FLUORIMETRY

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General principles

Fluorimetry is an analytical method based on the emission of ultraviolet or visible fluorescent radiation by some molecules in an **excited state**. Most frequently, an absorption of ultraviolet or visible radiation is applied to transfer molecules in the sample to the excited state (excitation of the sample). We limit here only to fluorimetry of diluted solutions of fluorescent compounds in solvents which absorb neither excitation nor emission radiation.

In **Fig.1, a** simplified scheme of energy levels in a molecule with even number of electrons is shown. In the ground state S_0, a pair of electrons with opposite spin usually occupies the same electronic state. Their spins compensate each other and so the total spin quantum number is zero. Hence, the ground state is a **singlet** state. Even after the excitation of a single valence electron to higher electron level, electrons may keep their total spin unchanged, which is expressed by a series of excited singlet states of the molecule S_1, S_2, etc. However, after the excitation, the electrons are no more paired and their total spin quantum number may be 1 too. Then, the molecule is in a **triplet** state. Again, there is a series of excited triplet states T_1, T_2,... . Molecular transitions between the singlet and triplet states are several orders of magnitude slower than similar transitions inside a series of singlet states or inside a series of triplet states.

Because of the vibrational motion, which increases the total energy of the molecule, each electronic state is formed by a series of **vibrational levels.** The vibrational level with the lowest energy is the ground vibrational level of the electronic state. In the state of thermal equilibrium, the majority of molecules is in the ground vibrational level of the state S_0. The energy of the absorbed photon increases the energy of the molecule which is thus transferred to one of the excited states (see transition 1 in **Fig.1**). Planck's equation holds for the emission of the photon

$$|\Delta E| = h\nu = \frac{hc}{\lambda} \qquad (1)$$

where ΔE is the difference in molecular energy between the final and the initial state, *h* is Planck's constant, ν is the frequency and λ the wavelength of the absorbed (emitted) radiation. Because the probability of such a transition is not the same for all energy levels, the ability of the molecule to absorb the radiation is wavelength dependent. This dependence is expressed in **absorption spectrum** of the compound, which is the dependence of absorbance of the solution or absorption coefficient of the

compound on the wavelength (see 'Liquid Chromatography and Absorption UV-spectrophotometry').

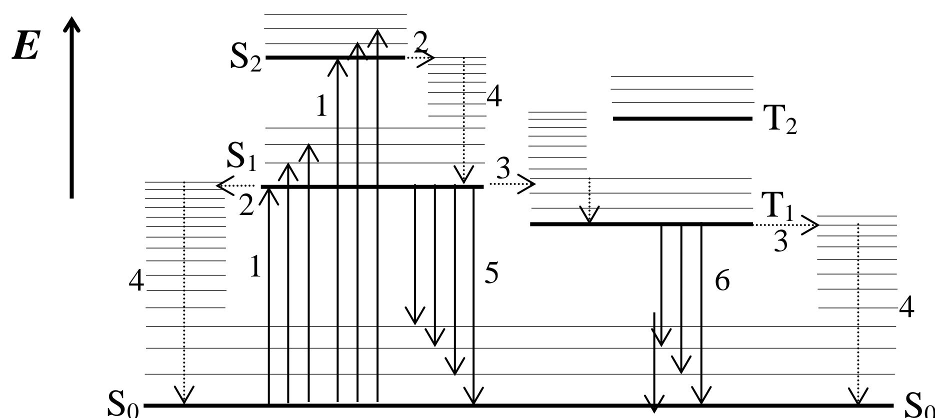

Fig.1: Energy levels of the molecule and transitions between them
S_0 - ground state, S_1, S_2…- excited singlet states, T_1, T_2…- excited triplet states.
1 - absorption of radiation, 2 - internal conversion, 3 - intersystem crossing, 4 - vibrational relaxation, 5 - fluorescence, 6 - phosphorescence

The excited molecule rapidly transfers the energy into its environment. The molecule step by step passes to the lower states by means of radiation-less transitions (internal conversion - transition 2 in **Fig.2** - and intersystem crossing - transition 3) followed by a vibrational relaxation (transition 4). Usually, only the radiation-less transition between the first excited singlet state S_1 and the ground state S_0 is slow enough, that also other transitions become important. The transition to the ground state accompanied with the emission of radiation is called **fluorescence** (transition 5). When the irradiation of the sample is stopped, the fluorescence decays have the lifetime of the order of 10^{-6} - 10^{-9} s. Also the intersystem crossing to some of the triplet states usually originates from the state S_1. The radiation transition from the ground vibrational level of the lowest triplet state T_1 to the ground state S_0 has the lifetime of the order 10^{-3} - 10^{1} s and is called **phosphorescence** (transition 6).

The molecule loses a part of the excitation photon energy prior to the fluorescence emission. The wavelength of the fluorescence radiation is therefore greater than that of the excitation radiation. Moreover, if the fluorescence occurs in the way shown in **Fig.1**, the wavelength of the fluorescence radiation is greater or equal to the wavelength of the first band in the absorption spectrum (transition between the ground vibrational levels of the states S_0 and S_1). Therefore, we search for the fluorescence emission of the compound with the known absorption spectrum nearby its first absorption band towards the longer wavelengths. Well known exception to this

rule is azulene and their derivatives, the fluorescence of which originates from the state S_2.

The ability of compounds to emit the fluorescence radiation is expressed by the **quantum yield of fluorescence** Y_F defined as the ratio of the number of photons emitted by the compound in the form of fluorescence n_F to the number of the absorbed photons n_A

$$Y_{\mathrm{F}} = \frac{n_{\mathrm{F}}}{n_{\mathrm{A}}} \quad (2)$$

The quantum yield determines the relationship between the **fluorescence radiation flux** expressed as the number of photons per unit time $\Phi_{\mathrm{p,F}}$ and the absorbed radiation flux $\Phi_{\mathrm{p,A}}$ as

$$\Phi_{\mathrm{p,F}} = Y_{\mathrm{F}}\,\Phi_{\mathrm{p,A}} \quad (3)$$

The quantum yield is given by the ratio of the fluorescence transition rate and the total rate of all transitions originating from the state S_1. Besides the transitions mentioned above, also other components present in the solution may deactivate the state S_1 and quench the fluorescence. Most common **quenchers** are molecular oxygen and also components containing elements with high atomic weight. Also thefluorescent compound alone may decrease the fluorescence quantum yield when present at higher concentrations. Then we speak about the concentration fluorescence quenching. Various intermolecular interactions such as the formation of molecular associates in the ground or excited state, energy transfer, etc. may participate in this process. Under normal conditions, phosphorescence is quenched very effectively because of its long lifetime. That is why phosphorescence appears only e.g. upon cooling down the samples (very frequently at liquid nitrogen temperature 77 K), when the external quenching processes are suppressed.

It is evident from **Fig.1** that fluorescence is not emitted at a single wavelength because the target state may belong to various vibrationally excited levels of the ground electronic state. The distribution of fluorescence radiation flux among the emitted wavelengths λ_{em} is described by the **emission spectrum**. Exactly, it is expressed as a **spectral radiation flux** $\Phi_{\mathrm{p,F},\lambda}(\lambda_{\mathrm{em}})$ which is a radiation flux emitted within a unit interval of wavelengths around the wavelength λ_{em}. The total fluorescence radiation flux is given as integral

$$\Phi_{\mathrm{p,F}} = \int_0^{\infty} \Phi_{\mathrm{p,F},\lambda}(\lambda_{\mathrm{em}})\,\mathrm{d}\lambda_{\mathrm{em}} \quad (4)$$

Another important conclusion can be derived from the scheme in **Fig.1.** The shape of the emission spectrum does not depend on the excitation wavelength. This follows from the fact that the florescence is determined only by the relationship

between the ground vibrational level of the state S_1 and the the vibrational levels of the state S_0.

If the vibrational levels of the states S_0 and S_1 were of similar structure then the vibrational structure of the emission spectrum would be similar to that of the first band in the absorption spectrum. In the frequency or wavenumber scale, the fluorescence spectrum would be a mirror image of the first absorption band with a mirror axis near the wavenumber of the transition between the ground vibrational levels of both states (so called mirror symmetry rule). Spectra of many polycyclic aromatic hydrocarbons such as anthracene (see **Figs. 3** and **4** below) are good examples of this relationship.

The shape of the emission spectrum does not depend on conditions of the excitation, but the total fluorescence radiation flux does. Therefore it is reasonable to characterise the emission properties of the compound by the spectral fluorescence flux divided by the spectral fluorescence flux at the maximum of the spectrum. The emission spectrum defined in this way is dimensionless and its maximum value is 1. Another possibility is to divide the spectral fluorescence flux by the total fluorescence flux

$$E_c(\lambda_{em}) = \frac{\Phi_{p,F,\lambda}(\lambda_{em})}{\Phi_{p,F}} \quad (5)$$

The emission spectrum defined in this way has the units of wavelength reciprocal value, e.g. nm^{-1}, and it's integral on interval (0, ∞) is one.

