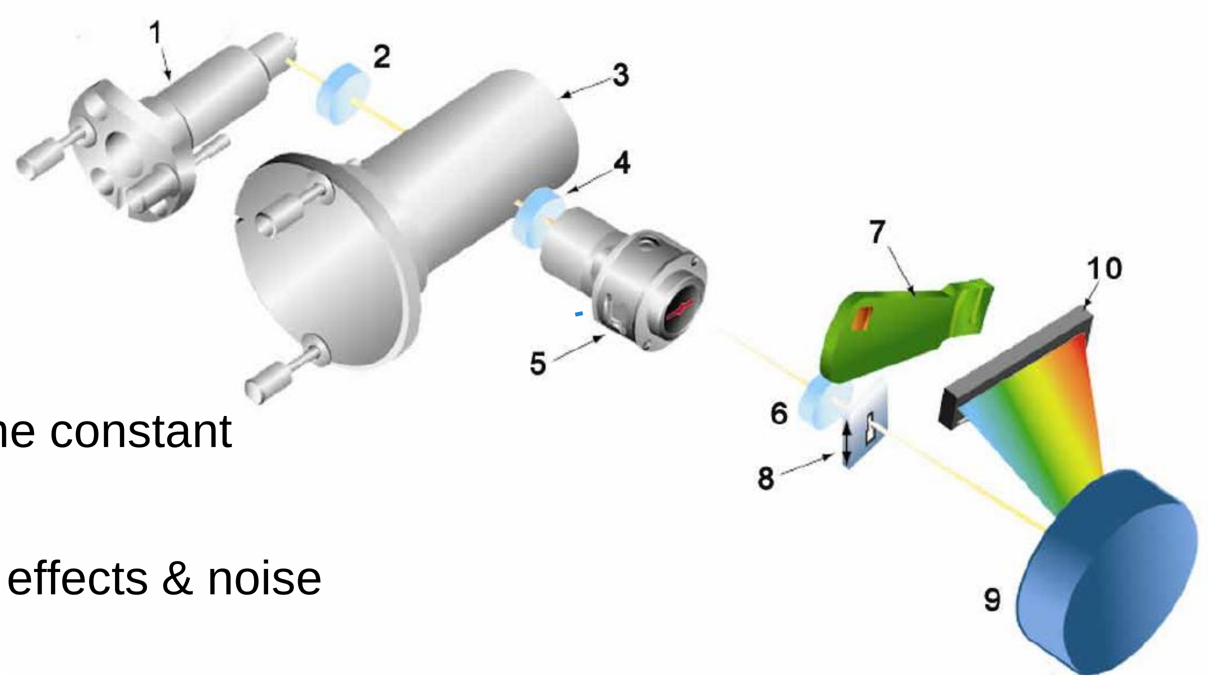

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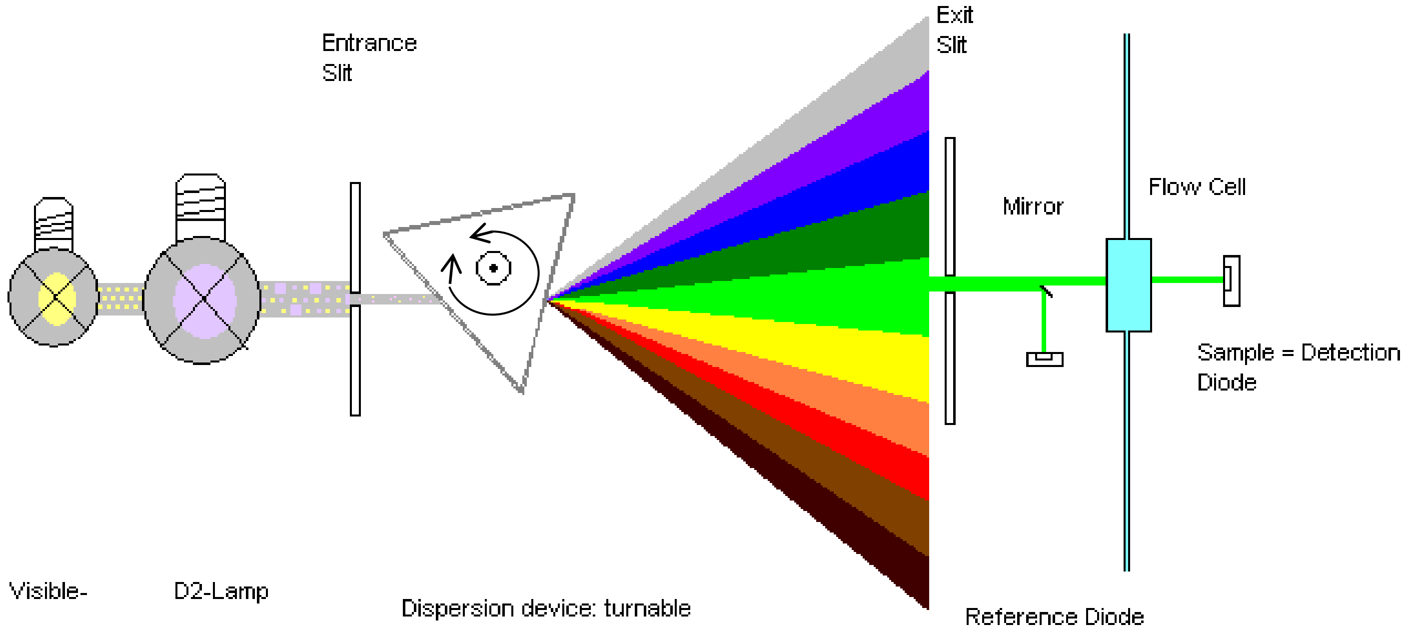
Operating Principle: Variable Wavelength Detector (VWD)

Forward optics design

- Only the selected wavelength passes the flow cell
- A part of the light beam is redirected to the reference diode

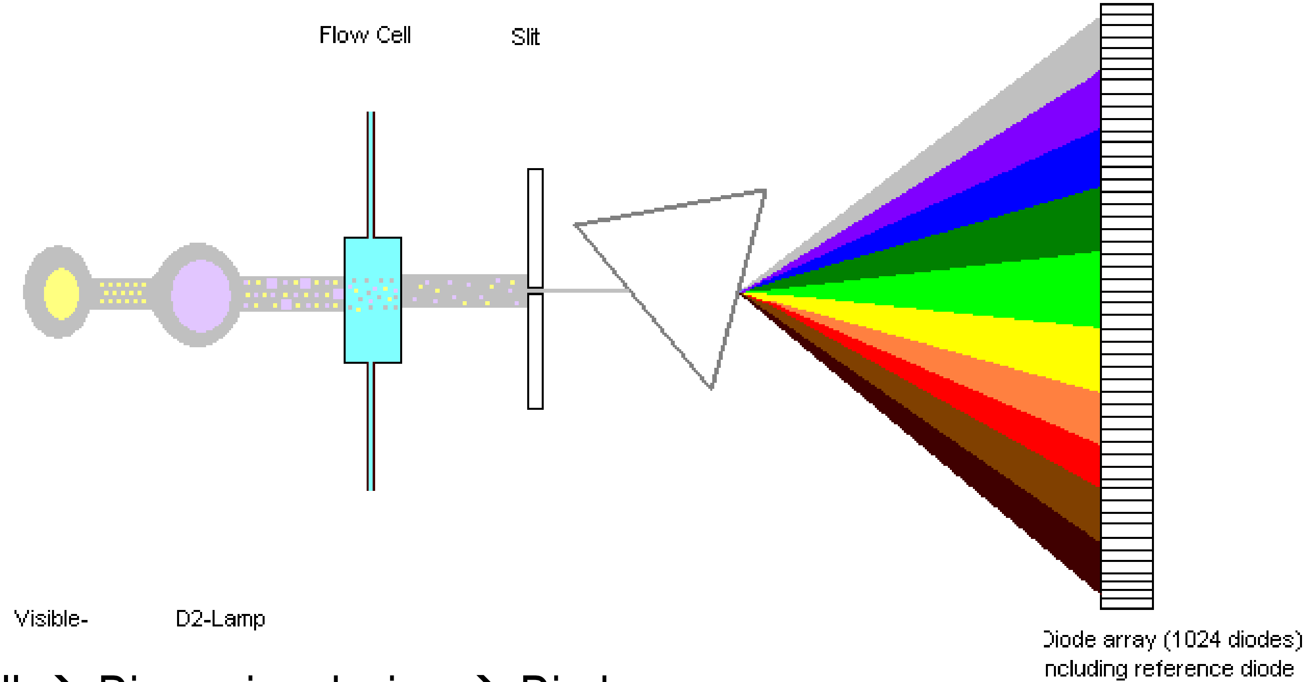


Light source → Dispersion device → Flow cell → Sample diode

Operating Principle: Wavelength Diode Array Detctor (DAD)

Reversed optics design

- Light beam passes the flow cell before being diffracted
  - No true reference signal can be obtained
- Any diode or bunch of diodes can be selected as a reference



Light source → Flow cell → Dispersion device → Diode array

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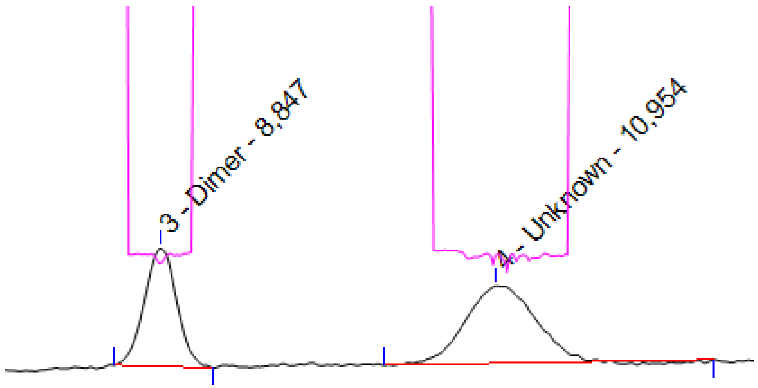
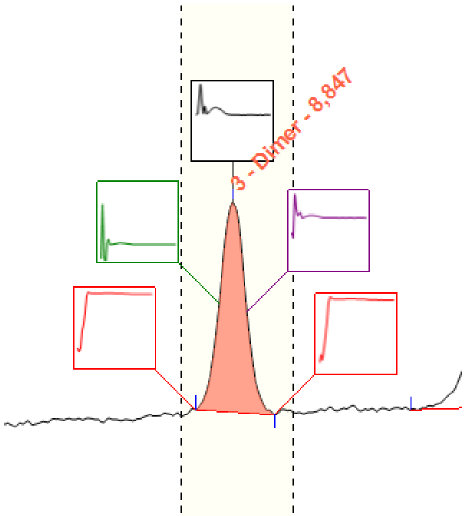
Uses of Diode Array Detectors



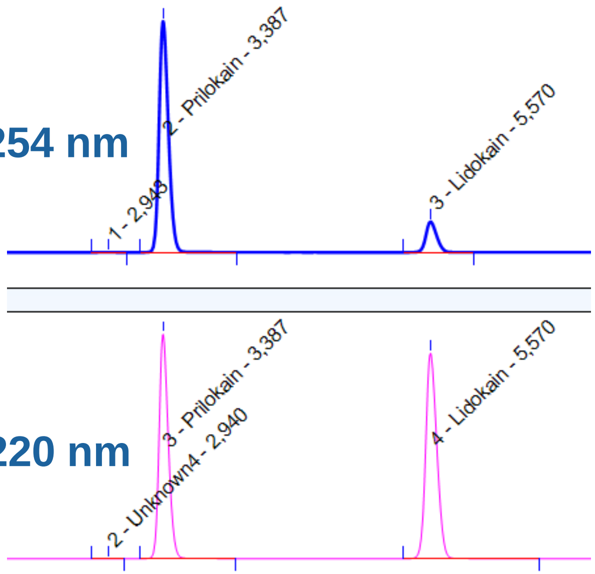
Peak purity measurement



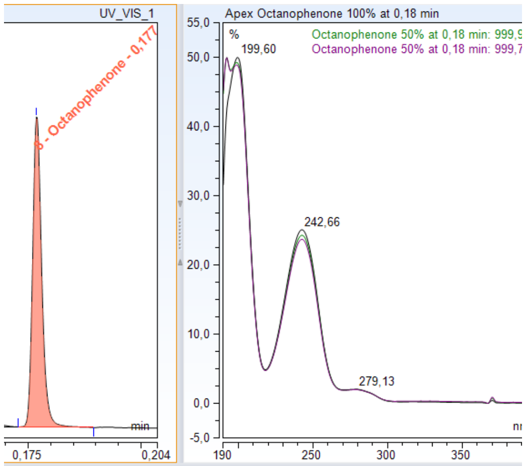
Dynamic spectral acquisition and identification

254 nm

220 nm

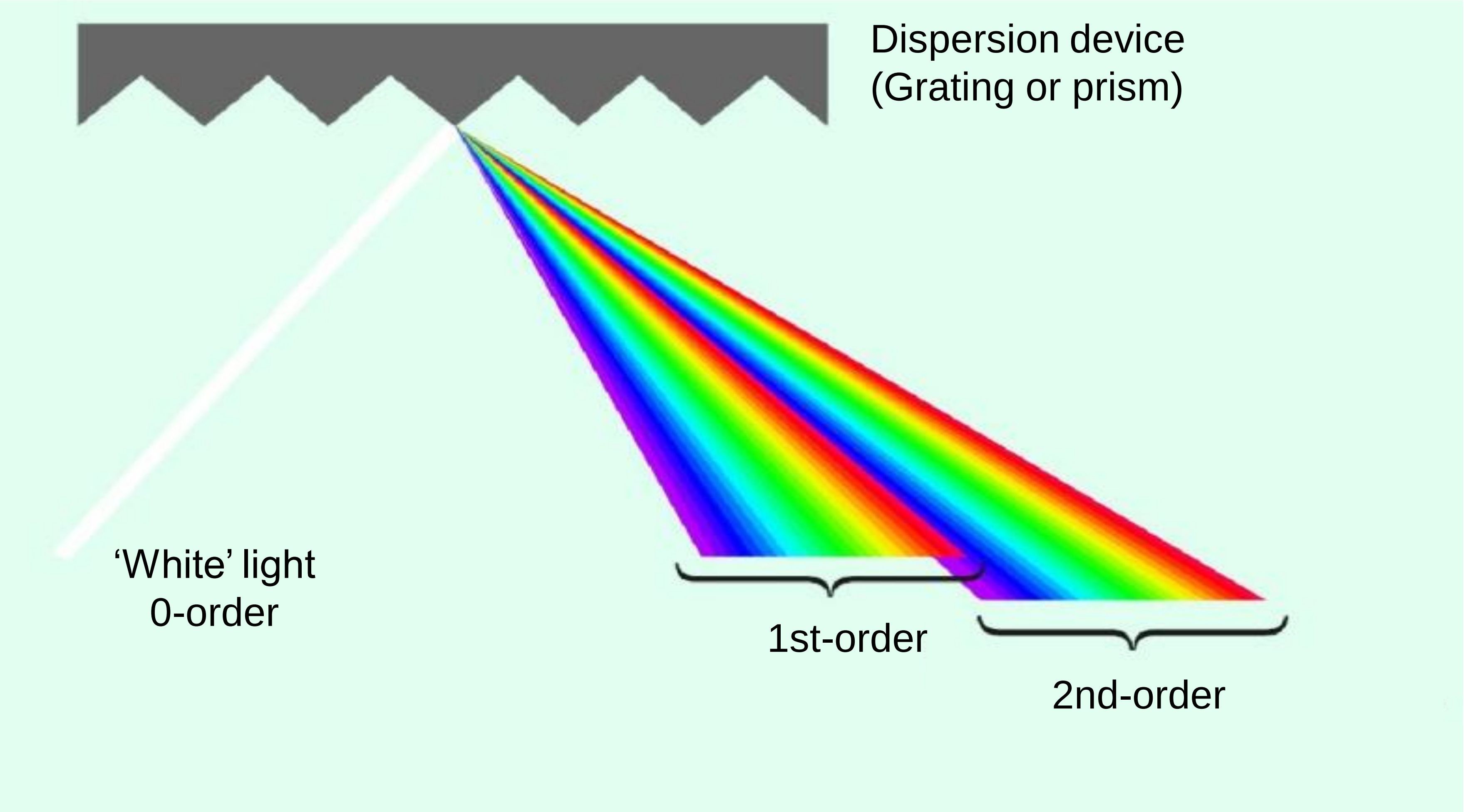


Signal deconvolution/ Multiple wavelength acquisition



Operating Principle – 2nd Order Filter

- Light diffraction at a dispersion device results always in different orders of light segmentation



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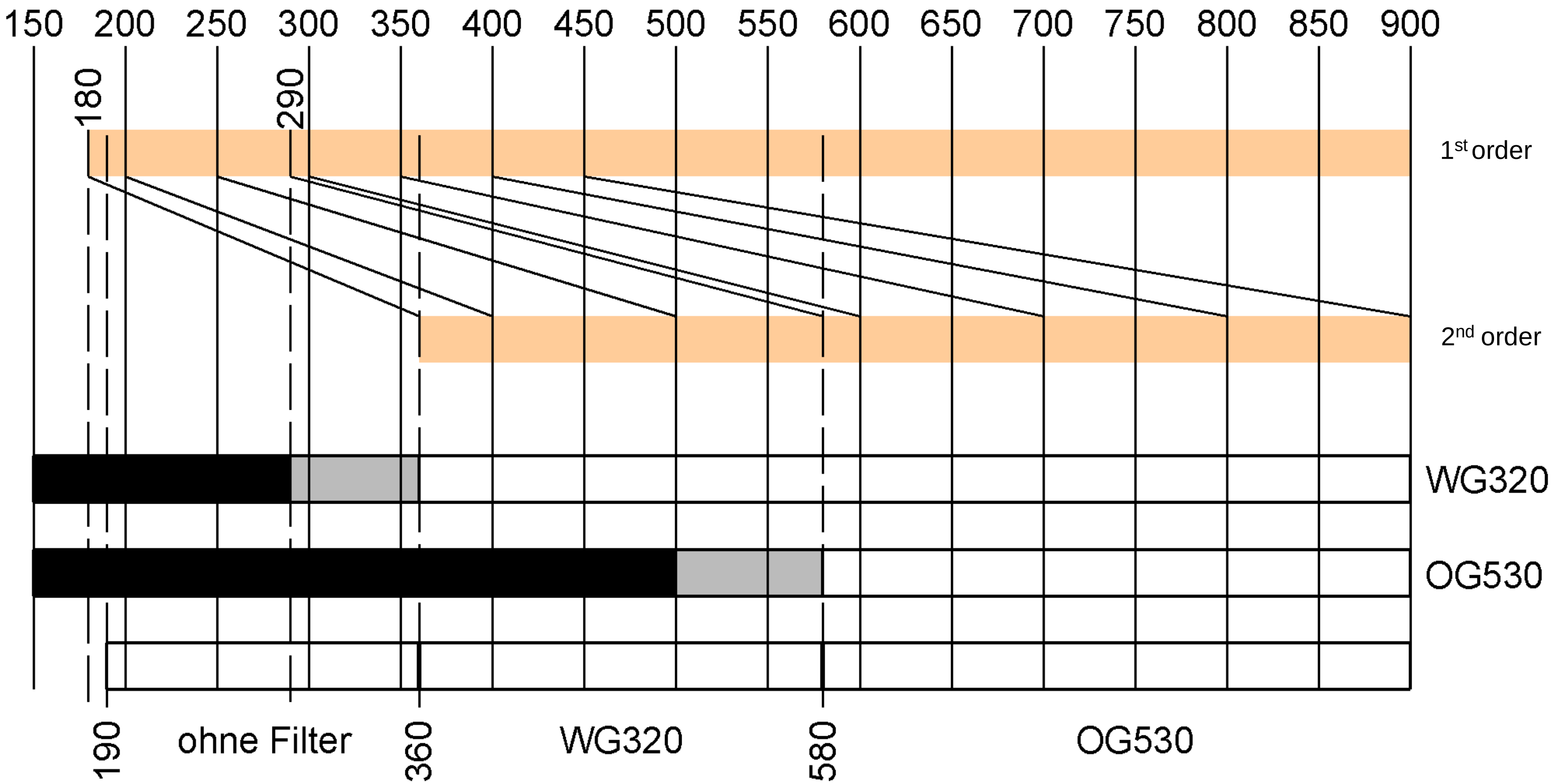

