LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies
Field: "Applications of physics in biology and medicine"
Specialization: "Molecular Biophysics"

Emission spectra of molecules (PPB6)

KAPITAŁ LUDZKI
NARODOWA STRATEGIA SPÓJNOŚCI

UNIA EUROPEJSKA
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PB6

Emission spectra of molecules

Each molecule has a characteristic energy levels: electronic, vibrational and rotational. As you have learned in the exercise concerning absorption spectroscopy, the distances between the various levels, and thus the relative energy necessary to excite them are as follows: electron energy > energy vibrational > rotational energy. At room temperature, in the absence of the external radiation, all molecules are in the ground electronic state and virtually all - in the ground vibrational state.

As a result of absorption of ultraviolet or visible radiation the molecule is excited to one of the electron excited states, and then releases in various ways the excess of energy. This decay may be either non-radiative or it may be associated with the emission of electromagnetic radiation (fluorescence and phosphorescence). Transitions between rotational levels can not be observed in liquids, since in liquid state collisions of molecules with each other and with solvent molecules occur very often which leads to widening of the rotational lines in the spectra. The processes of absorption and decay form the excites state is often depicted schematically in the so-called. Jabloński diagram (Figure 1). Only electronic and vibrational energy states are shown. Due to frequent collisions of molecules in the liquid state, the rotational levels are omitted.

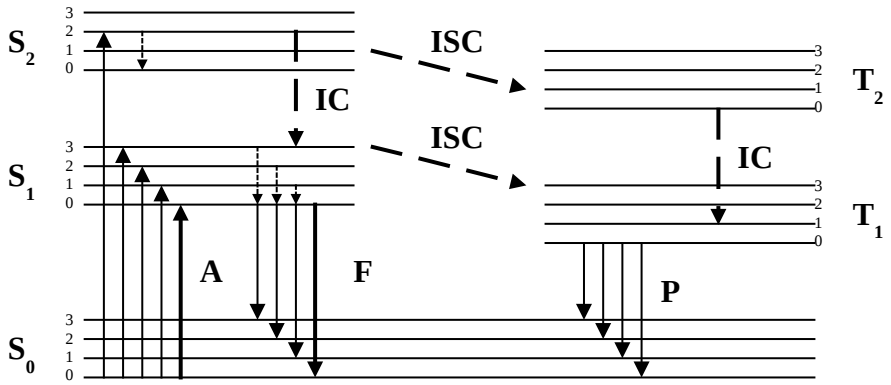


Figure 1. Jabłoński diagram. Legend: A – absorption, F – fluorescence, P –phosphorescence, IC – internal conversion, ISC – intersystem crossing (intercombination transitions), S_o, S_1, S_2, singlet electron states: ground state and two excited states, T_1, T_2, triplet excited states; numbers 0,1,2,3 denote vibrational states, arrows with continuous lines denote radiation transitions, , arrows with dashed lines – non-radiative transitions. The thick-line arrows represent the 0-0 transitions in absorption and in fluorescence. For the clarity of the figure rotational levels were omitted.

In liquids, at temperatures close to room temperature, practically only fluorescence is observed. Fluorescence spectrum consists of a series of bands of electron oscillation, and its shape is determined by the Franck-Condon rule, which says that during the act of absorption of a quantum of electromagnetic radiation (10^{-15} s), the atomic nuclei in the molecule does not significantly alter its position and mode of oscillation. Therefore, in the excited state, immediately after absorption, the configuration and mutual movements of nuclei are the same as in the basic state just prior to the absorption. The lifetime of the excited electronic state is of the order 10^{-4}-10^{-9} s. During this time, the molecule releases excess of vibrational and rotational energy in the non-radiate processes (like collisions with surrounding solute molecules) since characteristic times for the vibrational states relaxation, as well as for internal conversion, is much shorter, 10^{-11}-10^{-14} s. On the other hand electronic excitation energy is large and molecule could not so easily get rid of it in a non-radiate manner at temperatures close to room temperature. Therefore in some cases the radial mechanism is observed.

Since excess of vibrational energy was captured in the non-radiate processes, the emission occurs from zero oscillating state of the electronic excited state, and thus the emission spectrum is shifted toward a higher wavelength relative to the absorption spectrum (Stokes shift). If vibrational levels of the S1 electronic excited state and ground state are similar, the fluorescence emission spectrum is a mirror image of the long-term band in the absorption spectrum, and their only common area is the so called 0-0 transition (see the thick-line arrows on Figure 1).

