Experiment 1

Fluorescence and Absorption Spectra of Polycyclic Aromatic Hydrocarbons as a Tool for Quantitative Analysis

(Journal of Chemical Education, Vol. 81 No. 2 February 2004)

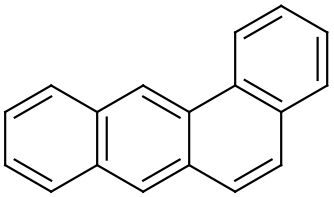
Background

Polycyclic aromatic hydrocarbons (PAHs)

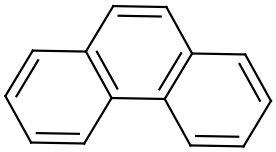
PAHs are chemical compounds that consist of fused aromatic rings and do not contain heteroatoms or carry substituents. PAHs occur in oil, coal, and tar deposits, and are produced as byproducts of fuel burning (whether fossil fuel or biomass). The U.S. Environmental Protection Agency (EPA) has identified 16 PAHs as priority pollutants. As a pollutant, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic. PAHs are also found in foods. Studies have shown that most food intake of PAHs comes from cereals, oils and fats. Smaller intakes come from vegetables and cooked meats.

PAHs generally absorb light in the 200–400 nm range and also strongly fluoresce. UV–vis absorption and fluorescence spectroscopic techniques have sensitivities for PAHs on the order of 0.1–1 μg/L and are widely used for analysis of PAHs.

The structures, names, molecular weights, and wavelengths of absorption and fluorescence maxima for two of the PAHs are shown in Figure 1.



Benz[*a*]anthracene
MW = 228.29 g/mol
abs = 288 nm
fluor = 387 nm



phenantrene
MW = 178.23 g/mol
abs = 251 nm
fluor = 366

Figure 1. Examples of polyaromatic hydrocarbons (PAH).

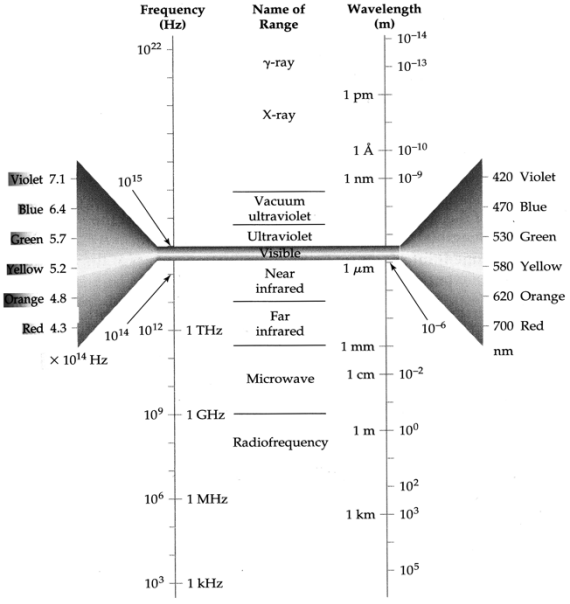


Figure 2. Diagram of electromagnetic radiation.

Absorption spectra

The absorption of a photon of energy by a molecule occurs due to an interaction of the oscillating electric field vector of the light wave with charges (electrons) in the molecule. It is an all or none phenomenon and can only happen with incident light of specific wavelengths known as absorption bands. If the absorbed photon contains more energy than is necessary for a simple

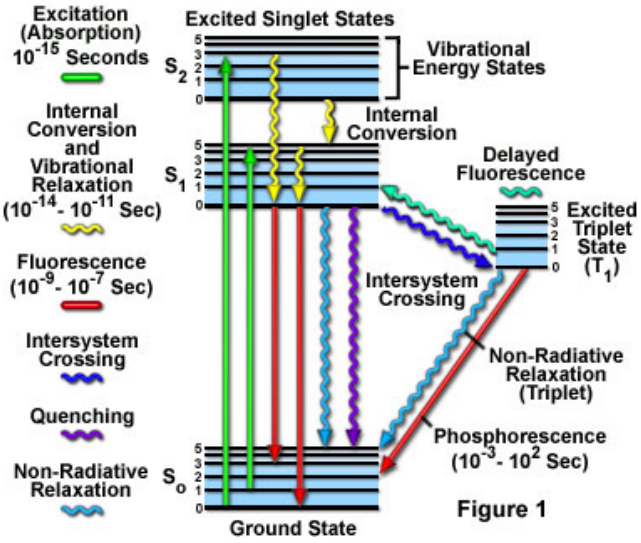


Figure 4. Jablonski Energy Diagram. (http://www.olympusconfocal.com/theory/fluoroexciteemit.html)