UV Vis Detectors

- The ideal detector ?
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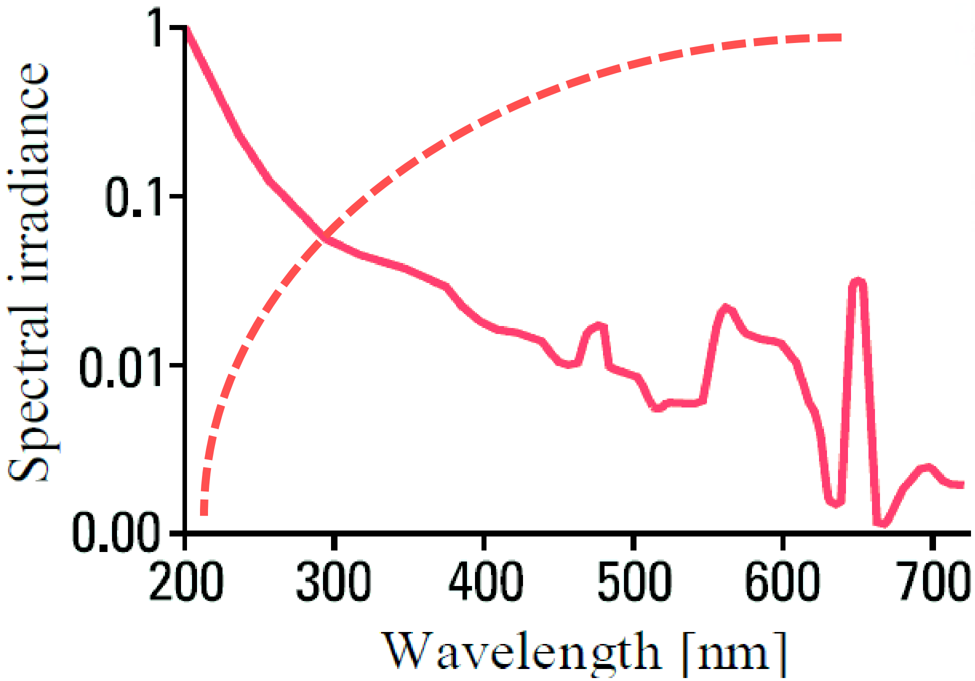
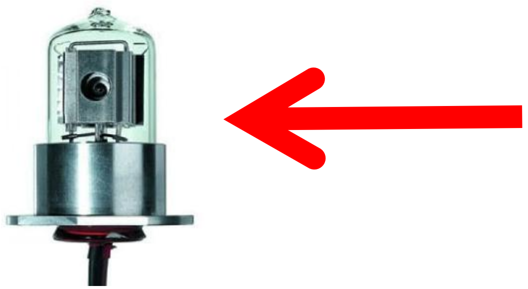
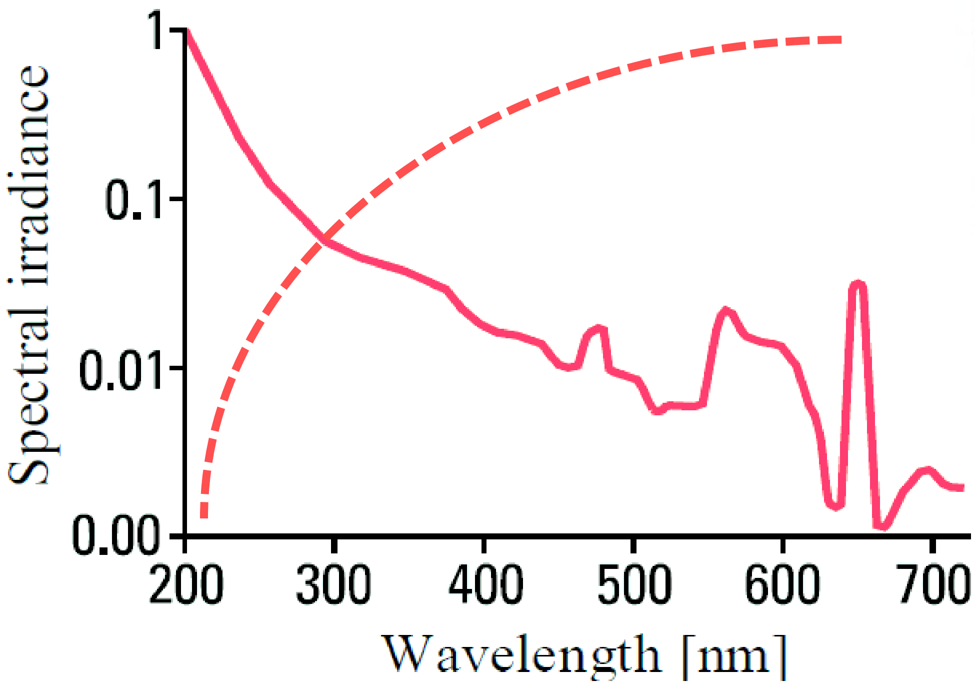
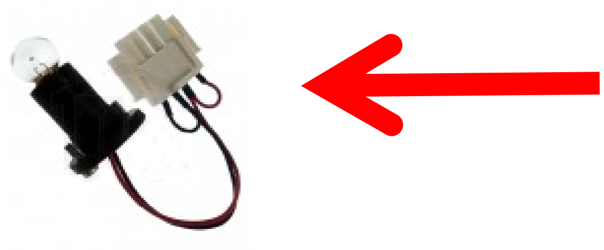
Source / UV Lamp





- Deuterium lamp for UV





- Tungsten halogen lamp for the longer wavelengths
- Light from both sources can be mixed to generate a single broadband source

- The Ideal detector ?
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  - Reference
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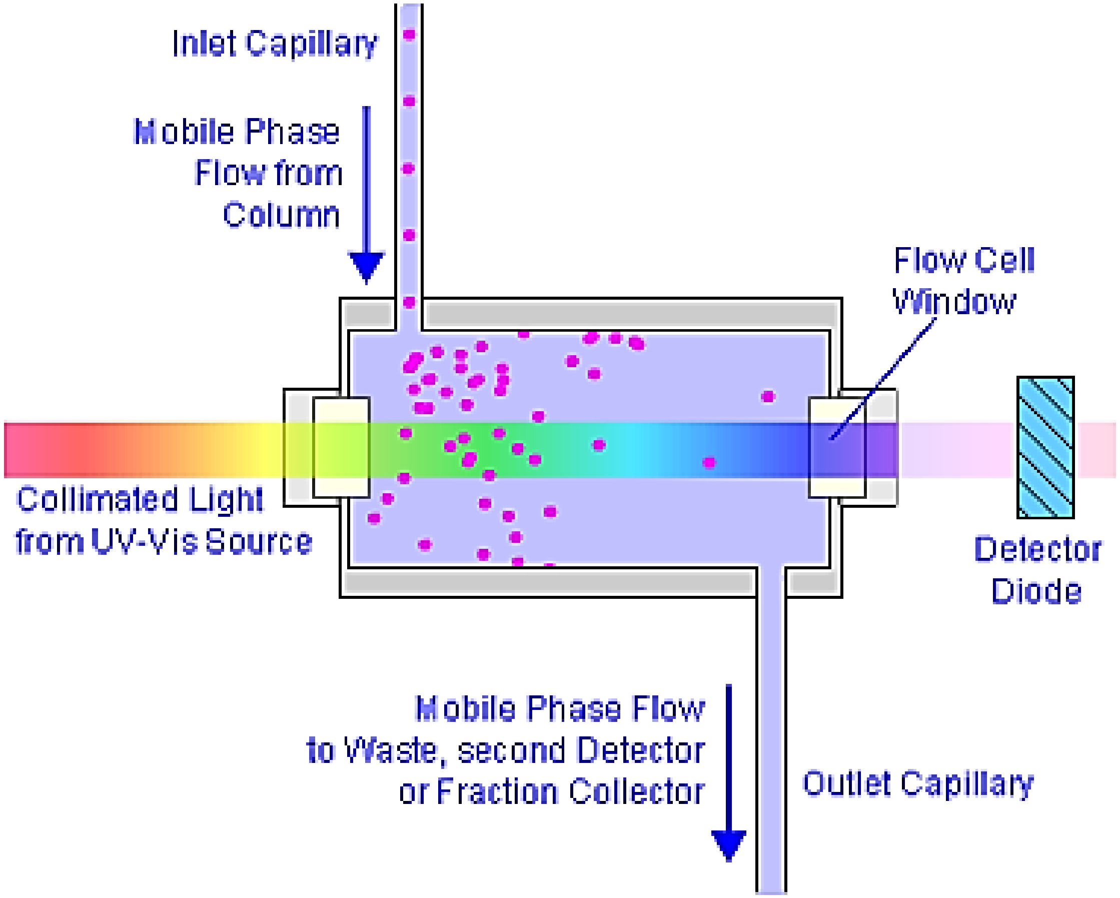
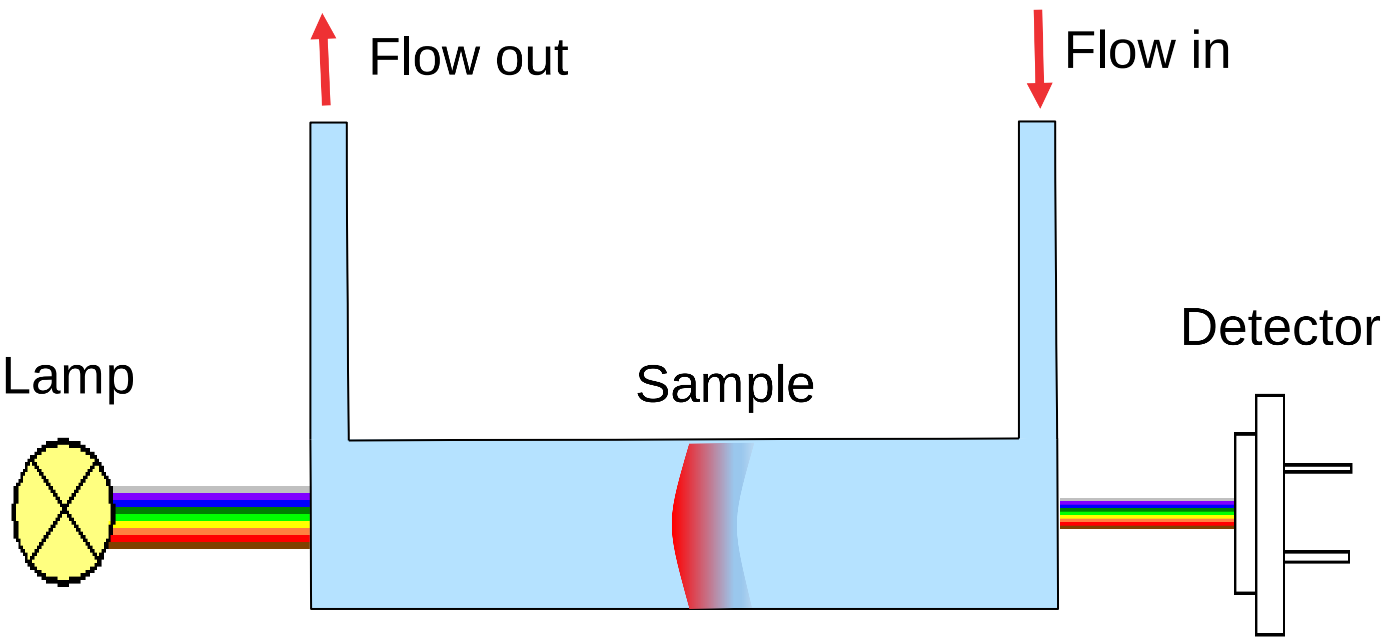
Flow Cell

- Response is proportional to the concentration of analyte in the flow cell
- Important to match the flow cell volume to the application



Signal height

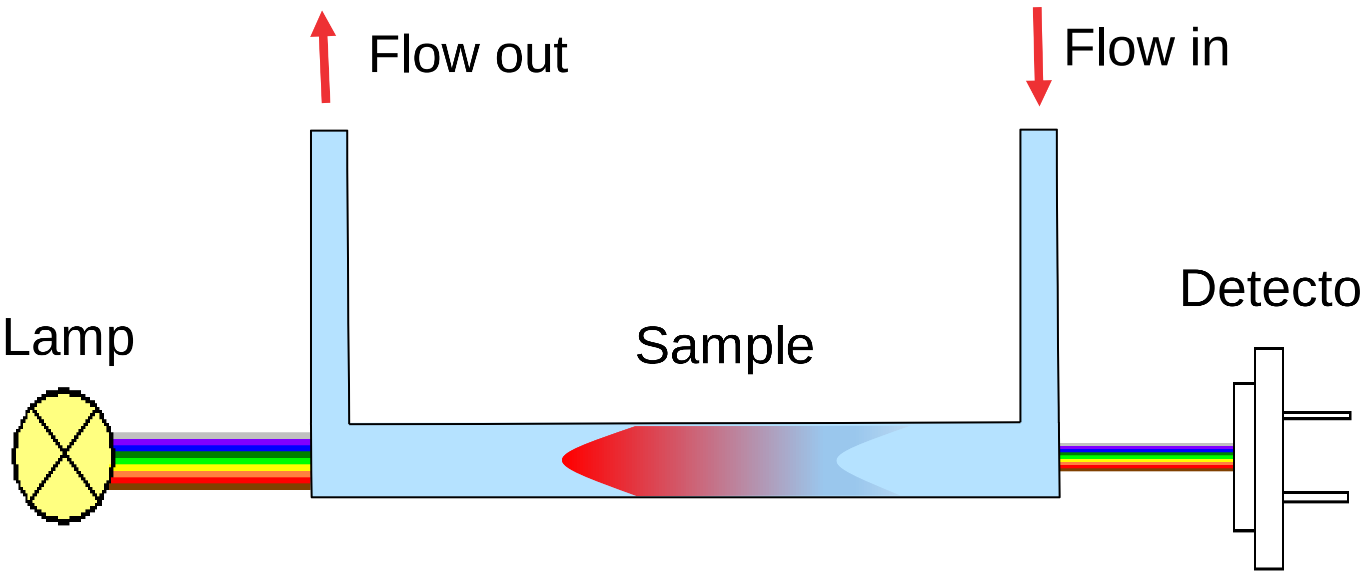
- Light path should be as long as possible