Quantum yield of various processes in the liquid state depends not only on the energy levels of the molecule, but also on the solvent and the temperature. Raising the temperature increases efficiency of non-radiate processes.

The intensity of the fluorescence, F is proportional to the intensity of the light absorbed by the sample and to the quantum yield of the molecule. A typical fluorescence measurement set-up is to measure the emitted radiation intensity at an angle of 90 ° relative to the excitation beam. In such a case, and if solvent does not absorb, the intensity of the fluorescence, F is expressed by the formula:

$$F(\lambda_{exc}, \lambda_{obs}) = I_o \Phi(\lambda_{exc})(1 - 10^{-\varepsilon(\lambda_{exc})lc}) f(\lambda_{obs}) \quad (1)$$

where: λ_{exc}, excitation wavelength, λ_{obs}, observation wavelength, I_o – intensity of the exciting light, c – concentration of the studied sample, l – path length, $\varepsilon(\lambda_{exc})$ – molar extinction coefficient of the studied sample, Φ, fluorescence quantum yield, f, normalized to 1 integrated shape of the emission fluorescence spectrum.

If the exponent $\varepsilon(\lambda_{exc})$lc is small, the formula is simplified to the form:

$$F(\lambda_{exc}, \lambda_{obs}) = I_o \Phi(\lambda_{exc}) \ln 10 \varepsilon(\lambda_{exc}) lc f(\lambda_{obs}) \quad (2)$$

and the observed fluorescence intensity is proportional to the concentration of the studied substance.

In general, however, a linear relationship is not observed, and this phenomenon is called the inner filter effect. Usually the limit of the exponent $\varepsilon(\lambda_{exc})$lc, above which the internal filter effect cannot be neglected is 0.05. The committed error is in such a case 5.5%, while if the value of the exponent is 0.1 the error is already on the level of 10.6%.

The fluorescence emission spectrum is the function $F(\lambda_{obs})$ at a fixed λ_{exc}, while the excitation spectrum is the function $F(\lambda_{exc})$ at a fixed λ_{obs}. As seen from the above formulas, when the exponent $\varepsilon(\lambda_{exc})$lc is small, the excitation spectrum must overlap with the absorption spectrum of the studied molecule. If it is not the case, although the exponent $\varepsilon(\lambda_{exc})$lc is small, it is a signal that we deal with a mixture of substances with different emission properties, eg. with two tautomers or two ionic forms of the compound, or simply with some contamination (impurities) of the studied substance. Thus, a comparison of the absorption spectrum of the excitation spectrum is a method to test homogeneity of the substance.

Another phenomenon that can affect the lack of a linear relationship between the fluorescence intensity and the concentration of the substance is the phenomenon called reabsorption. Because of the overlap, at least partly, of the absorption and fluorescence spectra, corresponding to the 0-0 transition, emitted radiation from this spectral range can be re-absorbed. This leads to a relative reduction of the intensity of the fluorescence emission spectra in the area corresponding to the 0-0 transition .

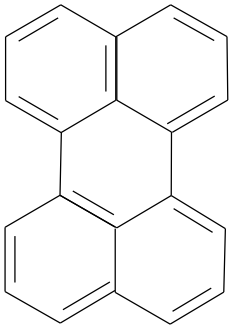
Fluorescence emission quantum yield is the ratio of quanta emitted by fluorescence to the number of quanta absorbed. We can determine quantum yield by integrating the emission spectrum (as a function of frequency, and not wavelength) obtained under conditions where

the exponent $\varepsilon(\lambda_{exc})lc$ is small, and therefore the equation (2) it is satisfied. Comparing the above integral, S, to the integral of a reference substance, S_{wz} and measuring the absorption at the excitation wavelength of the test substance and that of a reference substance, $A(\lambda_{exc})$ and $A_{wz}(\lambda_{exc})$, respectively, we can determine the quantum yield of the test molecule with the formula:

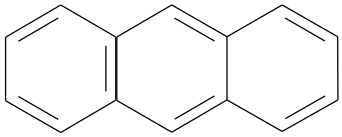
$$\Phi(\lambda_{exc}) = S\,A(\lambda_{exc}) / A_{wz}(\lambda_{exc}) S_{wz} \qquad (3)$$

The transition from the ground state of the molecule to the excited electronic S1 state may be accompanied by change of several physical properties of a molecule, such as dipole moment, the geometry of the molecule, the ability of the attachment and detachment of proton (acid-base properties). Therefore a number of secondary processes that bear on the emission properties of the compound may occur in the electronic state S1. However, to observe these processes the rapid kinetics is necessary, at least comparable with the lifetime of the S1 state (10^{-7} - 10^{-9}s).