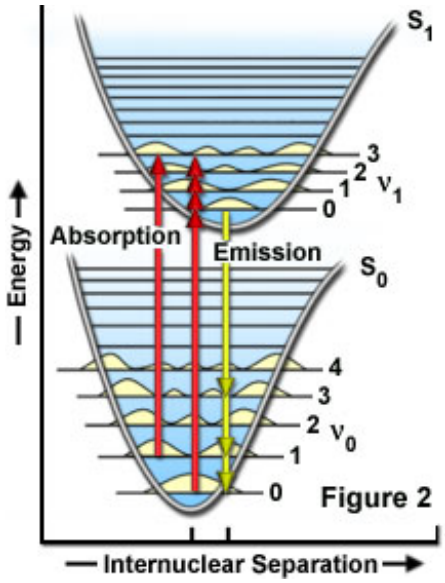


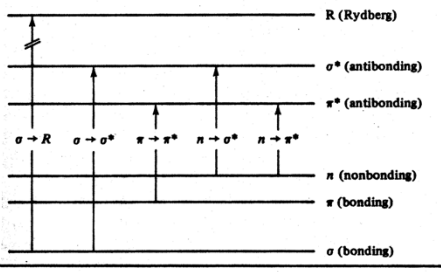
Figure 3. Franck-Condon Energy Diagram.

electronic transition, the excess energy is usually converted into vibrational and rotational energy. However, if a collision occurs between a molecule and a photon having insufficient energy to promote a transition, no absorption occurs. The spectrally broad absorption band arises from the closely spaced vibrational energy levels plus thermal motion that enables a range of photon energies to match a particular transition.

Absorption of energy (light) by molecules occurs in discrete amounts or packets called quanta on a very fast time scale approximately a femtosecond. The various energy levels involved in the absorption and emission of light by a molecule are classically presented by a Jablonski energy diagram (Figure 3)

UV-visible spectrophotometry is based on measuring the absorption of near-UV or visible radiation by molecules (Figure 2). Radiation in this wavelength region is responsible for electronic transitions. That is, absorption of a photon with the energy corresponding to the UV or visible light promotes electrons to higher energy levels. Typically, with ultraviolet or visible light molecules are excited to higher vibrational level of the first or second singlet electronic energy states. This corresponds to the green arrows in Figure 3. The wavelengths at which these transitions take place are characteristic of the molecular structure of the molecule. At room temperature, very few molecules have enough internal energy to exist in any state other than the lowest vibrational level of the ground state, and thus, excitation processes usually originate from this energy level. In a typical experiment, irradiation with a wide spectrum of wavelengths will generate an entire range of allowed transitions that populate the various vibrational energy levels of the excited states. Some of these transitions will have a much higher degree of probability than others, and when combined, will constitute the absorption spectrum of the molecule.

The probability of a transition occurring from the ground state S(0) to the excited singlet state S(1) depends on the degree of similarity between the vibrational and rotational energy states when an electron resides in the ground state versus those present in the excited state, as outlined in Figure 4. The Franck-Condon energy diagram illustrated in Figure 4 presents the vibrational energy probability distribution among the various vibrational levels in the ground S(0) and first excited S(1) states for a hypothetical molecule. Excitation transitions (red lines) from the ground to the excited state occur in such a short timeframe (femtoseconds)