Flow Cell – Signal to Noise

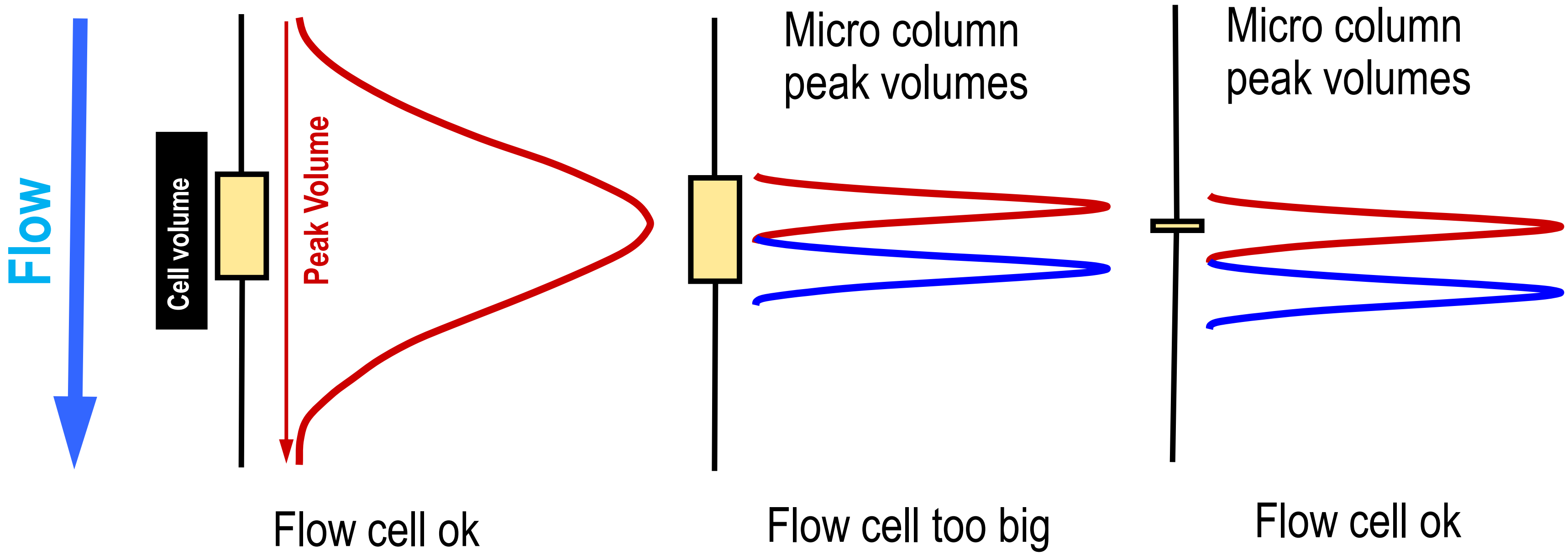
Signal height

- Light path should be as long as possible



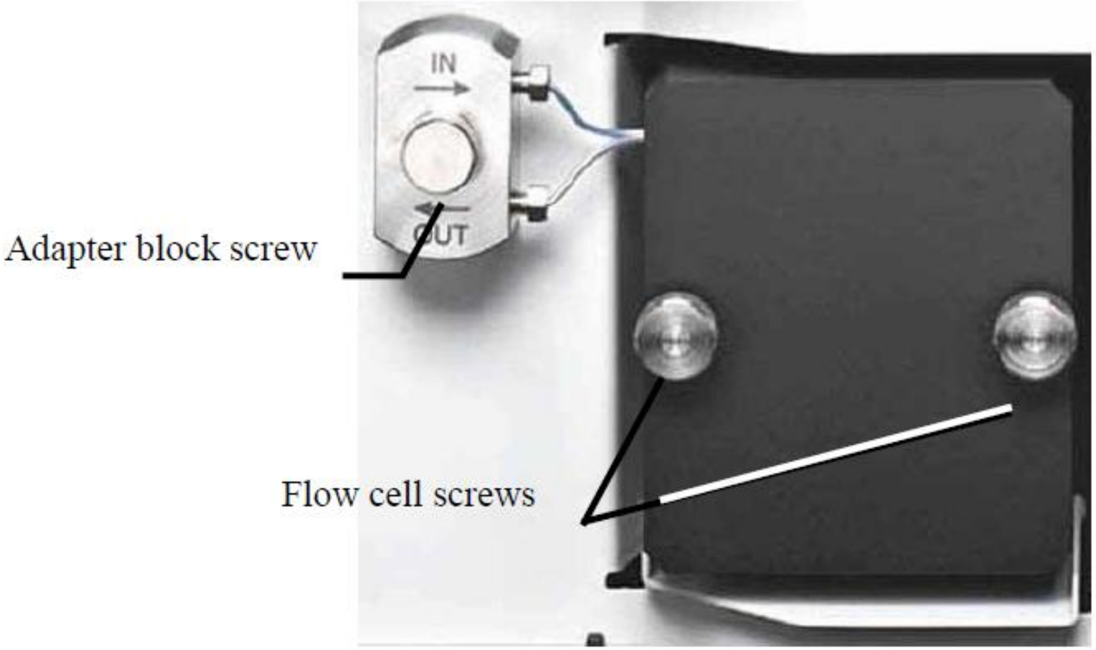
Flow Cell Volume

- Flow cell volume should not exceed 10% of the peak volume



Smaller cell volume → less light is passing through the flow cell

Flow Cells – Thermo Scientific™ UltiMate™ 3000 HPLC System



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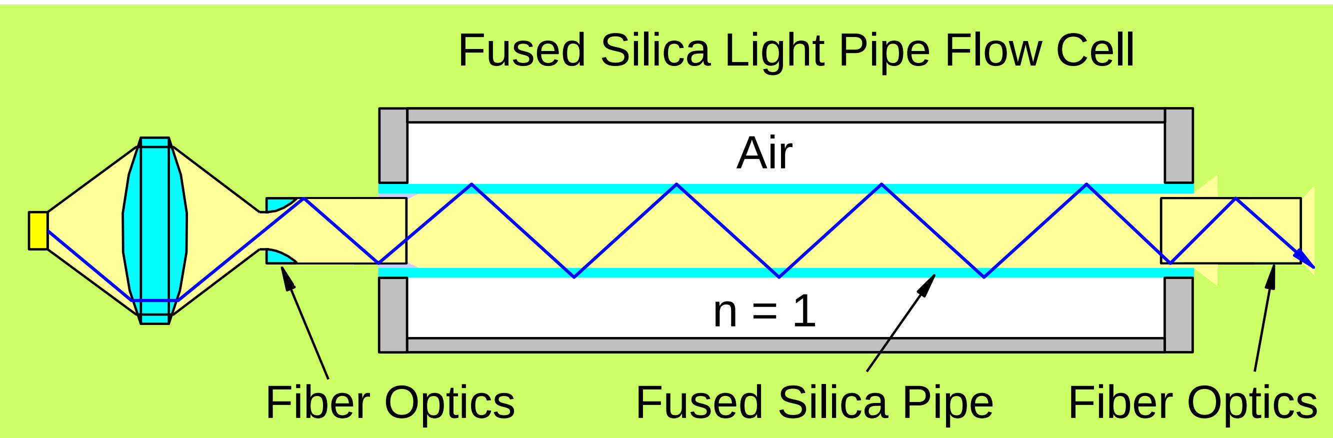
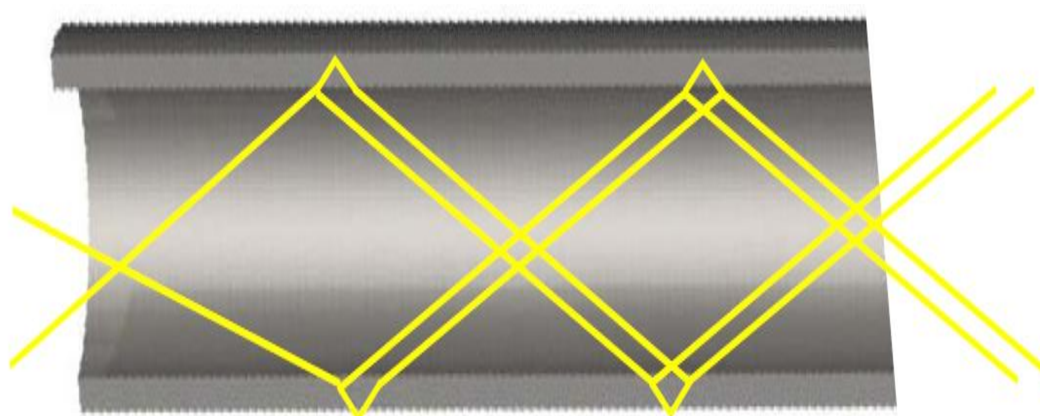
LightPipe Flow Cells – Fused Silica

Fused silica cells

- Very narrow silica walls (0.05 mm)
- Total reflection at silica – air interface





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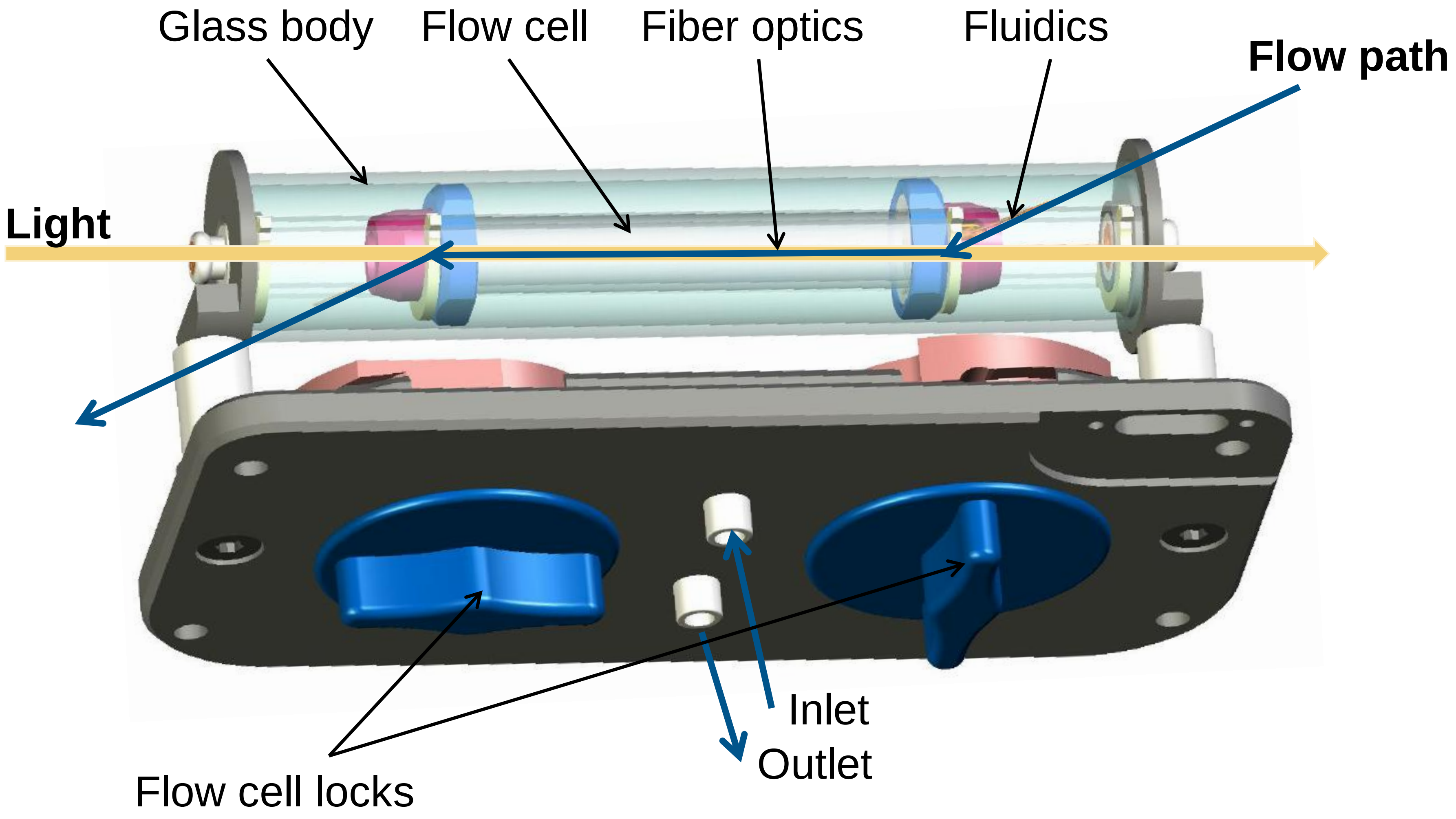
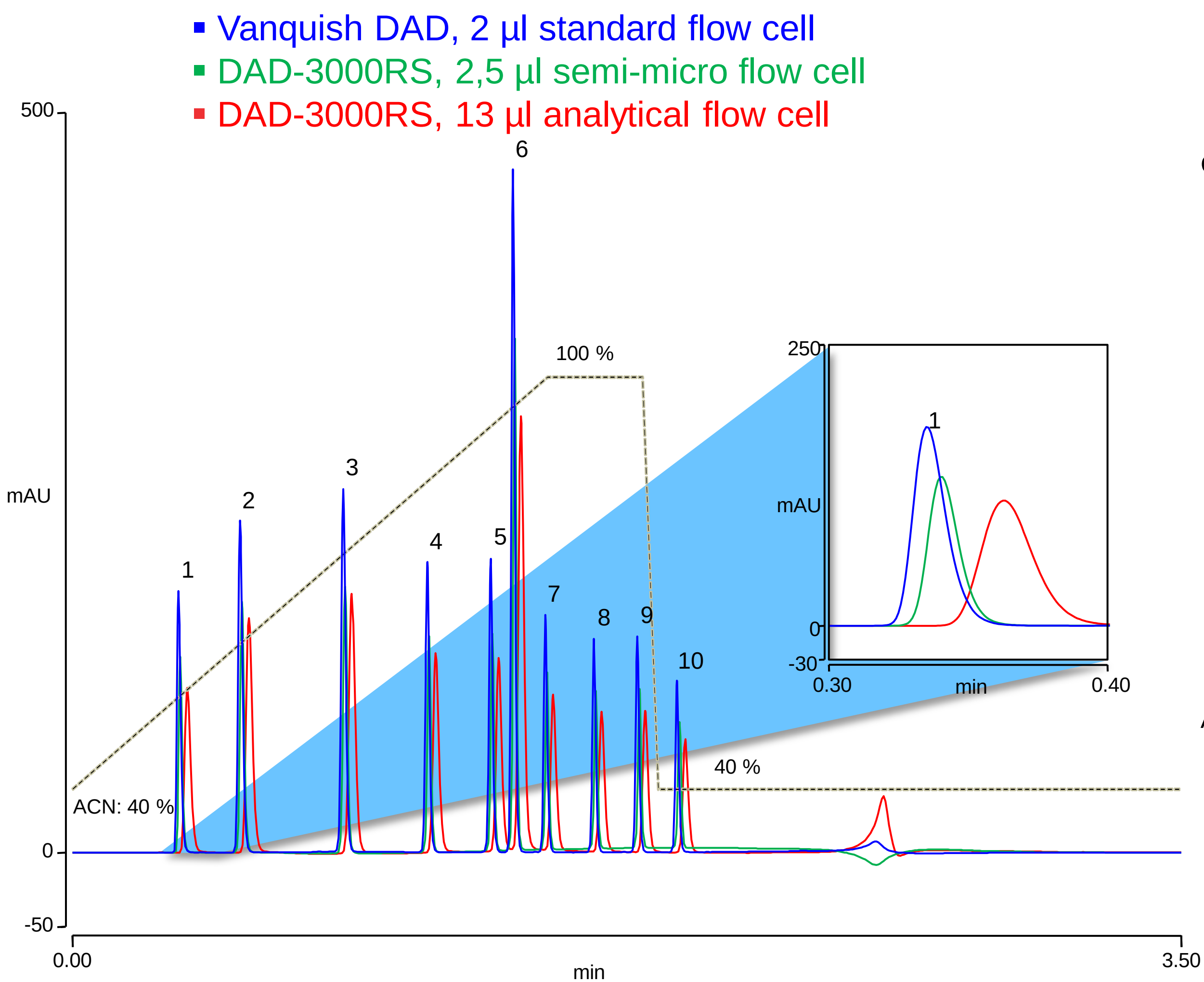
Thermo Scientific™ Vanquish™ LightPipe™ Flow Cells



Binary UHPLC Gradient Performance



Column:	Thermo Scientific™ Hypersil GOLD™, C18, 2.1 × 100 mm, 1.9 µm, P/N 25002- 102130
System:	Binary UltiMate 3000
Mixer vol.:	200 µL
Mobile phase:	A – Water B – ACN
Flow rate:	0.7 mL/min
Pressure:	630 bar (max)
Temperature:	35 ºC
Injection:	1 µL
Detection:	DAD-3000RS, semi-micro or analytical flow cell, wide slit (4 nm) Vanquish DAD with standard flow cell, 4 nm slit 254 nm, 4 nm bandwidth, 20 Hz, 0.2 s response time
Analytes:	1. Uracil 2. Acetanilide 3. – 10. Homologous Phenones 50 µg/mL each

- VDAD: higher (by 30-50%) and narrower peaks (by 30-35%)

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Flow Cells

- **VWD**
- 11µl 10mm standard analytical flow cell
- 2,5µl 5mm semi-micro flow cell
- 45nl 10mm capillary flow cell
- 3nl 10mm nano flow cell

- **DAD**
- 13µl 10mm standard analytical flow cell
- 5µl 7mm semi-analytical flow cell
- 2,5µl 5mm semi-micro flow cell

- **Vanquish**
- 2µl 10mm standard analytical LightPipe flow cell
- 13µl 60mm high sensitivity LightPipe flow cell

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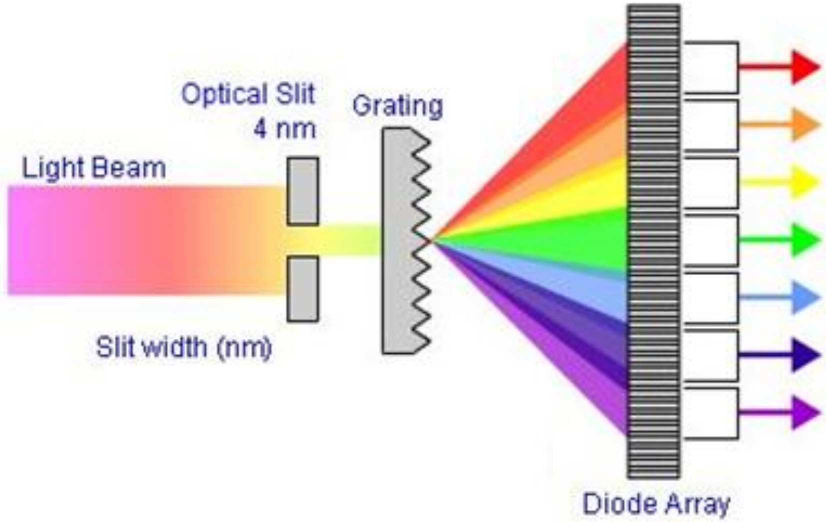
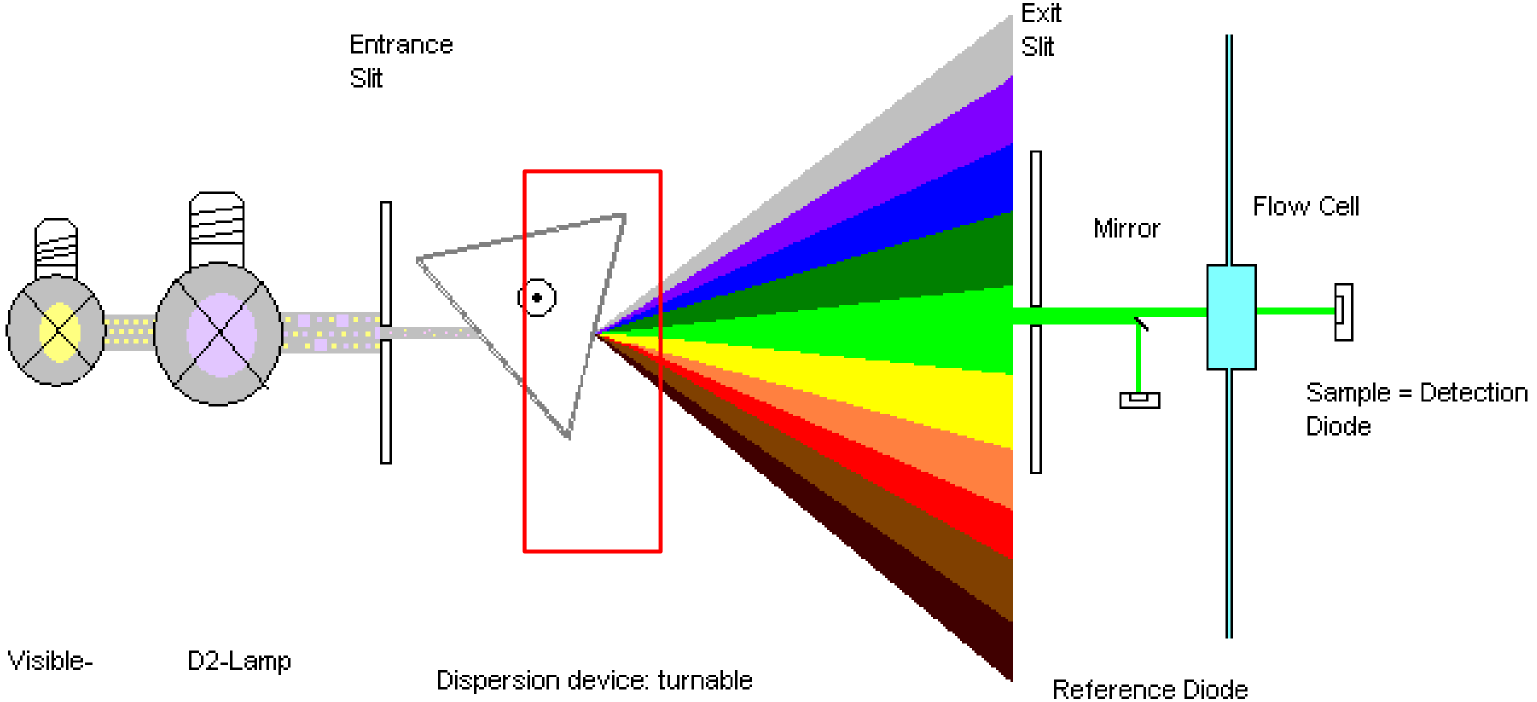


Operating Principle – Slit Width

- VWD detector
  - Bandwidth is defined by entrance slit
  - At 254nm the bandwidth is 6nm (254nm +/- 3nm)



