Practical course: Instrumental Bioanalytics

Fluorescence Spectroscopy

In the following experiment, the concentration of quinine in tonic water and bitter lemon is to be determined by means of a calibration line. To do this, various calibration and test solutions must first be produced. These are then measured in the fluorescence spectrometer and the calibration line is generated. This calibration line shows (under certain circumstances) a linear relationship between the fluorescence intensity and the concentration of the fluorophore (quinie) and can therefore also be used to determine the quinine concentration in the beverages.

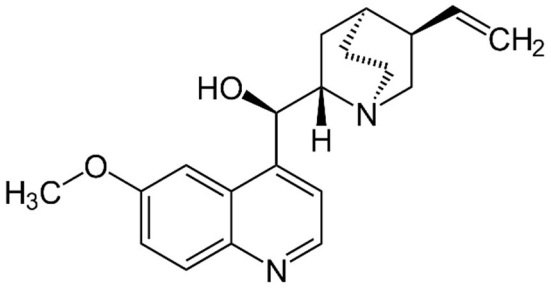

Abbildung 1: Strukturformel von Chinin

1 Theoretical foundations

In fluorescence spectroscopy, a sample is exited by electromagnetic radiation and the fluorescence (spontaneous emission) is observed. In contrast to absorption, fluorescence is measured perpendicular to the direction of irradiation (see Figure2). A monochromator in front of the sample selects the irradiated excitation wavelength and a second one in front of the detector determines the wavelength of the measured fluorescence.

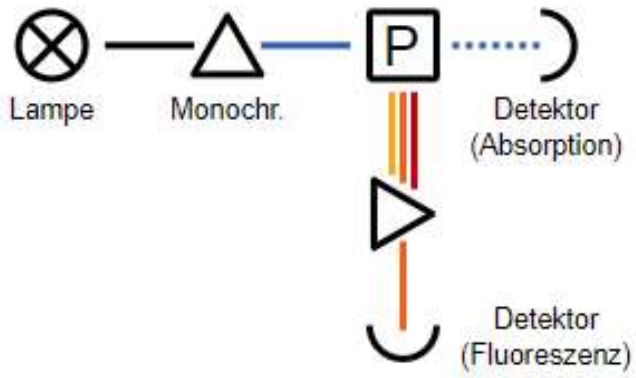

Abbildung 2: Aufbau eines Absorption- und Fluoreszenzspektrometers

1.1 Jablon'ski term scheme

The Jablon'ski term diagram (Figure 3) describes the electron excitation of a fluorophore and the processes by which the subsequent excitation into the electronic ground state can take place. The excitation initially takes place through the absorption of electromagnetic radiation and the fluorophore is raised from the electronic singlet ground state (S0) to an electronically excited state (here S1). In the process, electronic excitation can take place in different oscillation states of S1 (see Franck-Condon). Since the subsequent vibronic relaxation (VR) is usually orders of magnitude faster than the spontaneous emission, the fluorescence occurs from the oscillation ground state (Kasha rule). The emitted radiation is consequently lower in energy than the previously absorbed radiation (see Stokes shift). Under certain circumstances, the fluorophore can change into the triplet state (T1) by intersystem crossing (ISC). The spontaneous emission from the triplet state occurs again under spin reversal and is called phosphorescence. A further transition from S1 to the electronic ground state S0 takes place without radiation and is called internal conversion. In this process, the absorbed radiation is completely converted into thermal energy.