If the solution in the sample cell having asquare cross-section contains a single absorbing compound exhibiting fluorescence, then the absorption of the excitation radiation obeys Lambert-Beer's law

$$A = \varepsilon\, b\, c_f \quad (6)$$

where A is absorbance of the solution, ε is the molar absorption coefficient of the component, b is the cell thickness and a c_f is the concentration of the component in the solution. From this equation we can express the radiation flux absorbed by the solution $\Phi_{p,A}$ from the radiation flux reaching the cell $\Phi_{p,0}$ as

$$\Phi_{p,A} = \Phi_{p,0}(1 - 10^{-\varepsilon\, b\, c_f}) \quad (7)$$

For low absorbance values, we can approximate this dependence by a linear relationship

$$\Phi_{p,A} = 2{,}3\Phi_{p,0}\varepsilon\, b\, c_f \quad (8)$$

From Eq. (3), for the fluorescence radiation flux it follows

$$\Phi_{\mathrm{p,F}}(\lambda_{\mathrm{ex}}) = 2{,}3\ Y_{\mathrm{F}} \Phi_{\mathrm{p,0}}(\lambda_{\mathrm{ex}}) \varepsilon(\lambda_{\mathrm{ex}})\ b\ c_{\mathrm{f}} \tag{9}$$

where the parameter (λ_{ex}) expresses which quantities in this equation depend on the wavelength of the excitation radiation λ_{ex}. (In solutions, the quantum yield usually does not depend on the excitation wavelength.) For the spectral fluorescence radiation flux then results the relationship

$$\Phi_{\mathrm{p,F,\lambda}}(\lambda_{\mathrm{ex}}, \lambda_{\mathrm{em}}) = 2{,}3\ Y_{\mathrm{F}} E_c(\lambda_{\mathrm{em}}) \Phi_{\mathrm{p,0}}(\lambda_{\mathrm{ex}}) \varepsilon(\lambda_{\mathrm{ex}})\ b\ c_{\mathrm{f}} \tag{10}$$

The fluorescence spectral radiation flux therefore depends on the wavelength of the excitation radiation and on the emission wavelength. For solutions with low absorbance values containing a single component, it can be expressed as the product of two factors. First factor depends only on the excitation wavelength and the second one only on the emission wavelength. The dependence of the fluorescence radiation flux on the wavelength of the excitation radiation at constant conditions of the emission measurement is called the **excitation spectrum**. If the source of the excitation radiation flux independent on the wavelength were available, the excitation spectrum of a diluted solution containing a single component would be essentially the same as the absorption spectrum of this component. This would be true for all emission wavelengths. Similarly, also the emission spectrum does not depend on the excitation wavelength. Both the excitation and emission spectrum can be used for the compound identification by a comparison with the spectra of standards. When the solution contains several fluorescent compounds and their concentrations are so low that they does not mutually interact, their fluorescent radiation fluxes are added up.

For the quantitative analysis it follows from Eq. (10) that for solutions with low absorbance values, the fluorescence radiation flux increases linearly with the concentration of fluorescent component.

An instrument for the measurement of the fluorescence radiation is called fluorimeter. If the instrument allows for the measurement of the excitation and emission spectra then it is called **spectrofluorimeter**. A basic scheme of the fluorimeter can be seen in **Fig.2**.

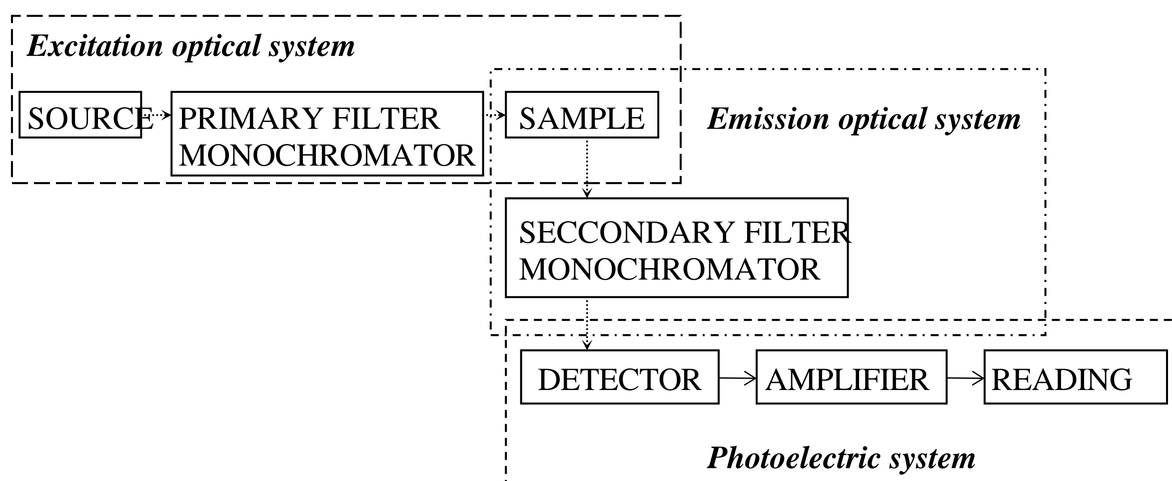

Fig.2: A basic scheme of the fluorimeter.

Radiation emitted by an intensive excitation source passes through a primary (excitation) filter or a monochromator, which isolates those wavelengths which are to be used for the excitation of the sample. This radiation is introduced into the sample and excites its florescence radiation which exits the sample in all directions. Only a part of the emitted radiation has the direction toward a secondary (emission) filter or monochromator, passes through it and is measured by a photoelectric detector. Here, it generates an electric signal which is amplified and measured. Its value corresponds to the fluorescence radiation flux emitted by the sample at the wavelengths isolated by the excitation filter or monochromator. Excitation conditions are given by the source of the excitation radiation and a primary filter or excitation monochromator setting. If the instrument is equipped with the excitation monochromator, we can continuously change the wavelength of the radiation transmitted by them and in this way to record the excitation spectrum of the sample. If the instrument is equipped with the emission monochromator, we can continuously change the wavelength of the radiation transmitted by them and in this way to record the emission spectrum of the sample.

Excitation and emission spectra measured directly in this way by spectrofluorimeters are distorted by instrument characteristics and are denoted as **uncorrected spectra**. Emission spectra are distorted primarily due to a spectral variability of the detector sensitivity. Also the excitation source has not the same intensity for all wavelengths, and therefore the excitation spectra are distorted. In order to approach the true shape of the spectra in the context of Eq.(10), we have to multiply the measured spectra for every wavelength by corresponding correction factors. So called **corrected spectra** are obtained in this way. The corrected spectra are important e.g. when we want to compare the spectra measured on various instruments. If possible, the corrected spectra should be therefore published in literature. The corrected excitation spectrum would be according to Eq.(10) identical with the

absorption spectrum of the compound. When used for analytical purposes, in most cases the measurement of uncorrected spectra is sufficient.

As an example, uncorrected excitation and emission spectra of anthracene are shown in **Fig.3**. In comparison with the absorption spectrum (**Fig.4**) the excitation band at 251 nm is relatively weaker, because the radiation of the excitation xenon lamp decreases considerably at short wavelengths. Otherwise, both spectra are rather similar because the spectrum of xenon arc lamp changes smoothly in this wavelength region. If the excitation source has a line spectrum (mercury tube and to a certain extent also a xenon arc lamp in the region 450-500 nm), also the excitation spectrum has partially line character and its similarity with the absorption spectrum disappears.

Other spectral distortion appears for samples absorbing significantly the radiation at the excitation or emission wavelength. If the excitation radiation is absorbed to such extent that the excitation radiation markedly diminishes within the sample, we speak about an **inner filter effect**. This effect causes that the calibration function is not linear at higher concentrations. Also the excitation spectra are distorted because the signal in the absorption maxima is more affected than that in the absorption minima. This effect is usually more pronounced for instruments with a right angle arrangement when the emission radiation is observed approximately in the middle of the cell in the direction perpendicular to the excitation beam. The curvature of the calibration function is in this case even more pronounced than it would correspond to Eq. (7). For high concentrations of the fluorescent compound, the signal approaches zero because the excitation beam is absorbed immediately in the surface layer of the sample and does not penetrate to its centre. Also the emitted radiation may be absorbed by the sample. This so called **reabsorption** causes a decrease of the emitted radiation and deformation of the emission spectra.

The best way to evaluate the possibility of inner filter effect or reabsorption is to measure the absorption spectrum of the sample. If the absorbance of the solution in the cell of 1 cm path-length is 0.04, than the beam radiation flux is reduced nearly by 5% at the distance 0.5 cm (half of the cell thickness). Therefore, the absorbance of the measured solutions should be lower than 0.04 both at the excitation and emission wavelength. If we have not the possibility to measure the absorption spectrum of the sample, we can compare fluorescence for several dilutions of the sample.

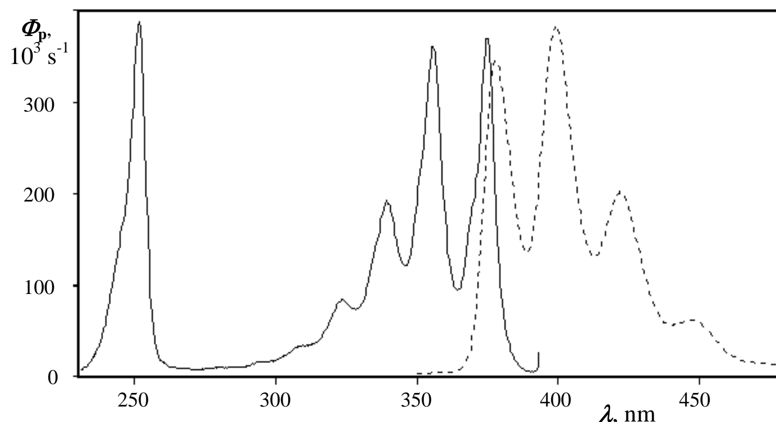

Fig.3: Uncorrected excitation and emission spectrum of anthracene in methanol

Instrument Fluoromax-2, excitation spectrum (――) recorded for the emission wavelength 399 nm, emission spectrum (- - -) was excited by the radiation with the wavelength of 251 nm. Concentration of anthracene 10^{-7} mol l^{-1}.

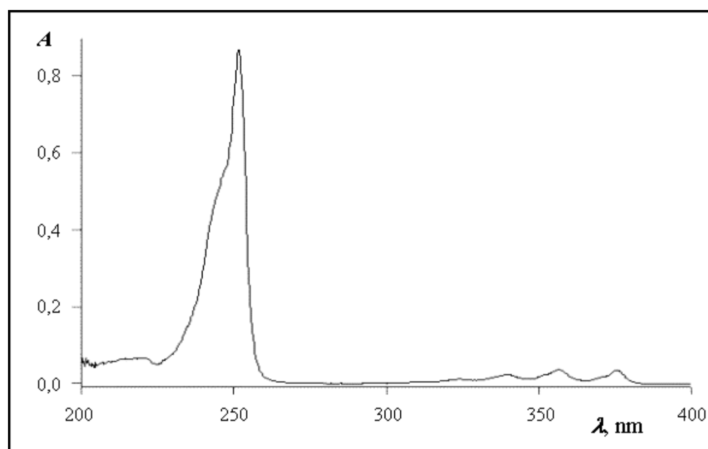

Fig.4: Absorption spectrum of anthracene in methanol solution.

Concentration of anthracene $5\cdot10^{-6}$ mol l^{-1}, cell 1 cm.