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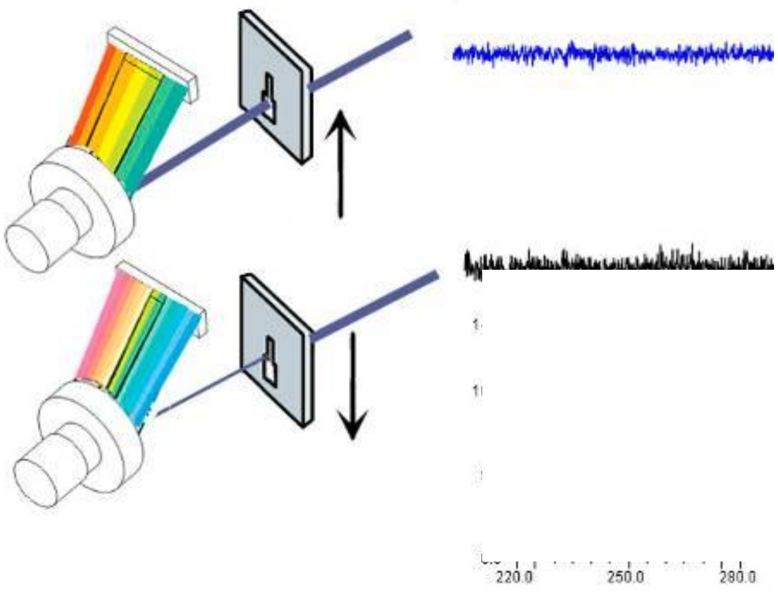
Operating Principle – Slit Width

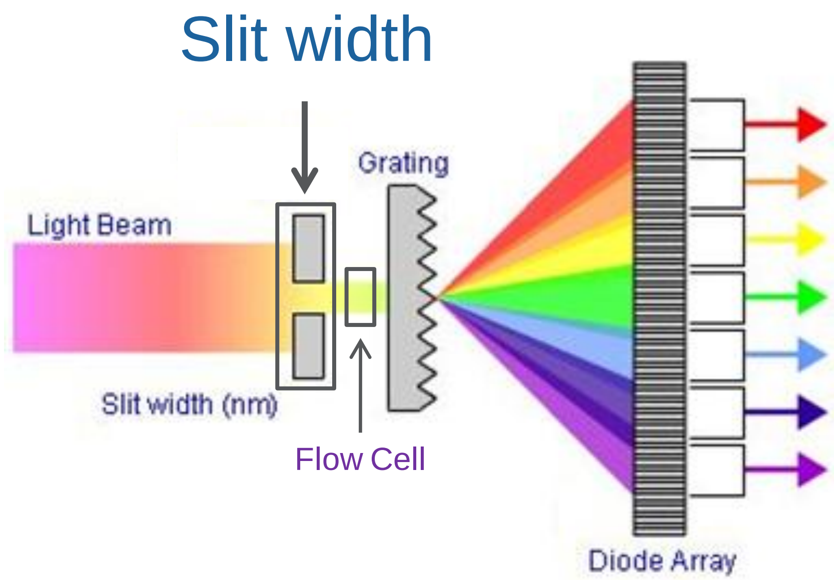
- UltiMate DAD RS modules offer two slit width positions
  - Narrow (default)
  - Wide



- Vanquish DAD offers four slit width positions
  - 1, 2, 4 and 8 nm

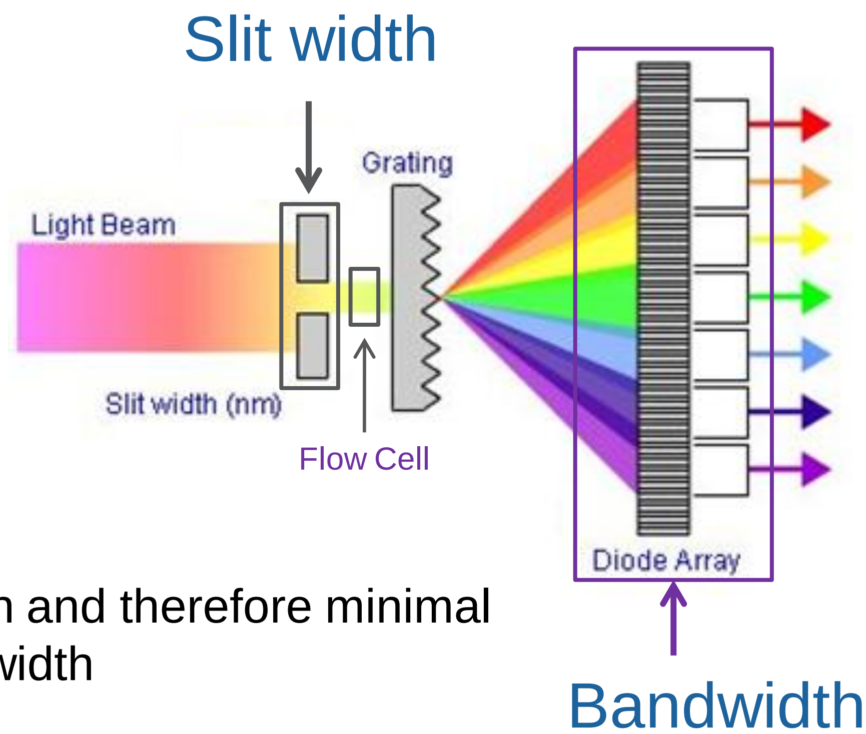
- UltiMate SD modules have a fixed slit
  (‘wide’ for modules with SN ≥ 8019060; up to that S/N ‘narrow’ slit was used)

Diode Array Detector



- Slit defines optical resolution and therefore minimal physically meaningful bandwidth

Diode Array Detector



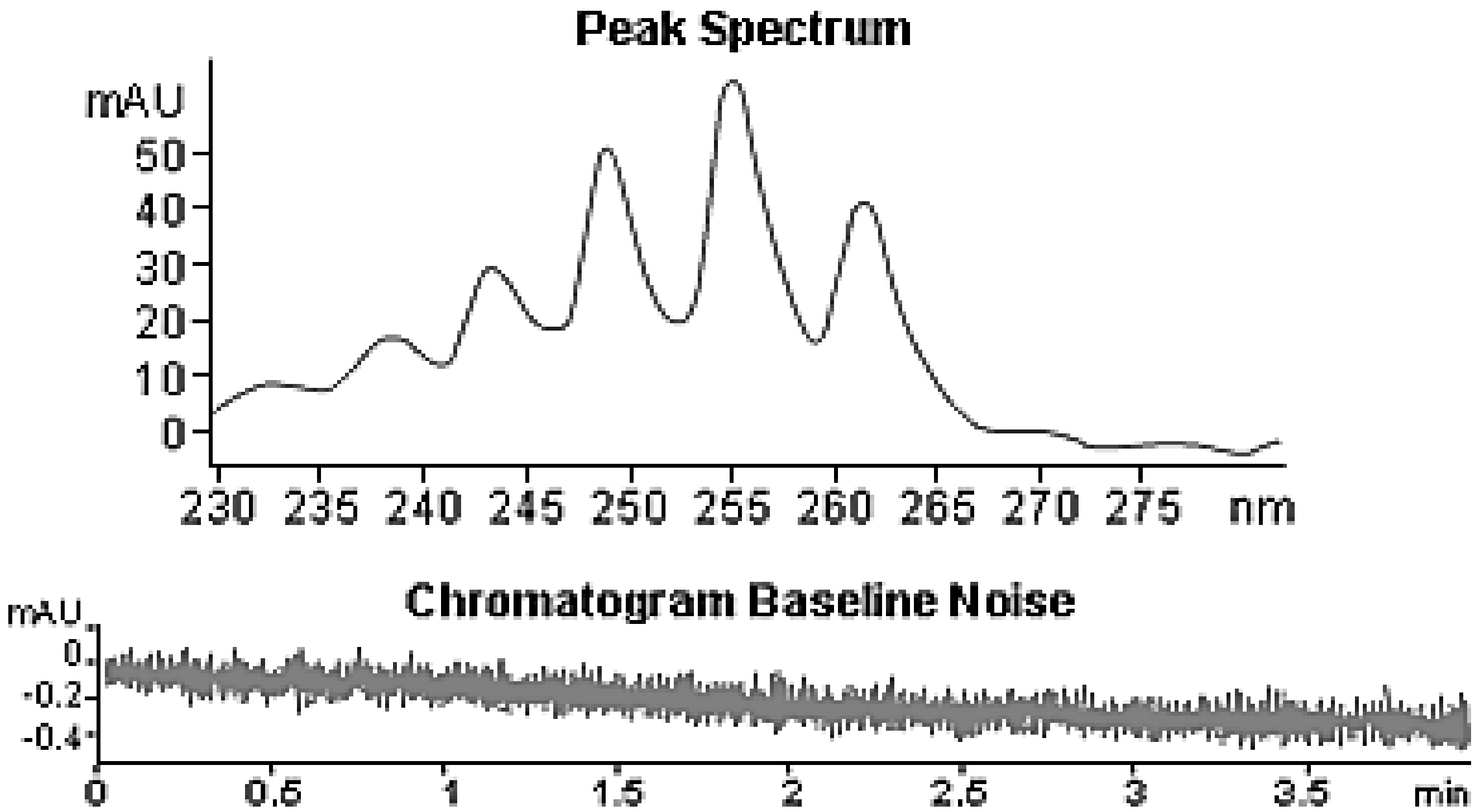
- Slit defines optical resolution and therefore minimal physically meaningful bandwidth

Slit width	Baseline noise	Spectral resolution
↓	↑	↑
↑	↓	↓

Bandwidth	S/N ratio	Spectral resolution
↑	↑	↓
↓	↓	↑

Effects of Slit Width

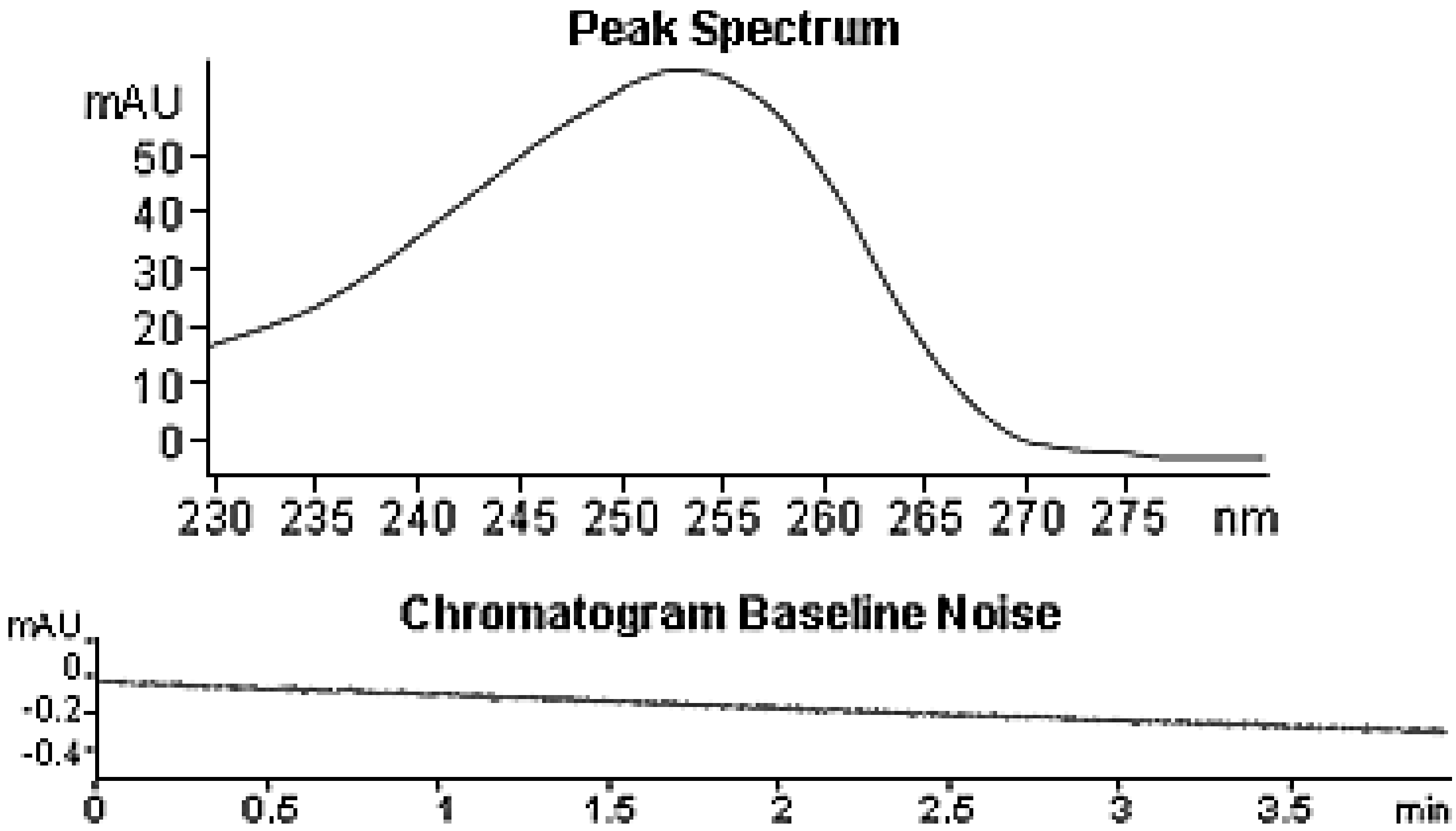
Slit width - 1nm



4 x light > 0.5 x noise

Effects of Slit Width

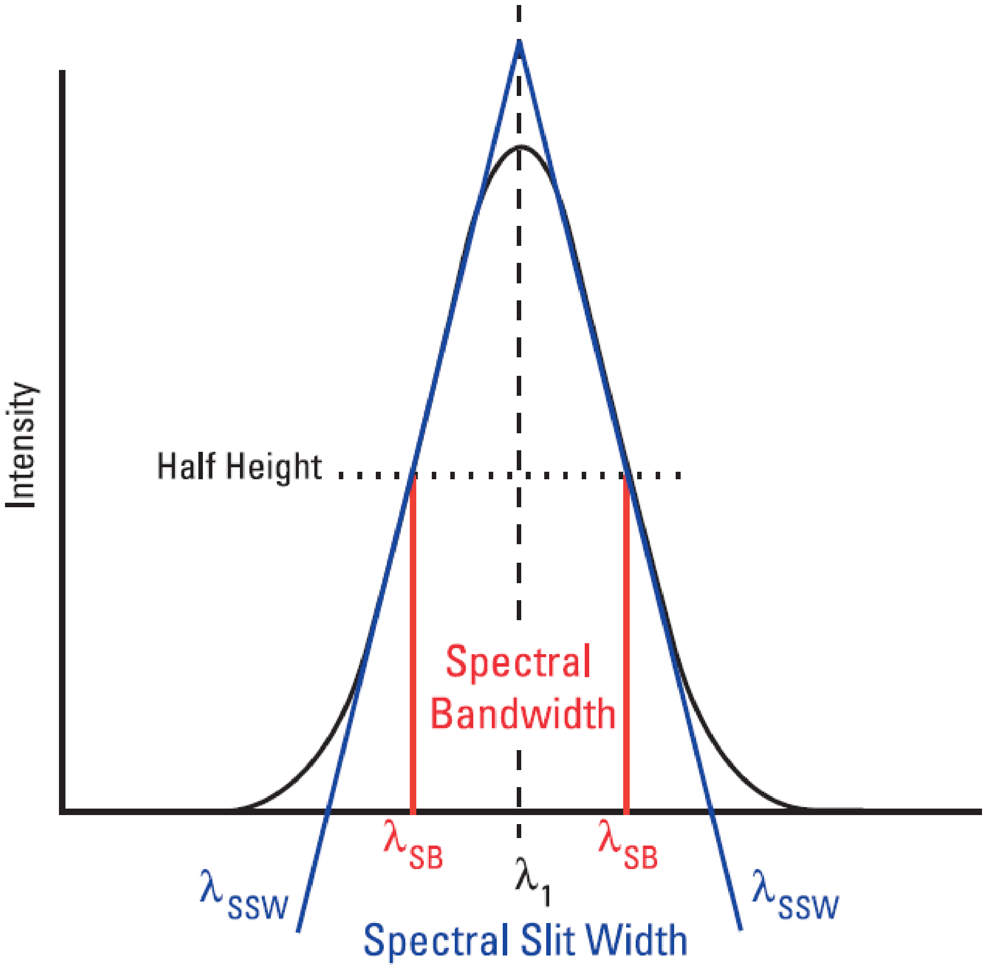
Slit width - 16nm



4 x light > 0.5 x noise

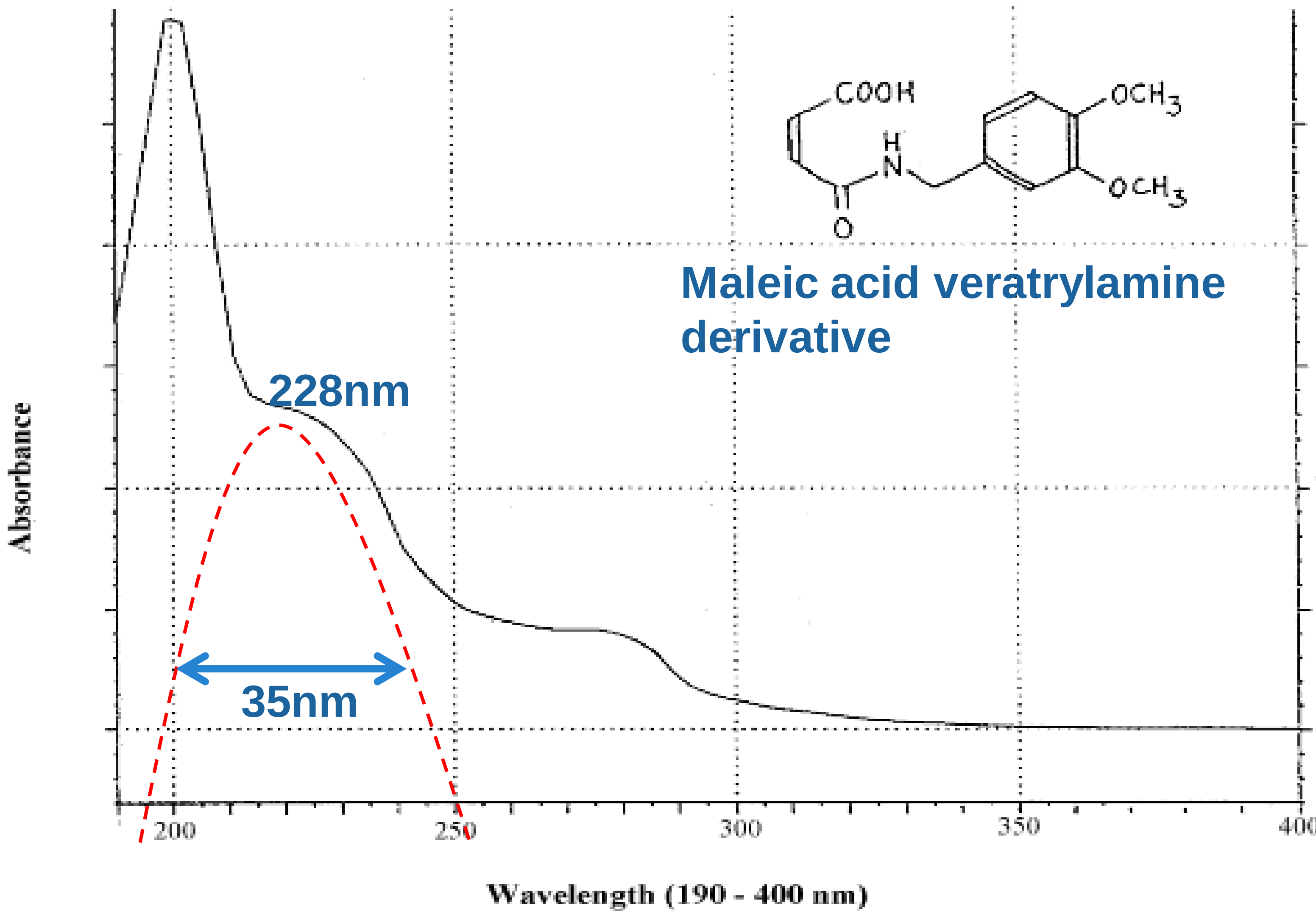
Bandwidth

- The light emerging from the exit slit will have a Gaussian distribution of wavelengths
- The 'Spectral bandwidth' is defined as the wavelength range at half height of the distribution
- Important to match the spectral bandwidth (SB) to the natural bandwidth (NB)



