15.2 Fluorescence Measurements

• description of the basic instrument
• an excitation spectrum measures intensity as a function of absorbed wavelengths
• an emission spectrum measures the spectral distribution of emitted fluorescence
• sample geometry influences the shape of the calibration curve
• sensitivity depends upon both the sample and instrumental parameters
• the limit of detection is determined by the spectroscopic blank
The Basic Instrument

- source is a Xe arc lamp if taking spectra or Hg for quantitation
- detector is always a photomultiplier
- the signal is either current (short time constants) or photon counts (long time constants)
- grating monochromators need a low f/#, reciprocal linear dispersion can be as poor as 5 nm mm\(^{-1}\), the excitation is blazed near 350 nm and the emission blazed near 500 nm
- double emission monochromators are used to reduce source scatter
- glass filters are commonly used for quantitation
- interference filters can be used for quantitation, and by tilting, obtained crude spectra
- the 90° geometry is typically used with dilute solutions - note how the cell edges are hidden because the cell itself fluoresces!
- the front surface geometry is used for solids and concentrated solutions - it suffers from the detector "looking at" the excitation
Strength of the Fluorescence Signal

The fluorescence signal depends upon the following parameters.

- the number of source photons at the excitation wavelength, $I_s(\lambda_{ex})\Delta\lambda_{ex}$
- the efficiency of transferring the photons from the source into the sample cell, $E_{ex}(\lambda_{ex})$
- the fraction of the excitation photons absorbed, $[1 - T(\lambda_{ex})]$, which when multiplied by the number of photons is equal to the number of excited states created
- the quantum yield, $\phi_f$, which is the fraction of excited states converted into fluorescence photons at all emission wavelengths
- the fraction of the fluorescence spectrum passed by the monochromator, $F(\lambda_{em})\Delta\lambda_{em}$ (note that $F(\lambda_{em})$ has units of dP/d\lambda!)
- the efficiency of collecting the fluorescence photons and transferring them to the detector, $E_{em}(\lambda_{em})$
- the efficiency of detecting the fluorescence photons and converting them into a measurable signal, $D(\lambda_{em})$, as current or counts

\[
I_f(\lambda_{ex}, \lambda_{em}) = I_s(\lambda_{ex})\Delta(\lambda_{ex})E_{ex}(\lambda_{ex})[1-T(\lambda_{ex})] \\
\times \phi_f F(\lambda_{em})\Delta\lambda_{em}E_{em}(\lambda_{em})D(\lambda_{em})
\]
Excitation Spectrum

• for an excitation spectrum, the emission monochromator is fixed at the fluorescence wavelength giving the largest signal, thus,

\[ \phi_f F(\lambda_{em}) \Delta \lambda_{em} E_{em}(\lambda_{em}) D(\lambda_{em}) = \text{constant} \]

\[ I_f(\lambda_{ex}) = I_s(\lambda_{ex}) \Delta \lambda_{ex} E_{ex}(\lambda_{ex}) [1 - T(\lambda_{ex})] \times \text{constant} \]

• for dilute solutions, \( A < 0.01 \), the transmission term can be replaced by absorption

\[ 1 - T(\lambda_{ex}) = 1 - e^{-2.3A(\lambda_{ex})} \approx 1 - [1 - 2.3A(\lambda_{ex})] = 2.3A(\lambda_{ex}) \]

\[ I_f(\lambda_{ex}) = I_s(\lambda_{ex}) \Delta \lambda_{ex} E_{ex}(\lambda_{ex}) 2.3A(\lambda_{ex}) \times \text{constant} \]

• the excitation spectrum should resemble the absorption spectrum, being different only by the wavelength-dependent source intensity and optical efficiency, lumped together as \( C(\lambda_{ex}) \)

\[ I_f(\lambda_{ex}) = C(\lambda_{ex}) A(\lambda_{ex}) \]
• a $6.3 \times 10^{-3}$ M solution of rhodamine-B is prepared in dry ethylene glycol
• in a 1-mm fluorimeter cell the solution has an absorption greater than 2 from 240 - 580 nm
• the fluorescence is examined in a front-surface configuration with the blocking filter rejecting wavelengths below 630 nm
• over the range 240 - 580 nm, $[1 - T(\lambda_{ex})] \sim 1$, making it possible to determine the source intensity and emission

$$C(\lambda_{ex}) = I_s(\lambda_{ex}) \Delta \lambda_{ex} E_{ex}(\lambda_{ex}) = I_{std}(\lambda_{ex})$$

$$A(\lambda_{ex}) = I_f(\lambda_{ex}) / C(\lambda_{ex})$$

• although the correction isn't perfect, it does get rid of the xenon lamp structure
Emission Spectrum

• for an emission spectrum, the excitation monochromator is fixed at the wavelength giving the largest signal, thus,

\[ I_s (\lambda_{ex}) \Delta(\lambda_{ex}) E_{ex} (\lambda_{ex}) [1 - T(\lambda_{ex})] \phi_f \text{ = constant} \]

\[ I_f (\lambda_{em}) = F(\lambda_{em}) \Delta \lambda_{em} E_{em} (\lambda_{em}) D(\lambda_{em}) \times \text{constant} \]