Transition	Mulliken's designation	λ (nm)	ε (L mol^{-1} cm^{-1})	Examples
$\sigma \rightarrow \sigma^*$	$N \rightarrow V$	<200	—	Saturated hydrocarbons
$\pi \rightarrow \pi^*$	$N \rightarrow V$	200–500	≈10^4	Alkenes, alkynes, aromatics
$n \rightarrow \sigma^*$	$N \rightarrow Q$	160–260	10^2–10^3	H_2O, CH_3OH, CH_3Cl, CH_3NH_2
$n \rightarrow \pi^*$	$N \rightarrow Q$	250–600	10^1–10^2	Carbonyls, nitro, nitrate, carboxyl

Figure 5. Characteristics of electronic transitions between σ, π, and n orbitals.

that the internuclear distance associated with the bonding orbitals does not have sufficient time to change, and thus the transitions are represented as vertical lines. This concept is referred to as the Franck–Condon Principle. The wavelength of maximum absorption (red line in the center in Figure 4) represents the most probable internuclear separation in the ground state to an allowed vibrational level in the excited state.

For polyatomic molecules it is possible to consider that the electronic excitations involve σ and π bonding and antibonding molecular orbitals (MO) as well as nonbonding (*n*) MOs as shown in Figure 4. This figure also shows the characteristics of Mulliken's symbolism for these transitions. It is also common to represent electronic states as singlet states (S_0, S_1, S_2, . . .) and triplet states (T_1, T_2, T_3, . . .), where the subscript indicates the energy ordering not the vibrational levels. Usually, the ground electronic state is the lowest-laying singlet state (S_0) with all the electrons paired in the ground-state electronic configuration.

The term spectrophotometry is used for absorption measurements because they involve measuring the ratio of the radiant power transmitted through a sample solution to that transmitted by a blank solution. This ratio, the transmittance (*T*), is used to calculate the absorbance ($A = -\log T$) which is directly proportional to the analyte concentration through Beer's law

$$A = abc \qquad (1)$$

Where *A* is absorbance, *a* – absorptivity, *b* – distance the light travels through the material (i.e. the path length), and *c* is the concentration. When *c* is expressed in mol L^{-1}, and *b* in cm then the *a* is called the molar absorptivity, ε, which has the units L mol^{-1} cm^{-1}.

Instruments used in UV–vis spectroscopy, spectrophotometers, can be based on single detectors or multichannel detectors, configured for single beam (SB) or double beam (DB) measurements, and designed for fixed-wavelength measurements or for acquiring complete absorption spectra. Some of the basic designed features are illustrated in Figure 4. In this figure a filter or monochromator transmits a narrow band of wavelength (single wavelength) to the sample. The transmitted radiant power is detected with a vacuum phototube, a photomultiplier tube, or a photodiode and converted

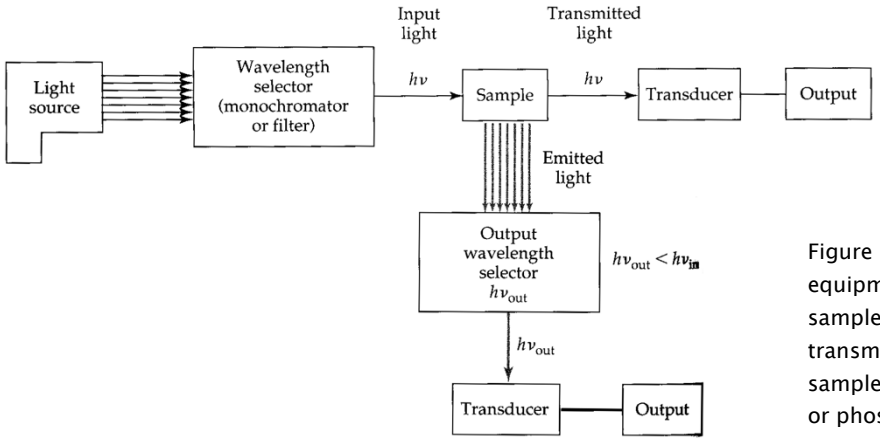


Figure 6. Generalized experimental equipment designed to measure sample absorption and/or transmittance (transmitted light) or sample luminescence (fluorescence or phosphorescence) (emitted light).

to a readout signal with a transducer. This is an example of a SB instrument in which one beam passes through one cell and is incident on the detector. In DB instruments the beam from wavelength selector is split, with a rotating sectored mirror, into two beams, which alternatively pass through a sample cell and reference cell. The sample and reference beams are then recombined to be incident on a single detector.