In this experiment some simple organic molecules are studied. Their structural formulas are shown in the diagram below. The main object of the study is perylene, and anthracene serve as a reference substance to determine the quantum yield of perylene.



perylene



anthracene

In the experiment the following phenomena will be studied and the following physical properties will be determined:

1. Electronic absorption: determination of the electronic spectra (with oscillation structure) for both substances, perylene and anthracene.
2. Fluorescence emission; determination of the fluorescence emission spectra of perylene (with oscillation structure) for several excitation wavelengths λ_{exc},

comparison of shape of the spectra, comparison of the shape of the fluorescence emission spectra with that of absorption spectra, observation of Stokes shift, shape of the spectra and how it is connected with the Franck-Condon principle.

3. Interactions of perylene molecules with solution molecules: compare 0-0 transition energy in absorption and in fluorescence spectra.
4. Fluorescence excitation spectra of perylene; spectra for several observation wavelengths wil be registered and compared with absorption spectra and fluorescence emission spectra.
5. Fluorescence quantum yield for perylene using anthracene as a reference substance will be determined.
6. Fluorescence emission spectra (with oscillation structure) for several solutions of perylene with various concentrations will be registered; the inner filter effect and reabsorption will be observed, which shows how these two phenomena affect the shape and intensity of the fluorescence emission spectra.

The requirements for the pre-test

The condition to start the experimental part of this exercise is to pass the pre-test.

The choice of how to carry out a pre-test, it means its form, written or verbal questions and answers, open or closed questions etc., depends on the person supervising students during the experiments.

1. The information concerning molecular spectroscopy and application of spectroscopy to study the properties of molecules, necessary to pass the pre-test, may be find in books such as:
   a) Banwell and McCash „*Fundamentals of molecular spectroscopy*",
   b) Barrow „*Introduction to molecular spectroscopy*"
   c) "*Physics of Atoms and Molecules*", Series Editors: P.G. Burke, H. Kleinpoppen

2. The information concerning molecular emission spectroscopy, necessary to pass the pre-test, may be find in books such as:
   a) Parker, *Photolumienscence of solutions*
   b) Lakowicz, *Principles of fluorescence spectroscopy*
   c) Underfriend *Fluorescence assay in Biology and Medicine*

From the above mentioned books (or from others specified at the Literature) one should read only those parts that are necessary to discuss the following issues:

1. Basic definitions concerning the electromagnetic radiation and the spectroscopic measurements:

- wavelength, frequency of radiation, wavenumber
- the intensity of radiation, the radiation density
- molar absorption coefficient (extinction coefficient), integral absorption coefficient
- ground state, excited state, the oscillator strength
- fluorescence quantum yield; methods of its determination

2. Energy of molecules:
   - types of energy, the characteristic energies of each type, relation between them,
   - quantization of energy, the simplest models (rigid rotator, harmonic oscillator)
   - diagram of the energy levels – Jablonski diagram, electronic, vibrational and rotational levels
   - degeneracy of energy levels
   - distribution of molecules on the energy levels in the thermal equilibrium (dependence of this distribution on temperature)

3. Measurement of fluorescence of molecules:
   - schematic representation of the typical set-up for emission measurements
   - what do we mean by emission spectrum?
   - what do we mean by excitation spectrum?
   - quantitative description of the emission
   - fluorescence quantum yield; methods of its determination
   - factors determining the contour shape and width of the emission band

4. Chemical structure of the molecules studied in this exercise:
   - draw the structural formulas perylene and anthracene,
   - define what we mean by σ, π, n orbitals, show σ, π, n orbitals in these structures

5. Electronic absorption and emission spectra with vibrational structure:
   -in which electromagnetic radiation region the electronic spectra are observed?
   -diagram of the energy levels – Jablonski diagram, electronic, vibrational and rotational levels
   - allowed transition between energy levels, selection rules
   - give the example of the absorption spectrum illustrating and explaining how the shape of the spectrum is connected with the structure of the electronic and vibrational energy levels and allowed transitions between these levels

6. Electronic states of molecules:
   - molecular orbitals, what is the meaning of symbols σ, σ^*, π, π^*, n
   - diagram of the energy levels – Jablonski diagram, electronic, vibrational and rotational levels
   - what are possible absorption transitions and what are the relative positions of the corresponding bands on the frequency scale of electromagnetic radiation
   - describe how excited molecule may capture the excess of the energy (radiate and non-radiate processes, characteristic rate constants of these processes)

Studied objects:

In the exercise the electronic absorption and florescence spectra of solutions of perylene and anthracene in ethanol will be recorded and analysed.

