FluSpec

A Simulated Experiment in Fluorescence Spectroscopy

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Electronic Excitation in Molecules

Electronic Transitions: Electronic excitation occurs when a molecule absorbs light from the ultraviolet (UV) or visible region of the spectrum[1,2]. This process involves the promotion of an electron from a molecular orbital of low energy to one of higher energy. Most commonly, an electron that is involved in a molecular orbital such as a non-bonding (n) or a pi-bonding (π) orbital is readily excited to a higher energy orbital such as a π^* or σ^* anti-bonding orbital to produce one of the four most common excited states that are depicted as follows:

Transition	Excited State
$n \rightarrow \pi^*$	(n, π^*)
$\pi \rightarrow \pi^*$	(π, π^*)
$n \rightarrow \sigma^*$	(n, σ^*)
$\pi \rightarrow \sigma^*$	(π, σ^*)

The energy of the excited state increases as one moves down the above list. Thus for example, the state designated (π, π^*) is of a higher energy than the (n, π^*) state. It is important to note however, that the (n, π^*) state is not always the lowest energy state such as in the case of simple aromatics (e.g. C_6H_6).

Singlet and Triplet States: A further complexity arises if one considers the spin of the promoted electron in relation to the spin of its partner that is left behind in the lower energy orbital. Two possibilities exist for the relative spin configurations and these, in turn, give rise to two possible excited states, namely the singlet and triplet states[3–5].

Singlet State — In this state (denoted "*S*") the spin of the excited electron is paired (i.e. antiparallel) with that of its partner. Most organic molecules that are dissolved and that have an even number of electrons that are paired exist as a singlet in their lowest possible energy state at room temperature (i.e. "ground state" or S_0 state).

Triplet State — In this state (denoted "*T*") the spin of the excited electron is unpaired (i.e. parallel) with that of its partner. The excited triplet state (T_1) is usually of lower energy than the excited singlet state (S_1) and can be attained through a quantum mechanical process known as intersystem crossing (ISC), which is in essence a conversion from the S_1 to the T_1 state.

Not all molecules have the S_0 state as the lowest possible energy. Indeed, molecules such as O_2 and NO_2 exist with triplet and doublet ground states respectively.

Vibrational Levels: Within each of the S_0, S_1, T_1, etc., electronic levels exist the different vibrational levels. Vibrational transitions within molecules are associated with much less energy compared with electronic transitions. In particular, vibrational transitions are such that they may occur upon absorption of light from the infrared (IR) region of the spectrum that has longer wavelengths (i.e. lower energy) compared with the UV-visible region (shorter wavelengths and hence higher energy). Indeed, a multitude of vibrational states are associated with each of the various electronic states.

If a molecule is vibrationally excited in the ground electronic state (S_0) it tends to lose this energy very rapidly by a process known as "vibrational relaxation" that efficiently returns the molecule to the lowest vibrational level in the S_0 state. This particular vibrational level is sometimes referred to as the "zero-point" level[6,7]. The efficient return of a vibrationally excited molecule[8] to the zero-point level in this way has the effect that, at room temperature and below, electronic excitation to the S_1 state is usually observed to occur from the zero-point level in the ground state.

In principle, the absorption of UV or visible light by a molecule will thus cause an electronic transition from the zero-point level in S_0 to any one of the vibrational levels within S_1. However, not all of these possible transitions are equally probable. This can be clearly observed if one examines a typical UV-visible absorption spectrum where it is apparent that not all wavelengths (energies) are absorbed to the same extent. The extent to which a given wavelength is absorbed is directly related to the probability with which its associated electronic transition occurs.

Franck-Condon Principle: According to the IUPAC Compendium of Chemical Terminology[9], the Franck-Condon principle is classically defined as "the approximation that an electronic transition is most likely to occur without changes in the positions of the nuclei in the molecular entity and its environment. The resulting state is called a Franck-Condon state, and the transition involved, a vertical transition. The quantum mechanical formulation of this principle is that the intensity of a vibronic transition is proportional to the square of the overlap integral between the vibrational wavefunctions of the two states that are involved in the transition." The Franck-Condon principle and the measurement of the Franck-Condon factors are discussed in the literature[8,10–15].

When the structure of the excited state is changed with respect to that of the ground state this will result in a different vibronic wavefunction for the excited state. This, in turn, will produce a different value of the overlap integral between the excited and ground states and a different probability of transition between the two states.

Assuming excitation occurs from the S_0 state, the most probable transition is therefore the one that corresponds to the greatest "overlap" between the wave functions of the zero-point vibrational level in S_0 and the particular vibrational level in S_1. This will be the transition that exhibits the greatest intensity in the spectrum.

Fate of an Electronically Excited Molecule

Photophysical Processes: The process of electronic excitation and the ways in which an electronically excited molecule can dissipate its energy is best summarized in the form of a Jablonski diagram[16,17]. Such diagrams depict deactivation processes as being either "radiative" (solid arrows), meaning that light in the UV or visible region of the spectrum is emitted by the excited molecule, or "non-radiative" (wavy arrows), meaning that deactivation occurs without emission of photons[18]. Non-radiative vibrational relaxation may occur by collisions between excited molecules and the solvent molecules leading to the heating of the solvent (this is the basis of photoacoustic spectroscopy).

In summary, the Jablonski diagram indicates the following main photophysical processes[19]:

Internal Conversion (IC)	Non-radiative deactivation between two states of the same multiplicity, e.g. S_1 ~~> S_0
Vibrational Relaxation (VR)	Non-radiative loss of vibrational energy within S_0, S_1, etc.
Intersystem Crossing (ISC)	Non-radiative deactivation involving a quantum mechanical change in multiplicity of the state, e.g. S_1 ~~> T_1, T_1 ~~> S_0, etc.
Phosphorescence	A (slow) radiative process between states of different multiplicity, typically $T_1 \rightarrow S_0$
Fluorescence	A (fast) radiative process between states of the same multiplicity, typically $S_1 \rightarrow S_0$

Phosphorescence and fluorescence are distinguishable in practice by the lifetime of the excited state. Fluorescence is a very fast process where the excited state is dissipated (decays) on the timescale typically of the order of nanoseconds to picoseconds. Phosphorescence, on the other hand, exhibits lifetimes in the order of microseconds to even minutes or longer[20].

Deactivation and Kasha's Rule: The excitation of a molecule to a particular vibrational level in S_1 is rapidly followed by rapid VR to the lowest vibrational level in S_1. Fluorescence will predominantly occur from the lowest vibrational level in S_1. Competing with fluorescence is ISC to populate the T_1 state. Rapid VR within the T_1 state will render the molecule in the lowest vibrational level in T_1 from which phosphorescence will predominantly occur. These observations are embodied in what is known as "Kasha's rule" which states that photon emission during fluorescence or phosphorescence occurs in appreciable yield only from the lowest excited state of a given multiplicity[21]. Thus overall, it can be said that in solution excitation occurs from the zero-point vibrational level in S_0 and emission occurs from the zero-point vibrational level in S_1 (or T_1).

The process of fluorescence (or phosphorescence) involves the emission of energy in the form of a photon from a molecule in the lowest vibrational level in S_1 (or T_1 in the case of phosphorescence) as it deactivates to one of the vibrational levels in the S_0 state. Clearly, there are many vibrational levels within S_0 to which the molecule can return so there are many possible transitions upon deactivation. The probability of a given transition occurring is again governed by the vibrational overlap integrals[10–14] and this explains why an emission spectrum (fluorescence or phosphorescence) exhibits different intensities at different wavelengths (energies). The history of the fluorescence and phosphorescence emission phenomena before the emergence of quantum theory has been discussed in the literature[22] and makes most interesting reading.

Stokes Shift: Consideration of the energetics of absorption and emission as revealed by the Jablonski diagram would suggest that the energy associated with the emission process is always less than the energy associated with the absorption process due to the non-radiative processes such as VR and IC. This means that fluorescence and phosphorescence emission of molecules in solution will always be observed at a longer wavelength (i.e. lower energy) than the absorption that produced the excited state in the first place[23]. The displacement between the absorption and emission maxima is known as the Stokes shift[16] and is often quoted in units of cm^{-1} to reflect that it is indeed a measure of energy difference.

The relationship between the absorption spectrum and the fluorescence emission spectrum, along with the Stokes shift is depicted in Figure 1. In this diagram the various vibrational states (designated with different values of the vibrational quantum number (ν) associated with each of the electronic levels) are indicated[24]. The transitions that give rise to the corresponding absorption and emission spectra are shown along with the so-called "O-O" band. The O-O band exhibits: (i) the lowest energy in absorption spectrum and (ii) the highest energy in emission spectrum. Furthermore, the O-O band need not be the most intense band in the spectrum. It may be so weak that it is undetectable. It can also be inferred from the schematic diagram that, in this hypothetical example, the 0-2 transition is the most probable one as it exhibits the greatest intensity.

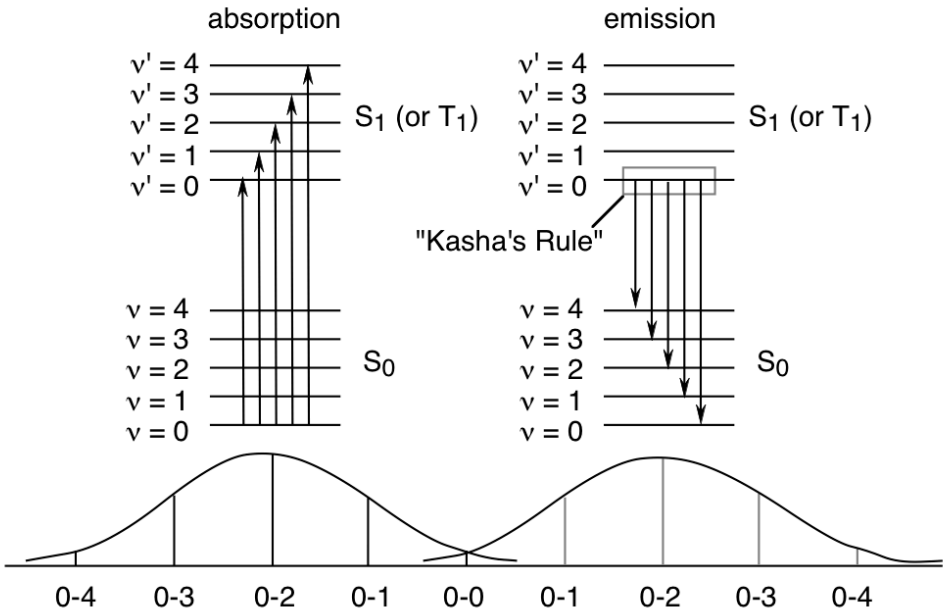

Figure 1. Schematic diagram showing relationship between absorption and emission.