Cary Eclipse Spectrofluorometer

Laboratories are equipped with the spectrophotometer Cary Eclipse Spectrofluorometer. A simplified functional scheme of this instrument is in **Fig.5**. It is equipped with grating excitation (2) and emission monochromator (6) with the wavelength adjustable in the range 190 – 1100 nm. With respect to the limited spectral sensitivity of photomultipliers, the useful range is only about 200 – 900 nm. The monochromators have 5 different slit widths that correspond to the selected spectral bandwidth 1.5, 2.5, 5, 10 a 20 nm. Both monochromators are supplemented with optional optical filters. Their purpose is to suppress the higher order spectra and disturbing stray light. Stray light results from the radiation scattered on walls and optical

elements in the monochromator. It passes through the exit slit of the monochromátor although its wavelength does not correspond to the monochromator setting.

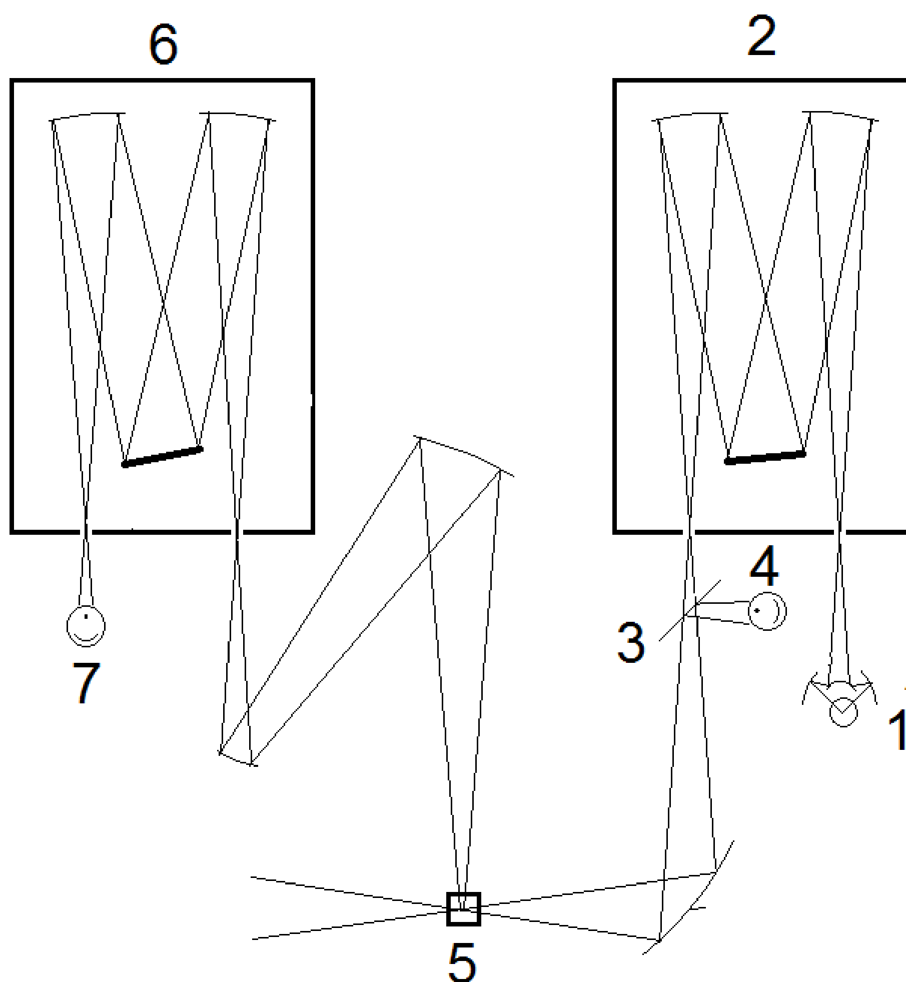

Fig. 5: Simplified optical scheme of Cary Eclipse spectrofluorometer
1-excitation source (xenon flash lamp), 2-excitation monochromator, 3-beam splitter 4-reference photomultiplier, 5-sample, 6-emission monochromator, 7-photomultiplier

Intense excitation radiation is produced by a xenon pulse lamp (1) working withfrequency 80 Hz and produces light pulses of 2 - 3 μs width. Radiation from the lamp is focused with a mirror condenser of the Schwartzschild type to the entrance slit of the monochromator (2). Part of the excitation radiation (about 8%) of the selected wavelength leaving the exit slit of the monochromator is reflected by a beam splitter (3) to the reference photomultiplier (4). Prior to incidence on photocathode of this photomultiplier it is about 10000-times attenuated by neutral colour filters. Main fraction of the radiation is concentrated to the sample in the sample compartment (5). Radiation emitted by the sample is partially focused to the entrance slit (6) of the emission monochromator. More or less monochromatic radiation leaving the exit slit of the emission monochromator is measured by a photomultiplier (7).

The necessity to use a reference photomultiplier follows from the relatively low repeatability of the excitation pulse intensity. When measuring the fluorescence, both the intensity of fluorescence pulse S detected by sample fluorescence (7) and the intensity of the excitation pulse R measured by the reference photomultiplier (4) are evaluated. A signal of non-illuminated photomultipliers is also measured (dark values) - S_t a R_t. As a result the ratio p is reported

$$p = 1000\ \frac{S - S_\mathrm{t}}{R - R_\mathrm{t}} \tag{11}$$

Reported values are limited to the range below 1000.

Besides the measurements of fluorescence and phosphorescence, Bio/Chemi-luminescence can be also measured. In the phosphorescence mode, the signal S is measured with an optional delay (*Delay Time*) against the excitation pulse and also the time of the emission measurement is optional (*Gate Time*). In Bio/Chemi-luminescence mode, the excitation lamp is not used and the photomultiplier (7) measures the light emitted solely by the sample.

Spectrofluorometer is equipped only with the main switch in lower part of the front panel. When switched on, it operates further only under the computer control using a program *Cary Eclipse* available from the desktop. There you can find several applications for various modes of measurement. In this laboratory training, only applications *Scan* and *Simple Reads* will be used. In the *Scan* application, the fluorescence spectra can be recorded. For the measurement of the fluorescence signal at predefined conditions, the application *Simple Reads* is used. In the *Scan* application, you will measure the spectra of quinine, compare them with the spectra of the analysed beverage and evluate which excitation and emission wavelengths are appropriate for the determination of the amount of quinine in the samples. The fluorescence intensities required for the determination will be measured in the *Simple Reads* application.

Most applications have similar structure of the control panel. Actual values of the wavelength and the measured signal are displayed in the upper part of the screen. From the menu line dialog, boxes *File*, *Edit*, *View*, *Commands*, *Setup*, *Graph* and *Help* are accessible. The spectra are displayed in the graphics area and the results are summarized in the report area. The text in the report area can be modified and edited. An instrument operation is controlled in the dialog box *Commands*. The most important commands may be activated also from the toolbar and by clicking command buttons situated above and next to the graphics area. For some commands, also keyboard shortcuts are available. A useful information about the instant state of the experiment can be found in the *Status display* which can be set in the *View* dialog box.

You can open several applications simultaneously but only one can control the spectrofluorometer. This application just executes the measurement or it is ready to do so. In this case, the button *Start* is displayed with a green stoplight in it. In an inactive mode, the applications show the button *Connect* instead of *Start*. By clicking this

button, the application can overtake control of the instrument unless it just measures in another application.

Conditions of the measurements are specified in the *Setup* dialog box. For the Scan application on the page Cary (**Fig. 6**):

- Mode of measurement *(Data Mode*: *Fluorescence*, *Bio/Chemi-luminescence*, *Phosphorescence)*
- Type of scan (*Excitation*, *Emission*, *Synchronous*, when the difference between the excitation and emission wavelength or wavenumber is kept constant)
- Wavelength range. For the excitation scan, set the constant emission wavelength and the initial and final excitation wavelengths. For the emission scan, set the constant excitation wavelength and the initial and the final emission wavelengths. It is not recommended to select the wavelength range so that the excitation and emission wavelengths will coincide in the course of the scan. The intensity of the excitation light scattered in the sample may be high and, at a high sensitivity setting, it can damage the photomultiplier. For this reason, the wavelength difference for both monochromators should be greater than the spectral bandwidth of both monochromators. In the *3-D mode,* it is possible to measure a series of the excitation, emission or synchronous spectra with successively increasing emission wavelength, excitation wavelength, or the wavelength difference (*Delta*). In this way, all the excitation-emission matrix can be recorded. In synchronous mode, we can obviate scattered excitation radiation by coincidence of excitation and emission wavelength.
- Spectral slit width of monochromators (*Excitation*/*Emission slit*). Wider slits increases the signal intensity at the cost of spectral resolution.
- Wavelength scan rate either from predefined options (from *Lowest* to *Survey*) or from *Manual* setting by selecting *Scan rate*, *Averaging Time* or *Data interval*. By changing one value, another is adjusted.

On the page *Option* (**Fig. 6**):

- Display options (*Overlay traces* is convenient in most cases; the spectra are displayed in one common graph,)
- Possibility to repeat spectrum measurement in selected time intervals (*Cycle mode*)
- Selection of auxiliary excitation and emission filters
- Photomultiplier voltage (7) for sample signal (Predefined values *Low*, *Medium*, *High* ie. 400, 600 and 800 V or *Manual* any value up to 1000 V.) Higher photomultiplier voltage increases its gain. Increasing the detector photomultiplier voltage by 10% will approximately double the fluorescence signal.

On the page *Auto-store,* the way in which the measured data are stored in the file with a dialog box either before or after the run is commenced. All the measured spectra

can be also saved in a single file in various formats (*File/Save as …*). Text file *Spreadsheet Ascii* with an extension *csv* is suitable for further data treatment in the *Excel*.

In the *Simple Reads* applications, similar parameters are to be set. Instead of wavelength intervals, only a combination of wavelengths remains and instead of the scan rate, parameters only time of single measurement (*Ave. Time*) remains. Intensity is measured after clicking *Read* (shortcut F9 or Alt R) and value together with serial number will appear in the Report.