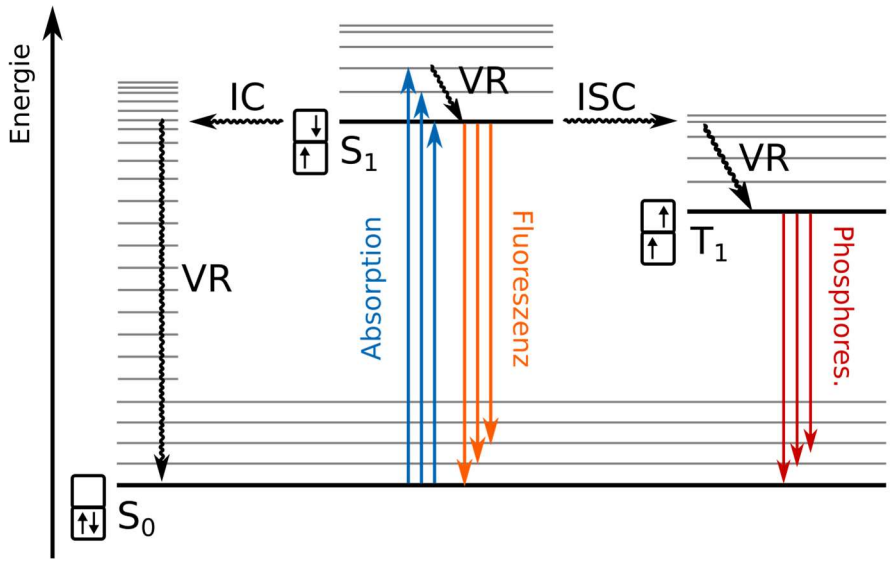

Abbildung 3: The Jablon'ski term diagram is shown with the following abbreviations: Electronic ground state (S0), first excited single state (S1), triplet state (T1), vibronic relaxation (VR), internal conversion (IC), intersystem crossing (ISC). The grey lines represent the oscillation states of the respective electronic states.

1.2 Franck-Condon principle

The Franck-Condon principle describes the influence of molecular oscillations on electronic excitation. Since the excitation of the fluorophore is very fast (approx. 1 fs), the core spacing does not change in the meantime (an oscillation is approx. 2 orders of magnitude slower). In figure 4, this excitation of S0 in S1 is therefore drawn as a vertical arrow - the core spacing remains constant during the excitation. However, such an excitation can only take place if both the initial and the final state have a certain state probability with a constant core spacing. Accordingly, a transition between two states is all the more probable the better their oscillation wave functions overlap.

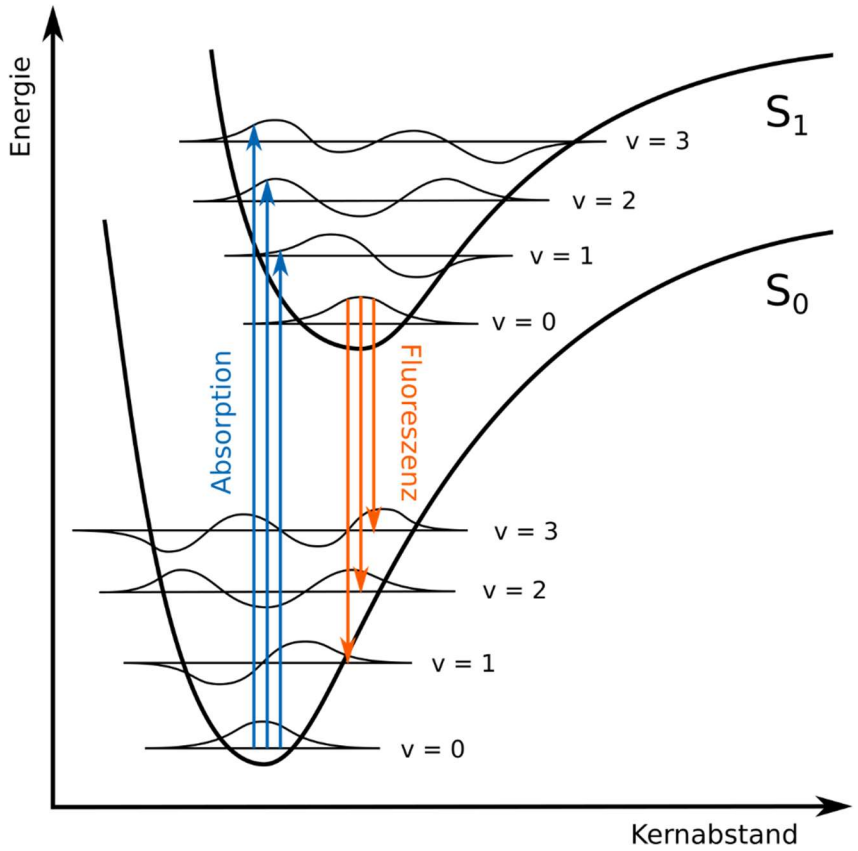

Abbildung 4: Schematic representation of the Franck-Condon principle. The molecular oscillation is based on the anharmonic oscillator and the first oscillation states of S0 and S1 are shown