Mirror Image Rule: In many cases the configuration of the molecule and the vibrational energy level spacing are similar in the S_0 and S_1 states. Thus the fluorescence emission and absorption spectra are often mirror images of each other. This is known as the "mirror image" rule[16,25]. Figure 1 also provides some insight into why this rule often applies when one considers that the probabilities associated with the various excitation transitions are often similar to the probabilities of the corresponding deactivation transitions.

Although some fluorescence spectra are broad and featureless (as inferred in the schematic diagram) others contain much more fine structure and can be used to identify compounds due to their distinctive shape and peak maxima. For example, the fluorescence emission spectrum of anthracene[25] contains very sharp peaks that occur at distinct wavelengths.

Spectrofluorimeters

One can detect, spectrally disperse, and record the fluorescence emission of a sample using an instrument known as a "spectrofluorimeter" or simply "fluorimeter" as a shortened version of the name[26–28]. There are other names given to this instrument, such as "fluorescence spectrophotometer", "fluorometer", etc., but the term "fluorimeter" is certainly amongst those most commonly used. The physical arrangement of the functional elements in a fluorimeter is such that fluorescence emission is collected and detected at right angles to the direction of the excitation beam. This feature explains why fluorimetric analysis is usually much more sensitive than colourimetric analysis because it is easier to measure low light intensities at right angles to the excitation beam than small differences in the intensity of, say, a transmitted beam in the case of colourimetric analysis.

In a fluorimeter, the excitation light source is commonly a xenon lamp[16,29]. Sometimes a combination of a deuterium lamp[30] that produces UV light along with a halogen lamp[16,31] that produces visible light is used to enable excitation wavelengths to be selected from anywhere in the UV and visible regions of the electromagnetic spectrum. Excitation wavelengths that lie in the blue and violet regions of the visible spectrum or in the UV region are common for many fluorimetric experiments. Light from the excitation source is passed through a monochromator[16,32] (excitation monochromator) set to an appropriate excitation wavelength (usually the absorption maximum of the fluorophore) and the excitation beam emerging from the monochromator is focused onto the sample. The sample is typically contained in a 1 cm path length fluorescence cell[33] mounted in a temperature-controlled holder. The fluorescence emission at right angles to the excitation beam is passed through another monochromator (emission monochromator) and detected by a photodetection device such as a photomultiplier tube (PMT)[16,34].

There are two basic modes in which a fluorimeter can be operated. In one of the modes a spectrum of the fluorescence emission (emission spectrum) can be recorded and in the other mode a fluorescence excitation spectrum can be recorded[16,27]. The instrumental set-up associated with each of these modes is as follows:

Emission Spectrum	Excitation monochomator is set to a fixed wavelength, λ(ex). Usually the excitation is the wavelength of maximum absorbance, λ_{max}(abs), of the analyte. Emission monochromator is scanned.
Excitation Spectrum	Emission monochomator is set to a fixed wavelength, λ(em). Usually the emission monochromator is set to the wavelength of maximum fluorescence emission, λ_{max}(em), of the analyte. Excitation monochromator is scanned.

The fluorescence excitation spectrum often corresponds to the absorption spectrum of the fluorophore and can be used to help identify fluorescent species[26]. Fluorescence excitation spectra can also be used to identify impurities provided the impurity is fluorescent and has an absorption spectrum different to that of the analyte.

Quantitative Fluorimetry

Fluorescence Quantum Yield: During steady-state fluorescence experiments the sample is excited using an excitation beam that has a constant intensity. This means that the sample is exposed to a constant number of photons per unit time per unit cross-sectional area. Now, under these conditions: (i) some photons are absorbed and excite sample molecules, (ii) some photons are not absorbed and are transmitted and (iii) some excited molecules may fluoresce whereas others may return to ground state *via* non-radiative pathways. Thus not all photons that are absorbed are re-emitted in the form of fluorescence.

The fluorescence quantum yield, ϕ_f, is defined as the fraction of the absorbed photons that are emitted as fluorescence[16,35,36]. Relative ϕ_f values are obtained by comparing integrated areas under the fluorescence emission spectra of an analyte and a reference. The two spectra must be run under identical instrumental conditions and the solutions should be sufficiently dilute (i.e. the absorbance should be less than about 0.05). Dilute solutions are required in order to avoid problems such as the significant attenuation of the incident excitation beam in the outer regions of the sample cell before it reaches the central part from which the fluorescent photons are collected by the optics of the instrument. Furthermore, the temperature of the sample compartment must be accurately controlled because the fluorescence quantum yield of most compounds is temperature-dependent[35].

One can derive the following expression that enables the fluorescence quantum yield, ϕ_f, of an analyte to be calculated from the area under its fluorescence emission spectrum and the area under the fluorescence emission spectrum of a reference substance:

$$\phi_s/\phi_r = [\alpha_s n_s^2 I_0(\lambda_{ex,r})(1 - 10^{-A_r})] / [\alpha_r n_r^2 I_0(\lambda_{ex,s})(1 - 10^{-A_s})] \quad (1)$$

where α is the area under the (corrected) fluorescence emission spectrum, n is the refractive index and the subscripts "s" and "r" represent the analyte (sample) and "reference" solution respectively. The refractive index terms correct for different transmissions of the excitation light intensity across the solution/cell interface if different solvents are used for the sample and reference. The quantities $I_0(\lambda_{ex,s})$ and $I_0(\lambda_{ex,r})$ are the respective intensities of the excitation beams used to excite the sample and reference solutions respectively and A_s and A_r are the respective absorbance values for these solutions measured at their respective excitation wavelengths.

Normally one would use a fluorescence standard such as quinine bisulfate (QBS) whose absolute value of ϕ_f has been accurately determined elsewhere using methods involving actinometry, integrating sphere detectors, etc. For QBS, ϕ_f(QBS, 0.5 M H_2SO_4) = 0.546 at 25°C and this value does not change with excitation wavelength[36–39]. The latter is normally a requirement of a fluorescence standard compound and implies that it is highly desirable for the fluorescence quantum yield of any such fluorescence standard to remain constant across a wide range of excitation wavelengths.

The shape of the recorded fluorescence spectrum will normally be distorted due to the varying wavelength sensitivity of the photomultiplier tube/emission monochromator combination. True corrected fluorescence spectra can be determined from correction factors obtained from the knowledge of the spectral response curves of the photomultiplier and monochromator or by recording the spectrum of known fluorescence standards or standard light sources. These correction methods have been well described in the literature[16]. In modern instruments the correction data is often incorporated into the software provided so corrected emission spectra are readily obtained.

To minimize inaccuracies that arise from applying correction factors across the operating range of the photomultiplier tube, the fluorescent reference substance is preferably chosen so that it can be excited at the same wavelength as the sample and has an emission spectrum covering a similar wavelength range. Clearly, in cases where the sample and reference are excited at the same wavelength equation (1) is simplified in that the intensity ratio $I_0(\lambda_{ex,r})/I_0(\lambda_{ex,s})$ becomes unity. Other possible problems that must be overcome for the accurate measurement of fluorescence quantum yields in the laboratory include instrument inner filter effects, wavelength effects on ϕ_f, polarization effects, impurities, photochemical instability as well as Raman and Rayleigh scattering. Further details relating to the accurate determination of fluorescence quantum yields in the laboratory can be found in the literature[35].

Analytical Fluorimetry: It can be shown that, for dilute solutions, the fluorescence emission intensity (expressed in terms of the integrated fluorescence intensity which is the area under the fluorescence emission spectrum) is proportional to the analytical concentration of the fluorophore[26,27]. Thus one can construct a calibration plot of fluorescence intensity, I_f, in terms of the area under the fluorescence emission spectrum versus concentration, c, and use this in an analogous way to other such calibration plots that are commonly constructed when utilizing various analytical techniques such as atomic absorption spectroscopy, gas chromatography, UV-visible spectroscopy, high performance liquid chromatography, etc.

Bimolecular Quenching: A quencher, Q, is a substance that accelerates the decay of an electronically excited state of molecule M, to either a lower excited state or the ground state. If the original excited state is luminescent, quenching is observed as a decrease in the intensity (quantum yield) of light emission. Quenching can be represented thus:

$$M^* + Q \rightarrow M + Q^* \qquad (2)$$

where M* and Q* are excited states of a molecule M and a quencher Q respectively[16,26,27]. The possible mechanisms for the quenching process include: (i) a rapid, reversible electron transfer occurring between M* and Q, and (ii) the formation of an excited-state encounter complex (M* . . . Q) and, when in this state of "collision", Q may promote rapid IC or ISC to a lower energy state. In many cases, the mechanism of quenching is not always fully understood.

A consideration of the kinetics of quenching enables the Stern-Volmer equation[8,16,26] to be derived:

$$\phi^\circ_f/\phi_f = 1 + k_q\tau_f[Q] \qquad (3)$$

where ϕ°_f and ϕ_f are the respective fluorescence quantum yields in the absence and presence of quencher, k_q is the second-order rate constant for the quenching process, τ_f is the fluorescence lifetime of M* in the absence of quencher Q and [Q] is the concentration of the quencher. The Stern-Volmer equation thus constitutes a mathematical relationship between the fluorescence quantum yield and the concentration of the quencher.

The Stern-Volmer equation can be verified by plotting ϕ°_f/ϕ_f versus [Q] which should yield a straight line passing through (0,1) and whose gradient is equal to the product $k_q\tau_f$. Such a plot enables the value of k_q to be determined if τ_f is known. The fluorescence lifetime of a molecule can be determined in a separate experiment utilizing one of a number of experimental techniques.

Diffusion-Controlled Quenching: Under the conditions of diffusion-controlled quenching one assumes that: (i) quenching occurs on every collision, (ii) the quenching process itself is very fast and (iii) the rate-determining step is the diffusion of Q to within the "active sphere" of M*, the excited molecule (see equation (2) above). In cases where the collision partners are of similar size and are not charged the diffusion-controlled rate constant, k_{diff}, can be calculated theoretically using the Debye-Smoluchowski and Stokes-Einstein equations[16,28]. These equations lead to the following:

$$k_{diff} = 8000RT/(3\eta) \qquad (4)$$

where R is the ideal gas constant, T is the absolute temperature and η is the viscosity of the solution.