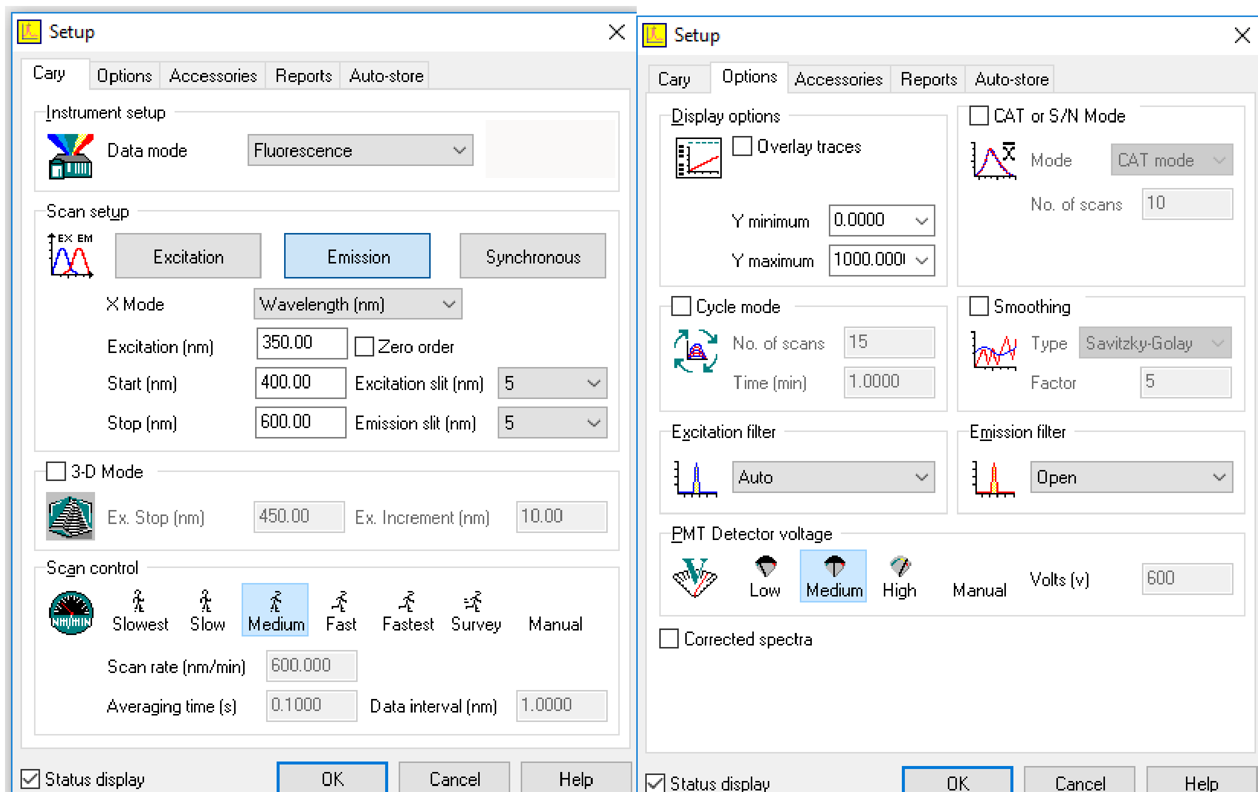

Fig. 6: Lists *Cary* and *Options* in *Setup* dialog box.

Instructions for the laboratory work

Fluorimetric determination of quinine in beverages

The aim of this work is the determination of quinine in a supplied liquid sample and in a sample of tonic water.

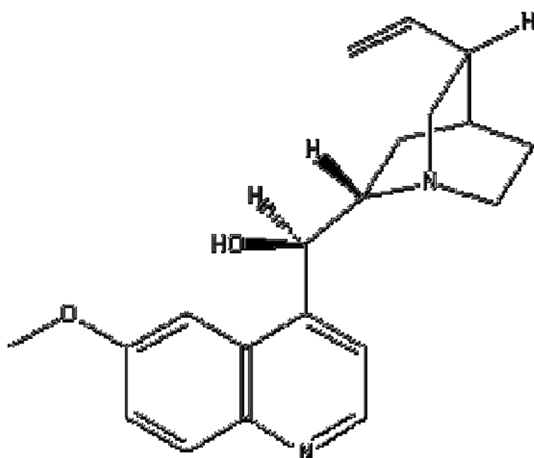

Fig.7: Formula of quinine

Quinine $C_{20}H_{24}N_2O_2$ (M_r=324.42) is an alkaloid with the structure given in **Fig. 7**. Quinine occurs in the bark of tropical trees of the gene *Cinchona*. Even in present days it is an important remedy for malaria. For its bitter taste it is added to some types of drinks. Both nitrogen atoms in the quinine molecule may be protonated. Backward dissociation of these protons has the dissociation constants about pK_1=4.3 (quinoline nitrogen) and pK_2=8.3 (quinuclidine nitrogen). Quinine forms salts with acids. It is usually supplied as a sulphate $(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$ (M_r=782.96), which is however partially hygroscopic and hence the water content in various batches may vary considerably. Solutions of quinine exhibit under certain circumstances bright fluorescence. Its emission spectrum lies at wavelengths above 400 nm with a wide maximum at about 460 nm. Fluorescent properties of quinine are relatively well estimated and hence it is frequently used as reference compound by fluorescence measurement. Fluorescence of quinine is partially quenched e.g. by chloride and bromide ions.

Absorption spectrum of protonated forms of quinine is in **Fig. 8**.

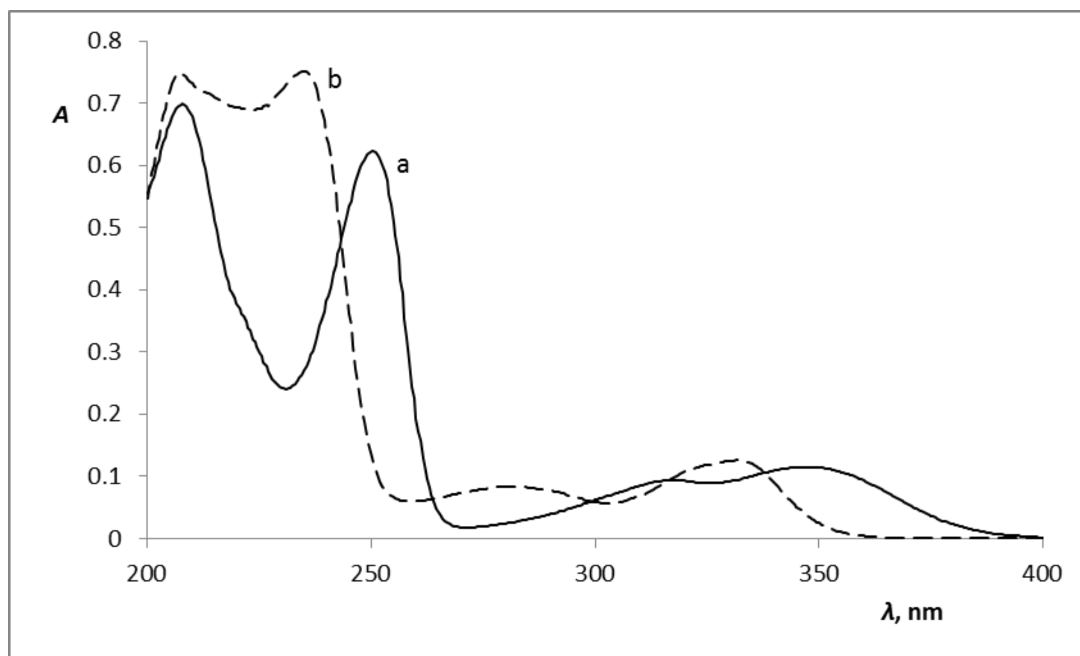

Fig.8: Absorption spectra of quinine solution in 0,05 M sulphuric acid (a) and in phosphate buffer pH=7 (b). Quinine concentration $2.2 \cdot 10^{-5}$ mol l^{-1}, cell thickness 1 cm.

Tasks

1. Measure the excitation and emission spectra of quinine in dependence on the solution acidity approximately in the range pH = 1 to pH = 7.
2. Based on the results from point 1, choose the medium for the set of calibration solutions and sample analysis.
3. For the medium selected in point 2, prepare solutions of both sample and tonic water and measure their excitation and emission spectra.
4. Measure an excitation-emission matrix for tonic water solution prepared in point 3.
5. Estimate the concentration range for the calibration dependence. Based on data from point 1 and from the preliminary measurement in point 3, estimate a suitable dilution of the samples. Prepare calibration solutions, three diluted solutions of the sample of the same concentration, diluted solution of tonic and diluted solution of tonic with added quinine standard. Measure the fluorescence signal of all solutions at constant excitation and emission wavelength.
6. Construct a calibration curve and determine the mass concentration of quinine in the sample and tonic water.
7. From the measurements in point 1, evaluate approximately the dissociation constant of quinine (optional).

Two students share the same instrument, but they work individually. Only in the point number 4 it is possible to measure the excitation-emission matrix only for single tonic solution.

Operating manual

1. *Preparing working solution of quinine*. From the quinine stock solution of concentration $1.25 \cdot 10^{-3}$ mol l^{-1}, which is available in the laboratory in the automatic burette, prepare a working solution of quinine of concentration $2.5 \cdot 10^{-5}$ mol l^{-1} in a volumetric flask of 250 ml.
2. *Preparing quinine solutions with various pH values.* Prepare a series of seven quinine solutions of concentration $1.0 \cdot 10^{-5}$ mol l^{-1} in seven volumetric flasks of 25 ml with pH values according to Table I.

Table I: Preparation 25 ml solution of approximate pH

pH	Volume of solution, ml			
	0.25 M H_2SO_4	0.0125 M H_2SO_4[a]	0.0005 M H_2SO_4[a]	0.02 M phosphate buffer pH=7.5
1.3	5.0	-	-	-
2.1	-	10.0	-	-
2.7	-	2.0	-	-
3.4	-	-	10.0	-
4.1	-	-	2.0	-
4.3	-	-	1.0	-
7.6	-	-	-	2.0

[a] Phosphate buffer and 0.25 M sulphuric acid are available in the laboratory. Diluted 0.0125 M sulphuric acid prepare by dilution of 0.25 M acid and 0.0005 M sulfuric acid by dilution of 0.0125 M sulfuric acid. The volume of 100 ml 0.0125 M sulfuric acid and 25 ml of 0.0005M sulfuric acid may be sufficient for all work.

