Molecular Spectroscopy:
Comprehensive Approach to UV-Vis & Fluorescence – Navigating why we test, how we test, and how to choose a system

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The Absorption Process

Electromagnetic Spectrum

- Penetrates Earth’s Atmosphere?
- Radiation Type: Radio, Microwave, Infrared, Visible, Ultraviolet, X-ray, Gamma ray
- Wavelength (m): $10^1$, $10^{-2}$, $10^{-5}$, $0.5 \times 10^{-3}$, $10^{-8}$, $10^{-10}$, $10^{-12}$

Wavelength → Frequency → Energy

Absorption

- Energy Loss
- Raman
- Fluorescence

$S_1$ (Excited State)

$S_0$ (Ground State)

vibrational levels
rotational levels
IR
The Measurement
This is an individual preference as to whether one likes the software or not. It depends a lot on ease of use and the types of spectral functionality required. Some individuals prefer individual modules others prefer it all in one application.
Questions to consider

What question am I trying to answer?

- What is it?
- How much is there?

What level of uncertainty do I need to make a decision?

- +/- 5%, +/- 1%, +/-0.1%

What limitations, restrictions, obstacles do I have to overcome?

- Concentration
- Physical form
- Amount of sample
- Etc.

We are not at the mercy of the machines (Instruments)
Instrument Platforms
Diode Array

- Simple optical and mechanical design ensures durability.
- Reliable. Only one moving part!
- Fast. Collect the full spectrum “instantly”
- Wavelength range 190 – 1100 nm
- Single Beam – Greater Drift than double beam instrument (4X)
- White light hit sample – Possible fluorescence interference
Fast Kinetics – Simultaneous $\lambda$ Collection

**Challenges:**
- Need to measure reactions that occur over seconds at more than one wavelength
- Need accurate temperature control to monitor biological reactions
- Use in multi-user laboratory. Must be easy to use and keep clean

**Features**
1. Photodiode array detector
2. No moving parts
3. Open sampling area
4. Easy to operate

**Benefits:**
1. Fast: measure the full spectrum in a single step and in $<1$ second.
2. Accurate and reliable results
3. Monitor multiple wavelength kinetics reactions
Double Beam UV-Vis

Single Monochromometer
1. Split the beam so that 50% of the source energy goes to the reference side and the other to the sample side
2. Simultaneous sample and reference measurement
3. Simultaneous measure of sample and blank
4. Wavelength range 190 – 1100 nm
5. Looses half source energy to sample
Ratio Double Beam

1. Pulsed lamp – long life, reduced cost of owners
2. Only about 5% of the energy diverted to reference detector
3. Higher energy through-put to the sample – lower detection limit
4. Focused beam for small sample volumes and fiber optics
5. Room Light Immunity
6. Fast data collection (80 pts/sec)
7. Wavelength range 190 to 1100 nm
8. No reference beam to monitor blank
Ratio Beam, Pulsed Lamp, Focused Beam
Fiber optics measurements for small sample volumes

Improve workflow and minimize sample preparation

Measure cold samples directly from the refrigerator!

Remotely and safely measure hot, cold, radioactive noxious samples

[Graph showing DNA concentration vs. absorbance]

y = 0.0198x
R² = 0.9996
Double Beam UV-Vis
Chopper System
Double Beam – Time Shared

1. Uses a chopper to alternate the beam between sample and reference
2. Most have higher ordinate capabilities due to reduced stray light due and PMT detector
3. Usually have variable slits when better resolution is required
4. Wavelength range 190 – 900 nm
Double Beam UV-Vis

Pre-Grating Monochromometer
Transmitted light with Increasing Absorbance

<table>
<thead>
<tr>
<th>Abs</th>
<th>%T</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>0.01</td>
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</table>

The amount of light transmitted with increasing absorbance.
Effect of Stray Light Photometric Accuracy