Three possible cases arise when one compares the experimentally observed quenching rate constant, k_{obs}, with the theoretical value of the diffusion-controlled rate constant, k_{diff}. These are: (i) if $k_{obs} = k_{diff}$ then the quenching process is diffusion-controlled, (ii) if $k_{obs} < k_{diff}$ then not every encounter between Q and M* leads to quenching and (iii) if $k_{obs} > k_{diff}$ then Q and M* are associated (i.e. "complexed") in the ground state[16,28]. In the last case, a diffusion-controlled quenching mechanism is clearly not operative.

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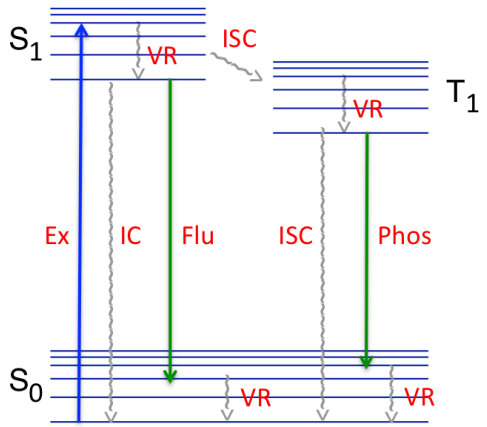
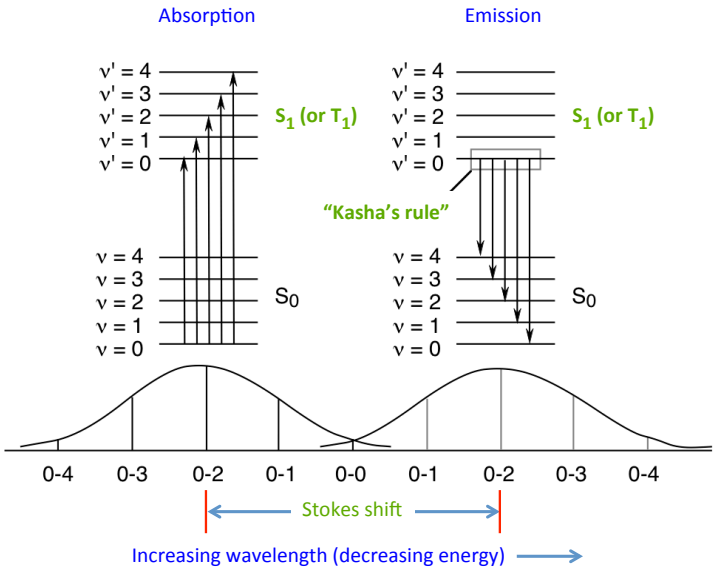
Jablonski Diagram



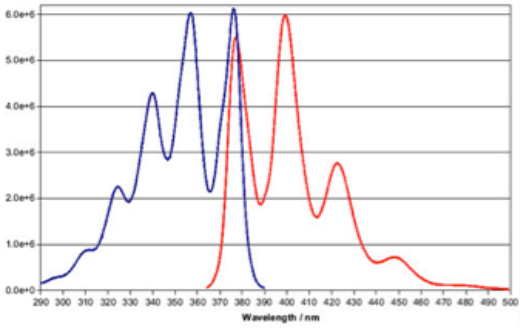
Excitation (Ex) always occurs from the lowest vibrational level ("zero point" level) of the S_0 ground singlet state because **vibrational relaxation** (VR) is a very fast process. The *most probable* transition from S_0 to the S_1 excited state will be that which has the maximum overlap of wave functions describing the ground and excited state (Frank-Condon principle). Vibrational relaxation also occurs quickly in S_1. In the absence of **inter-system crossing** (ISC) to produce the T_1 (triplet) state the molecule is rendered into the lowest vibrational level of S_1.

From the S_1 state the molecule can return to S_0 either by **internal conversion** (IC, a non-radiative transition) or by **fluorescence** (Flu) to a vibrational level in S_0, the latter again in accordance with the Frank-Condon principle. If ISC occurs in the S_1 rendering the T_1 state, the molecule can return to S_0 *via* **phosphorescence** (Phos) or ISC from T_1 to S_0 as indicated.

Schematic Absorption and Emission Spectra and Corresponding Transitions



Excitation and Emission Spectra of Anthracene



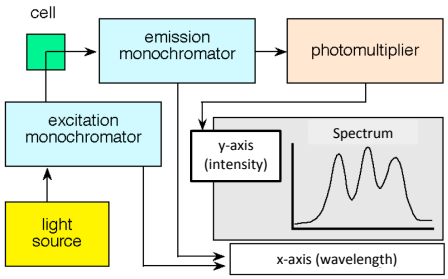
Excitation and emission spectra of 10^{-5} M anthracene in cyclohexane show a mirror image relationship. Note the strong overlap of the O-O excitation and emission bands. In such cases, the more advanced technique of synchronous fluorescence scanning (not available in the FluSpec simulator) can be used in quantitative analytical fluorimetry to improve the detection limits.

Sources:

Byron, C. M.; Werner, T. C. Experiments in Synchronous Fluorescence Spectroscopy for the Undergraduate Instrumental Chemistry Course *J. Chem. Educ.*, 1991, **68**, 433-436.

http://www.lambdaphoto.co.uk/products/115.800

Spectrofluorimeter



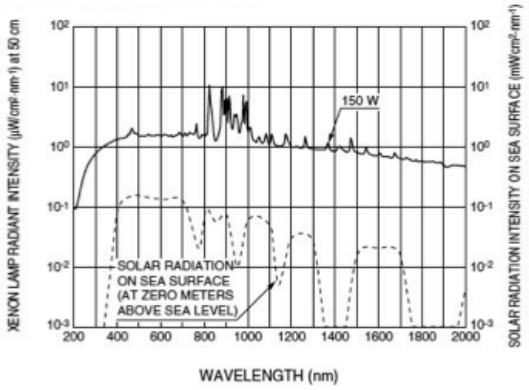
Fluorescence emission and excitation spectra can be detected and recorded using an instrument known as a "spectrofluorimeter" or simply "fluorimeter". In a fluorimeter the fluorescence is collected and detected at right angles to the direction of the excitation beam. Fluorimetric analysis is usually much more sensitive than colourimetric analysis. This is because it is easier to measure low light intensities at right angles to the excitation beam than small differences in the intensity of a transmitted beam. Although some fluorescence spectra are broad and featureless others contain much more fine structure and can be used to identify compounds due to their distinctive shape and peak maxima. The picture (left) shows a typical laboratory fluorimeter. The diagram (right) is a schematic representation of the functional elements of a fluorimeter.

Sources:

http://chemlab.truman.edu/CHEM322manual/perkinelmerls5b.htm

http://chemwiki.ucdavis.edu/Physical_Chemistry/Spectroscopy/Electronic_Spectroscopy/Electronic_Spectroscopy%3A_Application

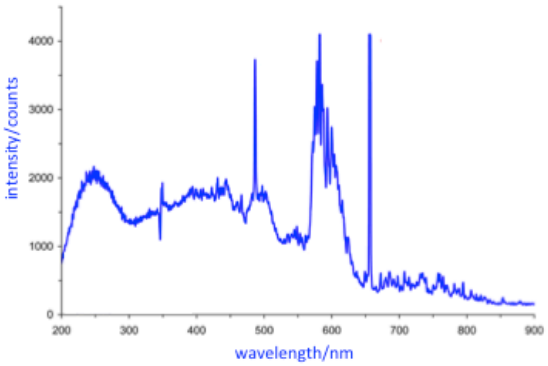
Xenon Lamp



A xenon arc lamp is a specialized gas discharge lamp that produces light by passing an electric current through ionized xenon gas at high pressure. The spectrum is close to that of natural sunlight (see above spectrum noting the logarithmic intensity scale) and so xenon lamps are commonly used in accelerated exposure experiments that test the light-fastness of plastics and textiles. Small xenon lamps can be found in laboratory spectrophotometers and produce wavelengths in the visible region and some of ultraviolet region of the electromagnetic spectrum.

Sources:
http://jp.hamamatsu.com/products/light-source/pd024/index_en.html
http://en.wikipedia.org/wiki/Xenon_arc_lamp

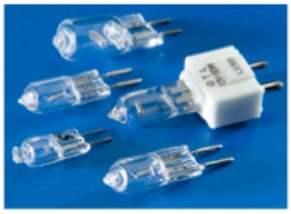
Deuterium Lamp

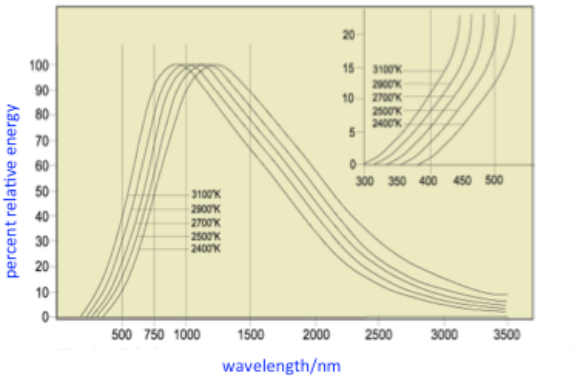


A deuterium arc lamp is a low-pressure gas discharge lamp that produces a continuous spectrum in the ultraviolet region. As such, it is often used in spectroscopy. The lamp emits wavelengths from 112 to 900 nm (note linear intensity scale) with its continuous spectrum considered to be from 180 to 370 nm (molecular deuterium continuum). The above spectrum shows also the characteristic hydrogen Balmer lines (486 and 656 nm) and the Fulcher band emission between *ca*. 560 to 640 nm.

Sources:
http://www.innovations-report.com/html/reports/energy_engineering/lamp_specialist_heraeus_noblelight_unveils_d_2_170664.html
http://en.wikipedia.org/wiki/File:Deuterium_lamp_1.png

Halogen Lamp

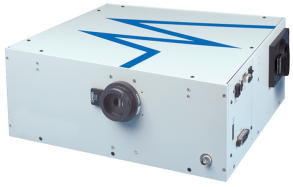


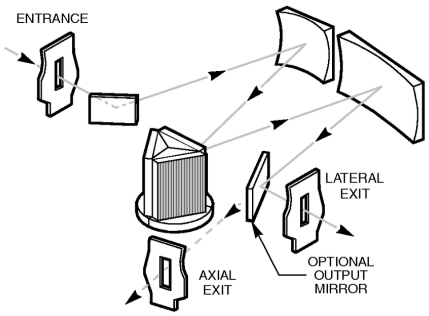


A halogen lamp (also known as a tungsten halogen lamp) is an incandescent lamp that comprises a tungsten filament contained within an inert gas and a small amount of a halogen (e.g. iodine or bromine). The added halogen increases the lifetime of the filament by re-depositing on the filament tungsten that would otherwise be deposited on the inside surface of the bulb. This also prevents darkening of the bulb. Halogen lamps have higher luminous efficiencies compared with other gas-filled counterparts and because of their smaller size can be advantageously used in optical systems such as spectrophotometers, etc. [Note: linear intensity scale on above spectra.]