Emission spectra

Luminescence (fluorescence and phosphorescence) spectrum is measured by passing a monochromatic light beam through the sample and measuring the light emitted from the sample as a function of a wavelength (Figure 4). Usually, fluorescence emission is measured at right angles to the incident beam, to avoid measuring the incident radiation (Figure 6). Phosphorescence and fluorescence are emission processes in which atoms or molecules are excited by absorption of a beam of electromagnetic radiation. The excited species then relax to the ground state giving up the excess of energy as photons emitted in all directions and at longer wavelengths. The emission is one of the two general modes of deactivation of an excited species, i.e. radiative deactivation. The second possibility involves nonradiative deactivation in which the excess electronic energy is converted to translational, rotational, or vibrational energy with no emission of radiation.

The various **activation and deactivation processes** are shown in a Jablonski diagram (Figure 5). The *absorption* or *excitation* process (a) is very rapid on the order of 10^{-15} s. In Figure 7 the excitation from the ground vibrational level of the ground singlet electronic state (S_0) to different vibrational levels in the first and second excited electronic states (S_1 and S_2) is designated by (*a*). Molecules in excited vibrational states rapidly dissipate their excess vibrational energy and relax to the ground vibrational level in a given electronic state (b). The excess vibrational energy is transferred into thermal and vibrational motion of solvent molecules during collisions between excited molecules and molecules of the solvent. This process is so efficient that the average lifetime of an excited vibrational state in only about 10^{-15} s. This process is referred to as *vibrational relaxation* and indicated by wavy arrows in Figure 5. The crossover between two states of the same multiplicity is a nonradiative transition called *internal conversion* (c). It can occur between excited states (e.g. $S_1 \rightarrow S_2$) or between the first excited state and the ground electronic state (e.g. $S_1 \rightarrow S_0$). The internal conversion between excieted states is generally rapid (10^{-12} s) while the internal conversion from S_1 to S_0 is usually less efficient if there is a wide energy separation between S_1 and S_0. *Fluorescence* (d) is

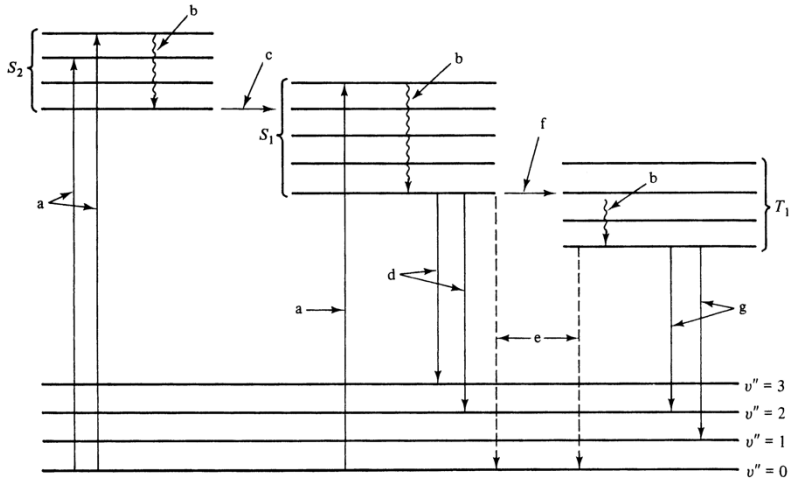


Figure 7. Deactivation process for an excited molecule. a, absorption; b, vibrational relaxation; c, internal conversion; d, fluorescence; e, external conversion; f, intersystem crossing; g, phosphorescence.