SBw/NBw ratio of 0.1 or less will yield measurement with accuracy of 99.5 % or better

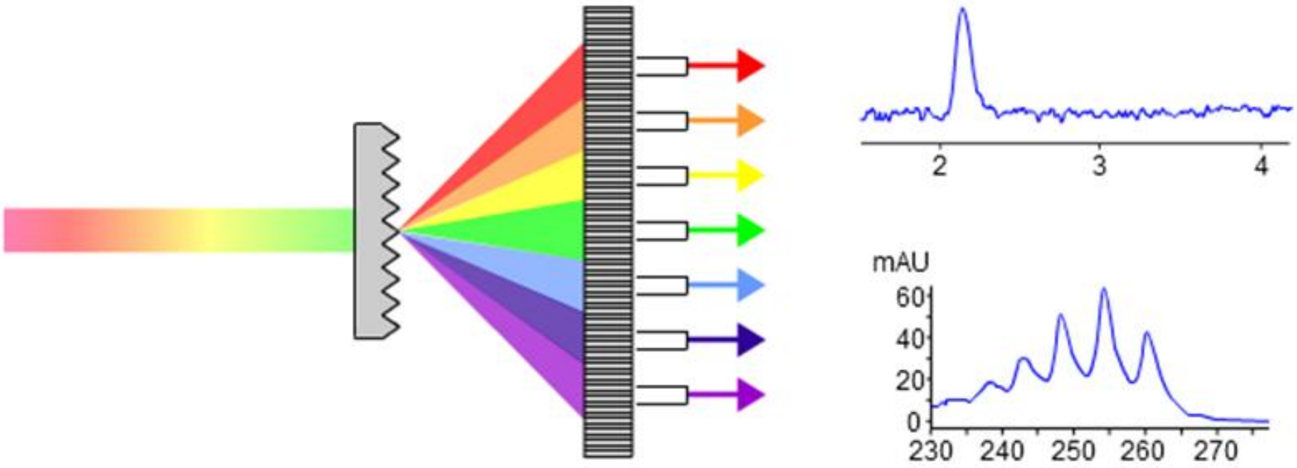
Setting Bandwidth



Setting Bandwidth

Bandwidth 4 nm

s/n = 5



4 x light = < 0.5 noise

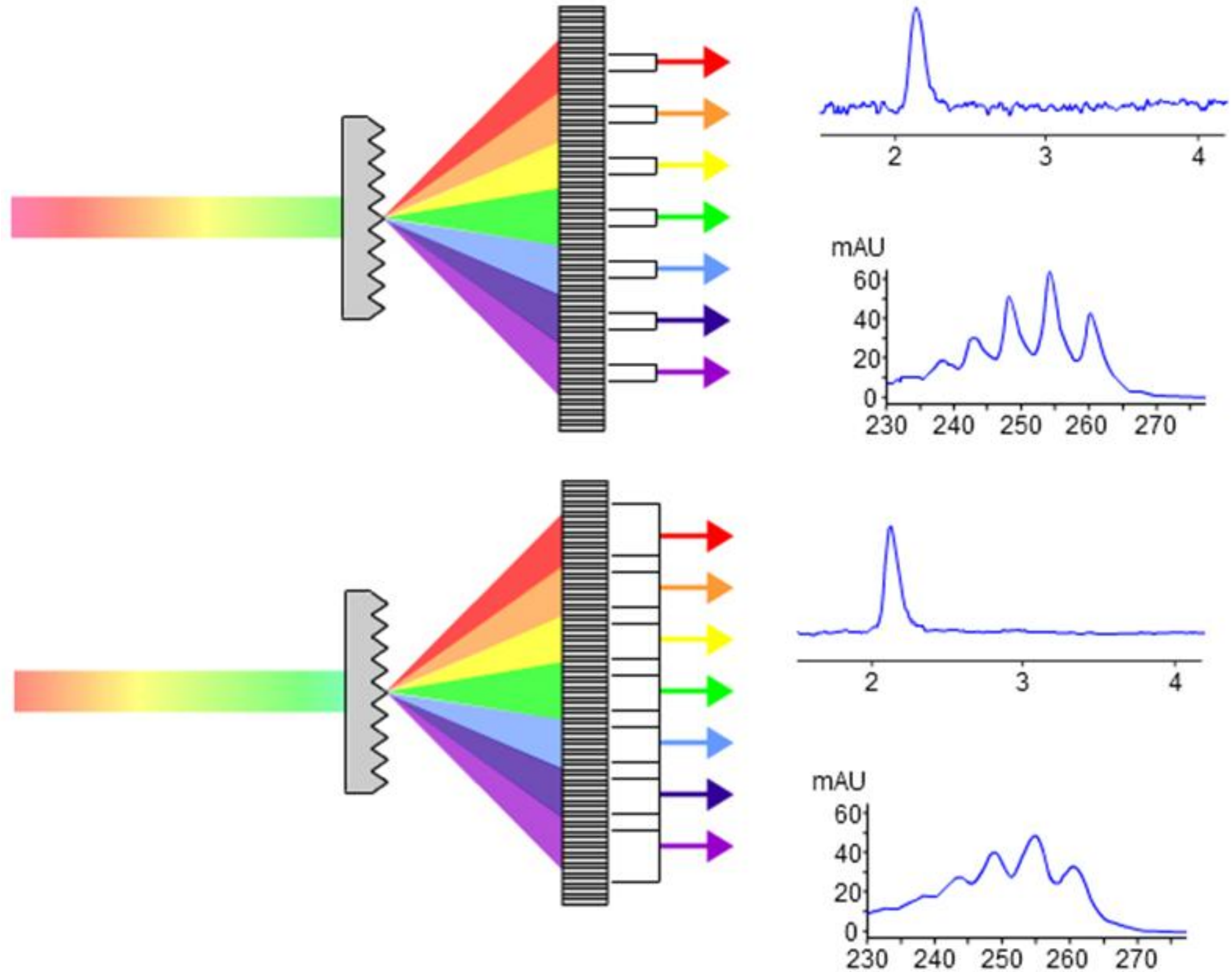
Setting Bandwidth

Bandwidth 4 nm

s/n = 5

Bandwidth 30 nm

s/n = 25



4 x light = < 0.5 noise

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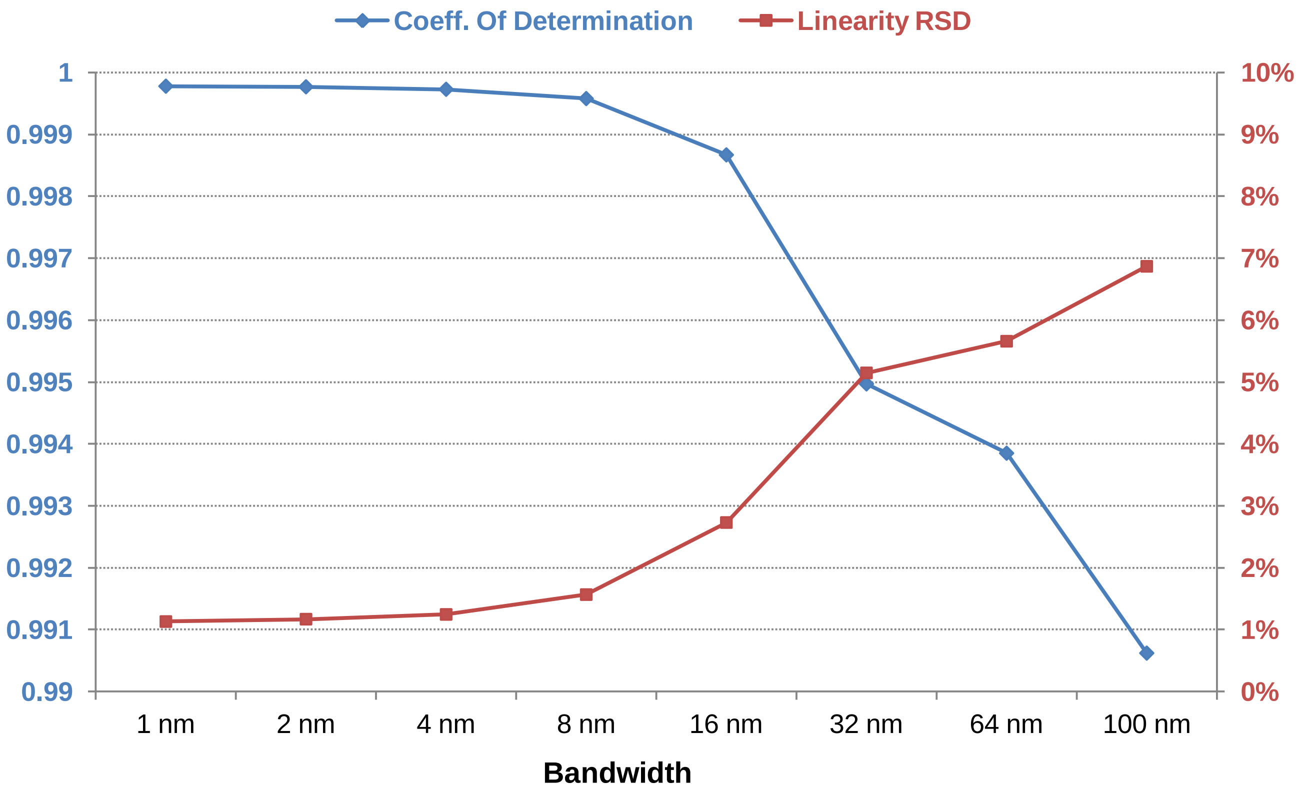
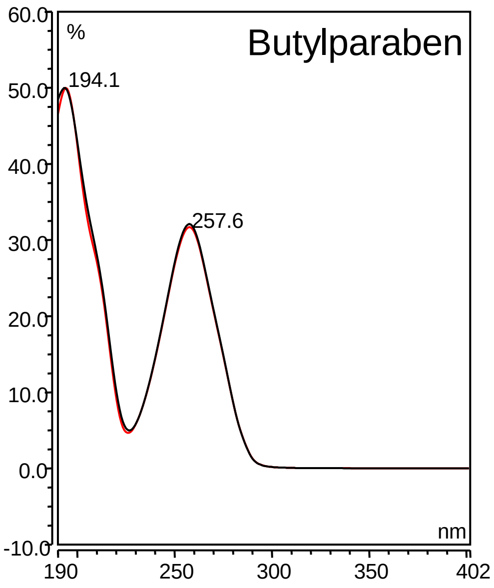
Influence on Linearity – Bandwidth

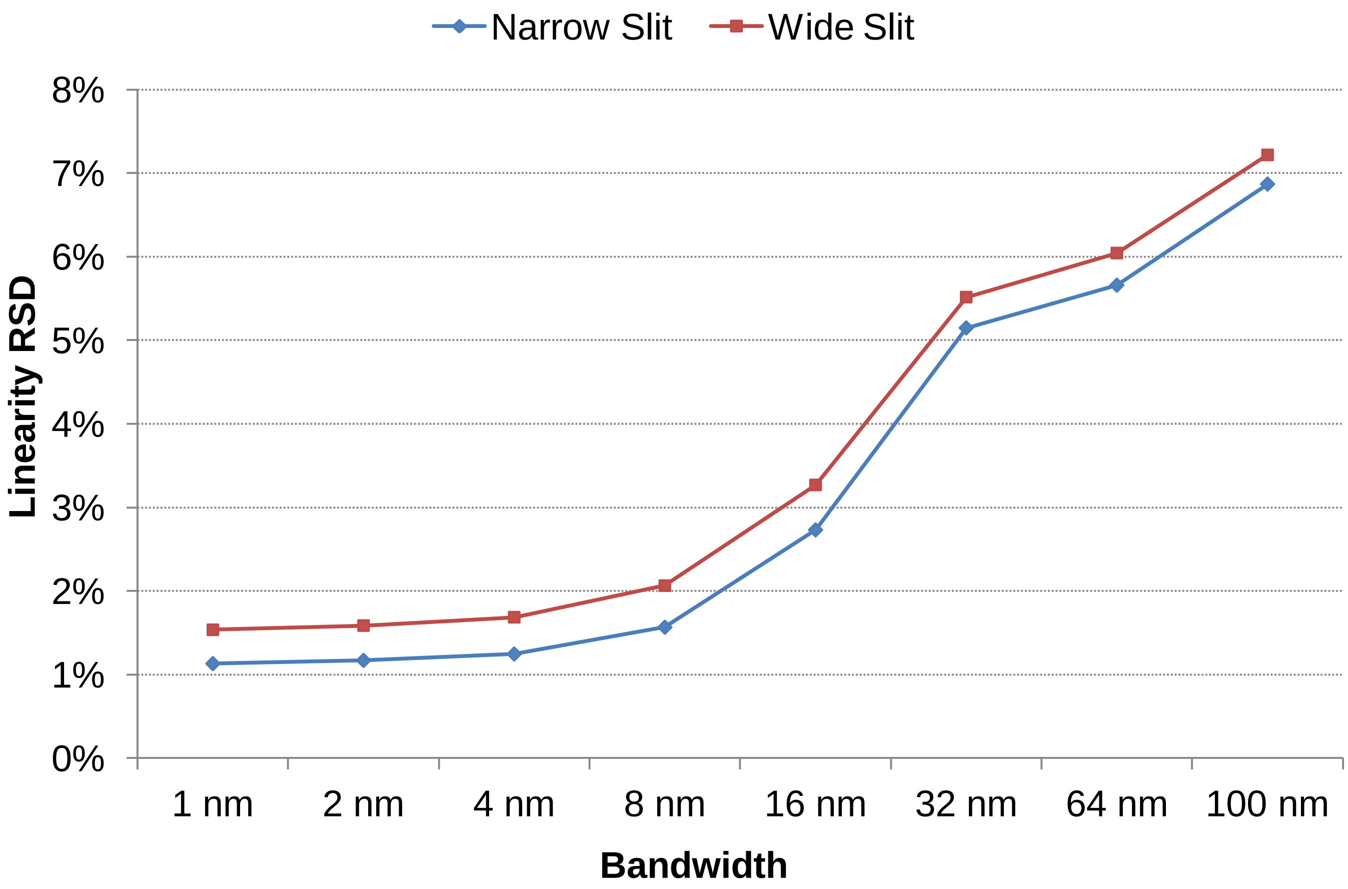
Linearity of butylparaben





Influence on Linearity – Slit Width

Linearity of butylparaben



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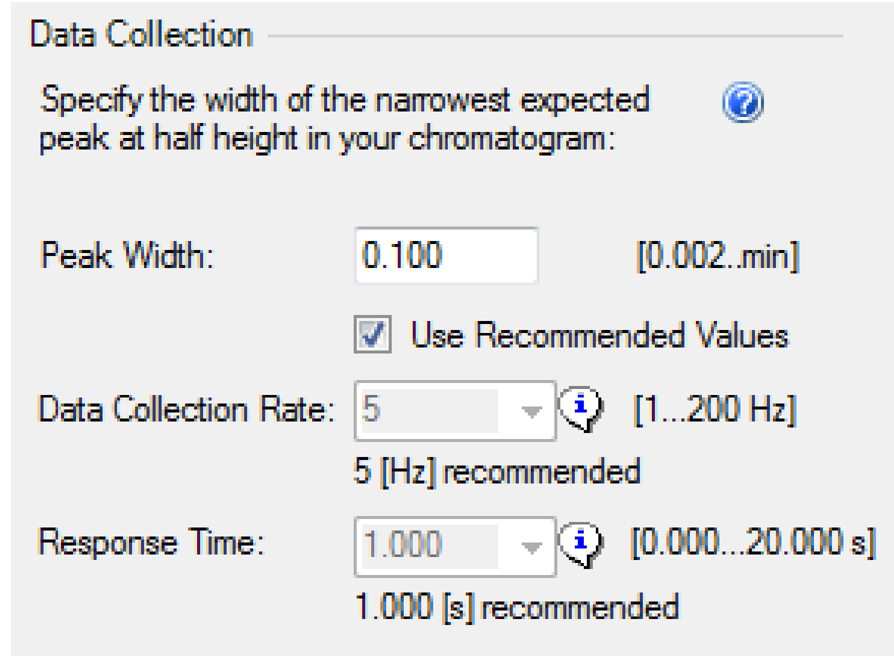
  - Band and slit width
  - Data collection rate and time constant
  - Reference
  - Stray light, refractive index effects & noise