3. *Switching the spectrofluorimeter on and measurements of the spectra.*

Switch instrument and computer on using the main switch and open *Cary Eclipse* and start the *Scan* application. In *Setup* set the fluorescence mode, measurement of the excitation or emission spectra and slits on both monochromators to 5 nm. Insert the sample cell filled with quinine solution of pH = 1.3 to the cell holder. If the initialization of the spectrometer was finished, push the button *Start* (it lights in green) and click on the preliminary measurement *Prescan*. In the dialog box then select *Rayleigh scatter*, *Raman scatter* and *2nd Order* to display ranges affected by the scattered radiation in the emission spectrum. *Prescan* procedure does not depend on the type of scan. In the first step, the emission grating monochromator is set to the zero order position where the light of all wavelengths is transmitted to the photomultiplier. Then the excitation spectrum is recorded at medium photomultiplier voltage. If the signal oversteps the range of the spectrometer, the scan is repeated with lower photomultiplier voltage. In the next step, the excitation monochromator is set to the wavelength of the excitation maximum and the emission spectrum is measured. This step is eventually repeated at increased photomultiplier voltage. Unless the measured sample is a complex mixture of fluorescent component, this approach usually yields

basic information about the wavelengths of the maxima on the spectra and shows positions of disturbing scattered radiation. You will take it in the account when selecting the excitation and emission wavelengths for the measurement of emission and excitation spectra, respectively. According to Fig. 8, two maxima are expected in the excitation spectrum of quinine, namely at about 250 and 345 nm. For 250 nm, the excitation radiation is partially absorbed by plastic cell. Also the presence in the tonic of components absorbing at this wavelength is likely. For the determination of quinine, the excitation at about 345 nm is therefore preferable. Photomultiplier voltage set so that the maximum intensity of emission spectrum is at about 500 (halve of the range). It is convenient to describe the measured spectra with description of sample, type of scan (emission resp. excitation), constant excitation resp. emission wavelength and eventually photomultiplier voltage. Do not use comma in the description as comma is used as a separator by export of spectra to the text file. Measure spectra with the same instrument setting for all solutions prepared according to the Table I. The position of excitation and emission maxima may shift in some solutions. In that case repeat the spectra measurement also for new maxima. To see the list of recorded spectra click the leftmost pushbutton in the toolbar for *Trace preferences dialog box*. Local menu is activated by right mouse button. In the left column of the list, select the spectra to be displayed. In the second column or by clicking on the spectrum in the graph area one spectrum becomes focused (red). For this spectrum range of axis can be modified using series of pushbuttons in the toolbar and spectrum can be included in mathematical operations. In the course of the measurement, maxima in the spectra are identified and written to the graph and *Report*. At the end, save all measured spectra in one file (*Batch*) with extension FBSW. It can be opened again only in *Scan* application. Also export spectra to the text file (*Spreadsheet Ascii*) with extension *csv,* which can be opened and further handled in the *Excel*. **Only spectra actually displayed are saved to the files!** Copy *Report* to Notepad using clipboard and save. Files and all other measurement save to your own directory on the disc F. Use directory name QXyymmdd. where X = A to D marks spectrofluorimeter (from entrance door left to right) and yymmdd is date. E.g. for the second spectrofluorimeter from the left on October 18th 2018 use directory name QB181023.

Note 1: For samples plastic cells with square cross-section with inner dimension 1 cm and height 4 cm are used. Al four surfaces are smooth and partially transparent also in UV range to about 270 nm. For precise measurement it is important, that region where excitation and measured beams pass through it, are perfectly transparent. Every contamination or scratch on the surface attenuate beam intensity. Therefore do not touch the cell in lower part up to about 3 cm and hold it rather by edges in the upper part. Fill the cell with solution to sufficient height about 2.5 to 3 cm so that excitation beam does not strike the liquid surface. Clean the cell only by rinsing with water eventually with little drop of detergent. Do not rub the cell. Their surface can be easily scratched and so damaged. You may gently drain droplets on the surface by the rim of filter paper. Wash cell several times (at least 3 times) with measured solution prior to measurement. If possible, do not wet outer surface of the cell. It usually change light transmittance. Check cleanliness of the cell visually against light and dark background. Even little contamination, droplets or fingerprints distort fluorescence measurement. Keep orientation of the cell in

the cell holder because properties of cell windows may differ. Plastic cells are not usable for organic solvents which at least etch their surface.

4. *Estimation of pH suitable for quinine determination*. Based on previous measurements select the most appropriate medium for quinine determination This medium will be used in all the following measurements. You need not to restrict just on media mentioned in the Table I. Select medium in such a way that the intensity of fluorescence is high and does not change much with pH change in order to achieve good robustness of the method. Take into account also simple preparation of solutions.

5. *Approximate estimation of fluorescence signal for samples.* Pipette 1 ml of sample to 25 ml volumetric flasks and 1 ml of tonic to another volumetric flask of 25 ml. In both flasks, modify the solutions according to the step number 4. Measure the excitation and emission spectra of these solutions. Look for the emission of impurities which could interfere with quinin determination. Save the spectra together with that measured in step 3.

6. *Measurement of excitation-emission matrix of the tonic solution.* While you will prepare solutions according the following step, let the instrument measure the whole excitation-emission matrix of the tonic solution. Delete all spectra measured until now (and saved) from the list. Insert the sample cell with tonic solution from the previous step to the cell holder. All conditions for the measurement will be the same as for the spectra measurement. Only in the case that the signal exceeded the range, decrease adequately the photomultiplier voltage. Then, select the **3D-mode**, ***Emission*** scan in the range (***Start***) **300 nm** to (***Stop***) **600 nm** with ***Data interval*** **5 nm, *Excitation* 250 nm** with final value (***Ex. Stop***) **600 nm** and step (***Ex. Increment***) **5 nm**. Measurement will take about 10 to 15 minutes with ***Averaging time*** **0.1 s**. Save measured spectra both as batch file (FBSW) and *3D Ascii* (csv). Spectra can be displayed in three dimensions either as *Contour Graph* or in *3D* perspective using program *Grams*. First axis X in the graph is emission wavelength of measured spectra specified by values *Start, Stop* and *Data interval*, axis Y corresponds to intensity and Z axis to the second (excitation) wavelength. Its final value and step is inserted in section *3D* on the list *Cary* in *Setup* dialog box. The same values are to be inserted for axis Z in the dialog box *Contour* or *Grams 3D*.

Note 2: With these conditions excitation-emission matrix covers also scattered light. In this case in tis possible because we measure relatively concentrated solution of compound with intense fluorescence and relatively low sensitivity (with low photomultiplier voltage) of the instrument is sufficient. Scattered light in clear (transparent) solution is in this case of similar intensity as fluorescence and does not exceed range of instrument. We could eliminate first order scattered radiation in *3D* synchronous mode. However, synchronous *3D* spectra ale less illustrative because excitation and emission spectra are represented by diagonal sections of the 3D graph. In our graphs excitation and emission spectra are represented by sections parallel with wavelength axes and may be easily displayed in selected points both in *Contour Graph* and *3D* view in *Grams*. Moreover, *3D* view in *Grams* may be rotated in any direction.

7. *Preparation of calibration solutions and sample dilution*. Prepare 6 calibration solutions with **evenly** increasing concentrations of quinine from zero to a maximum value. Even for the most concentrated calibration solution, the effect of inner filter

should be negligible. For this reason, its absorbance should be lower than about 0.03 for the light path-length of 1 cm. However, the fluorescence intensity should not be too weak. For this reason, the absorbance of the calibration solution with the highest concentration should be higher than about 0.02. An absorption spectrum of quinine is in **Fig. 8**. In acid solution, molar absorption coefficient of the quinine at wavelength 348 nm is 5700 l mol^{-1} cm^{-1}).

Based on results from the step number 5, dilute the sample to volumetric flasks of 25 ml in such a way, that quinine concentration of these solutions will be in the upper halve of the calibration dependence, where lower relative uncertainty of result is expected. Prepare three parallel solutions of the given sample with the same concentration. Use standard addition method to analyse tonic water. To two volumetric flasks of 25 ml (eventually 50 ml or 100 ml) measure such a volume of tonic water that the concentration of quinine will be approximately in the middle of the calibration line (also solution with added standard should be in the range of calibration). To the second flask (25 ml) add 2 ml of working quinine solution $2.5 \cdot 10^{-5}$ mol l^{-1}. Modify the medium in all diluted samples just as in the calibration solutions, fill the flasks to the mark and mix well.

Note 3: Micropipette with adjustable volume up to 1 ml is available in the laboratory for pipetting sample and quinine working solution. It is good practise to prepare calibration and sample solutions by repeated pipetting with the same micropipette setting. In this way eventual systematic inaccuracy in measured volume and micropipette setting is eliminated. Supplier of micropipette reports precision better than 0.15% by sampling 1 ml. However, some precaution summarised in Appendix 2 should be observed.

9. *Determination of quinine in the sample.* Start and activate (*Connect*) application *Simple Reads*. Set the excitation and emission wavelength according to previous measurements. All other conditions (slits, filters and photomultiplier voltage) remain as for the spectra acquisition. Measure (at least) 10 values of the intensity for each solution. Measure solutions in the order: calibration solutions from zero to maximum concentration, three solution of the sample, solution of tonic alone and solution of tonic with addition of standard. Open *Notepad*, copy there the values from the *Report* and save as text file. In the root directory on the disc F you can find the file QX19mmdd.xls which is pre-prepared for evaluation of sample concentrations and their uncertainties using the method of weighted linear regression. Open the file in the program *LibreOffice Calc* and *Save as* with modified name in your directory. There are two identical sheets for two students working on the instrument. Copy using clipboard measured signals from *Notepad* to free space on the sheet. Separate signal from serial number (*Data/Text to Columns*). Move 10 values for every solution side by side in the order given above. Transfer resulting matrix with transposition to corresponding cells D10:M20 on the sheet (*Copy. Edit/Paste Special*). Average values of signals are computed and both linear and quadratic calibration functions are evaluated using the method of weighted linear regression (see Appendix 1). When volumes of volumetric flasks and pipetted solutions are entered to the corresponding cells also concentrations of samples are computed in the column N with uncertainties in the column O. Based on the statistical test of the coefficient of quadratic term (range Q20:R50) assess

whether your measurement displays curvature in calibration function. Select corresponding type of calibration function to calculate mass concentration of the quinine in samples. **In the laboratory report, summarise the average fluorescence signals for the calibration solutions and samples and the parameters of the applied calibration function. Based on these vales calculate in the report the concentrations, apparent recovery for the tonic and concentration in the undiluted sample. Evaluate whether the tonic water matrix affects the fluorescence intensity of quinine (see Appendix 2). Give the final results with corresponding number of significant digits including the combined uncertainty or expanded uncertainty and coverage factor. Transfer uncertainties from the calculations in *LibreOffice Calc* or *Excel* file.** Uncertainty is reported at most with two digits and measured value with the same number of decimal places as uncertainty.