Sources:
http://www.intl-lighttech.com/applications/light-source-apps/halogen-lamps/halogen-lamps-index
http://en.wikipedia.org/wiki/Halogen_lamp

Monochromators

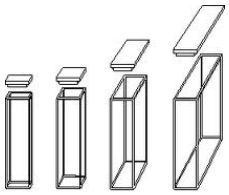


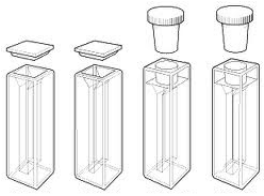


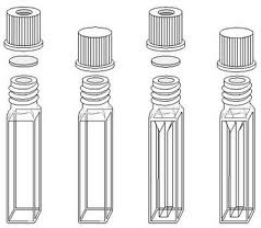
A monochromator is a device that selects a single wavelength or wavelength band from light that is incident on its entrance slit and transmits it through an exit slit. The incident light beam is collimated onto a diffraction grating. The relative motion of the grating with respect to the entrance and exit slit optics enables different wavelengths to be selected and as such a spectrum can be scanned. A common type of mono-chromator is the Czerny-Turner type depicted in the schematic diagram (above right) and can be used in the optics of a spectrophotometer. The picture (above left) shows a stand-alone monochromator that can be mounted, say, on an optical bench.

Sources:
http://search.newport.com/?x2=sku&q2=74126
http://gratings.newport.com/library/handbook/chapter6.asp

Fluorescence Cells



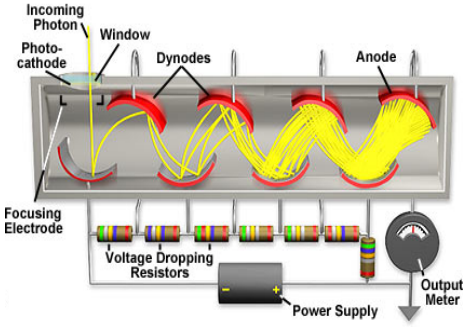




The picture and diagrams give an indication of the wide range of available fluorescence cells ranging from the common 1-cm square based cell that has four optical glass or quarts walls and an inert stopper to other types that have different optical path lengths and stopper systems.

Sources:
http://www.made-in-china.com/showroom/minyotec/product-detailNbixMvmThyhQ/China-Fluorometer-Cell-With-Stopper-TYPE-13-.html
http://shop.spectrecology.com/Quartz-cells-for-Fluorescence_c33.htm
http://www.optiglass.com/ukhome/d_cells/d_cells_f/T003.html
https://wbphotonics.com/index.php/starna-cells/fluorometer-cells.html

Photomultiplier Tubes



In a photomultiplier tube (PMT) the absorption of a photon results in the emission of an electron from a photoactive material (photocathode). The emitted electrons are amplified by electrodes known as metal channel dynodes and electrons are collected by the anode at the end of the chain. The flux of incident photons (intensity) thus produces a proportional electric current over a large range of incident photon fluxes. Photomultiplier tubes produce a signal in the absence of incident light due to a small amount of thermal emission of electrons from the photocathode (dark current) and other sources that create electronic noise. The spectral sensitivity of a PMT depends on the chemical composition of its photocathode. Gallium-arsenide materials are typically used for photocathodes and provide good sensitivity in the range of 300 to 800 nm.

Sources:
http://learn.hamamatsu.com/articles/photomultipliers.html
http://www.hofstragroup.com/product/rca-6342-photomultiplier-tube-2-10-stage-s-11/

Derivation of Equation for the Fluorescence Quantum Yield

One can derive an expression for ϕ_f by realizing that the fluorescence emission intensity, I_f, is proportional to I_a, the intensity of light that is absorbed:

$$I_f \propto I_a \text{ and } I_f = \phi_f \times I_a \quad (1)$$
$$A = \log_{10}(I_0/I_t) \text{ and so } I_t = I_0 \times 10^{-A} \quad (2)$$
$$I_a = I_0 - I_t = I_0(1 - 10^{-A}) \quad (3)$$

where A is the optical absorbance; I_0 and I_t are the respective intensities of the incident and transmitted beams. From equations (1) and (3):

$$\phi_f = I_f/I_a = I_f/[I_0(1 - 10^{-A})] \quad (4)$$

Now $I_f = k\alpha$ where α = area under the fluorescence emission spectrum and k is an instrument constant so:

$$\phi_f = k\alpha/[I_0(1 - 10^{-A})] \quad (5)$$

The constant, k, is one that is associated with a given instrument. In particular it is an instrument factor that links the proportionality between the actual fluorescence intensity and the area (arbitrary units) under the fluorescence emission spectrum produced by the instrument. It is assumed that the value of k does not not vary for a given instrument.

. . . Continued next page

Derivation of Equation for the Fluorescence Quantum Yield (cont'd)

If the analyte is denoted "s" and reference denoted "r" then a form of equation (5) can be written for both the sample and reference solutions:

$$\phi_s = k\alpha_s/[I_0(\lambda_{ex,s})(1 - 10^{-As})] \quad (6)$$
$$\phi_r = k\alpha_r/[I_0(\lambda_{ex,r})(1 - 10^{-Ar})] \quad (7)$$

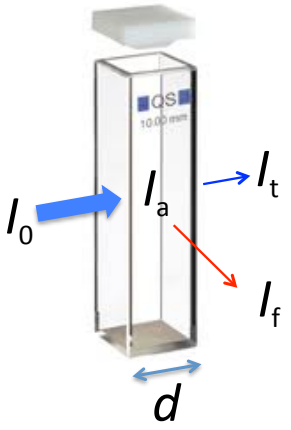
where $I_0(\lambda_{ex,s})$ and $I_0(\lambda_{ex,s})$ are the respective intensities of the excitation beam used to excite the sample and reference solutions respectively; A_s and A_r are the respective absorbance values for these solutions measured at their respective excitation wavelengths.

Dividing equation (6) by equation (7) yields:

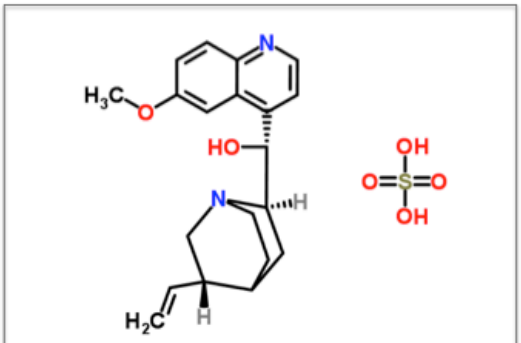
$$\phi_s/\phi_r = [\alpha_s I_0(\lambda_{ex,r})(1 - 10^{-As})]/[\alpha_r I_0(\lambda_{ex,s})(1 - 10^{-As})] \quad (8)$$

A correction for the differences of the refractive index of the analyte and reference solutions (n_r and n_s respectively) can be applied to equation (8) in which case the following general equation is obtained:

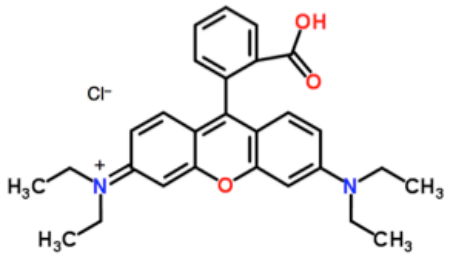
$$\phi_s/\phi_r = [\alpha_s n_s^2 I_0(\lambda_{ex,r})(1 - 10^{-As})]/[\alpha_r n_r^2 I_0(\lambda_{ex,s})(1 - 10^{-As})]$$



Structures of Quinine Bisulfate and Rhodamine B



Quinine Bisulfate: hydrogen sulfate; (R)-(6-methoxy-4-quinolyl)-[(2R,5S)-5-vinylquinuclidin-2-yl]methanol



Rhodamine B: 9-(2-Carboxyphenyl)-6-(diethylamino)-N,N-diethyl-3H-xanthen-3-iminium chloride

Sources:
http://www.chemspider.com/Chemical-Structure.20473857.html
http://www.chemspider.com/Chemical-Structure.6439.html?rid=ced8500d-d909-48eb-a554-889c01e59f62

Fluorescence Emission Intensity and Analyte Concentration

One can demonstrate that at low concentrations the fluorescence emission intensity is proportional to the concentration of the analyte and so fluorimetry can be used as a quantitative analytical technique. Consider the Beer-Lambert law:

$$A = \log_{10}(I_0/I_t) = \varepsilon cd \qquad (1)$$

where I_0 and I_t are the intensities of the incident and transmitted beams respectively, ε is the extinction coefficient (molar absorptivity), c is the concentration of the analyte and d is the optical path length of the cell. From equation (1):

$$\ln(I_0/I_t) = \ln(10) \times \log_{10}(I_0/I_t) = 2.303\varepsilon cd$$
$$I_t/I_0 = \exp(-kc), \text{ where } k = 2.303\varepsilon cd$$
$$I_t = I_0\exp(-kc) \qquad (2)$$

Use the relation $I_a = I_0 - I_t$ and substitute for I_t in equation (2):

$$I_a = I_0[1 - \exp(-kc)] \qquad (3)$$

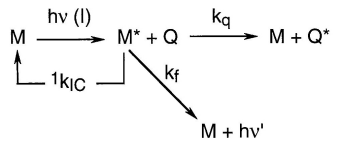
Now since $I_f = \phi_f \times I_a$ then from equation (3):

$$I_f = \phi_f I_0(1 - \exp(-kc)) \text{ and so } I_f \approx \phi_f I_0 kc$$

The latter approximation is valid since $\exp(-x) \approx 1 - x$ if x is small. Whence I_f is proportional to c at low concentrations, an important analytical result.