<table>
<thead>
<tr>
<th>Level of Stray</th>
<th>Abs</th>
<th>DM</th>
<th>DGM</th>
<th>SM</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs</td>
<td>0.0001</td>
<td>%E</td>
<td>0.0005</td>
<td>%E</td>
<td>0.02</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0000</td>
<td>.004</td>
<td>1.0000</td>
<td>0.002</td>
<td>0.999</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0000</td>
<td>.002</td>
<td>1.997</td>
<td>0.017</td>
<td>1.991</td>
</tr>
<tr>
<td>3.0</td>
<td>2.999</td>
<td>.010</td>
<td>2.997</td>
<td>0.072</td>
<td>2.920</td>
</tr>
<tr>
<td>4.0</td>
<td>3.995</td>
<td>.110</td>
<td>3.978</td>
<td>0.529</td>
<td>3.522</td>
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<tr>
<td>5.0</td>
<td>4.958</td>
<td>.827</td>
<td>4.823</td>
<td>3.521</td>
<td>3.677</td>
</tr>
</tbody>
</table>

*Where DM = Double Monochromator, DGM = Double Grating Monochromator, SM = Single Monochromator*
Exceeding the Linear Range of the Instrument

The “grass like” fringe at peak indicates the linear range of the instrument has been exceeded.
Pre-Grating Double Beam

Reduces the stray light of the instrument and eliminates the filter wheel. However, system needs a more sensitive detector (PMT) to realize the benefit of the reduced stray light and higher ordinate readings (5A).
Double Beam UV-Vis

Double Monochromometer
Double Beam Double Monochromator

1. Wavelength range 175 – 3300 nm
2. Absorbance range 8A

Double monochromator and optical isolation reduces stray light
Qualitative Analysis

Trading Rules of Spectroscopy

Applies to Uv/Vis and Fluorescence
Selection of Optimal Wavelength

Absorbance

Wavelength

200 300 400 500

A B C
**Resolution** - Ability to detect the presence of two spectral features next to one another.

For qualitative analysis you want to resolve the spectral peaks as much as possible to give the best spectral characterization.

For quantitative you want to resolve the spectral peaks as much as possible to eliminate spectral overlap.

The slit should be 1/10 the half band width of the spectral peak.
Insufficient resolution can cause a reduction of the analytical peak and a spectral shift.
The scan speed and data interval are linked. Faster scan speeds limit the data interval that can be taken affecting resolution and signal/noise since the instrument is measuring the signal for a shorter period of time.
Sample Handling Considerations
Common Solvent Cut-offs (based on a 10mm cuvette filled with solvent reading 1A/ 10%T)
# Cuvette Transmission Characteristics

<table>
<thead>
<tr>
<th>Material</th>
<th>Trademarks</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical glass</td>
<td>OG</td>
<td>360 nm – 2500 nm</td>
</tr>
<tr>
<td>Borofloat®</td>
<td>BF</td>
<td>330 nm – 2500 nm</td>
</tr>
<tr>
<td>Special optical glass</td>
<td>OS</td>
<td>320 nm – 2500 nm</td>
</tr>
<tr>
<td>HOQ 310H</td>
<td>UV</td>
<td>260 nm – 2500 nm</td>
</tr>
<tr>
<td>Quartz SUPRASIL®</td>
<td>QS</td>
<td>200 nm – 2500 nm</td>
</tr>
<tr>
<td>Quartz SUPRASIL® 300</td>
<td>QX</td>
<td>200 nm – 3500 nm</td>
</tr>
</tbody>
</table>

Transmission of empty cells made of different materials

![Graph showing transmission of empty cells made of different materials](image.png)
Sample Handling & Cuvettes

Materials

Quartz sample cells (cuvettes) or sample cells with quartz face plates are required if you want to use the full 190 to 1100 nm wavelength range of your spectrophotometer. If you plan on working only in the visible and/or short-wave near-infrared range of 350 to 1100 nm, you can use good quality glass cells.

Aperture cuvettes

When using aperture cuvettes it is important that the walls of the cell are black so that light passes only through the sample itself and light does not pass through the walls of the cuvette.

Disposable plastic

The quality of these cells varies and should not be used below ~ 325 nm. For better uniformity, some manufacturers place an arrow on the front of the cell so they are measured with the same orientation.
Sample Handling & Cuvettes

Flow Cells

Using a flow cell eliminates the necessity of removing the cell between sample measurement. The design of the flow cell should minimize entrapment of bubbles and flow channeling to provide the most reliable results. Small volume and ease of cleaning are also desirable features.