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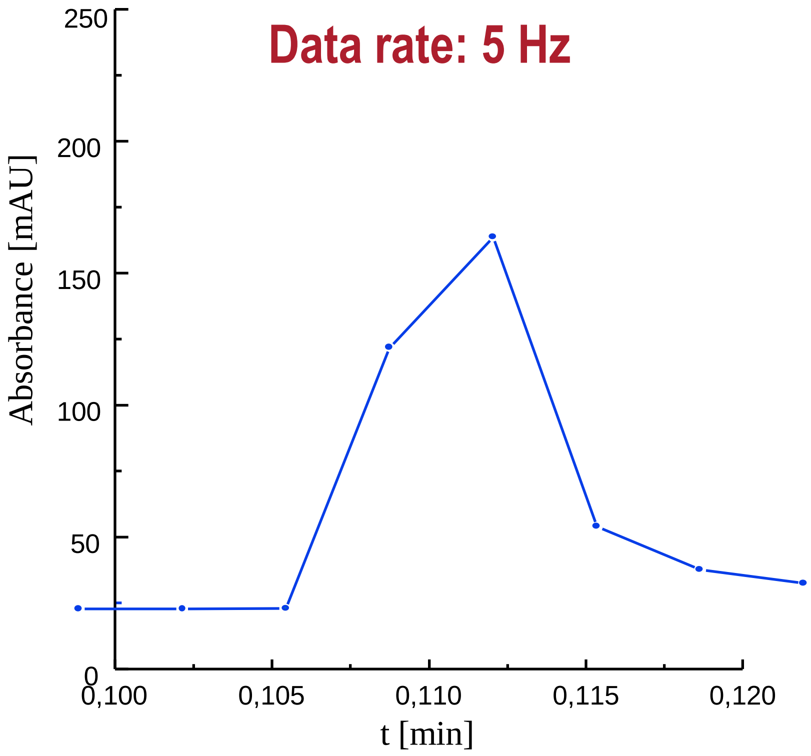
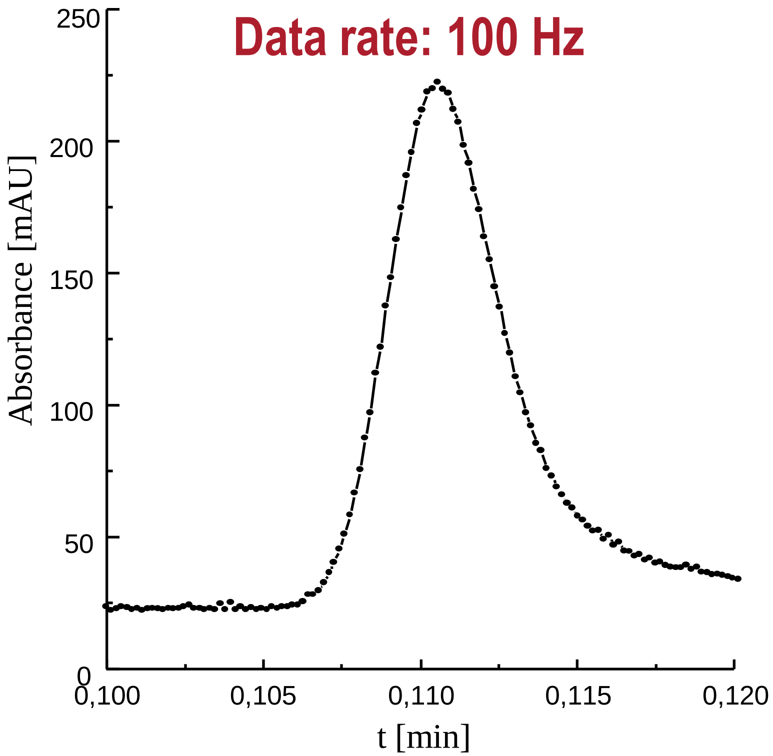
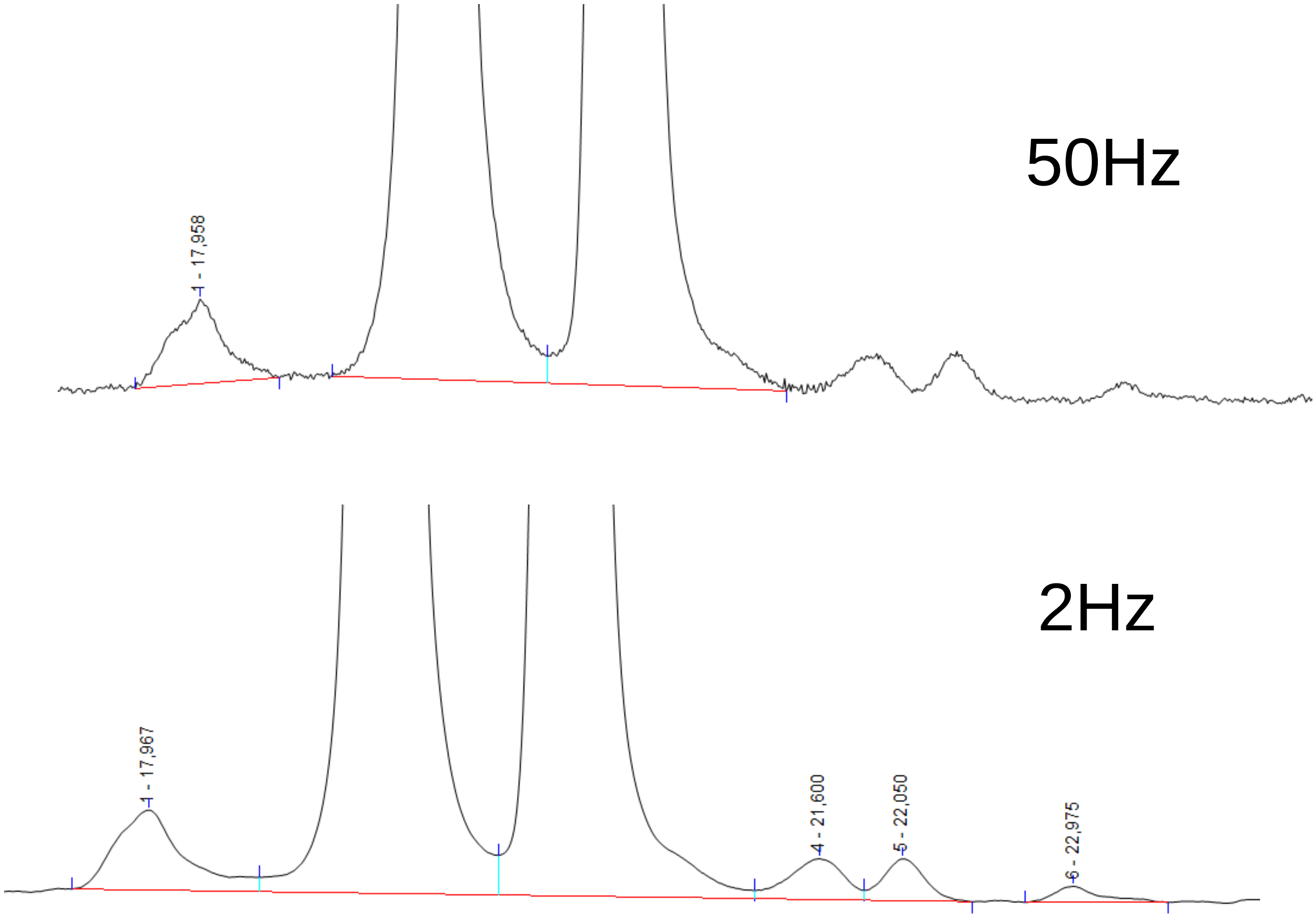
Recommended Parameters: Data Acquisition





- Too few data points effect peak form, reproducibility and area precision
- A minimum of 20, ideally 30-40 data points/peak is required

Data Collection Rate



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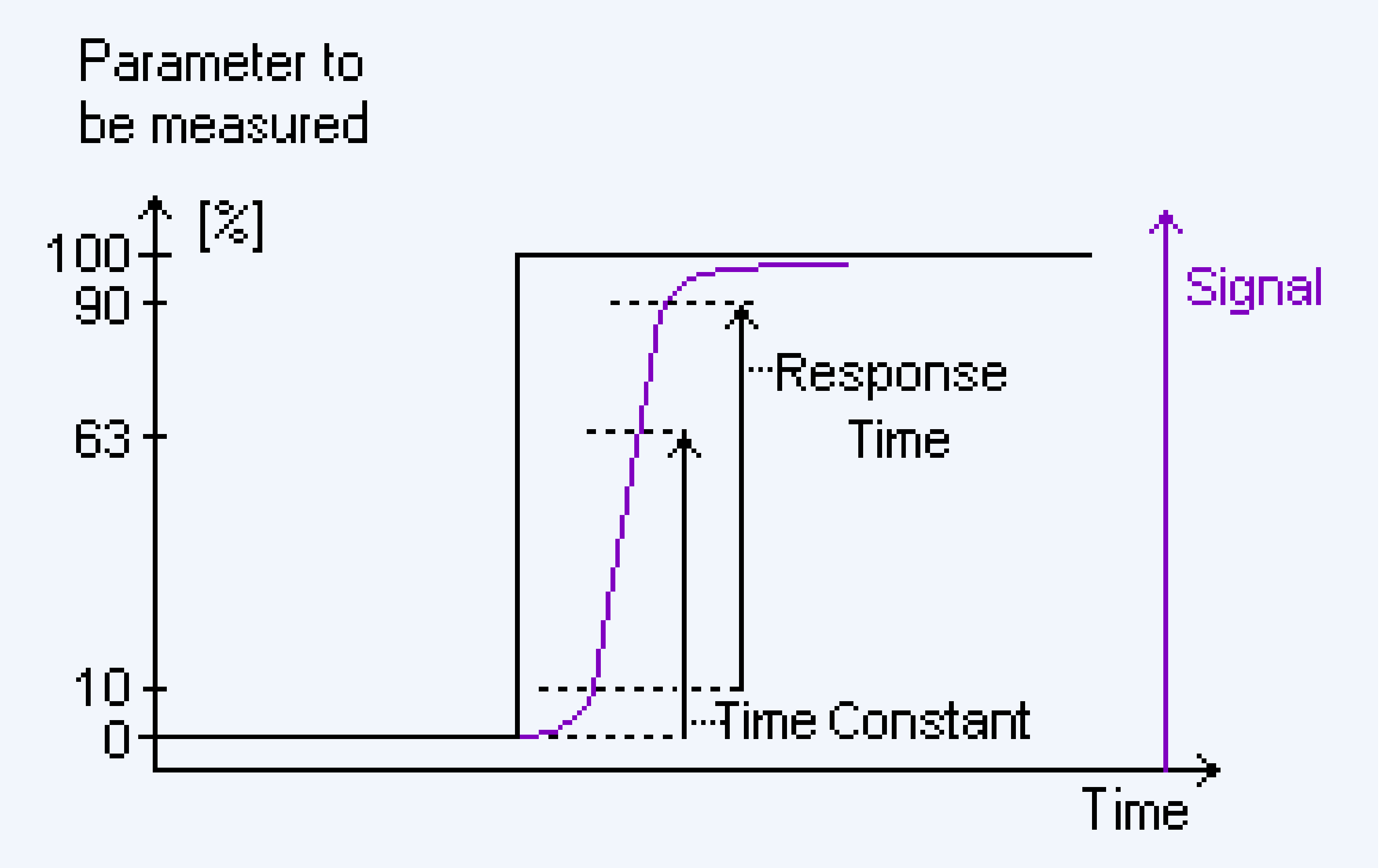
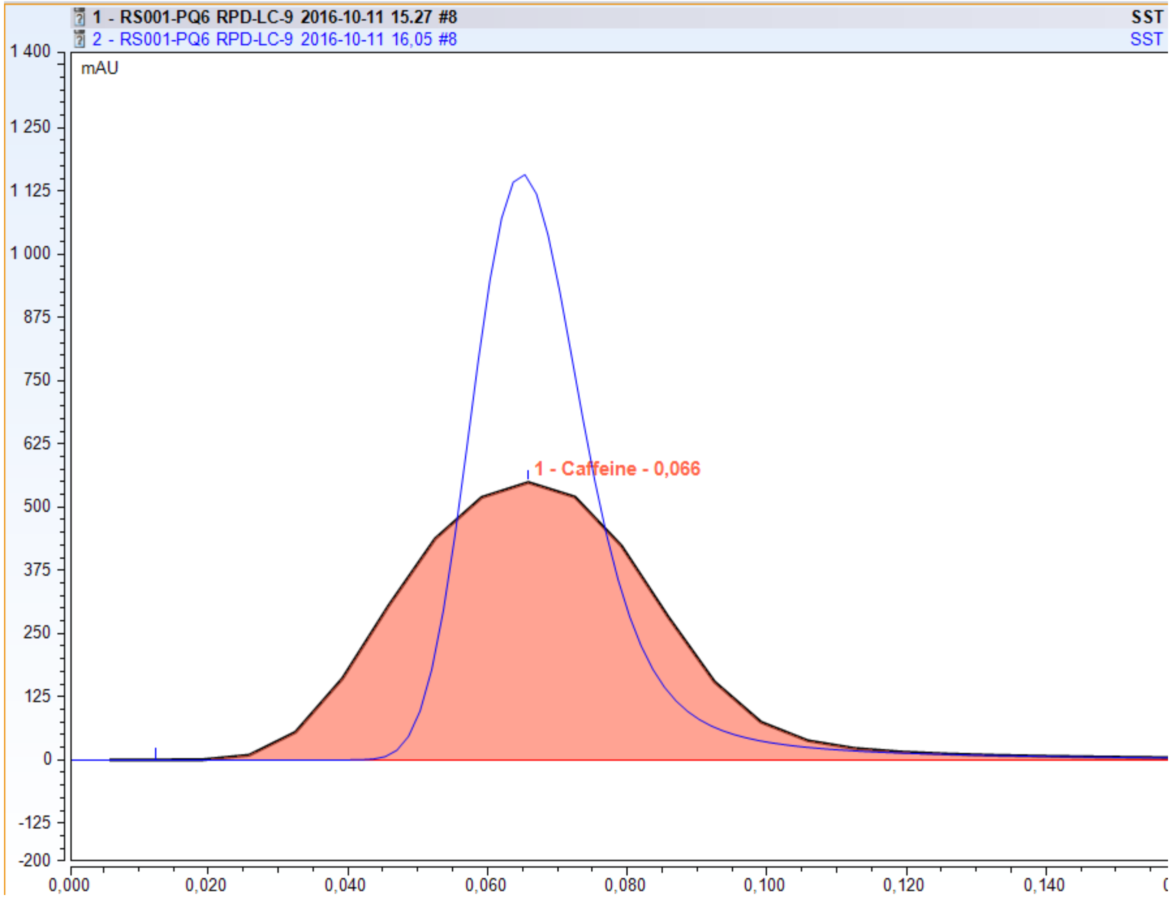
Time Constant



- The rise time (response time) is closely related to the time constant: Rise time = 2,2 x Time constant

Sampling and Rise Time

- The same instrument, back pressure loop, eluent and sample. The area is the same – the peakshape is very different.
- 2,5 Hz 2s response time
- 10 Hz 0,5s response time



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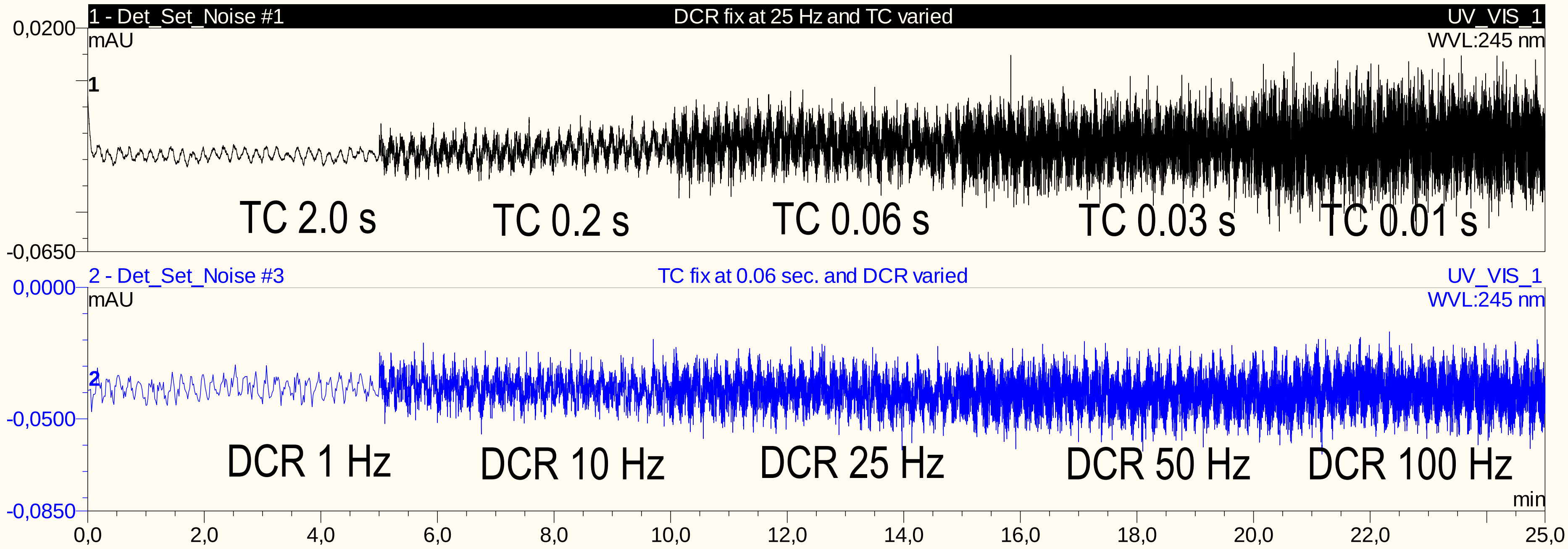
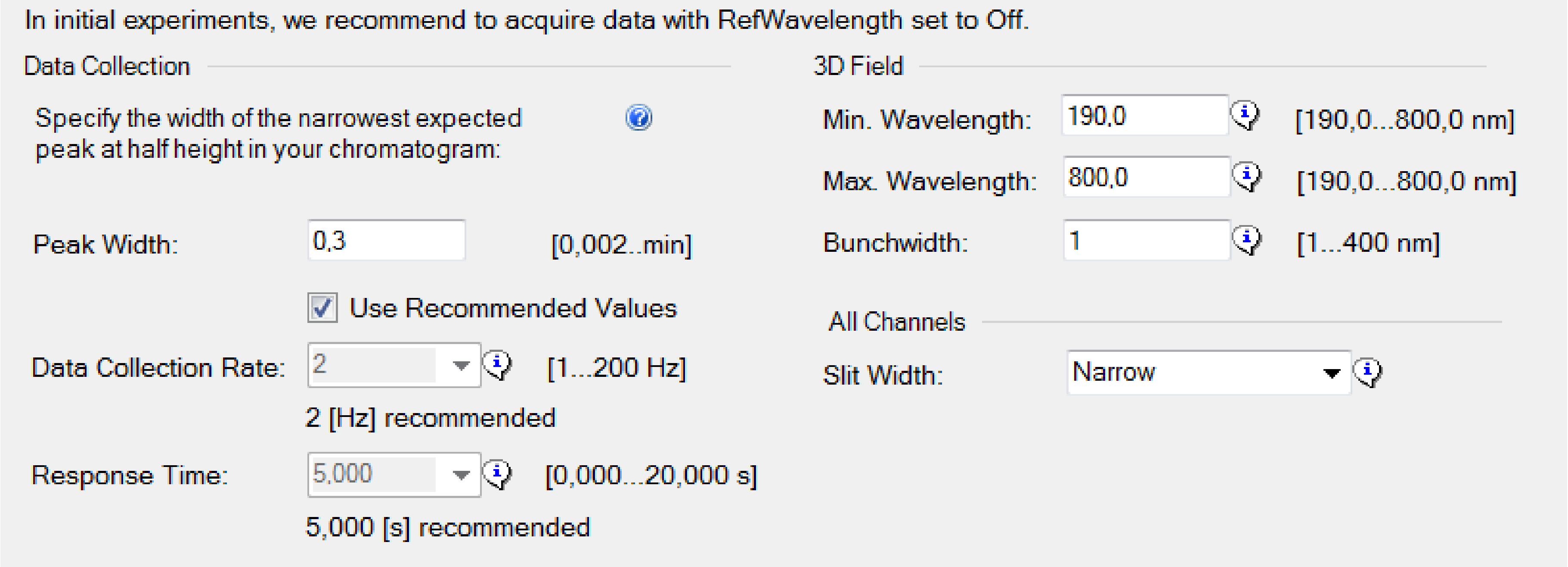
Data Collection Rate and Time Constant