a radiational transition between electronic states of the same multiplicity. For most molecules, the electrons are paired in the ground state so that fluorescence involves singlet–singlet transition. Because internal conversion to S_1 and vibrational relaxation are more rapid processes than fluorescence, fluorescence usually occurs from the ground vibrational state of S_1 to various vibrational levels in S_0. For this reason, only one fluorescence band is normally observed even if absorption to different excited singlet states occurs. Typically fluorescence requires 10^{-10} to 10^{-6} s to occur. Fluorescence usually appears at longer wavelengths than absorption because absorption transitions are to higher excited electronic states or to higher vibrational levels in the S_1 manifold. Notice that the transition from the S_1 to the lowest laying vibrational states of the ground state has the highest energy of all the transitions in the band and other lines that terminate in higher vibrational levels of the ground state are lower in energy and produce fluorescence emission at longer wavelengths. That is, molecular fluorescence bands consist largely of lines that are longer in wavelength than the band of absorbed radiation responsible for their excitation (Stokes shift).

The term *external conversion* refers to nonradiative process in which excited states transfer their excess energy to other species such as solvent or solute molecules.

Although in this experiment we will be dealing only with singlet excited states the triplet state, designated by T, can also be populated from excited singlet states by a process of *intersystem crossing*, which is a crossover between electronic states having different multiplicities, usually $S_1 \rightarrow T_1$ (absorption transitions to triplet states are forbidden by symmetry $\Delta S = 0$). After intersystem crossing, a molecule in the T_1, state deactivates by vibrational relaxation to the ground vibrational level of T_1. Normally, the triplet state deactivates by external conversion or intersystem crossing to the ground state ($T_1 \rightarrow S_0$). The triplet state can also deactivate by emission of a photon. This radiational deactivation process between electronic states of different multiplicity is called *phosphorescence*. Usually, phosphorescence takes 10^{-4} to 10^{4} to occur because the process is spin forbidden.

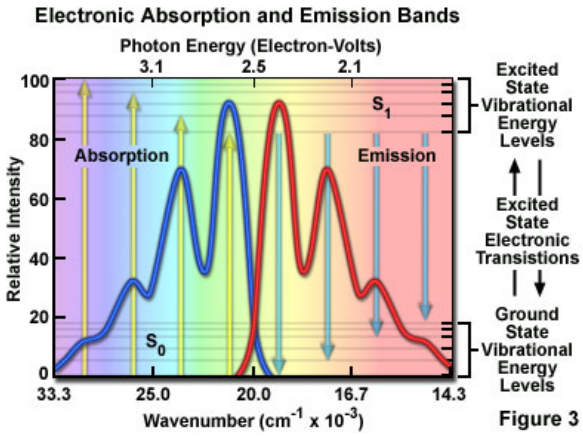


Figure 8. Absorption and Emission Spectra.

Following photon absorption, an excited molecule will quickly undergo relaxation to the lowest vibrational energy level of the excited state. An important consequence of this rapid internal conversion is that all subsequent relaxation pathways (fluorescence, nonradiative relaxation, intersystem crossing, etc.) proceed from the lowest vibrational level of the excited state S(1). As with absorption, the probability that an electron in the excited state will return to a particular vibrational energy level in the ground state is proportional to the overlap between the energy levels in the respective states (Figure 4). Return transitions to the ground state S(0) usually occur to a higher vibrational level (Figure 8), which subsequently reaches thermal equilibrium (vibrational relaxation). Because emission of a photon often leaves the molecule in a higher vibrational ground state, the emission spectrum is typically a mirror image of the absorption spectrum resulting from the ground to first excited state transition. In effect, the probability of an electron returning to a particular vibrational energy level in the ground state is similar to the probability of that electron's position in the ground state before excitation. This concept, known as the Mirror Image Rule, is illustrated in Figure 8 for the emission transitions (blue lines) from the lowest vibrational energy level of the excited state back to various vibrational levels in ground state. The resulting emission spectrum (red band) is a mirror image of the absorption spectrum displayed by the hypothetical chromophore.