Cleanliness

The oils in fingerprints are significant absorbers in the UV region and, if left on optical surfaces, can cause erroneous results. Wipe off all fingerprints and contaminants before using a sample cell. Lens tissues for glasses or other uses often contain detergents or lubricants which can affect your measurements. Once a blank measurement has been made, however, do not clean the faces of your cell unless you plan on taking a new blank measurement.
Sample Handling & Cuvettes

Working with Cells

A sample cell should be rinsed three to five times with your intended solvent before you fill it with the pure solvent that will be used in the measurement. Turning the cell upside down on a small stack of absorbent tissues will help remove any residual solvent. This treatment will minimize contamination from previous experiments.

Always install a cell so that it faces the same direction to minimize problems with cell non-uniformity.

Stirring and Temperature Control

Solution uniformity can be a problem, especially for viscous solutions. There are cases where, due to convection induced gradients, rapid absorbance changes may invalidate data. To minimize convection effects keep the temperature of your sample the same as the cell holder or environmental temperature.
Sample Handling & Cuvettes

Mixing

A similar effect can occur in cases of incomplete mixing. This is especially true where the specific gravities or miscibilities of the solvent and analyte are quite different. Again, stirring is a way to prevent this kind of problem.

Photodegradation

In an unstirred cell, it is sometimes possible to observe local photodegradation of sensitive analytes. Because the actual volume of the sample in the light path is very small, stirring the sample will reduce the time any given analyte molecule is in the light path. This minimizes the photodegradation and increases uniformity.
Beer-Lambert Law

What parameter can we change to effect better quantitative analysis?

Absorbance = $\varepsilon \cdot c \cdot l$

$\varepsilon$ = Molar Absorptivity

c = Concentration (moles/L)

$l$ = Path length (cm)
What can we do?

You are presented with the following sample situations:

1. Small sample volume (1 - 2 Abs, ~ 0.5 ml)

   ![Small Sample Volumes](image)

2. High concentration sample (~ 5 Abs, > 10 ml)

   ![High Concentration Samples](image)
What can we do?

3. Multiple Samples ( > 100, Abs 0.1 to 2, < 2 ml)

4. Low concentration sample (< 0.01 Abs, > 10 ml)

Long Pathlength Cells up to 100 mm

Rectangular Long Pathlength Cell
Effect of Pathlength on Absorbance – Long Path Cells

- 100 mm Lpath.Sample: 1.3857 A
- 50 mm Lpath.Sample: 0.68666 A
- 10 mm Lpath: 0.14409 A

Correlation: \( R = 0.999974395 \)

\( \Sigma \beta^2 = 0.31749848 \)
The Tray Cell can read samples as small as 1-2 µl
Quantitative Analysis
Accuracy and Precision

One can be precisely wrong!
Why do we quantitative in absorbance when the instrument measures %T?

- Concentration is linear with absorbance not % T
- Historically, a straight line relationship was easier to plot than a polynomial
- With computers, we are able to fit the curve to a number of curve fitting models

\[ Y = mx + b \]
Steps in Developing Standard Concentration Curve

1. Scan the solvent to identify any cut-offs
2. Scan the sample in the solvent to confirm the analytical wavelength
3. Initially, run a serial dilution to establish the upper and lower detection limits
4. Keep the absorbance range of the standards between 0.2 and 2.0 A
5. Make up individual standards from stock solution and bracket sample range of unknowns
6. Use at least three standards to construct the regression curve (Linear, Quadratic, Polynomial, Cubic, Multicomponent Analysis, Etc.)
7. Validate the method

\[ Y = mx + b \]
Factors Causing Deviation From Beer’s law

- Gravimetric / Diametric Errors
- Incomplete Spectral Resolution
- Ionizable Species
- Temperature Effects
- Stray Light
- Solvent Absorption
- Aggregation
- Sensitivity
- Scattering
- Adsorption
Response Time and Curve Fitting Algorithms

Noise is random and self cancels while the signal is constant. A longer response time will reduce the noise in the measurement yielding better precision.