Note 4: Program *Cary Eclipse* offer also application *Calibration* which evaluate calibration and reports concentrations. However it uses the method of un-weighted regression, suppose linearity of the calibration and gives no information about result uncertainty. Procedure given here seems at the moment to be the most practicable for estimation of this information.

For the task 7 describe in the protocol how the dissociation constant was evaluated and resulting value of pK_1. Estimation of pKa may follow solution of example 10.13 on p. 88 in "*Příklady z analytické chemie pro bakaláře*" (Volka K. a kol.. VŠCHT Praha. 2nd ed. ver. 1.0. 2010) (https://vydavatelstvi.vscht.cz/katalog/publikace?uid=uid_isbn-978-80-7080-743-9). In the upper part of p. 88 also guideline on solution can be found. For fluorescence substitute ratio of fluorescence intensity to concentration for absorption coefficients. This ratio for ions BH_2^{2+} can be roughly obtained from spectrum measured at pH=2.7 and for the form BH^+ from the spectrum measured at pH=7.4.

Questions

1. What is it emission fluorescence spectrum?
2. What is it excitation fluorescence spectrum? Describe the way in which emission and excitation spectrum is measured on spectrofluorimeter with both excitation and emission monochromators.
3. What are the transitions passing in molecules of fluorescing compounds by excitation and by emission of the fluorescence radiation? What inequality therefore holds for the wavelength of excitation radiation and emitted radiation?
4. What are the changes in emission spectrum induced by changes of excitation wavelength for single fluorescing compound and for a mixture of fluorescing compounds?
5. How the shape of excitation spectrum depends on emission wavelength for single fluorescing compound and for a mixture of fluorescing compounds?
6. What is the mirror symmetry rule?
7. How fluorescence signal depends on concentration of fluorescing compound in solution?
8. What is the relationship between absorption and excitation spectrum?
9. What are the effects of inner filter and reabsorption?
10. What is the correction of fluorescence spectra and what are the main reasons of distortion of uncorrected excitation and emission spectra?

Appendix 1: Evaluation of Calibration Function in Laboratory Training on Fluorimetry

In this work the common method of linear regression is not well suited for calibration function and uncertainties estimation. It uses an assumption of homoscedasticity, i.e. of uniform uncertainty of dependent variable y in all range of values (**Fig.9a**). This assumption is not usually fulfilled in fluorimetry and pattern of experimental points resembles more that in **Fig.9b**. In these (heteroscedastic) cases the method of weighted linear regression is usually applied which takes into account differences in variability of y. However. it requires knowledge of uncertainties for all data points. If sufficiently large set of experimental points is available, like in **Fig.9b**. we can assess variability of y from these data. In this work we need to describe calibration function from 6 experimental points only. In such case we have to use knowledge gained by validation of the method or apply a reasonable model for uncertainties.

In fluorimetry, the most important contributions to signal uncertainty are approximately proportional to the signal. Such factors as excitation source instability.

presence of other components absorbing excitation of emitted radiation, cell positioning, light loss due to reflections on cell walls and impurities and partially also uncertainty of measuring photometer belong to this category. Also uncertainty of concentrations of measured solutions is projected to the uncertainty of measured signal. Linear regression supposes this uncertainty to be negligible, but in fact it needs not to be true. By dilutions rather relative uncertainty of resulting concentration is constant. Factors like variable temperature, adjustment of volumetric flasks to the mark, uncertainty of volumetric vessels calibration, fraction of liquid remaining on the walls of pipettes and in their tips, evaporation of solvent and residues and impurities residing on the rim of vessels which dissolve in the liquid when poured from the vessel are of this type.

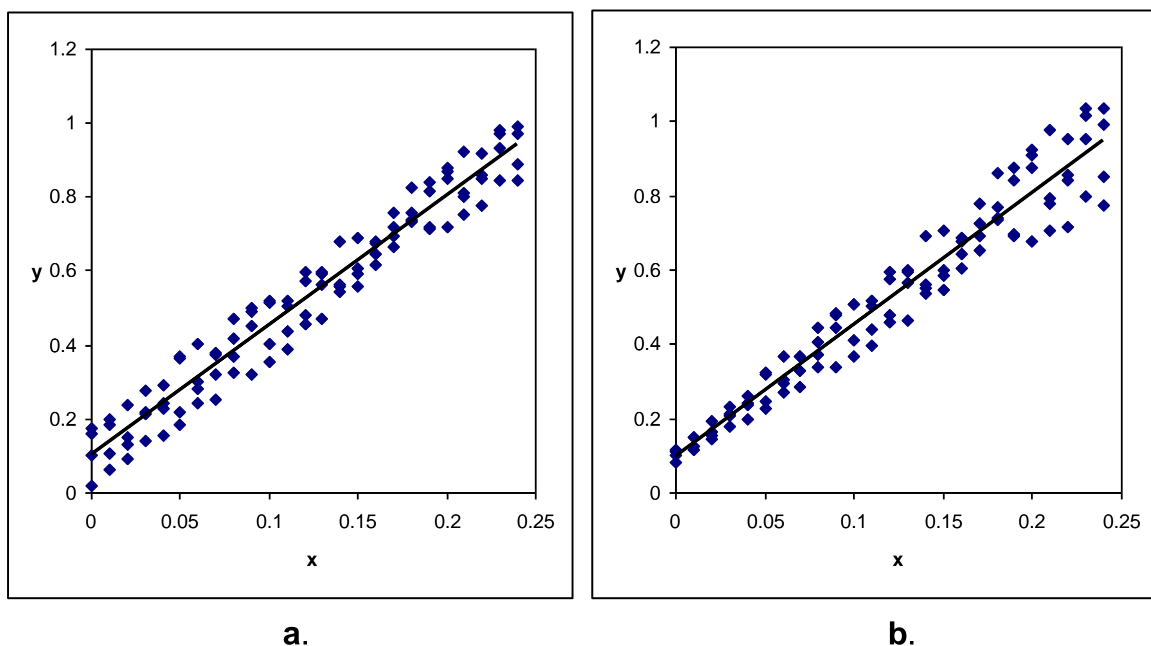

Fig.9: Linear dependence with constant uncertainty of y (a) and with uncertainty proportional to this quantity (b)

At low radiation flux its uncertainty is proportional to the square root of the signal. It is true when signal is generated by low number of photons because they obey Poisson's statistics.

Only a part of measured signal uncertainty does not depend on its level. We can mention scattered radiation, fluorescence of impurities, in part uncertainty of measuring photometer and light from environment which penetrate to the photometric detector. For this reasons linear dependence of uncertainty on signal level seems to be adequate model.

Estimate of function parameters by linear regression

Linear regression suppose linear dependence between y and one or several factors (variables) x_k in the form

$$y = \sum_{k=1}^{p} a_k \, x_k \qquad (12)$$

where a_k are constant coefficients and p is the number of factors. Linear regression method yields estimate of coefficients a_k and other parameters on the basis of a series of n values y_i for n sets of factors x_{ki} (i=1,..,n). Uncertainty of y_i values is considered constant in all range of y. Uncertainty of factors x_{ki} is supposed to be zero. Linear regression method estimates parameters $\hat{a}_k$ yielding minimal value of sum of squared residuals, i.e. differences of y_i and values $\hat{y}_i$ computed using formula (2)

$$\hat{y} = \sum_{k=1}^{p} \hat{a}_k \, x_{ki} \qquad i = 1 \ldots . n \qquad (13)$$

Minimisation of the sum of square residuals

$$S_{\text{res}} = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 \; = \sum_{i=1}^{n} \left(y_i - \sum_{k=1}^{p} \hat{a}_k \, x_{ki} \right)^2 \qquad (14)$$

requires partial derivatives by all coefficients $\hat{a}_l$ to be zero. These conditions yield set of linear equations for estimates $\hat{a}_k$ in the form

$$\sum_{i=1}^{n} y_i \, x_{li} = \sum_{k=1}^{p} \hat{a}_k \sum_{i=1}^{n} x_{ki} \, x_{li} \qquad l = 1, .., p \qquad (15)$$

respectively

$$S_l = \sum_{k=1}^{p} \hat{a}_k S_{kl} \qquad l = 1, .., p \qquad (16)$$

where sums

$$S_l = \sum_{i=1}^{n} y_i x_{li} \qquad (17)$$

are linear function of y_i, whereas matrix of the set of equations

$$S_{kl} = \sum_{i=1}^{n} x_{ki} x_{li} \qquad k.l = 1, .., p \qquad (18)$$

is given only by x_{ki} factors and does not depend on values of y_i. Hence, also inversion matrix S^{inv} does not depend on y values and solution

$$\hat{a}_k = \sum_{l=1}^{p} S_l S_{kl}^{\text{inv}} \qquad (19)$$

is through sums S_l linear function of y_i values. Uncertainties of estimates $\hat{a}_k$ can be therefore evaluated by applying simple rules for uncertainty propagation supposing that we know uncertainty of y_j values. If this uncertainty is not known we apply its estimate s_y based on the sum of squared residuals S_{res} from equation

$$\hat{s}_y^2 = \frac{S_{\mathrm{res}}}{n-p} \tag{20}$$

Uncertainty of estimates $\hat{a}_k$ is given by diagonal elements of inversion matrix S^{inv}

$$u(\hat{a}_k) = \hat{s}_y \sqrt{S_{kk}^{\mathrm{inv}}} \tag{21}$$

Similarly. value $\hat{y}_0$ corresponding to given values of factors $x_{k.0}$ (k=1..p) is estimated with uncertainty given by relationship

$$u(\hat{y}_0) = \hat{s}_y \sqrt{\sum_{k=1}^{p} \sum_{l=1}^{p} x_{k.0} S_{kk}^{\mathrm{inv}} x_{l.0}} \tag{22}$$

The way of evaluation described above is applied also in analytical chemistry in order to evaluate calibration functions which describe dependence of analytical signal y on analyte content x. This function is frequently linear in x. but it may take curved form, which can be described by additional terms, e.g. by higher powers of x. Calibration function is then approximated by polynomial as sum of terms 1, x, x^2,.., x^d multiplied by coefficients a_0, a_1, a_2,..,a_d where d is the degree of polynomial. The first coefficient gives absolute term a_0.