Execution of the experiment – overall remarks:

Students receive a previously prepared solutions of the tested compounds. Concentration of solutions is known and appropriate for this experiment, namely:

Perylene: stock solution 0.5 mg/100 ml and five diluted solutions with the following concentration with respect to the stock solution: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}

Anthracene: stock solution 1 mg/80 ml and solution 10^{-1} of the stock

All spectra are recorded for the solutions in quartz cuvettes with a path length of 1 cm x 1 cm.

The volume of the solution in the cuvette (for absorption and for emission) should be 3 ml.

Absorption cuvette has only two walls polished, while in that for emission has all walls are polished, which allows measurement of the intensity of the emitted radiation at 90 degrees with respect to the exciting radiation.

Exercise is carried out on a UV-VIS spectrofluorimeter, Perkin-Elmer, or Shimadzu.

How to conduct the experiment:

Remark 1: when spectra is registered pour back the solution form the cuvette into the vessel from which it was obtained

Remark 2: perylene and anthracene show strong fluorescence. Therefore, very carefull washing of the cuvettes before the next measurement is necessary. First, twice rinse the cuvette with ethanol, then 8 times rinse it with distilled water and finally again once with ethanol.

1. Register absorption spectra of perylene and anthracene stock solutions in the range 190-800 nm.

2. Introduction to spectra collection using the UV-VIS spectrofluorimeter (Perkin-Elmer or Shimadzu)

3. Fluorescence emission of perylene. Register spectra for:
   a) Several excitation wavelength λ_{exc}: 246, 253, 387, 408, 436 nm (excitation and emission slits should be 5 nm)
   b) Several combinations of excitation and emission slits: 5 nm/3 nm, 5 nm/10nm, 5 nm/20 nm, 10 nm/3 nm, 10 nm/10nm, 10 nm/20 nm (λ_{exc} 436 nm)

   The solution should be chosen in such a way, that its absorption in the excitation wavelength does not exceed 0.05, since only when this condition is fulfilled the inner filter effect may be neglected. Please decide, based on results obtained in point 1, which of your solutions, you should be used.

4. Excitation spectra: Register spectra for several observation wavelengths λ_{obs}: 450, 480, 500, 520 nm (excitation and emission slits should have 5 nm)).

The solution should be chosen so that the absorption in all excitation wavelengths does not exceed 0.05, since only when this condition is fulfilled the inner filter effect may be neglected. Can we use the same solution as in point 3?

5. Determination of the fluorescence quantum yield of perylene using anthracene as a reference. Register emission spectra of solutions of both compounds using the same excitation wavelength (366 nm) and determine absorption of both solutions at excitation wavelength. Based on the previous measurements choose the solution, for which absorption at excitation wavelength (366 nm) in not greater than 0.05, so the inner filter effect may be neglected. The 10^{-1} solution probably fulfills this condition.

6. Register fluorescence emission spectra for several perylene concentrations to observe the inner filter effect and reabsoption and their influence on the shape and intensity of the spectra.

   Register spectra of solutions 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} placing them in the cuvette one by one starting from 10^{-5}. Excitation wavelength should be λ_{exc} 408 nm, both slits 5 nm. In the beginning solvent (ethanol) spectra should be determined using the same parameters of registration.

7. When the experimental part is finished all the files containing the recorded spectra have to be sent to your e-mail account or copied on your own USB Flash drive (or other external memory device). In most of the cases recorded files should be first converted into the text format.

How to make the report

The report should start from the Introduction, in which general spectroscopic issues relevant to the task performed are discussed, followed by Materials and Methods, in which equipment used, compounds studied and measurement conditions should be described. Then, in the Results and Discussion one should show, evaluate, describe and interpret the recorded spectra. The following issues should be considered in this part.