- Noise is much more influenced by time constant than by data collection rate

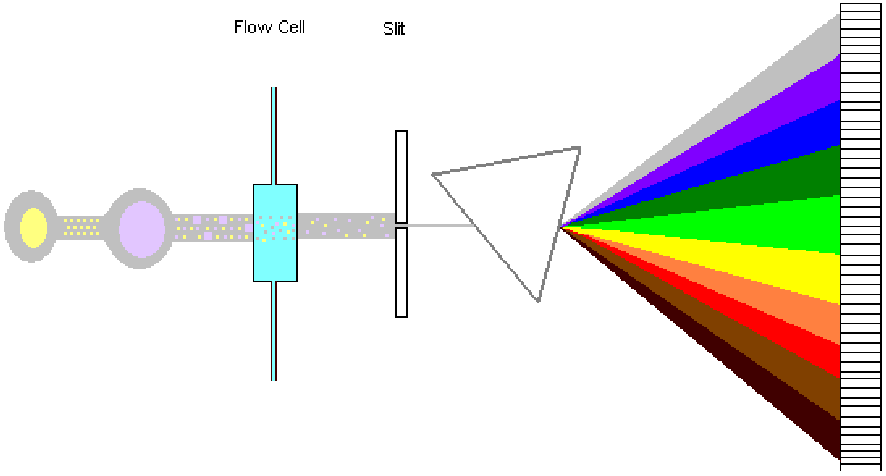
Automated Settings

- The program wizard of Thermo Scientific™ Chromeleon™ 7.2 CDS has a dedicated step for setting the correct ‘Data collection rate’ and ‘Time constant’



UV Vis Detectors

- The ideal detector ?
- Why do we get a signal?
- Optics
  - Lamps
  - Flow cell
  - Band and slit width
  - Data collection rate and time constant
  - Reference
  - Stray light, refractive index effects & noise



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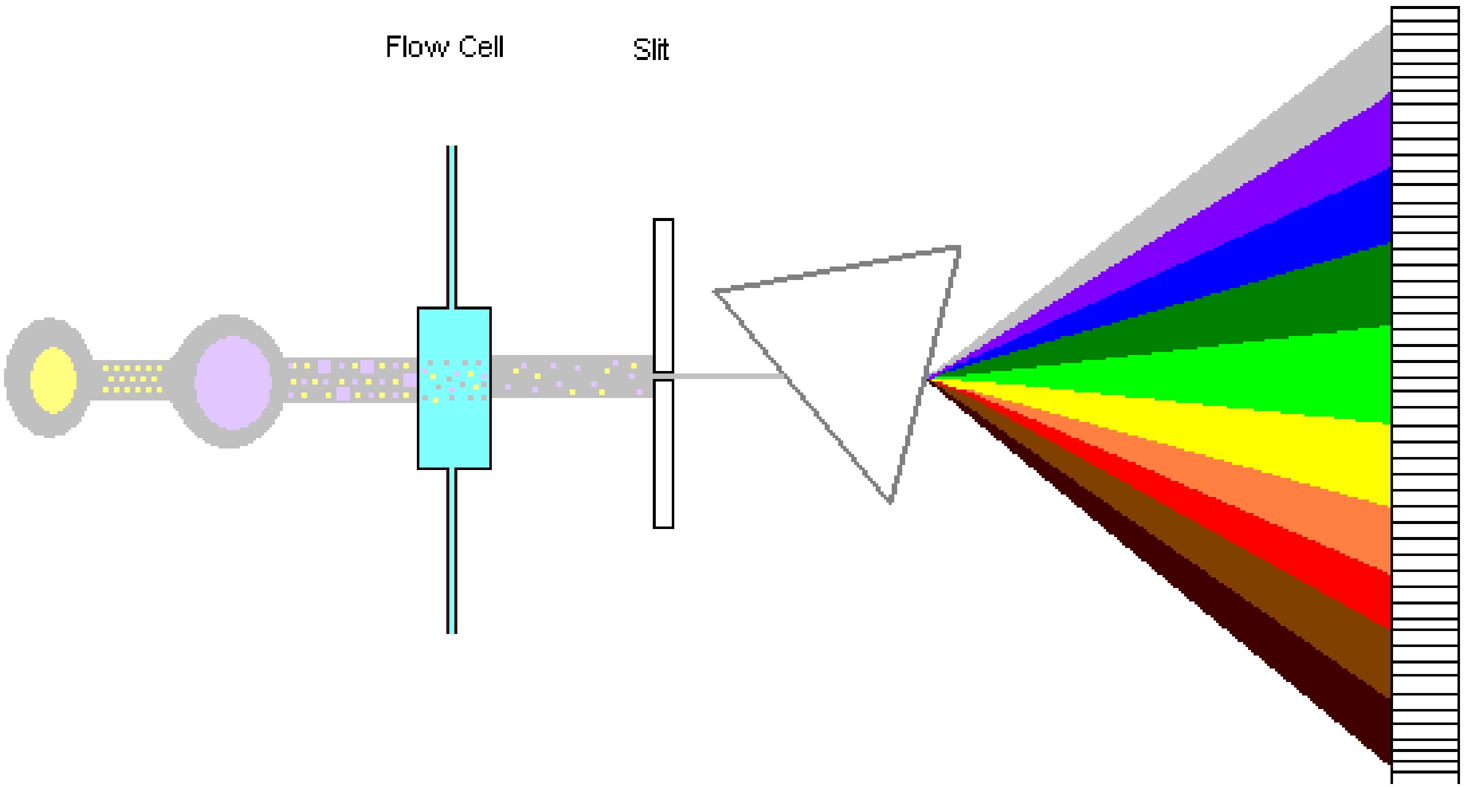
Operating Principle – Reference on a DAD

- Light beam passes the flow cell before being diffracted
  No true reference signal can be obtained

- Any diode or bunch diodes can be selected as a reference



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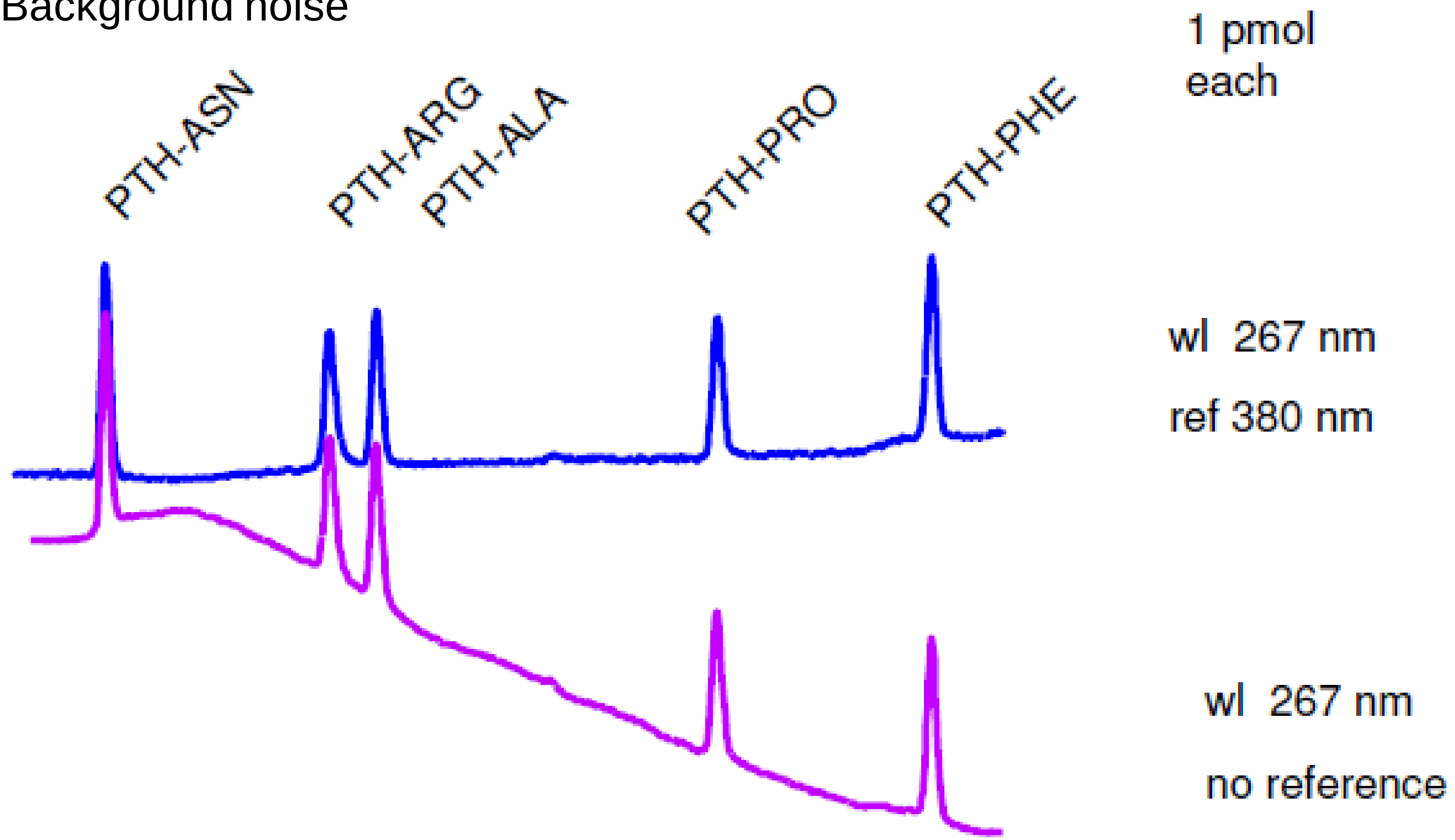
Operating Principle – Reference on a DAD

- A reference can compensate for
  - Fluctuations in lamp intensity
  - Changes in absorbance/refractive index during gradient analysis
  - Background noise



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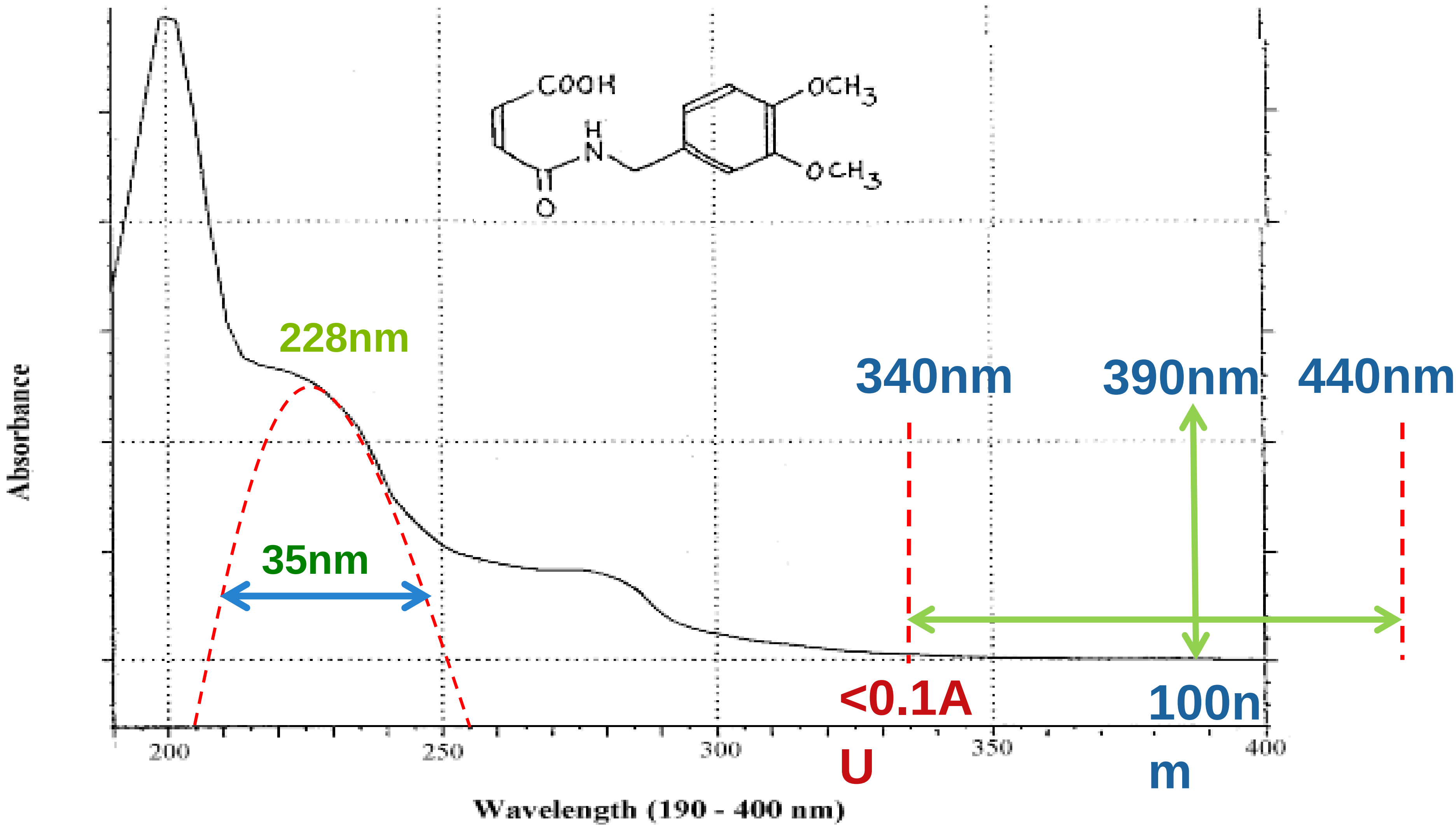
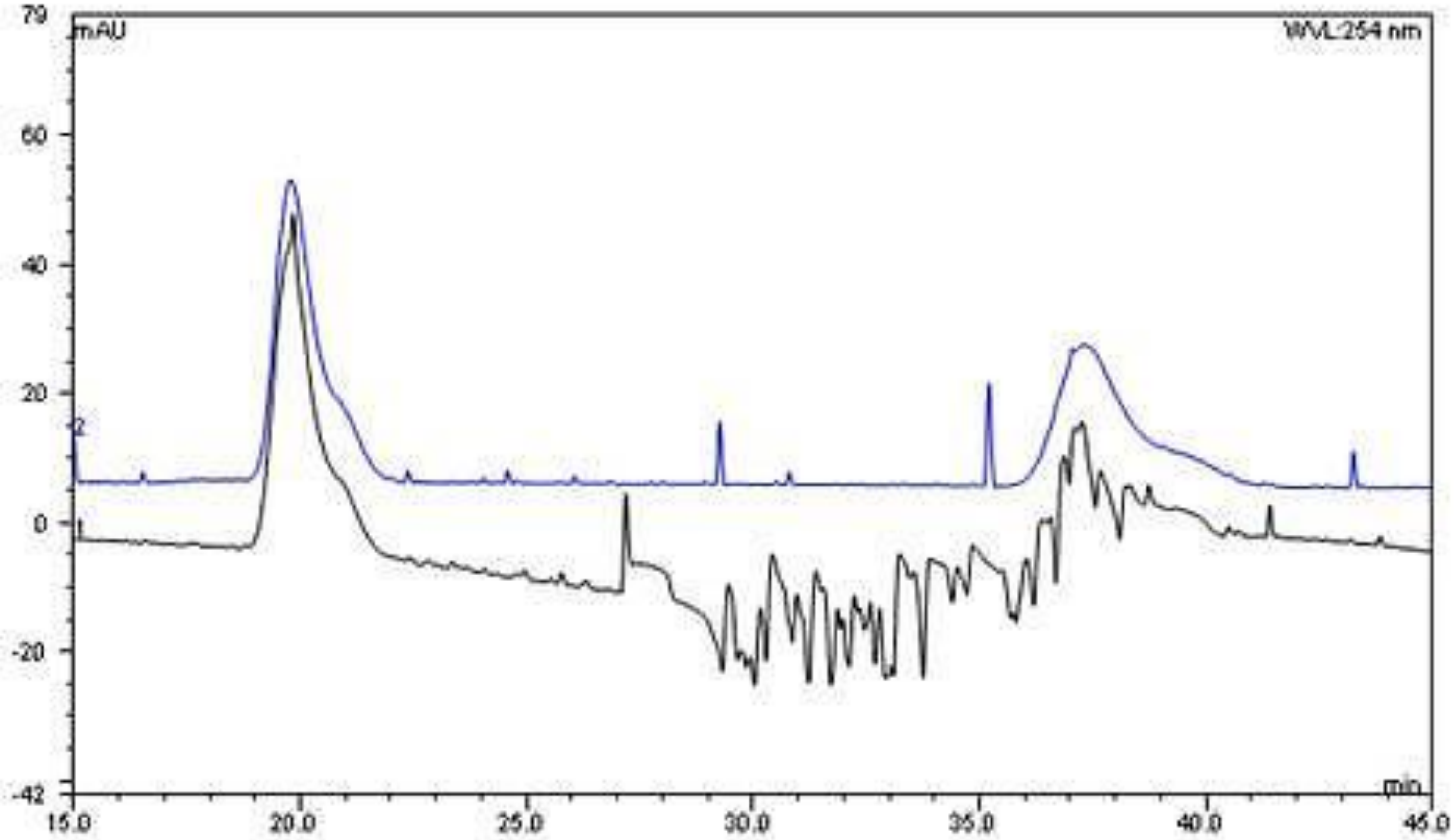
Reference Settings

Sample 228nm, Bw 35nm : Reference 390nm, Bw 100nm



Issues with Reference Wavelength

- Wavelength: 254 nm
- Both the UV and Vis lamps turned on
- Reference wavelength set to 600 nm (80 nm bandwidth)

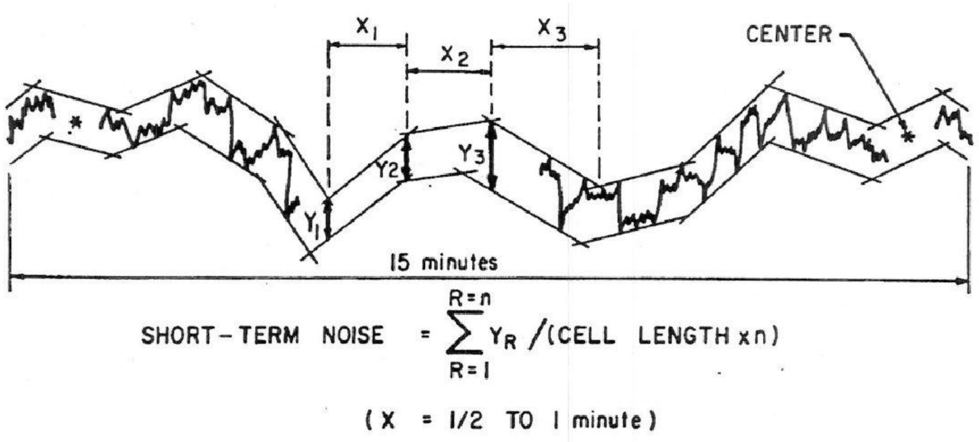


***Blue chromatogram:** Without reference* ***Black chromatogram:** With reference*

Issues with Reference Wavelength

- First, always try to develop a method without a reference wavelength
- Do not use narrow bandwidth with high reference wavelengths
- If you experience intense noise in your UV detection try without the reference wavelength
- Sometimes the problems are caused by the Vis lamp
- If only the UV lamp is on, do not use high reference wavelength settings
- Always run the sample with and without reference during method development

- The Ideal detector ?
- Why do we get a signal?
- Optics
  - Lamps
  - Flow cell
  - Band and slit width
  - Data collection rate & time constant
  - Reference
  - Stray light, refractive index effects & noise