Calibration function is used to determine value of content $\hat{X}$ corresponding to the mastered value of signal Y. Uncertainty of $\hat{X}$ is affected both by uncertainty of calibration according to equation (22) and uncertainty of measured signal Y. Uncertainty of $\hat{X}$ is calculated as ratio of total uncertainty of signal Y a to the slope of calibration $\hat{a}$ in a given point. For calibration in the form of polynomial of the degree d is

$$u(\hat{X})^2 = \frac{1}{\hat{a}^2} \left[u(Y)^2 + \hat{s}_y^2 \sum_{k=0}^{d} \sum_{l=0}^{d} \hat{X}^k S_{kl}^{\mathrm{inv}} \hat{X}^l \right] \tag{23}$$

For linear function y on x this procedure gives known equation (24) for uncertainty of content $\hat{X}$ estimated from average signal Y obtained from m measurements

$$u(\hat{X})^2 = \frac{\hat{s}_y^2}{\hat{a}_1^2} \left(\frac{1}{m} + \frac{1}{n} + \frac{(Y-\bar{y})^2}{\hat{a}_1^2 Q_{xx}} \right) \tag{24}$$

where $\hat{a}_1$ is (estimated) slope, quantity

$$\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n} \tag{25}$$

is the mean value of calibration signals y and Q_{xx} variance of calibration x_i values around mean value $\bar{x}$:

$$Q_{xx} = \sum_{i=1}^{n} (x_i - \bar{x})^2 \tag{26}$$

Estimate of function parameters by weighted linear regression

Weighted linear regression estimates coefficients and other parameters by minimisation of weighted sum of residuals

$$S_{\text{res.w}} = \sum_{i=1}^{n} w_i (y_i - \hat{y}_i)^2 \;\; = \sum_{i=1}^{n} w_i \left(y_i - \sum_{k=1}^{p} \hat{a}_k \, x_{ki}\right)^2 \tag{27}$$

where w_i are weights ascribed to individual points. Higher weights have points with lower uncertainty of y according to equation

$$w_i = \frac{K}{u(y_i)^2} \tag{28}$$

Coefficient K is so adjusted that sum of weights equals to number of points n. So,

$$K = \frac{n}{\sum_{i=1}^{n} \frac{1}{u(y_i)^2}} \tag{29}$$

and also hold relations

$$K = \frac{\sum_{i=1}^{n} w_i \, u(y_i)^2}{n} = \frac{\sum_{i=1}^{n} w_i \, u(y_i)^2}{\sum_{i=1}^{n} w_i} \tag{30}$$

It means that coefficient K equals to weighted squared uncertainty, i.e. product of squared uncertainty and weight for each point and also to their weighted average and substitutes single value of uncertainty by linear regression with uniform uncertainty. It is worth mentioning that weights, and so also estimates of functional parameters, do not depend on absolute uncertainties for individual points, but only on their ratio. If we change all uncertainties in the same ratio weights and estimates of parameters remain the same. Even minimised sum of squared residuals will not change but uncertainties of some derived quantities may change.

Procedure is further quite similar to that of linear regression with constant uncertainty. Partial derivatives lead to linear equations for coefficients $\hat{a}_k$ in the form

$$\sum_{i=1}^{n} w_i\, y_i\, x_{li} = \sum_{k=1}^{p} \hat{a}_k \sum_{i=1}^{n} w_i\, x_{ki}\, x_{li} \qquad l = 1,..,p \tag{31}$$

which are the same as equations (5). but with weighted sums

$$S_{l.\mathrm{w}} = \sum_{i=1}^{n} w_i\, y_i x_{li} \tag{32}$$

which are again linear function in y_i. whereas matrix

$$S_{kl.\mathrm{w}} = \sum_{i=1}^{n} w_i\, x_{ki} x_{li} \tag{33}$$

depends only on values x_{ki} and weights w_i and does not depend on values of y_i. It means that estimates $\hat{a}_k$ are computed in analogy with non-weighted regression (Equation 19). Even uncertainties of coefficients are obtained in the same way, only squared uncertainty of y is substituted with weighted squared uncertainty K. which is in analogy with equation (20) derived from weighted sum of squared residuals (27)

$$K = s_{y.\mathrm{w}}^2 = \frac{S_{\mathrm{res.w}}}{n-p} \tag{34}$$

However, when uncertainties of values derived from regression function are estimated, also uncertainty of y for relevant range is required. E.g. Equation (24) is transformed to

$$u(\hat{X})^2 = \frac{1}{\hat{a}_1^2}\left(\frac{u(Y)^2}{m} + \frac{s_{y.w}^2}{n} + \frac{s_{y.w}^2 (Y-\bar{y}_w)^2}{\hat{a}_1^2 Q_{xx.\mathrm{w}}}\right) \tag{35}$$

Hence, to evaluate uncertainty of $\hat{X}$ we need also uncertainty of Y as given by the applied model with specified value of constant K.

Use of Excel file in the laboratory

On computers in laboratory pre-programmed file is available for calibration data handling in *Excel* or *LibreOffice Calc*. It contains two identical sheets. Rename one of them using your name. You will not modify sheets of your colleagues. Modified file should be saved with original filename.

Input data are to be typed to cells with yellow background. Concentrations of calibration solutions in the range C10:C15 are calculated from pipetted volumes of quinine working solution which you input to the range B10:B15. Ten values randomly recorded from fluctuating display of fluorimeter for every calibration and sample solution are in the range D10:M20. Their averages are computed in the range N10:N20 and are used for further treatment. Measured volumes are entered to the range

N21:N27. First graph on the sheet shows calibration evaluated using common (un-weighted) linear regression. Second graph presents standard deviation of the average of ten measured signal values plotted against average signal values.

Estimates of coefficients are computed in the range N34:N34 (N48:N50) for linear (quadratic) calibration function. From these values and measured signal for the sample. the concentration of measured sample solution is estimated in the cell N37 (N52). Finally, it is recalculated for dilution and molecular weight in the range N38:N39 (N53:N54).

Concentrations of tonic water solution and tonic water solution with added standard as estimated from calibration function are in the range N40:N41 (N55:N56). From these values apparent recovery R is computed in the cell N42 (N57) as the ratio of their difference to the added quinine concentration. The concentration of the tonic solution is then divided by this value and recalculated for dilution and molecular weight in the range N43:N44 (N58:N59). In the cell N45 (N60) recalculated value for tonic water alone is reported for comparison. This value is the result of analysis if tonic water matrix doesn’t affect fluorescence signal of quinine ($R = 1$)

To estimate parameters of calibration function, linear dependence of signal uncertainty on signal value is supposed. In the cell Q2 we can change the value which specifies ratio of uncertainty of minimal calibration signal to that of maximal signal. (When value 1 is inserted to this cell. uncertainty of y will be the same for all data and evaluation is transformed to common un-weighted regression.) Based on this dependence relative values of y uncertainties are computed in cells Q10:Q20 and then weights of calibration points in cells R10:R15. These are used by weighted linear regression and yield estimates of coefficients as well as the value of $s_{y.w}$ in the cell N36 (N51). Based on these values constant K is computed in the cell O6 which is applied to the computation of model y uncertainties in the range O10:O20. The value in the cell O2 controls whether model for linear or quadratic calibration function is produced (for 0 the model is computed for linear calibration. non-zero value selects quadratic calibration). Based on this model uncertainties of concentrations computed from sample signals are evaluated in ranges O37:O45 (O52:O60). Uncertainties of volumes applied by dilution are inserted in the range O21:O27.

In the range T37:T60 uncertainties are also evaluated using Kragten's (T7:AL60) scheme and are applied to the results obtained using standard addition method. In the range F37:G41(F58:J62) parameters of calibration function are computed using common un-weighted regression.

In the cells Q34:Q35 and Q48:Q50 statistical importance of calibration coefficients is tested. If for quadratic calibration function (cell O2≠0) in the cell Q50 will be "NEPRAVDA" ("FALSE"), then quadratic coefficient is not statistically different from zero and we use results from linear calibration, of course after zero is inserted to the cell O2.

APPENDIX 2: COMMENT ON THE STANDARD ADDITION METHOD

For many samples with complex matrix, isolation of analyte prior to its determination is necessary. Methods like extraction, chromatography, electrolysis and the like are used. In these procedures analyte is more or less lost. Parameter called recover R of the method is used to characterize efficiency of the isolation procedure. Recovery R is defined as the ratio of amount of analyte in isolated fraction n_1 to the analyte amount which was present in the original sample n_x. Various so called reference materials, for which precise content of analyte is known, are used for recovery estimation. Another possibility is the application of standard addition method. In this method beside analysis of the sample also the same amount of the sample, to which known amount of analyte n_s (standard) was added, is also analysed. For the first part holds

$$n_1 = Rn_\mathrm{x} \tag{36}$$

If added standard is isolated from the sample with the same recovery as analyte contained in the sample, then amount of analyte isolated from the second part of the sample n_2 is

$$n_2 = R(n_\mathrm{x} + n_\mathrm{s}) \tag{37}$$

By subtracting both equations we gain

$$n_2 - n_1 = Rn_\mathrm{s} \tag{38}$$

and for recovery R

$$R = \frac{n_2 - n_1}{n_\mathrm{s}} \tag{39}$$

Content of the analyte in the sample is obtained from the result for the first n_1 after correction for recovery

$$n_\mathrm{x} = \frac{n_1}{R} \tag{40}$$

The same formalism can be applied to the cases where other components in the sample matrix affect slope of the calibration function. In our case fluorescence of quinine may be partially quenched by some components in the tonic water and then concentration derived from the unquenched calibration function will be lower than correct value. The ratio of the concentration obtained in this way to the concentration of the analyte present in the sample is called Apparent Recovery. Also in this case it is the method of standard addition which is convenient for concentration evaluation. In

this laboratory training we use two volumetric flasks of the same volume and the same volume of sample is pipetted to the both. To the second flask known amount of standard is added. Apparent recovery is evaluated as

$$R = \frac{c_2 - c_1}{c_{\mathrm{s}}^{\cdot}} \qquad (41)$$

where difference of analyte concentrations c_2 a c_1 measured for both flasks is in the nominator and concentration increase in the second flask due to addition of standard $c_{\mathrm{s}}^{\cdot}$. is in the denominator. Possible fluorescence quenching is demonstrated by the apparent recovery which is significantly lower than 1.