• in order to compare spectra from one lab to another, or to compare a spectrum to theory, what is desired is a plot of \( F(\lambda_{em}) \) versus \( \lambda_{em} \)
• the true and measured spectra are related to each other through the wavelength-dependent efficiency terms

\[ I_f (\lambda_{em}) = C(\lambda_{em}) F(\lambda_{em}) \Delta \lambda_{em} \]

as long as \( \Delta \lambda_{em} \) is constant and smaller than a vibronic peak width it can be ignored
• for comparing spectra taken on the same instrument, there is seldom a need to convert the "raw" spectrum to a "corrected" spectrum
the wavelength-dependent optical efficiencies can be determined over the range of 400 - 700 nm by using standard emitters provided by the National Institute for Standards and Technology (NIST)

\[
I_f (\lambda_{em}) = C(\lambda_{em}) F_{NIST} (\lambda_{em})
\]

\[
C(\lambda_{em}) = I_f (\lambda_{em}) / F_{NIST} (\lambda_{em})
\]

• corrected spectrum can then be computed using the calibration constants

\[
F(\lambda_{em}) = I_f (\lambda_{em}) / C(\lambda_{em})
\]

In the figure, the solid line is the "raw" spectrum and the dashed line is the corrected spectrum. The increase at smaller energies (2.0 \( \mu^{-1} = 20,000 \text{ cm}^{-1} \)) is due to decreased photomultiplier and monochromator efficiencies.
Sample Geometry

The line labeled 2.3A is the calibration curve that would result if \((1 - T)\) were proportional to concentration.

When \(2.3A < 0.1\) the calibration curve is reasonably linear and independent of sample geometry. Note that the relationship is never exactly linear!

With front surface geometry, FS, the calibration curve asymptotically approaches a fixed value. This is because the fluorescence intensity is proportional to \((1 - T)\).

With 90° geometry, D90, the emission intensity is not uniform across the cell. The signal is proportional to \((1 - T)\times10^{-A/2}\). This is disconcerting because it produces a two-valued calibration graph. That is, two concentrations give the same signal!
Component values with a xenon arc lamp and single monochromators

- source: \( I_s(\lambda_{ex})\Delta \lambda_{ex} = 10^{13} \text{ photons at 400 nm} \) (includes the lens)
- excitation efficiency: \( E_{ex}(\lambda_{ex}) = 0.5 \)
- sample parameters: \( (1-T) \sim 2.3\varepsilon/C = 2.3\times10^4\times1\times C \)
- quantum yield: \( \phi_f = 1 \)
- fraction of fluorescence observed: \( F(\lambda_{em})\Delta \lambda_{em} = 0.1 \)
- emission efficiency: \( E_{em}(\lambda_{em}) = 0.025 \) (includes the lens)
- detector efficiency: \( D(\lambda_{em}) = 0.2 \)

Overall equation with values inserted: \( I_f = (5.75\times10^{13} \text{ photons M}^{-1})\times C \)

The detection limit is defined as that concentration yielding a signal to noise ratio of 3. When the signal has units of photons, this is achieved with 9 photons. The concentration producing 9 photons can be computed from the above equation as \( 1.57\times10^{-13} \text{ M} \).

This concentration cannot be reached without a laser because of the blank. With a laser it can only be reached by examining a very small volume of solution with a highly focused beam.
Scatter Contributions to the Blank

The blank is the measured response when no analyte is present in the sample cell. The blank is determined by making a measurement with only solvent, buffer, and other fixed sample components present. In quantitative work, the blank is removed from the sample measurement by subtraction.

A major component of the blank is scattered light.
- **Rayleigh** scatter appears at the excitation wavelength and is due to microscopic inhomogeneities in the solvent refractive index - it is minimized by pointing the electric vector of the excitation beam toward the detector
- **Mie** scatter appears at the excitation wavelength and is due to light interacting with small particles, such as dust or biological cells - it is minimized by centrifugation
- **Raman** scatter appears at longer wavelengths than the excitation and is due to vibrational levels of the solvent - it is minimized by moving the excitation wavelength far from the fluorescence
Interfering fluorescence can come from a necessary sample component or an unwanted sample component. Additionally, most solvents exhibit a very low level of "white" fluorescence.

When doing trace analysis the interference can come from molecules having very low quantum yields. For example, assume the analyte is present at $10^{-9}$ M and has a quantum yield of 1. An equal amount of fluorescence will come from a buffer at 10 mM when it has a quantum yield as small as $10^{-7}$ (which would ordinarily be considered a non-fluorescent molecule).

Two approaches often decrease the interference blank - (1) use an emission monochromator to find a wavelength region that minimizes the interference/sample overlap, and (2), use a highly focused laser to decrease the volume of sample observed.

A double emission monochromator can be used to reduce Rayleigh and Mie scatter contributions to the blank.