Curve fitting algorithms can give better results for non-linear systems.
Peak Measurement – Minimizing Matrix Interferences

- Peak Height
- 1 point
- 2 point
- Area
- Derivative (Matrix Interference)

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Fluorescence Spectroscopy
Fluorescence and the Particle Nature of Light

The absorption of light increases the velocity of an electron primarily by changing its direction, not its speed.

Thus the electron will undergo an electronic transition from the ground state to an excited state.

Three things can happen while it is in this excited state:

1. It may undergo a radiationless loss (vibrational relaxation) of electronic energy through collisions and other interactions.
   a. Most molecules do not fluoresce because of this deactivation process.

2. It can emit a photon of light (fluoresce).

3. It can undergo a transition to a metastable state (triplet) and phosphoresce.
Why Fluorescence?

The benefits of fluorescence spectroscopy

- **Sensitivity** - Measure target species with extremely high sensitivity (pb, ppt)
- **Selectivity** – Measure an individual component in a complex matrix without the need for chromatography
- **High Information** – Information on emitting species, concentration, reaction kinetics, interactions, molecular motion, etc.
- **Non Destructive** – fluorescence allows one to run preliminary tests then reanalyze the same sample with a complimentary technique
Fluorescence vs. Absorbance - Sensitivity

Absorbance measurements are made by detecting small changes in large amounts of light passing through a sample

- A calculated is obtained which has units
- The value is the same on any instrument
- Optimal range .5 to 2.5 Å

Fluorescence measurements are made by detecting a relatively small amount of light emitted by the sample

- The value is relative and without units
- The value can be different on every instrument
- Optimal Range < .05 Abs
Fluorescence Advantage - Selectivity

- Specificity in luminescence arises from two factors. Only about 10% of all substances that absorb radiation re-emit it as light and there are two selectable wavelengths (excitation and emission) in luminescence as opposed to only one in absorbance.

- It is highly unlikely that two substances will share both a similar excitation and emission. This is because the difference between excitation and emission peaks can range anywhere from several nanometers to hundreds of nanometers.

- No separation chemistry needed.
Fluorescence Spectrophotometer - Sensitivity

- **Light source**
- **Excitation** monochromator
- **Sample**
- **Emission** monochromator
- **Detector (PMT)** — measured at 90 deg to avoid interference of transmitted light

Equations:
- $I_0$
- $I_t$
- $I_f$
- Excitation spectrum
- Emission spectrum
Pulsed Lamp vs Continuum

- Fluorescence intensity is directly proportional to the incident light.
- Large Uv output where most fluorophores absorb
- The Schwarzschild collection optics capture a large portion of the light from the powerful xenon flash lamp and directs it through the sample,
- There is no warm-up time for the lamp
Other Advantages of a Pulsed Lamp

Multiple measurement modes
  - Fluorescence
  - Phosphorescence
  - Lifetimes
  - Bio & Chemi-luminescence
  - Time Delayed Fluorescence

Minimizes Photodegradation

Room-Light Immunity

There is no warm-up time for the lamp
What If My Sample Is Photosensitive

The pulsed lamp reduces the exposure time of the fluorophore to the incident eliminating or reducing any photodecomposition of the sample. The lamp only flashes when a measurement is being made.

Some pulsed lamp unit pulse the lamp continually.

**Figure** (a) and (b). Emission wavelength vs intensity for BFP following 370 nm excitation. A negligible drop in peak BFP emission (450 nm) was recorded after 10 successive scans at a scan rate of 120 nm/min (total exposure time 12 min 30 s) using the Cary Eclipse (a), whereas photobleaching of approximately 20% was observed using a commercially available instrument fitted with a xenon arc lamp (b)
Horizontal Beam Image

If one is sample limited, the horizontal beam allows one to use far less sample while having the full beam image impinging on the sample for maximum sensitivity and minimal amount of sample.
**Phosphorescence**

Fluorescence is a nanosecond phenomena in that fluorescence occurs as soon as the light goes on and disappears as soon as the light goes off.