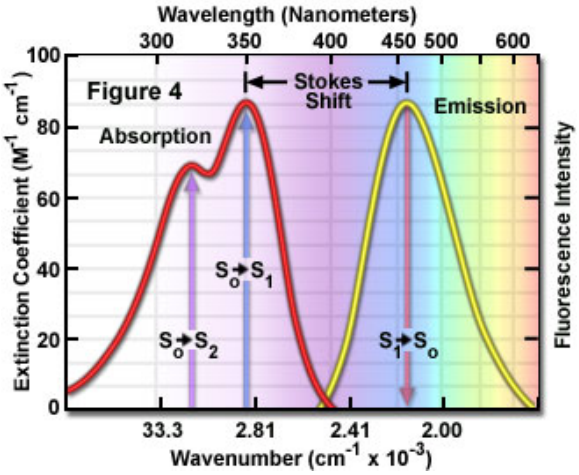


Figure 9. Absorption and Emission Spectra of Quinine,

In many cases, excitation by high energy photons leads to the population of higher electronic and vibrational levels (S(2), S(3), etc.), which quickly lose excess energy as the fluorophore relaxes to the lowest vibrational level of the first excited state (Figure 4). Because of this rapid relaxation process, emission spectra are generally independent of the excitation wavelength (some fluorophores emit from higher energy states, but such activity is rare). For this reason, emission is the mirror image of the ground state to lowest excited state transitions, but not of the entire absorption spectrum, which may include transitions to higher energy levels. An excellent test of the mirror image rule is to examine absorption and emission spectra in a linear plot of the wavenumber (the reciprocal of wavelength or the number of waves per centimeter), which is directly proportional to the frequency and quantum energy. When presented in this manner (Figure 8), symmetry between extinction coefficients and intensity of the excitation and emission spectra as a function of energy yield mirrored spectra when reciprocal transitions are involved.

Example of a compound which does not adhere to the mirror image rule is quinine (Figure 9). Fluorescence emission of quinine occurs exclusively from the lowest excited singlet state S(1), resulting in a spectrum that mirrors the ground to first excited state transition (350 nanometer peak) in quinine and not the entire absorption spectrum. Because the energy associated with fluorescence emission transitions is typically less than that of absorption, the resulting emitted photons have less energy and are shifted to longer wavelengths. This phenomenon is generally known as Stokes Shift The primary origin of the Stokes shift is the rapid decay of excited electrons to the lowest vibrational energy level of the S(1) excited state. In addition, fluorescence emission is usually accompanied by transitions to higher vibrational energy levels of the ground state, resulting in further loss of excitation energy to thermal equilibration of the excess vibrational energy. Other events, such as solvent orientation effects, excited-state reactions, complex formation, and resonance energy transfer can also contribute to longer emission wavelengths.

Quenching of excited states

The measured fluorescence signal can be reduced by the presence of concomitants through several mechanisms. One of them, dynamic quenching, involves non-radiative energy transfer from excited species to other molecules before photon emission. It requires contact between the excited chromophore and quenching species, the quencher (Q) and results in the electronic energy being converted first to vibrational and rotational energy, and ultimately to heat. Particularly effective in promoting this conversion of electronic to thermal energy are large atoms such as bromide and iodide, the so called “heavy atom effect”. The quenching of the fluorescence is often described by what is known as the Stern-Volmer equation. When a photon of sufficiently large energy (usually in the UV or visible region of the spectrum) is absorbed by a molecule in its ground state, A, it promotes an electron to make the excited state A*:

$$A + h\nu_1 \rightarrow A^* \qquad (2)$$

The excited molecule A* can then return to its ground state A by emitting a photon of light, *i.e.* fluorescence:

$$A^* \xrightarrow{k_f} A + h\nu_2 \qquad (3)$$

Collisional quenching occurs when another molecule interacts with A* and takes away the excess electronic energy so that fluorescence is not emitted, i.e. fluorescence is "quenched":

$$A^* + Q \xrightarrow{k_Q} A + Q \quad (4)$$

The relaxation of the excited molecule by emission of light (Eq. 3) is first order with rate constant k_f. On the other hand, the interaction of the excited molecule with the quencher (Eq. 4) is 2nd order with rate constant k_Q. These two reactions compete with each other. The larger the concentration of the quencher "Q", the more A* is quenched and the less fluorescence is observed. The Stern-Volmer relation, (Eq. 5) relates the decrease in the measured fluorescence intensity to the increasing concentration of the quencher Q, where I_a is related to the intensity of light absorbed:

$$\frac{1}{I_f} = \frac{1}{I_a}\left(1 + \frac{k_Q}{k_f}[Q]\right) \quad (5)$$

Thus, the slope of a plot of $1/I_f$ versus [Q] is $\{k_Q/(I_a \cdot k_f)\}$ and the intercept is $1/I_a$. The ratio of the slope to the intercept then gives k_Q/k_f, the ratio of the quenching rate constant to the fluorescence rate constant. An alternate, but equivalent, formulation of the Stern-Volmer relationship is given by (Eq. 6)

$$\frac{\Phi_0}{\Phi} = \frac{F_0}{F} = 1 + K_{SV}[Q] \quad (6)$$

where Φ_0 and Φ are the fluorescence quantum yields in the absence and presence of quencher at concentration [Q] respectively, F_o and F are the fluorescence intensities in the absence of quencher and in the presence of quencher at concentration [Q], and $K_{SV} = k_Q/k_f$ is the Stern-Volmer quenching constant.

Quenching is important to consider when using fluorescence as an analytical technique. You want to avoid the use of certain solvents which are strong quenchers of electronically excited states. In addition, in complex mixtures, there may be some unrecognized quenchers present; in this case, calibration with standards in pure solvent may be incorrect, and the method of standard additions would be more appropriate for calibration.

Limit of detection

Limit of detection (LD) is the lowest concentration of analyte that can be reported as being present in the sample with a specified limit of confidence. In practice, it is the lowest concentration of analyte that can be distinguished from the blank with a standard degree of confidence. The LD is defined as the analyte concentration yielding an analytical signal equal to some confidence factor k times the standard deviation (SD) of the blank measurement (s_{bk}).

In our case the limits of detection for the absorption and fluorescence techniques can be identified through the measurement of the variability in the signal after repetitively collecting a number of spectra. From the average of 20 measurements, an average "blank" signal S_{bl} and a standard deviation for this "blank" signal s_{bl} are produced. The minimum detectable analytical signal, S_M, is then equal to the blank signal, S_{bl}, plus 3 times the standard deviation of the blank

$$S_M = S_{bl} + 3s_{bl} \quad (7)$$

The limits of detection are obtained by applying

$$LD = \frac{S_M - S_{bl}}{m} = \frac{3s_{bl}}{m} \quad (8)$$

where m is the slope of the calibration plots (either absorbance or fluorescence).

Experimental

Part A: Absorption Spectra

NOTE: All glassware must be thoroughly cleaned before preparation of solutions. A CD spectrometer (J-815) operation manual will be available as a guide for using the instrument software.

1. If not available, prepare a stock solution of benzo[a]anthracene with the concentration of 200 $mg \cdot L^{-1}$.

   Warning: PAHs have been shown to be hazardous (carcinogenic/mutagenic) to human health. You will be working with dilute solutions in heptanes. However, always use the hood and wear the appropriate gloves when handling solutions. Remember to dispose of all solutions properly.
2. Measure the absorption spectrum of the blank (just heptane) in the 225-400 nm wavelength range.
3. Prepare 100 mL solution of the benzo[a]anthracene at concentration of 5 mg L^{-1} by diluting the stock solution in heptanes. Then, using this 5 mg L^{-1} so-

lution, prepare a series of increasingly dilute solutions, in 5 mL volumetric flasks with concentrations as shown in the table below

	Vol of 5 mg L^{-1} solution (mL)	Volume of heptane (mL)	Concentration of PAH (mg L^{-1})
1	1.00	4.00	1.0
2	0.80	4.20	0.8
3	0.60	4.40	0.6
4	0.40	4.60	0.4
5	0.20	4.80	0.2

4. Obtain the absorption spectrum of the benzo[a]anthracene in the range 225–300 nm for solutions 1 through 4. Record the absorption spectrum of solution 5 (0.2 mg/L) in the 225–400 nm range. (PAHs typically absorb in the 200 to 600 nm region).
5. Record the wavelength at which the maximum absorption occurs and corresponding absorbance.
6. Collect 10 spectra of heptane solvent "blank" by recording the value of the absorption of the blank at the maximum absorption wavelength of benzoanthracene. Limit the wavelength range for the blanks to maximum absorption wavelength – 1nm to maximum absorption wavelength +1 nm range.