A Derivation of the Stern-Volmer Equation

The fate of an electronically excited molecule, M, in the presence and absence of quencher, Q, is summarized in the following scheme:



In the absence of quencher: $\phi^\circ_f = k_f/\Sigma^1 k$ (1)

In the presence of quencher: $\phi_f = k_f/(\Sigma^1 k + k_q[Q])$ (2)

where ϕ°_f and ϕ_f are the respective fluorescence quantum yields in the absence and presence of quencher, k_f is the first-order rate constant for the fluorescence process, $\Sigma^1 k$ is the sum of all first order processes that deactivate the M* state (i.e. in this case $\Sigma^1 k = k_f + {}^1k_{IC}$), k_q is the second-order rate constant for quenching and [Q] is the concentration of quencher. Dividing equation (1) by (2) yields:

$$\phi^\circ_f/\phi_f = (\Sigma^1 k + k_q[Q])/\Sigma^1 k = 1 + k_q[Q]/\Sigma^1 k \qquad (3)$$

Thus:

$$\phi^\circ_f/\phi_f = 1 + k_q\tau_f[Q] \qquad (4)$$

where τ_f is known as the fluorescence lifetime of M* in the absence of Q. Clearly, $\tau_f = 1/\Sigma^1 k$. Equation (4) is known as the Stern-Volmer equation.

Diffusion-Controlled Rate Constant

Debye-Smoluchowski Equation

$$k_{diff} = 4\pi\sigma_{MQ}N_A(D_M + D_Q)\ m^3\ mol^{-1}\ s^{-1}$$
$$= 4\pi\sigma_{MQ}N_A(D_M + D_Q) \times 10^3\ M^{-1}\ s^{-1} \quad (1)$$

where: σ_{MQ} = collision diameter of M* and Q [units: m]
N_A = Avogadro constant [units: mol^{-1}]
D_M, D_Q = diffusion coefficients of M* and Q [units: $m^2\ s^{-1}$]

Stokes-Einstein Equation

$$D = kT/(6\pi r\eta) \quad (2)$$

where: k = Boltzmann constant [units: $J\ K^{-1}$]
r = radius of (spherical) particle [units: m]
η = solvent viscosity [units: $kg\ m^{-1}\ s^{-1}$]

Substituting equation (2) in (1), with the assumptions that $\sigma_{MQ} = 2r$ and $D_M = D_Q = D$

$$k_{diff} = 4\pi \times 2r \times N_A\ [2kT \times 10^3/(6\pi \times r \times \eta)]$$

$$k_{diff} = 8000RT/(3\eta)\ [\text{units: } M^{-1}\ s^{-1}]$$

FluSpec

A Simulated Experiment in Fluorescence Spectroscopy

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Exercise 1 – Exploring the Fluorescence Spectrophotometer Simulator and Lab Book

This first exercise is intended as a guide that familiarizes the user with the simulator and encourages him/her to explore its various controls. The user should therefore "experiment" with the simulator before embarking upon the formally structured experiments that follow.

(1) Click the **Flu Spec** tab and enter the fluorescence spectrometer (fluorimeter) simulator. When you enter the simulator after booting, it is automatically set to run the fluorescence emission spectrum of a 2.4×10^{-7} M solution of Rhodamine B (RhB) in ethanol solvent. Note that moving the active **RhB Conc** sliding control to the left will decrease the concentration of the RhB.

(2) Verify the current settings of the monochromators by clicking the **Scan Guide** button. This opens a window that provides relevant information and the suggested wavelength ranges for scanning the samples. In the case of RhB one can observe that it has an absorption maximum of 540 nm (this will be later confirmed experimentally by running its fluorescence excitation spectrum) and the suggested wavelength scanning range to use in observing the emission of RhB is from 560 to 750 nm. Thus by default the fluorimeter is set to scan the fluorescence emission of RhB in accordance with the **Scan Guide** suggestions.

(3) One may put away the guide information either by clicking on its OK button or by clicking the **Scan Guide** button again. Do not be concerned if you forget to do this before starting a subsequent run because the information window will be automatically hidden when scanning commences.

(4) Click the **Scan** button to commence scanning the spectrum. Notice that the label of this button changes to "**Abort**" as soon as scanning commences and so this button can also be used to abort a run.

(5) Observe that during the emission scanning process the fluorescence of the RhB sample in the cell can be seen as a steady orange "glow". This fluorescence results from the excitation of the RhB by the light emerging from the excitation monochromator. Note also that the excitation beam is maintained at a fixed wavelength of 540 nm. The absorption maximum of RhB (measured separately using a UV-visible spectrophotometer) is found to be 540 nm. Hence this wavelength has been recommended and chosen as the excitation wavelength in the current settings.

(6) Notice that during the scan the emission monochromator is sequentially stepped through the wavelengths in the set emission range (i.e. the emission from the sample is being

dispersed into its component wavelengths and as such is said to be "scanned") and the intensity signal from the photomultiplier tube (PMT) detector is being displayed as a function of the wavelength to produce the "fluorescence emission spectrum" in the output window.

(7) Once the scanning is complete one will observe that the integrated area under the entire spectrum is displayed in the control panel in arbitrary units or "arb units". This information will later be used in the subsequent exercises.

(8) Click the **Show Cursor** button to reveal the cursor and drag it along the horizontal axis to confirm that the fluorescence emission maximum of RhB in ethanol occurs at *ca*. 625 nm in accordance with the information given in the **Scan Guide**. Notice that the name of this button changes to "**Hide Cursor**" when the cursor is revealed and can thus be used to toggle the appearance and disappearance of the cursor.

(9) At any time after scanning is complete, a hard copy of the screen with its current contents can be printed. To do this, click the **Print** button that will display the usual system print menu to enable printing to occur.

(10) Each time the **Capture** button is clicked the **Clipboard** is updated with the data that comprise the spectrum along with the relevant instrument settings and conditions under which the spectrum was recorded. This enables the data to be pasted into a suitable spreadsheet program for further display and manipulation if the user desires.

(11) The **Capture** button also activates a file dialog window that displays a suggested, sequentially numbered filename (the suggested filename can be overwritten if desired). This gives the user the option to export the data already in the **Clipboard** as a tab delimited text file that can later be read into a spreadsheet program. Once a text file is created a summary of its contents is written in the **Lab Book**.

(12) The **Clear** button erases all spectra that are currently displayed in the simulator output window. This button does not erase any data files that have been created using the **Capture** button nor does it affect the entries appearing in the **Lab Book**.

(13) Wavelength settings for the monochromators can be made either by typing the required setting into the appropriate edit field or by clicking the up (∧) or down (∨) control buttons that appear at the side of the edit fields. These controls will increase or decrease the setting in steps of 1 nm. The simulator only accepts wavelengths that are within the range of 250 to 750 nm (inclusive).

(14) The sample can be changed to a solution of 4.4×10^{-6} M quinine bisulfate (QBS) made up in 0.5 M H_2SO_4 (optical absorbance at 348 nm is less than 0.05 as measured in a 1 cm path length cuvette) by clicking the sample pull down menu. When QBS is selected as the sample the quencher concentration sliding control becomes active. This control enables the quencher NaCl to be added to the test solution at concentrations from zero to 0.100 M.

(15) Click the **Lab Book** tab to access the contents of the **Lab Book** and to access typical results that are obtained in the various exercises. Separate instructions for the **Lab Book** control buttons appear within that section of the simulator.

(16) Try scanning the emission spectrum of RhB again (2.4×10^{-7} M RhB; excitation wavelength 540 nm) but this time monitor the emission over the wavelength range from, say, 520 nm to 750 nm. You will see a very high intensity peak centered at 540 nm. This is the so-called Rayleigh scattering peak and results from scattering of the excitation beam by the solution in the sample cell. As the simulator integrates all of the area under the fluorescence emission spectrum (see step 1(7) above) it is important that this scattering peak be avoided when making measurements where the area under the fluorescence emission curve is required for subsequent calculations.

Exercise 2 – Exploring Fluorescence Emission and Excitation Spectra

2.1 Fluorescence Emission Spectrum of Rhodamine B

(1) After having become familiar with the operation of the simulator and explored its various functions in the previous exercise, clear any entries in the **Lab Book** and any displayed spectra that may be left in the simulator output window.

(2) Refer to the **Scan Guide** and/or the previous exercise (Ex. 1) and set the instrument to record the fluorescence **emission** spectrum of 2.4×10^{-7} M RhB in ethanol. Note that the spectrum corresponds to that of RhB dissolved in ethanol only without the addition of acid or base where such subsequent pH adjustment shifts the absorption and emission maxima of this fluorophore. Recall that the **Scan Guide** recommends recording this spectrum using the wavelength of maximum absorption (λ_{max}(abs) = 540 nm) of RhB as the **excitation** wavelength.

(3) After the settings have been made, scan the spectrum. When the scanning is in progress note which monochromator is scanning and observe the fluorescence intensity or "glow" of the sample during the scan.

(4) When scanning is complete, capture the data and then use the cursor to determine the wavelength corresponding to the emission maximum, λ_{max}(em).

(5) Observe the effect on the fluorescence emission spectrum if wavelengths either side of the absorption maximum of RhB are used to excite the sample. For example, try scanning using excitation wavelengths of 500, 525, 555 and 565 nm. Use the **Capture** button to record all of these data for future analysis.

[**Note**: In order to avoid recording the Rayleigh scattering peaks in the spectra excited at 555 nm and 565 nm, set the starting wavelength to 590 nm for each of these scans.]

Questions and Exercises

(1) During the collection of a fluorescence emission spectrum state: (i) which monochromator is scanning and (ii) what happens to the intensity of fluorescence emission emanating from the sample during the run.

(2) Compare the magnitude of λ_{max}(abs) with λ_{max}(em). With reference to a Jablonski diagram explain this difference and make a generalization in relation to the wavelength

range over which fluorescence is normally observed compared to the wavelength range over which absorption occurs for a given analyte.

(3) Use the data files created during this exercise to produce a plot showing each of the collected emission spectra superimposed on each other. Label each spectrum with the corresponding excitation wavelength.

(4) What effect does the excitation wavelength have on the emission spectrum?

(5) On the basis of your observations made in Question 2.1(4) explain why it is preferable to select the wavelength of maximum absorbance of an analyte as the excitation wavelength when one is recording fluorescence emission spectra.

2.2 Fluorescence Excitation Spectrum of Rhodamine B

(1) Refer to the **Scan Guide** and/or the previous exercise (Ex. 2.1) and set the instrument to record the fluorescence **excitation** spectrum of 2.4×10^{-7} M RhB in ethanol. Recall that the wavelength of maximum emission of RhB is *ca*. 625 nm and so the **emission** monochromator will need to be fixed at this wavelength.