Stray Light

- Stray light is radiation emerging from the monochromator of all wavelengths other than the bandwidth at the selected wavelength
- Arise from imperfections in the grating, optical surfaces, diffraction effects as well as wider bandwidth and slit width settings

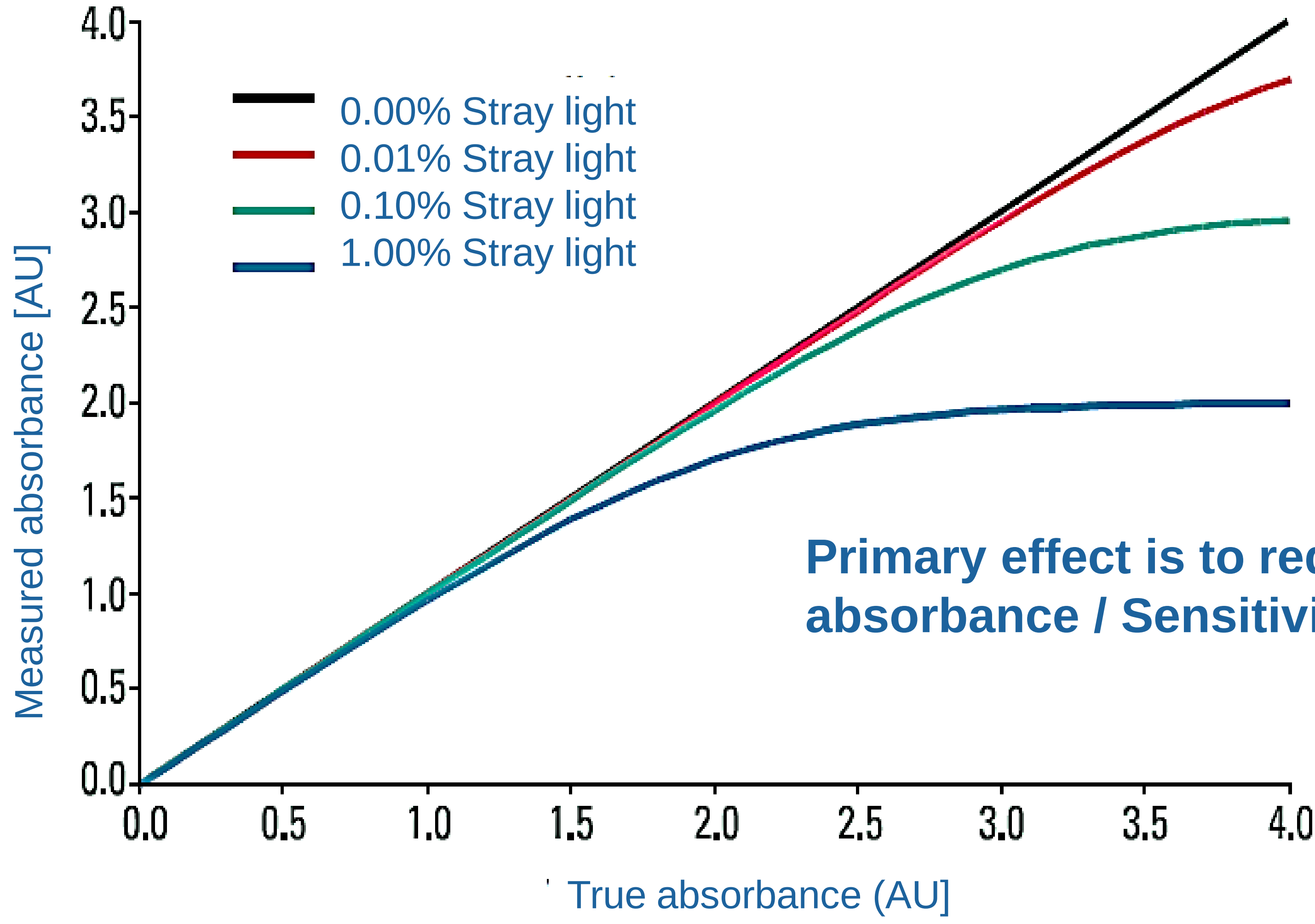
$$A = \varepsilon l c$$

ε = Molar absorption coefficient ($dm^3\ mol^{-1}\ cm^{-1}$)

l = Path length (cm)

c = Concentration ($mol\ dm^{-3}$)

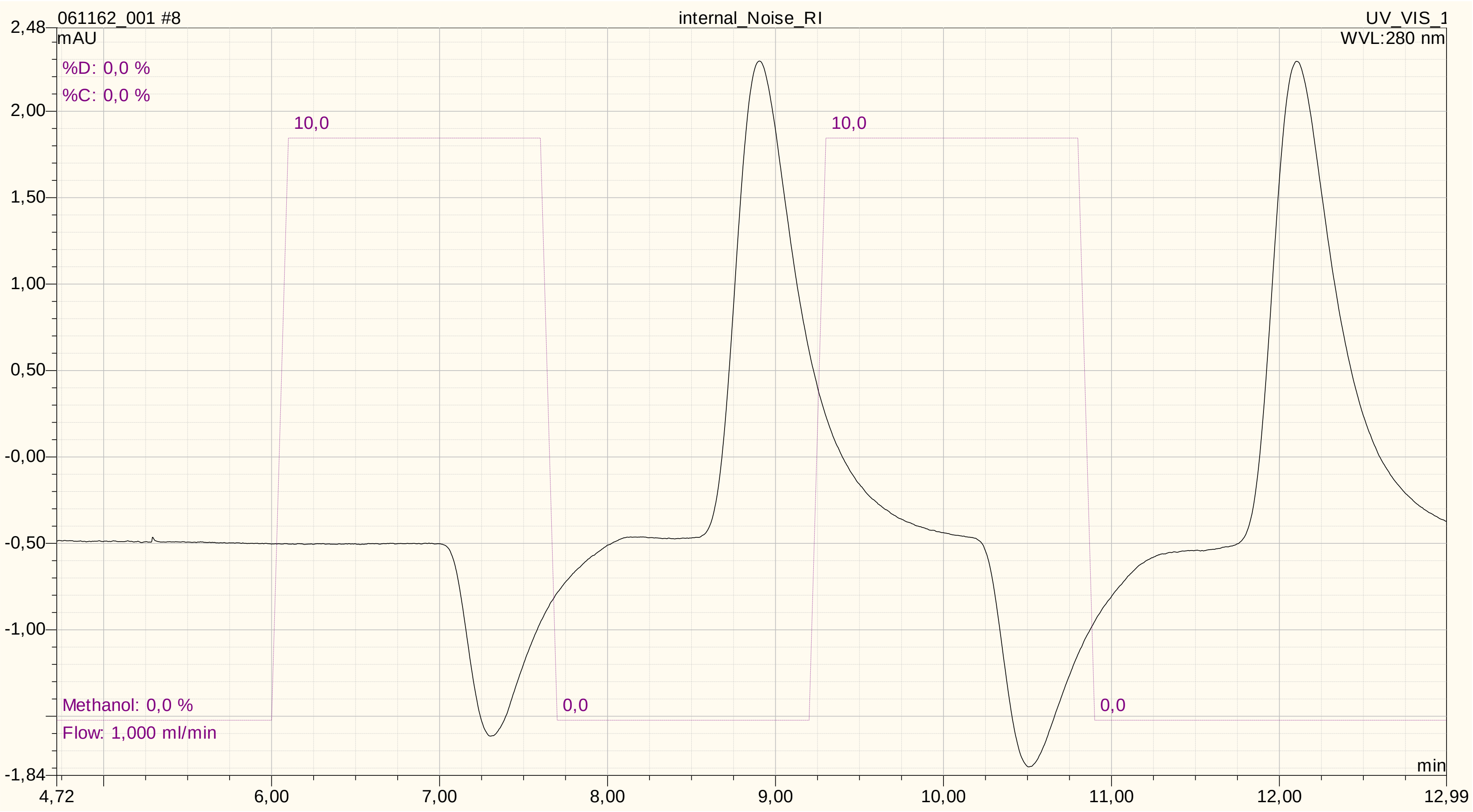
Stray Light



Primary effect is to reduce analyte absorbance / Sensitivity

Refractive Index Effects

- Eluents have different refractive index (RI)
- The flow profile within the flow cell causes RI gradients



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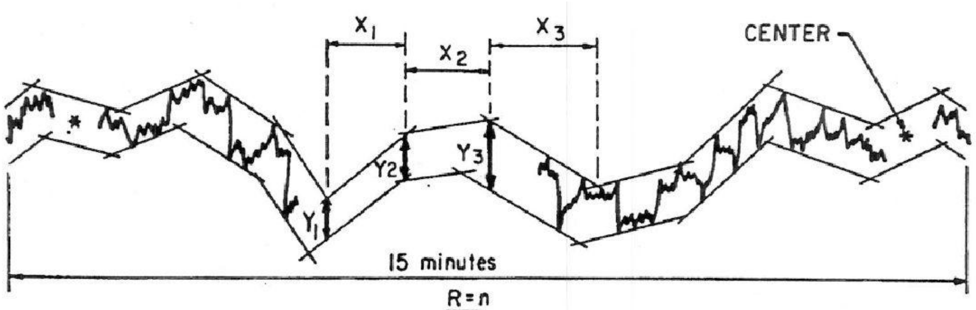
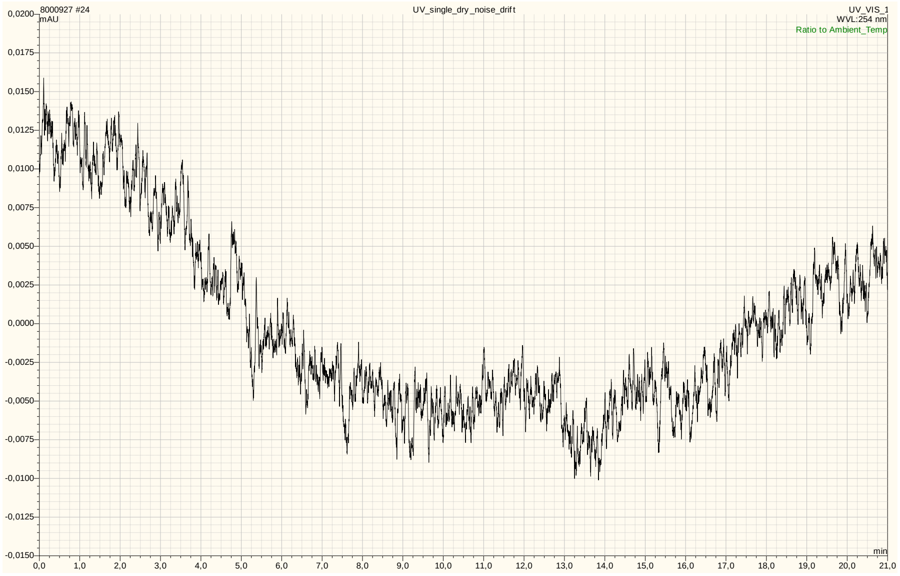
Noise: Definition



$$\text{SHORT-TERM NOISE} = \sum_{R=1}^{R=n} Y_R \,/\, (\text{CELL LENGTH} \times n)$$

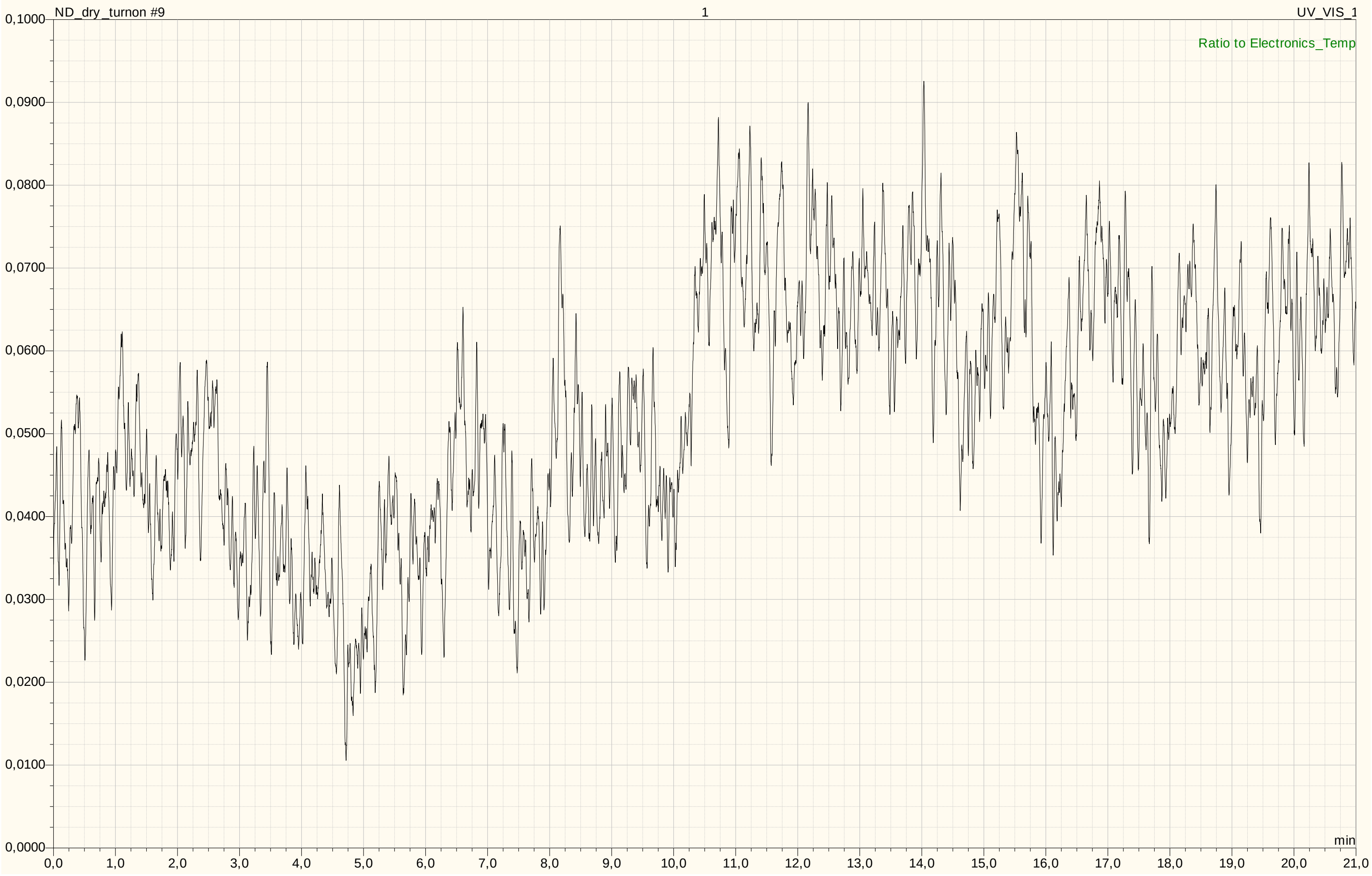
(X = 1/2 TO 1 minute)

- Short term (Statistical) signal changes
- Defined in ASTM
- Defines LOD and detection accuracy

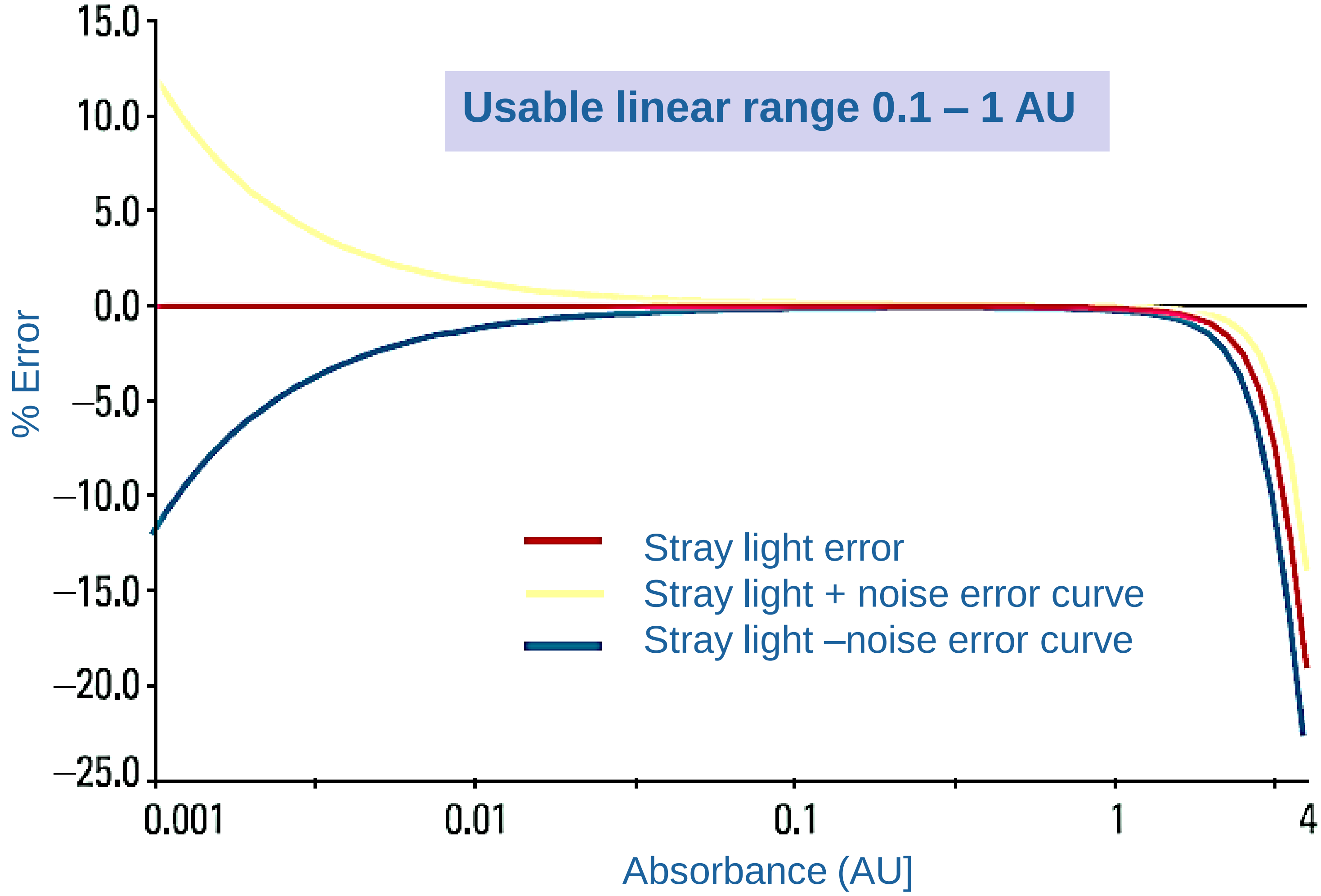


Noise

- Effects measurements on analytes with low absorbance
- Also affects high absorbance samples



Noise



Usable linear range 0.1 – 1 AU
15.0
10.0
5.0
0.0
–5.0
–10.0
–15.0
–20.0
–25.0
% Error
Stray light error
Stray light + noise error curve
Stray light –noise error curve
0.001
0.01
0.1
1
4
Absorbance (AU]

Thank You for Your Attention! Any Questions?

Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an email to
analyze.eu@thermofisher.com
and we will get back to you.