1. Shape of absorption spectra, identification of bands responsible for transition to S1 electronic state, if possible also to S2, S3 states, analysis of possible vibrational „coarse" structure of bands.

2. Analyze how registered spectra correspond with the chemical structure of the studied molecules, it means on the number and relative location of the π bonds. Why perylene solution has colour, while that of anthracene is colourless ?

3. Determine molar extinction coefficients for wavelengths corresponding to maxima of spectra for perylene and anthracene. Compare with literature values (see table below).

4. Analyze how shape and intensity of perylene fluorescence emission spectra depend on:
   a) Excitation wavelength
   b) Spectral bandwidth of emission end excitation slits

5. Analyze how shape and intensity of perylene excitation spectra depend on observation wavelength

6. Compare shape of absorption, fluorescence emission and excitation spectra, analyze interactions of perylene molecules with solvent molecules, compare 0-0 transition energy in absorption and florescence emission spectra.

7. Analyze how shape and intensity of fluorescence spectra depend on concentration of studied molecule, i.e. perylene

8. Determine the fluorescence quantum yield for perylene.

Literature

C.N. Banwell and E.M. McCash „*Fundamentals of molecular spectroscopy*”,
C.A. Parker, *Photolumienscence of solutions*
G.M. Barrow „*Introduction to molecular spectroscopy*”
“*Physics of Atoms and Molecules”* Series Editors: Burke, Philip George, Kleinpoppen, Hans
J. A. Barltrop i J. D. Coyle, *Principles of photochemistry*
Banwell and McCash „*Fundamentals of molecular spectroscopy*”,
J. Fisher and J. Arnold *Chemistry for biologists*, *Instant Notes*

And also, if there is a need to red more:

J.R. Lakowicz, *Principles of fluorescence spectroscopy*
S. Underfriend *Fluorescence assay in Biology and Medicine*
H. Haken, H. C. Wolf, Molecular Physics and Elements of Quantum Chemistry : *Introduction to Experiments and Theory*
H. Haken, H. C. Wolf, *The physics of atoms and quanta. Introduction to experiments and theory*
H. A. Staab, *Introduciton to Theoretical Organic Chemistry*
D. Kealey, D.J. Haines *Instant Notes in Analytical Chemistry*
J. Simons, *Photochemistryand spectroscopy*
H. D. Forsterling and H. Kuhn, *Principles of Physical Chemistry*

Extinction coefficients and molecular weight of the studied compounds

Coefficients are from:
H. Du, R. A. Fuh, J. Li, A. Corkan, J. S. Lindsey, PhotochemCAD: A computer-aided design and research tool in photochemistry," *Photochemistry and Photobiology,* 68, 141-142, 1998].
I. B. Berlman, *Handbook of Fluorescence Spectra of Aromatic Molecules*, Academic Press, 1971
Quantum yield values are from:
C.A. Parker, *Photoluminescence of solutions*, Elsevier, 1968, pp. 266 i 267

Molecule	MW [g mol^{-1}]	λ [nm]	ε [$M^{-1}cm^{-1}$]	Solvent (for absorption spectrum	λ_{exc} [nm]	Φ	Solvent (for emission spectrum)
Anhtracene	178.23	366	2 315	cyclohexane	366	0.30	ethanol
		356	9 700	cyclohexane			
Perylene	252.31	245.5	36 376	cyclohexane	366	0.87	ethanol
		252.75	52 299	cyclohexane			
		360.5 (sh)	3 510	cyclohexane			
		387	13 014	cyclohexane			
		408.25	28 096	cyclohexane			
		430.25 (sh)	30 896	cyclohexane			
		435.75	38 500	cyclohexane			

Tips for the trainer

Prepare the following solutions, all in ethanol:

Perylene

Stock solution	0.5 mg/100 ml
Solution 10^{-1}	5 ml of the stock solution + 45 ml ethanol
Solution 10^{-2}	5 ml of the stock solution 10^{-1} + 45 ml ethanol
Solution 10^{-3}	5 ml of the stock solution 10^{-2} + 45 ml ethanol
Solution 10^{-4}	5 ml of the stock solution 10^{-3} + 45 ml ethanol
Solution 10^{-5}	5 ml of the stock solution 10^{-4} + 45 ml ethanol

Anthracene

Stock solution	1 mg/80 ml
Solution 10^{-1}	5 ml of the stock solution + 45 ml ethanol

Each group should get 8 ml of each solution