The advantage of delayed fluorescence and phosphorescence is that the lamp is off when the measurement is made.

So there is no background light, and it eliminates any competing fluorescence in the system by other fluorophores.

**Chemiluminescence**

Emission resulting from a chemical reaction

No instrument light source required

Capable of measuring emission well into the red region of the spectrum

Application note: Measuring chemiluminescence of a ruthenium complex
Time Delayed Fluorescence

Diagram showing the process of fluorescence and phosphorescence with labels for various stages such as absorption, internal conversion, intersystem crossing, and nuclear relaxation.
Time Delayed Fluorescence & Phosphorescent Lifetimes

- best performance >100 ms lifetime, can as low as do 50 ms

Total decay time (TDT) represents the time over which the decay will be measured.

Delay Time (DT) represents the time elapsed between the flash the lamp and the emission measurement.

Gate Time (GT) represents the overall reading time of the emission signal, where the fraction of light collected depends on the GT.
Time Delayed Fluorescence - Peroxidase enhanced lanthanide luminescence

Dotted line: standard fluorescence spectrum. Solid line: time resolved fluorescence spectrum (delay time).
The Measurement

Light source → \( \lambda \) excitation

Excitation monochromator

Sample → \( I_0 \) → \( I_t \)

Emission monochromator → \( \lambda \) emission

Detector (PMT) – measured at 90 deg to avoid interference of transmitted light

Spectrum

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Prescan Function

Select the type of scatter for which to search.

- Rayleigh scatter
- 2nd Order
- Raman scatter

Accept changes

Prescan Changes

Update the current method with the new prescan settings:
- Excitation Wavelength: 337.96
- Emission Wavelength: 472.98
- PMT Voltage: 760

OK  Cancel

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The Software

- Instrument setup
  - Data mode: Fluorescence

- Scan setup
  - Excitation: 350.00 nm
  - Start: 400.00 nm
  - Stop: 600.00 nm
  - X Mode: Wavelength (nm)

- 3-D Mode
  - Ex. Slope (nm): 450.00
  - Ex. Increment (nm): 10.00

- Scan control
  - Scan rate (nm/min): 24000.000
  - Averaging time (s): 0.0125
  - Data interval (nm): 5.0000

- Display options
  - Overlay traces
  - Y minimum: 0.0000
  - Y maximum: 1000.000

- Cycle mode
  - No. of scans: 15
  - Time (min): 1.0000

- CAT or S/N Mode
  - Mode: CAT mode
  - Number of scans: 10

- Smoothing
  - Type: Savitzky-Golay
  - Factor: 5

- Excitation filter
  - Auto

- Emission filter
  - Open

- PMT Detector voltage
  - Manual: Volts (v): 600

- Corrected spectra

- Status display:
  - OK
  - Cancel
  - Help
Fluorescence "Rules-Of-Thumb"

The excitation spectrum is identical in shape to the absorbance spectrum.

The emission spectrum is the mirror image of the longest wavelength absorption band and occurs at wavelengths longer than the absorption spectrum.
Characteristics of Fluorescence Analysis

Inversely Proportional to Temperature
Fluorescence Intensity vs Excitation Slit Width
Effect of Emission Slit on Resolution
Quantitative Analysis of a Fluorophore

Fluorescence is a relative measurement while Uv/Vis is an absolute measurement
Interferences in Fluorescence Analysis
Interferences in Fluorescence

Rayleigh – Tyndall Peak

Raman Peak  2\textsuperscript{nd} Order Band
Eliminating the Raman Peak Interference

How to eliminate Raman band interference
Concentration Quenching

Quenching refers to any process that reduces the fluorescence intensity of a given substance.

This may occur due to various factors like pH, Concentration, temperature, viscosity, presence of oxygen, heavy metals or, specific chemical substances etc..
Other Factors Effecting Fluorescence Intensity

- Concentration
- Quantum yield of fluorophore
- Intensity of Incident light
- Adsorption
- Oxygen
- Ph
- Temperature
- Viscosity
- Photodecomposition
- Quenchers
- Scatter
The End

Questions?