[**Note**: In order to minimize the time required in performing multiple scans, the **Scan Guide** suggests scanning the excitation spectrum of RhB between 440 nm and 590 nm whereas the complete excitation spectrum of this compound extends below 440 nm in the UV region. A shorter starting wavelength can be used if the full spectrum is required.]

(2) After the settings have been made, clear the output window and scan the spectrum. Whilst the scanning is in progress note which monochromator is scanning and observe the fluorescence intensity or "glow" of the sample.

(2) When scanning is complete, capture the data and then use the cursor to determine the wavelength at which the maximum intensity in the excitation spectrum occurs, $\lambda_{max}(ex)$.

(3) Observe the effect on the fluorescence excitation spectrum if wavelengths either side of the emission maximum of RhB are used in monitoring the signal during the scan. For example, try scanning and use emission wavelengths of 600, 615, 645 and 700 nm. Record all of these data for analysis.

Questions and Exercises

(1) During the collection of a fluorescence excitation spectrum state: (i) which monochromator is scanning and (ii) what happens to the intensity of fluorescence emission emanating from the sample during the run.

(2) Explain your observation made in relation to Question 2.2(1)(ii) above.

(3) Use the data files created during this exercise to produce a plot showing each of the collected excitation spectra superimposed on each other. Label each spectrum with the corresponding emission wavelength.

(4) What effect does changing the emission wavelength have on the observed excitation spectrum?

(5) On the basis of your observations made in Question 2.2(4) explain why it is preferable to select the wavelength of maximum emission of an analyte as the monitoring wavelength when one is recording fluorescence excitation spectra.

2.3 Relationship Between RhB Emission and Excitation Spectra

(1) Clear the output window and set the instrument to record the fluorescence excitation spectrum of 2.4×10^{-7} M RhB in ethanol with maximum intensity.

(2) Scan the excitation spectrum and capture the data.

(3) Without clearing the output window set the instrument to record the fluorescence emission spectrum of 2.4×10^{-7} M RhB in ethanol with maximum intensity.

(4) Scan the emission spectrum and capture the data.

(5) Use the cursor to note λ_{max}(ex) and λ_{max}(em).

Questions and Exercises

(1) Confirm that λ_{max}(ex) corresponds to λ_{max}(abs) and state why this should be so.

(2) Use the two data files created during this exercise to produce a plot showing each of the collected spectra plotted on the same set of axes. Label each spectrum and indicate λ_{max}(ex) and λ_{max}(em).

(3) Calculate the Stokes shift for the RhB sample in units of cm^{-1}.

(4) Does the sample obey the "mirror image" rule? Explain.

2.4 Fluorescence Excitation and Emission of Quinine Bisulfate

In this exercise the experiments that were performed in the preceding exercises using RhB are repeated, this time using a solution of 4.4×10^{-6} M quinine bisulfate (QBS) in 0.5 M H_2SO_4 where the concentration of QBS has been adjusted to produce a solution whose optical absorbance at 348 nm is less than 0.05 as measured in a 1 cm path length cell.

The instructions are therefore condensed and the user is referred to Exs. 2.1 to 2.3 above for more detail in relation to individual steps.

(1) **Clear** the output window of the simulator and set the sample to QBS. Ensure that the NaCl quencher concentration is set to zero.

(2) Refer to the **Scan Guide** and set the instrument to scan the fluorescence emission spectrum of QBS. Scan the spectrum and when scanning is complete capture the data.

Use the **Cursor** to determine the wavelength at which the maximum intensity is observed.

(3) Investigate the effect of the excitation wavelength on the emission spectrum by exciting at 280, 300, 375 and 390 nm. **Capture** the data associated with each run and note the wavelength/s at which the maxima occur.

[**Note**: In order to avoid recording the Rayleigh scattering peaks in the spectra excited at 375 nm and 390 nm, set the starting wavelength to 410 nm for each of these scans.]

(4) Refer to the **Scan Guide** and set the instrument to scan the fluorescence excitation spectrum of QBS. Scan the spectrum and when scanning is complete capture the data. Use the **Cursor** to determine the wavelength at which the maximum intensity is observed.

(5) Investigate the effect on the fluorescence excitation spectrum of monitoring at different emission wavelengths. Try monitoring at 410, 430, 500 and 550 nm. **Capture** the data associated with each run and note the wavelength/s at which the maxima occur.

Questions and Exercises

(1) From your observations made using QBS as the sample, state in the case of *both* a fluorescence emission spectrum and a fluorescence excitation spectrum: (i) which monochromator is scanning during its collection and (ii) what happens to the intensity of fluorescence emission emanating from the sample during the run. In relation to part (ii) of this question, explain your answer in each of the cases.

(2) Use the data files created during this exercise to produce a plot showing the collected emission and excitation spectra superimposed on each other. Label each spectrum with the corresponding excitation or emission wavelength at which is was recorded. Also label λ_{max}(ex) and λ_{max}(em) and indicate the Stokes shift.

(3) Confirm that λ_{max}(ex) corresponds to λ_{max}(abs) and state why this should be so. Compare the magnitude of λ_{max}(abs) with λ_{max}(em) and with reference to a Jablonski diagram: (i) explain this difference and (ii) make a generalization in relation to the wavelength range over which fluorescence is normally observed compared to the wavelength range over which absorption occurs for a given analyte.

(4) State the effect of: (i) changing the excitation wavelength on the emission spectrum and (ii) changing the emission wavelength on the excitation spectrum.

(5) On the basis of your observations made in Question 2.4(4) explain why it is preferable to select: (i) λ_{max}(abs) as the excitation wavelength when one is recording fluorescence emission spectra and (ii) λ_{max}(em) of an analyte as the monitoring wavelength when one is recording fluorescence excitation spectra.

(6) Calculate the Stokes shift for the QBS sample in units of cm^{-1}.

(7) Does the sample obey the "mirror image" rule? Explain.

Exercise 3 – Fluorescence Quantum Yield

In this exercise, the fluorescence quantum yield of RhB will be determined using QBS as a fluorescence standard. The system chosen for study involves a sample and reference that absorb and emit in different regions of the spectrum. This choice has been made in order to illustrate the general case where a correction factor for the different excitation intensities has to be made. In practice, it is usually more convenient to choose the reference such that it can be excited at the same wavelength as the sample thereby eliminating the need for such a correction.

(1) Clear the output window of the simulator and set the sample to RhB.

(2) Refer to the **Scan Guide** and/or the relevant previous exercises and set the instrument to record the fluorescence emission spectrum of 2.4×10^{-7} M RhB in ethanol. Scan the spectrum and when scanning is complete capture the data. In particular, ensure that you have noted the area under the emission spectrum.

(3) Change the sample to QBS and ensure the quencher concentration in set to zero.

(4) Refer to the **Scan Guide** and/or relevant previous exercises and set the instrument to record the fluorescence emission spectrum of QBS. Scan the spectrum, capture the data and again make a note of the area under the emission spectrum.

Questions and Exercises

(1) Plot the fluorescence emission spectrum of RhB and that of QBS on the same set of axes. For the purposes of this experiment these spectra are considered to be the "corrected" fluorescence emission spectra.

(2) Given that the 4.4×10^{-6} M QBS solution was made up in 0.5 M H_2SO_4, the absorbance of the QBS solution is 0.0251 at 348 nm, ϕ_f(QBS, 0.5 M H_2SO_4) = 0.546 at 25°C, and the absorbance of the 2.4×10^{-7} M RhB in ethanol solution is 0.0254 at 540 nm, use your collected data along with equation (1) in the theory notes to calculate the fluorescence quantum yield of RhB in ethanol.

[**Note**: The 0.5 M H_2SO_4 solution is sufficiently dilute so the refractive index of this solution may be taken to be the same as that of water, 1.333; the refractive index of ethanol is 1.360. For the simulator instrument the ratio of the excitation intensities is I_0(348 nm)/I_0(540 nm) = 2.198.]

(3) Compare your result in Question 3(2) with that given in the literature. See, for example: López Arbeloa, F.; Ruiz Ojeda, P.; López Arbeloa, I. Fluorescence Self-Quenching of the Molecular Forms of Rhodamine B in Aqueous and Ethanolic Solutions *J. Luminesc*. **1989,** *44*, 105-112.

(4) When using a real instrument to conduct fluorimetric measurements it is important to work with "corrected" emission spectra when calculating, say, the fluorescence quantum yield. What is meant by "corrected" fluorescence emission spectrum?

(5) In the case of a real instrument, it is important to ensure that "identical instrumental conditions" are used when recording the sample and reference emission spectra. What

is meant by this expression and why is it important to maintain "identical instrumental conditions" between the runs?

Exercise 4 – Quantitative Analytical Fluorimetry

(1) Clear the output window of the simulator and set the sample to RhB.

(2) Refer to the **Scan Guide** and/or the relevant previous exercises and set the instrument to record the fluorescence emission spectrum of 2.4×10^{-7} M RhB in ethanol. Scan the spectrum and when scanning is complete capture the data. In particular, ensure that you have noted the area under the emission spectrum.

(3) Repeat the experiment using RhB concentrations of 1.8, 1.2, 0.6 and 0.1×10^{-7} M. In each case make sure you have recorded the area under the emission spectrum.

(4) Repeat steps 4(2) and 4(3) using an excitation wavelength of: (i) 525 nm and (ii) 558 nm.

Questions and Exercises

(1) For each case where a different excitation wavelength was used in the experiment (i.e. cases where $\lambda(ex) = \lambda_{max}(ex)$, 525 nm or 558 nm) plot, on the same set of axes, the area under the fluorescence emission spectrum versus the concentration of RhB. Label clearly each plot.

(2) From your data comment on the use of fluorescence emission measurements as a quantitative analytical technique for fluorescence analytes at low concentrations.

(3) What effect does the excitation wavelength have on the sensitivity of the method? Explain.

Exercise 5 – Fluorescence Quenching

(1) Clear the output window of the simulator and set the sample to QBS.

(2) Refer to the **Scan Guide** and/or the relevant previous exercises and set the instrument to record the fluorescence emission spectrum of QBS in 0.5 M H_2SO_4. Ensure the quencher concentration is set to zero and scan the spectrum.

(3) When scanning is complete capture the data. In particular, ensure that you have noted the area under the emission spectrum.