Part B: Fluorescence spectra

1. Use the 5 mg L^{-1} solution of benzo[a]anthracene from part A to prepare 50 mL of 1 mg L^{-1} solution.
2. Identify the optimum wavelength for exciting the benzo[a]anthracene (the wavelength at which the maximum absorbance occurs). Use this as the excitation wavelength and record the fluorescence spectrum of the 1 mg L^{-1} solution of benzo[a]anthracene in the 350–460 nm wavelength range.
3. Dilute the 1 mg L^{-1} solution using heptane to obtain 5 mL solutions at concentrations of 0.2, 0.15, 0.1, and 0.05 mg L^{-1}. Record the fluorescence spectra of these solutions in the 350–460 nm wavelength range.

   Record the wavelength at which the maximum fluorescence intensity occurs and corresponding intensity for each solution.
4. Collect 10 fluorescence spectra of the heptane solvent (blank). As for absorption, find the limits of detection for the benzo[a]anthracene by measuring the "fluorescence signal" at the wavelength where each PAH should exhibit maximum fluorescence.

Part C: Fluorescence Quenching

1. Dilute 2.0 mL of the 1 mg L^{-1} solution of benzo[a]anthracene using a combination of heptane and 1-bromoheptane

	Vol of 1 mg L^{-1} benzo[a]anthracene (mL)	Approximate volume of heptane (mL)	Vol of 1-bromoheptane (mL)
1	2.0	3.0	0
2	2.0	2.8	0.2
3	2.0	2.6	0.4
4	2.0	2.4	0.6
5	2.0	2.2	0.8
6	2.0	2.0	1.0

2. Record the fluorescence spectrum of each solution using the peak absorption wavelength as the excitation wavelength. Plot the results in the form of the Stern-Volmer plot using equation (5) and calculate the ratio k_Q/k_f.

   The density of 1-bromoheptane is 1.140 g/mL

Calculations and Disscussion

For the absorption spectra plot the absorbance at the maximum absorption wavelength as a function of concentration (in units of mol L^{-1}) for each of the benzo[a]anthracene solution. Using a least-square method calculate the absorbance calibration curve for the points you just plotted. The molar absorptivity ε (in units of L mol−1 cm−1) for benzo[a]anthracene at the wavelength of maximum absorption will be the slope of the calibration curve. You can use a spreadsheet to carry out the calculations. Be sure to carry out error analysis. Plot the fluorescence intensity as a function of concentration (in units of mol L^{-1}) for each of the benzo[a]anthracene solutions used in fluorescence measurements. Use a least-square analysis to calculate the fluorescence calibration curve.

From the calibration plots of absorbance and fluorescence signals and the corresponding spectra of heptane solvent (blank) calculate the limits of detection of benzo[a]anthracene for the two spectroscopic methods

used in this experiment. Comment on the sensitivity of the two methods.

Reference (1) reports the molar absorption coefficients ε of benzo[a]anthracene at 288 nm to be $8.9 \cdot 10^4$ Mol^{-1} cm^{-1}. Compare the results you obtained with the published values. By what percent do they differ? Is this within your error limits?

Compare the absorption and fluorescence spectra of the 0.2 mg/L solution of Benzanthracene. Are they approximate "mirror images" of each other? Can you explain why? Use the wavelength at which the absorpthion and emission spectra overlap to estimate the energy difference between the ground S_0 and first excited S_1 states. The spectra should be normalized before performing the analysis.

From the fluorescence spectra obtained in Part C calculate the Stern-Volmer quenching constant, k_Q / k_f, in the presence of 1-bromoheptane. Comment on the implications of the presence of a quencher for measurements of PAHs by fluorescence in complex mixtures such as environmental samples where unknown quenchers might be present. Is it possible to take this into account in the experimental procedure?

1. **Karcher, W, et al.** *Spectral Atlas of Polycyclic Aromatic Hydrocarbons.* Dordrecht : D. Reidel Publishing Company, 1985.