**Practical No 36**

# WAVE ABSORPTION IN SOLUTIONS OF ORGANIC DYES. ANALYSIS OF SOLUTION COMPOSITION

## Equipment

1. Spectrophotometer
2. Solution of fluorescein (FL) 2 x $10^{-4}$ mol/$dm^3$ (200 µM)
3. Solution of Bengali rose (BR) 2 x $10^{-4}$ mol/$dm^3$ (200 µM)

## Course of the practical

I. PREPARATION OF DYE SOLUTIONS.

1. You are given water solutions of two dyes: fluorescein (FL) and Bengali rose (BR). The concentration of both stock solutions is 2 x $10^{-4}$ mol/$dm^3$ (200 µM).
2. Dilute stock solution of each dye with distilled water so that you obtain the series of eight solutions (concentrations 2, 3, 4, 5, 6, 7 and 8 µmol/$dm^3$). Total volume of each solution is 10 $cm^3$. Mix all the solutions carefully. (Put a rubber stopper into the test tube and invert the tube several times. Before transferring the stopper to the next solution dry the stopper with a paper tissue).

II. FINDING MOLAR ABSORPTION COEFFICIENTS OF STUDIED DYES

**A. Measurements**

Turn on the spectrophotometer with the switch on its back. Wait at least 15 minutes. Use this time to prepare dye solutions.

**ATTENTION 1: keep the spectrophotometer's chamber closed while turning the device on.**

**ATTENTION 2: the cuvettes can be touched only at side (opaque) walls.**

1. Place a cuvette filled with distilled water inside the spectrophotometer. Check the position of the cuvette – a small triangle on it should overlap a white line inside the measurement chamber. Close the lid.
2. Set the measurement mode to Absorption using "A/T/C" button. You should see a letter A in the lower right corner of the display.
3. Using the figures of absorption spectra find the analytical wavelengths (with accuracy of 1 nm) for the monomer of fluorescein (FL) and Bengali rose (BR) and write them down to the Table 1.
4. Set the wavelength chosen in the point II.3 for FL using the buttons "100", "010" and "001" for setting hundreds, tens and units, respectively.
5. Accept the wavelength with the button "BLANK".
6. Remove the cuvette with distilled water from the spectrophotometer.
7. Fill the second, dry cuvette with the solution of FL of the lowest concentration. Place the cuvette into the spectrophotometer's chamber. Close the lid. After a few seconds read off the absorption intensity from the display and write it down in the Table 2.
8. Pour the solution off the cuvette, dry it by pressing against a paper towel. Fill the cuvette with the FL of the next higher concentration and repeat point 7.
9. Perform the analogous measurements for Bengali rose.

**B. Graphical presentation of the results**

1. Based on the data from the Table 2 plot the dependence A(c) = $\varepsilon \cdot l \cdot c$ (absorbance A versus the concentration c), for both dyes.
2. Based on the above plots calculate molar absorption coefficients ($\varepsilon$) for both dyes. Write the obtained values down to the boxes below the Table 2.

III. ANALYSIS OF MIXTURE OF DYES.

1. In a clean test tube prepare dye mixture by mixing two **different** volumes of FL and BR solutions (concentration 8 μmol/$dm^3$). Use higher volume of FL solution and lower volume of BR solution. Write these volumes down to the Table 3.1.

**ATTENTION: keep the rest of FL and BR solutions (8 μmol/$dm^3$) used to prepare the mixture, they are necessary for further measurements.**

2. **Finding the concentrations of FL and BR in the dye mixture.** The analysis of absorption spectra of both dyes gives us some important pieces of information:
   a. absorption of FL solution is equal to 0 in the wavelength range 530 – 560 nm,
   b. for the wavelength range between ca. 450 nm and ca. 520 nm the absorption of a mixture is a sum of absorptions of FL and BR solutions. Because of this fact in order to calculate the concentrations of fluorescein ($x_{FL}$) and Bengali rose ($x_{BR}$) in the mixture one should prepare **three different solutions in three cuvettes:** 1) the mixture, 2) BR solution (8 μmol/$dm^3$) and 3) FL solution (8 μmol/$dm^3$) and measure the absorbance of these solutions at two wavelengths**: $\lambda_1$ = 490 nm and $\lambda_2$ = 495 nm. In addition, measure the absorbance of a mixture at the wavelength of a maximal absorption of the BR solution.**

- write the measured values down to the Tables 3.2 and 3.3,
- calculate the values missing in the Table 3.2 and write their values down to the proper boxes.

Taking into account the additivity of absorption of both dyes, the measured absorptions fulfil the equation:

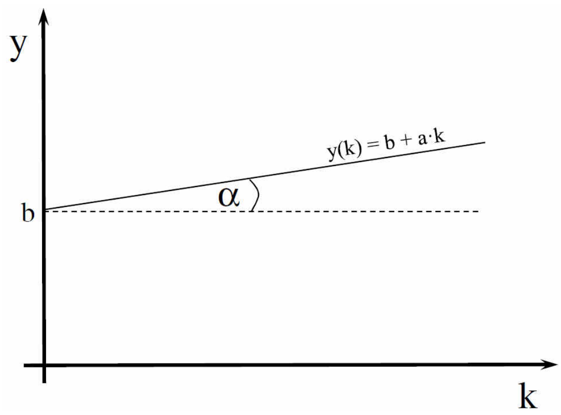
$$\frac{A(\lambda)}{A_{FL}(\lambda)} = \frac{x_{FL}}{c_{FL}} + \frac{x_{RB}}{c_{RB}} \frac{A_{RB}(\lambda)}{A_{FL}(\lambda)}$$

   c. One should notice that the equation shown above presents a linear function y(k) = b + a·k, where:

$$y = \frac{A(\lambda)}{A_{FL}(\lambda)}, \qquad b = \frac{x_{FL}}{c_{FL}}, \qquad a = \frac{x_{BR}}{c_{BR}}, \qquad k = \frac{A_{BR}(\lambda)}{A_{FL}(\lambda)}$$

The correctly plotted line has a slope tg α = $\frac{x_{BR}}{c_{BR}}$ and crosses vertical axis at point b = $\frac{x_{FL}}{c_{FL}}$ .