(4) Repeat steps 5(2) and 5(3) but use quencher concentrations of 0.006, 0.018, 0.050 and 0.100 M.

Questions and Exercises

(1) Plot α_0/α versus [NaCl] where α_0 is the area under the QBS fluorescence emission spectrum in the absence of quencher and α is the area when the quencher is present in

the system. With reference to your plot, comment on the applicability of the Stern-Volmer equation to this system.

(2) The Stern-Volmer equation is expressed in terms of the ratio of quantum yields ϕ°_f/ϕ_f whereas in this exercise α_0/α is plotted as a function of the concentration of quencher. Explain why the ratio of areas can be used in place of the ratio of quantum yields in constructing the Stern-Volmer plot.

(3) Given that the fluorescence lifetime of QBS in 0.5 M H_2SO_4 solution is 19.02 ns (see: Meech, S. R.; Phillips, D. Photophysics of Some Common Fluorescence Standards *J. Photochem.* **1983***, 23*, 193–217) calculate the second-order rate constant, k_q for the quenching of QBS by NaCl.

(4) Use the equation derived from the Debye-Smoluchowski and Stokes-Einstein equations to obtain an estimate of the diffusion-limited rate constant for the quenching process, k_{diff}, at 20°C.

(5) What assumptions are inherent in the calculation performed in Question 5(4)?

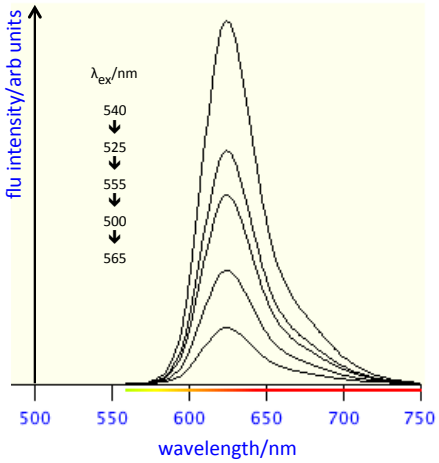
(6) Given that the diffusion coefficients of the QBS and Cl^- species in aqueous solution are 0.70×10^{-9} m^2 s^{-1} and 2.03×10^{-9} m^2 s^{-1} respectively, the surface area of QBS is 359.06 $Angstrom^2$ and the radius of the Cl^- ion is 1.81×10^{-10} m, obtain a more accurate estimate of k_{diff} by using only the Debye-Smoluchowski equation.

(7) Compare k_q with the calculated k_{diff} values and interpret what is implied by the relative magnitudes of these constants.

Acknowledgement

The authors are grateful to Dr Efrat Eilam, College of Education, Victoria University, for her expert advice on the pedagogical approach used in this work and her assistance in proofing the Windows version of the software.

Exercise 2.1 – Fluorescence Emission Spectrum of 2.4 × 10^{-6} M RhB in Ethanol



(1) During the collection of a fluorescence **emission** spectrum the emission monochromator is **scanning** and the intensity of fluorescence emission from the sample remains **constant**.

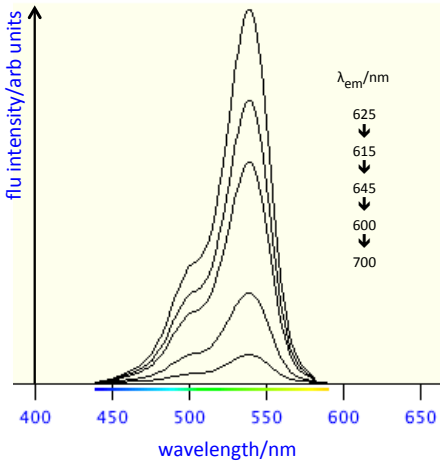
(2) For RhB in EtOH it is clear that λ_{max}(abs) = 540 nm and λ_{max}(em) = 625 nm. Thus fluorescence emission occurs at a **longer** wavelength (i.e. lower energy) compared with absorption. In terms of the Jablonski diagram this energy difference is explained by the energy associated with vibrational relaxation in the S_1 and S_0 states as the molecule returns to the lowest vibrational level of S_0.

(3) Refer to the emission spectra (left). Corresponding excitation wavelengths are indicated.

(4) The results suggest that the maximum fluorescence emission intensity is obtained when the sample is excited at its wavelength of maximum absorbance.

(5) For fluorescence emission spectra, it is preferable to excite at λ_{max}(abs) as this will produce the most intense signal and hence optimize the **sensitivity** of the technique.

Exercise 2.2 – Fluorescence Excitation Spectrum of 2.4 × 10^{-6} M RhB in Ethanol



(1) During the collection of a fluorescence **excitation** spectrum the excitation monochromator is **scanning** and the intensity of fluorescence emission from the sample **varies**.

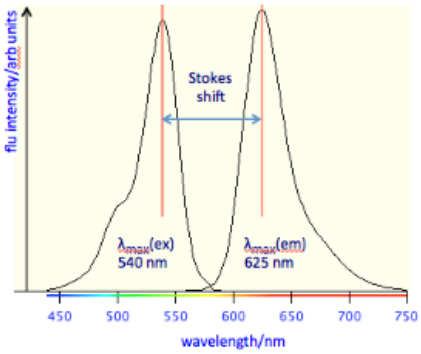
(2) Under steady-state conditions the intensity of fluorescence is directly related to the absorbance. Thus when the sample is excited with a wavelength that is strongly absorbed a strong fluorescence is observed and vicé vèrsa. The fluorescence **excitation** spectrum thus corresponds to the **absorption** spectrum of the analyte.

(3) Refer to the excitation spectra (left). Corresponding emission wavelengths are indicated.

(4) The results suggest that the fluorescence excitation spectrum is most intense when it is recorded using the wavelength of maximum emission of the analyte, as the monitoring (fixed) wavelength.

(5) For fluorescence excitation spectra, it is preferable to monitor at λ_{max}(em) as this will produce the most intense signal and hence optimize the **sensitivity** of the technique.

Exercise 2.3 – Relationship Between RhB Emission and Excitation Spectra



(1) Analysis of the fluorescence excitation spectrum shows that at $\lambda_{max}(ex) = 540$ nm. This corresponds to the given absorption maximum of RhB (i.e. 540 nm) as expected because the fluorescence excitation spectrum should corresponds to the absorption spectrum of the analyte.

(2) Refer to the excitation and emission spectra (left). Corresponding maximum wavelengths are indicated.

(3) Stokes shift $= (1/540 - 1/625)/(10^{-9} \times 100)\ cm^{-1}$
$= 2518\ cm^{-1}$

(4) If one assumes that the excitation spectrum of RhB is the same as its absorption spectrum then the results suggest the emission spectrum of RhB is almost a mirror image of its absorption spectrum. In many cases the probability of an electron returning to a particular vibrational level in the ground state is similar to the probability of that electron's position in the ground state before excitation and so fluorescence emission and absorption spectra are often mirror images of each other. This is known as the "**mirror image rule**". The RhB sample appears to obey this rule reasonably well.

Exercise 2.4 – Fluorescence Excitation and Emission of Quinine Bisulfate

(1) During the collection of a fluorescence **emission** spectrum the emission monochromator is **scanning** and the intensity of fluorescence emission from the sample remains **constant**. During the collection of a fluorescence **excitation** spectrum the excitation monochromator is **scanning** and the intensity of fluorescence emission from the sample **varies**. Under steady-state conditions the intensity of fluorescence is directly related to the absorbance. Thus when the sample is excited with a wavelength that is strongly absorbed a strong fluorescence is observed and vicé vèrsa. The fluorescence **excitation** spectrum thus corresponds to the **absorption** spectrum of the analyte.

(2) Refer to the excitation and emission spectra. Corresponding excitation/emission wavelengths and maxima are indicated.

(3) Analysis of the fluorescence excitation spectrum shows that at $\lambda_{max}(ex) = 348$ nm. This corresponds to the given absorption maximum of QBS (i.e. 348 nm). The fluorescence excitation spectrum correspond to the absorption spectrum of the analyte in cases where the quantum yield of fluorescence is constant over the recommended scan wavelength ranges in the chosen solvents. However, it should be noted that this is not always the case in general. For QBS $\lambda_{max}(abs) = 348$ nm and $\lambda_{max}(em) = 452$ nm. Thus fluorescence emission occurs at a **longer** wavelength (i.e. lower energy) compared with absorption. In terms of the Jablonski diagram, this energy difference is explained by the energy associated with vibrational relaxation in the S_1 and S_0 states as the molecule returns to the lowest vibrational level of S_0.

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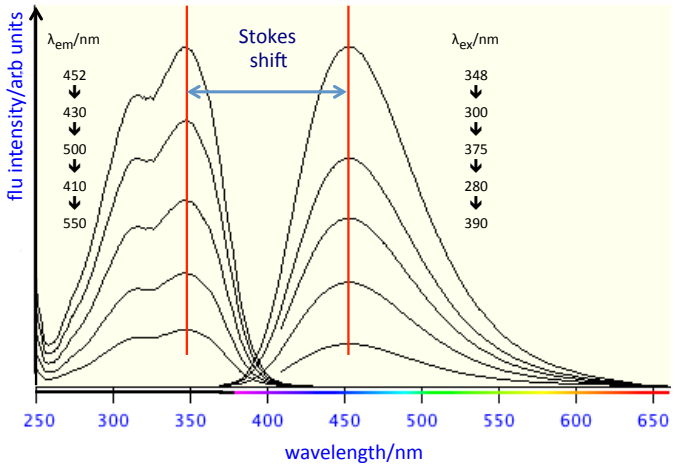
Exercise 2.4 – Fluorescence Excitation and Emission of Quinine Bisulfate (cont'd)

(4) The results suggest that: (i) maximum fluorescence emission intensity occurs when the sample is excited at its wavelength of maximum absorbance and (ii) the fluorescence excitation spectrum is most intense when it is recorded using the wavelength of maximum emission of the analyte, as the monitoring wavelength.

(5) For fluorescence emission spectra, it is preferable to excite at λ_{max}(abs) as this will produce the most intense signal and hence optimize the **sensitivity** of the technique. Similarly, for fluorescence excitation spectra, it is preferable to monitor at λ_{max}(em) as this too will produce the most intense signal and hence optimize the **sensitivity** of the technique.