To include uncertainty of pipetted volumes to the combined uncertainty of the result e.g. using Kragten method, we have to consider more general case, where volumes of both volumetric flasks as well as volumes of sample pipetted to both flasks is not the same. For the first solution measured analyte concentration c_1 will be

$$c_1 = R\frac{c_{\mathrm{v}} V_{\mathrm{v1}}}{V_{\mathrm{o1}}} \qquad (42)$$

where c_{v} is wanted concentration of analyte in the sample solution, V_{v1} is volume of sample solution pipetted to the first volumetric flask of volume V_{o1} and R is the value of apparent recovery. For the second solution measured analyte concentration c_2 will be

$$c_2 = R\frac{c_{\mathrm{v}} V_{\mathrm{v2}} + c_{\mathrm{s}} V_{\mathrm{s}}}{V_{\mathrm{o2}}} \qquad (43)$$

kde V_{v2} is volume of sample solution pipetted to the second volumetric flask of volume V_{o2}. c_{s} is concentration of added standard solution and V_{s} is its volume.
By rearrangement from equation (42)

$$c_1\frac{V_{\mathrm{o1}}}{V_{\mathrm{v1}}} = Rc_{\mathrm{v}} \qquad (44)$$

and from equation (43)

$$c_2\frac{V_{\mathrm{o2}}}{V_{\mathrm{v2}}} = Rc_{\mathrm{v}} + Rc_{\mathrm{s}}\frac{V_{\mathrm{s}}}{V_{\mathrm{v2}}} \qquad (45)$$

From difference of both equations it follows for recovery R

$$R = \frac{c_2\frac{V_{\mathrm{o2}}}{V_{\mathrm{v2}}} - c_1\frac{V_{\mathrm{o1}}}{V_{\mathrm{v1}}}}{c_{\mathrm{s}}\frac{V_{\mathrm{s}}}{V_{\mathrm{v2}}}} \qquad (46)$$

This value of apparent recovery is used for evaluation of analyte concentration in the sample solution c_v from the equation (42).

If volumes of sample are the same for both volumetric flasks ($V_{v1}=V_{v2}$), the equation (46) becomes

$$R = \frac{c_2 V_{o2} - c_1 V_{o1}}{c_s V_s} = \frac{n_2 - n_1}{n_s} \tag{47}$$

Here measured amount increment in the nominator is compared with added analyte amount in the denominator. If volumes of both volumetric flasks are the same ($V_{o1}=V_{o2}$), equation (42) is further simplified to equation (41), because

$$R = \frac{c_2 - c_1}{c_s \frac{V_s}{V_{o2}}} = \frac{c_2 - c_1}{c_s^{\cdot}} \tag{48}$$

where

$$c_s^{\cdot} = c_s \frac{V_s}{V_{o2}} \tag{49}$$

is just concentration increase in the second flask due to standard addition.

APPENDIX 3: FUNDAMENTALS OF MANIPULATION WITH MICROPIPETTE

The micropipettes in this laboratory measure liquids volume in the range from 0.1 to 1 ml. Liquid is drawn to exchangeable plastic tip attached to the shaft by means of a plunger. Movement of the plunger is transferred to the liquid through column of air. Measured volume is given by stroke of the plunger which is adjusted by screw-driven mechanism. To change the set volume, turn the knurled knob in the handle or with lower part of pushbutton, which otherwise serve for eventual calibration. The set volume is displayed in the window in the centre of the micropipette body. The plunger will stop at two different positions when it is depressed. The first of these stopping points is the point of initial resistance and is the level of depression that will result in the desired volume of solution being transferred. The second stopping point is when the plunger is depressed beyond the initial resistance until it is in contact with the body of the micropipette. This second stopping point is only used for the complete discharging of solutions from the plastic tip.

Several rules should be observed by use of micropipette

Press and release the push-button slowly and smoothly. There should be not “snapping” noises.

Liquid must not penetrate to the inner parts of the micropipette. For this reason, piston movement should be slow and micropipette must not be turned upside down or

left flat on the bench, especially with content in the tip. Place the micropipette vertically in a stand after use.

The volume must not be adjusted out of specified range (100-1000 µl). For precise adjustment, approach the desired volume from upper values at least about one third of the screw turn.

Micropipette should be used in vertical position. When tilted, volumes are measured less precisely.

Immerse the tip into the aspired liquid to minimum depth depending on the pipette volume, for micropipette in the laboratory only 2 to 3 mm. Inserting the tip too deeply into the liquid causes excess droplets to cling to the outside surface of a tip. Also hydrostatic pressure may compress air in the micropipette mechanism.

Liquid in the tip must be free of air bubbles.

If droplets are to be removed from the tip surface, do not touch end of the tip with the tissue. Otherwise liquid may be withdrawn from the tip interior.

Press the tip on the shaft firmly using a slight twisting motion to ensure a positive, airtight seal. Pre-rinse the tip by aspirating and expelling the liquid two or three times to wet surface of the tip and to saturate vapour above liquid.

Check the tightness of the micropipette mechanism and of the tip. Draw liquid to the tip and observe for a while eventual leak of the liquid. Any leak means that volume measurement is not reliable.

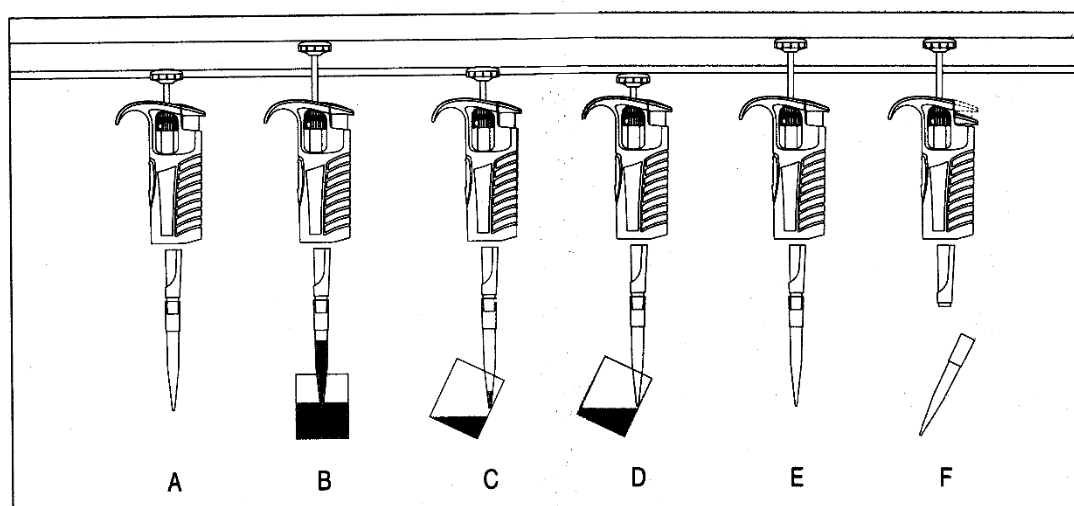

Fig. 10: Pipetting using forward technique.

When pipetting and mixing a sample or reagent into another liquid, forward technique is usually used - **Fig. 10.** Press the pushbutton to the first stop (A). Then dip the tip into the solution to a depth of 2 – 3 mm, and slowly release the pushbutton (B). Withdraw the tip from the liquid, touching it against the edge of the reservoir to

remove excess liquid from the tip exterior. Touch end of the tip against wall of the receiving vessel at the angel 10 to 40 degrees and dispense the liquid by gently pressing the pushbutton to the first stop(C). After one second, press the pushbutton to the second stop (D). This action will empty the tip. Remove the tip from the vessel, sliding it along the wall of the vessel and release the pushbutton to the ready position (E). If necessary, e.g. before pipetting liquid of very different composition, release the tip by pressing white button on the micropipette (F).

Inhomogeneous liquids like blood or serum are pipetted by modified procedure. Dip the tip filled with the liquid into the target solution. Make sure the tip is sufficiently below the surface. Press the pushbutton to the first stop and release it slowly to the ready position. Do not remove the tip from the solution. Repeat this process until the interior wall of the tip is clear. Remove the tip from the solution by sliding it along the wall of the vessel. Press the pushbutton to the second stop, and completely empty the tip.

For viscous liquids or liquids easily forming foam reverse technique of pipetting is recommended. Press the pushbutton to the second stop, dip the tip into the solution to a depth of 2 – 3 mm, and slowly release the operating button. Wait until the liquid will fill the tip and withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid from the tip exterior. To the end of the tip must not enter air bubble. Dispense the liquid into the receiving vessel by depressing the pushbutton gently and steadily to the first stop. Hold the button in this position until liquid stop flowing from the tip. Some liquid will remain in the tip, and this should not be dispensed. The liquid remaining in the tip can be pipetted back into the original solution or thrown away with the tip.

Similar technique can be used by repeated pipetting of the same volume. Press the pushbutton to the second stop. Dip the tip into the solution to a depth of 2 – 3 mm, and slowly release the operating button. Withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid. Dispense the liquid into the receiving vessel by gently pressing the pushbutton to the first stop. Hold the button in this position. Some liquid will remain in the tip, and this should not be dispensed. Repeat pipetting by filling the tip with solution.

In the chapter the following text was partially used:

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