For correct measurements of dye mixture, the line connecting both previously designated points should form the angle α with the axis k, as shown below:




3. FL and BR concentrations ($x_{FL}$ and $x_{BR}$) in dye mixture can be found as:

$$x_{FL} = bc_{FL} \qquad\qquad x_{BR} = \text{tg}\,\alpha\, c_{BR}$$

   where: $c_{FL}$ and $c_{BR}$ are concentrations of FL and BR solutions used to prepare the mixture.
   Write these concentrations down below the Table 3.2.

4. **Finding BR concentration in the dye mixture using the calibration plot.** Since absorption of FL solution is equal to 0 in the wavelength range 530 – 560 nm, the concentration of BR in the mixture ($x'_{BR}$) can be read off the calibration curve prepared previously for BR (point II.B.1).
   a. mark this absorption value on the calibration plot obtained in point II.B.1,
   b. read the BR concentration in dye mixture off this plot and write it down to the Table 3.3.

**The concentration of BR found in this way should be treated as a verification of the value of this concentration estimated in the point III.2.**

5. Knowing the volumes and concentrations of FL and BR solutions used to prepare a mixture, calculate theoretical FL and BR concentrations ($x_{FL}^{theoret}$ and $x_{BR}^{theoret}$) in the mixture. Write these concentrations down to the Table 3.4. The values $x_{FL}^{theoret}$ and $x_{BR}^{theoret}$ should approximately be the same as the values of $x_{FL}$ and $x_{BR}$ estimated according to the procedure described in the point III.2 (Table 3.2).

**<u>Required theoretical knowledge:</u>**

1. What is spectroscopic analytical method ?
2. Types of chemical bonds in organic compounds and names of molecular orbitals.
3. What is a chromophore in a structure of organic molecule.
4. Ground and excited state of a molecule, mechanism of excitation.
5. Types of electronic transitions in molecules.
6. What is UV-VIS spectroscopy ?
7. Light absorption laws:
   a) Lambert Law (I absorption law)
   b) Lambert-Beer law (II absorption law)
   c) absorption addition law (III absorption law)
8. What is electronic absorption spectrum and what are its parameters ?
9. What is a monomer and what is an aggregate of organic dye in a water solution.
10. Condition, at which molecules of organic dye in a solution exist in a monomeric phase.
11. Main reasons of deviations from Lambert-Beer law.
12. Spectral analysis of mixture of two dyes:
    a) interpretation of equation about additivity of absorption of two organic dyes
    b) describe the method of determination of unknown concentration of fluorescein (FL) and bengali rose (RB) in a mixture

| **Wrocław Medical University Department of Biophysics and Neuroscience** | **Practical No 36**<br><br>**Wave absorption in solutions of organic dyes. Analysis of solution composition** |
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| Grade: | Tutor's signature: |

I. Analysis of dyes' absorption spectra

Table 1. Analytical wavelength

| $\lambda_{max}$ (nm) FL | $\lambda_{max}$ (nm) BR |
|---|---|
| | |

II. Graphical presentation of the results

Table 2. Dependence of absorbance on dye concentration

| No. | FL concentration mol/dm$^3$ | A (FL) | BR concentration mol/dm$^3$ | A (BR) |
|---|---|---|---|---|
| **1.** | | | | |
| **2.** | | | | |
| **3.** | | | | |
| **4.** | | | | |
| **5.** | | | | |
| **6.** | | | | |
| **7.** | | | | |

| $\varepsilon_{FL}$ = | $\varepsilon_{BR}$ = |
|---|---|

III. Analysis of mixture of dyes

Table 3.1. Dyes' volumes used to prepare the mixture

| FL volume | BR volume |
|---|---|
| | |

Table 3.2. Finding FL and BR concentration in the dye mixture

| λ (nm) | Absorbance of the dye mixture $A(\lambda)$ | Absorbance of 8 μmol/dm$^3$ FL solution $A_{FL}(\lambda)$ | Absorbance of 8 μmol/dm$^3$ BR solution $A_{BR}(\lambda)$ | $\frac{A(\lambda)}{A_{FL}(\lambda)}$ | $\frac{A_{BR}(\lambda)}{A_{FL}(\lambda)}$ |
|---|---|---|---|---|---|
| 490 | | | | | |
| 495 | | | | | |

$X_{FL}$ =

$X_{BR}$ =

Table 3.3. Finding BR concentration in the dye mixture using calibration curve

| λ (nm) | A(λ) | $x'_{BR}$ |
|---|---|---|
| | | |

Table 3.4. Theoretical FL and BR concentrations in dye mixture.

$x_{FL}^{theoret}$ =

$x_{BR}^{theoret}$ =