(6) Stokes shift $= (1/348 - 1/452)/(10^{-9} \times 100)\ cm^{-1}$
$= 6612\ cm^{-1}$

(7) In many cases the probability of an electron returning to a particular vibrational level in the ground state is similar to the probability of that electron's position in the ground state before excitation and so fluorescence emission and absorption spectra are often mirror images of each other. This is known as the "**mirror image rule**". If one assumes that the excitation spectrum of QBS is the same as its absorption spectrum then the results suggest the emission spectrum of QBS is almost a mirror image of its absorption spectrum but not quite. The QBS excitation spectrum exhibits a second (shoulder) peak at *ca.* 317 nm that corresponds to an excitation to the S_2 state. However, in QBS the S_2 state rapidly relaxes to the S_1 state and so emission from S_2 is not observed. Whence, the emission spectrum of QBS is comprised of only one broad band centred at 452 nm. Thus QBS does not conform to the mirror image rule.



Exercise 3.0 – Fluorescence Quantum Yield of RhB in Ethanol

(1) The fluorescence emission spectra of RhB and QBS run under identical conditions can be viewed by clicking here or on the link at the bottom of this page.

(2) From equation (1) in the Theory section including intensity factor correction):

$$\phi_f(\text{RhB})/\phi_f(\text{QBS}) = \{\alpha_{\text{RhB}} n_{\text{EtOH}}^2(1 - 10^{-A_{\text{QBS}}})/[\alpha_{\text{QBS}} n_{\text{water}}^2(1 - 10^{-A_{\text{RhB}}})]\} \times I_0(348\text{ nm})/I_0(540\text{ nm}) \quad (1)$$

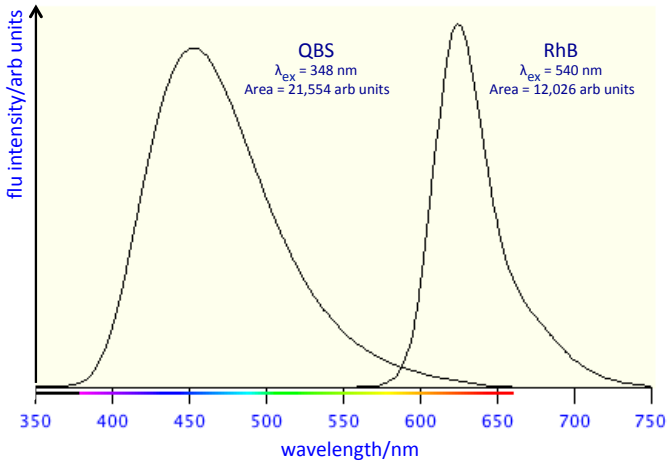
where $\phi_f(\text{QBS}) = 0.546$, $\alpha_{\text{RhB}} = 11789$, $n_{\text{EtOH}} = 1.360$, $A_{\text{QBS}} = 0.0251$, $\alpha_{\text{QBS}} = 21362$, $n_{\text{water}} = 1.333$, $A_{\text{RhB}} = 0.0254$ and $I_0(348\text{ nm})/I_0(540\text{ nm}) = 2.230$. Thus:

$$\begin{aligned}\phi_f(\text{RhB}) &= \phi_f(\text{QBS})\{\alpha_{\text{RhB}} n_{\text{EtOH}}^2(1 - 10^{-A_{\text{QBS}}})/[\alpha_{\text{QBS}} n_{\text{water}}^2(1 - 10^{-A_{\text{RhB}}})]\} \times I_0(348\text{ nm})/I_0(540\text{ nm}) \\ &= 0.546 \times \{12026 \times (1.360)^2(1 - 10^{-0.0251})/[21554 \times (1.333)^2(1 - 10^{-0.0254})]\} \times 2.198 \\ &= 0.69\end{aligned}$$

(3) The previous (typical) result compares favourably with the literature value of 0.7 obtained by López Arbeloa, F.; Ruiz Ojeda, P.; López Arbeloa, I. Fluorescence Self-Quenching of the Molecular Forms of Rhodamine B in Aqueous and Ethanolic Solutions *J. Luminesc.* **1989**, *44*, 105-112.

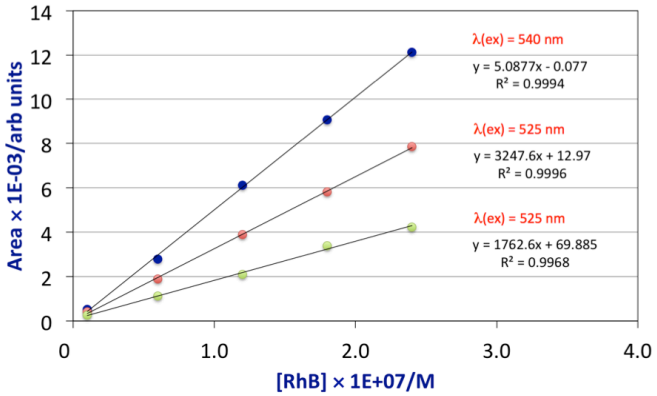
(4) When using a real instrument corrections may sometimes need to be made to the fluorescence emission spectrum to take account of differences in light intensity transmissions through the instrumental optics (monochromators) that are wavelength-dependent.

(5) It is important to maintain identical instrumental conditions between runs so that the sample and reference emission spectral areas can be compared and utilized in accordance with equation (1).



Exercise 4.0 – Quantitative Analytical Fluorimetry

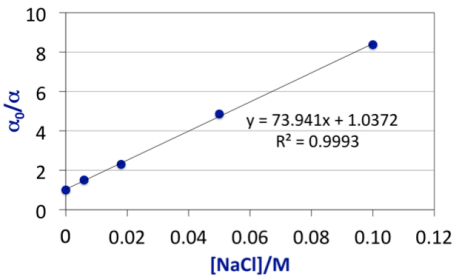
(1) Plots of the area under the fluorescence emission spectrum versus the concentration of RhB are shown below. All of these are linear and pass close to the origin as expected.



(2) The linearity of the above plots confirms that fluorescence emission measurements can be used as a quantitative analytical technique for fluorescent analytes at low concentrations.

(3) The above data suggest that the sensitivity of the technique (as indicated by the gradients of the plots) is at a maximum when excitation is performed using the wavelength of maximum absorption.

Exercise 5.0 – Fluorescence Quenching



(1) The Stern-Volmer plot is shown (left). Its linearity and the y-axis intercept being close to unity confirms the applicability of the Stern-Volmer equation in this case.

(2) The fluorescence quantum yield is proportional to the area under the (corrected) fluorescence emission spectrum. Thus the ratio of quantum yields in the Stern-Volmer equation can be replaced by the ratio of areas provided the same instrumental conditions are maintained throughout the runs.

(3) Gradient of Stern-Volmer plot = $k_q \times \tau_f$

$$k_q = 73.941/(19.02 \times 10^{-9}) = 3.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$$

(4) At 20°C and assuming η(0.5 M H_2SO_4) = η(water) = 1.002×10^{-3} kg m^{-1} s^{-1} then:

$$k_{diff} = 8000RT/3\eta$$
$$= 8000 \times 8.314 \times (20 + 273)/(3 \times 1 \times 10^{-3})$$
$$= 6.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$$

Exercise 5.0 – Fluorescence Quenching (cont'd)

(5) The assumptions inherent in the previous calculation in Question 5(4) are: (i) the viscosity of the 0.5 M H_2SO_4 solution of QBS is the same as that of water at the temperature of the experiment, (ii) the size of the quencher (Q) and excited state molecules (M*) are the same and (iii) the Q and M* species are not charged.

(6) Debye-Smoluchowski equation:

$k_{diff} = 4\pi N_A \sigma_{MQ}(D_M + D_Q)$ [units: $m^3\ mol^{-1}\ s^{-1}$]
σ_{MQ} = collision diameter of M* and Q [units: m]
N_A = Avogadro constant = $6.02 \times 10^{23}\ mol^{-1}$
D_M, D_Q = diffusion coefficients of M* and Q [units: $m^2\ s^{-1}$]

Assume: $\sigma_{MQ} = r_M + r_Q$
r_M, r_Q = radius of M, Q (assuming spherical geometry)
$D_M = D(QBS) = 0.70 \times 10^{-9}\ m^2\ s^{-1}$ [Ref 1]
$DQ = D(Cl^-) = 2.03 \times 10^{-9}\ m^2\ s^{-1}$ [Ref 2]
Surface area QBS = 359.06 $Angstrom^2$ [Ref 3]
$r_M = r(QBS) = \sqrt{(359.06/4\pi)} = 5.345 \times 10^{-10}$ m
$r_Q = r(Cl^-) = 1.81 \times 10^{-10}$ m [Ref 4]

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Sources:
Ref 1: Grossman, P. D.; Colburn, J. C. (eds.) *Capillary Electrophoresis: Theory and Practice*; Academic Press: London, 1992; p 302.
Ref 2: Cussler, E. L. *Diffusion: Mass Transfer in Fluid Systems*, 2nd ed.; Cambridge University Press: UK, 2003; p 143.
Ref 3: Schardein J. L.; Macina, O. T. *Human Developmental Toxicants: Aspects of Toxicology and Chemistry*, CRC Press: Boca Raton, USA, 2007; p 183.
Ref 4: Cotton, E. A.; Wilkinson, G.; Gaus, P. L. *Basic Inorganic Chemistry*, 2nd ed.; Wiley: New York, 1987; p 128.

Exercise 5.0 – Fluorescence Quenching (cont'd)

(6) cont'd

Now:

$k_{diff} = 4\pi N_A(r_M + r_Q)(D_M + D_Q) \times 10^3\ M^{-1}\ s^{-1}$
$= 4\pi \times 6.02 \times 10^{23} \times (5.35 + 1.81) \times 10^{-10} \times (0.70 + 2.03) \times 10^{-9} \times 10^3\ M^{-1}\ s^{-1}$
$= 1.478 \times 10^{10}\ M^{-1}\ s^{-1}$

(7) Comparing k_q and k_{diff} (the latter having been obtained by either of the methods explored in this exercise) it is clear that $k_q < k_{diff}$. This result can be interpreted to mean that in 0.5 M H_2SO_4 not every encounter between excited QBS molecules and the quencher results in quenching.

[**Note**: Since both QBS (in 0.5 M H2SO4) and the quencher (Cl^-) are charged, the effect of the ion-ion interaction on the rate of diffusion of the particles together may also be considered for greatest accuracy. Straightforward equations for doing that are presented in the literature. See for example, Steinfeld, J. I.; Francisco, J. S.; Hase, W. I. *Chemical Kinetics and Dynamics*, 2nd ed.; Prentice-Hall: New Jersey , 1998; pp 130-132.]