1.3 Stokes shift

It is initially surprising that the energy of the emitted radiation does not correspond to the energy of the absorbed radiation. It was found that the emitted light has a longer wavelength, i.e. it has less energy than the previously absorbed light. Two processes are described here that contribute to the so-called Stokes shift.

Vibronic relaxation

As shown in figure 3, both absorption and fluorescence can take place in an excited oscillation state. This increases the required energy of the absorbed radiation and decreases the energy of the emitted radiation. Both lead to the fact that the emission takes place at a higher wavelength than the absorption.

Solvent Relaxation

The excitation of the fluorophore into an electronically excited state also changes its charge distribution. This leads to the solvent molecules, which were initially energetically favourably oriented around the fluorophore, becoming energetically unfavourably oriented after excitation (figure 5, left). The excitation occurs so quickly (analogous to Franck-Condon) that the solvent only reorients itself afterwards and assumes a more energetically favourable state. With subsequent fluorescence, reorientation of the solvent is again necessary (figure 5, right). Here, too, both effects lead to a Stokes shift.

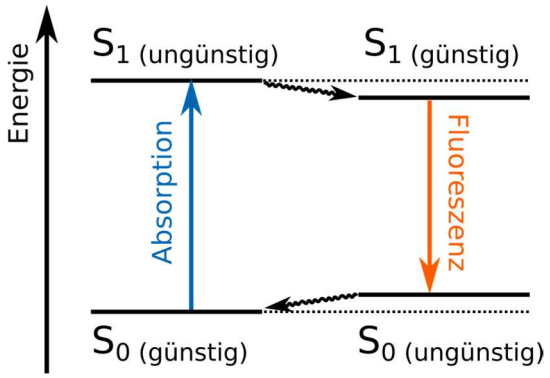

Abbildung 5: The relaxation by reorientation of the solvent is illustrated. It should be noted that a favourable arrangement of the solvent molecules always leads to an energy reduction of the state. energetic lowering of the state.

1.4 Decay of the excited state S1

In the following section, the decay of the excited state S1, which is qualitatively summarised in the Jablon'ski term scheme (Figure 3), will be examined in more detail. After the absorption of light, the temporal evolution of S1 can typically be described by first-order kinetics. Consequently, its population *n* at time *t* is given by

$$n(t) = n_0 * \exp\{-k_{S1} * t\}$$

Here, k_{S1} is the rate constant that determined the speed of decay. It is composed of further rates whose competing process (fluorescence, internal conversion, intersystem crossing) are known from Jablon'ski term scheme:

$$k_{S1} = k_F + k_{IC} + k_{ISC}$$

From k_{S1}, the lifetime τ_{S1} of the excited state can be calculated as follows:

$$\tau_{S1} = \frac{1}{k_{S1}}$$

1.5 Fluorescence quantum yield

The fluorescence quantum yield Φ_F is defined as the ratio of emitted to absorbed radiation. It can also be represented by corresponding rates:

$$\Phi_F = \frac{emitted\ photons}{absorbed\ photons} = \frac{k_F}{k_F + k_{IC} + k_{ISC}}$$

Another process that leads to the excitation of the S1 is called fluorescence quenching and is described in more detail in the following chapter.

1.6 Fluorescence quenching

In the presence of a so-called quencher, there are further processes that cause a decay of S1 and equation (1.1) must be extended by one term:

$$k_{S1} = k_F + k_{IC} + k_{ISC} + k_Q - [Q]$$

Here [Q] is the concentration of the quencher and k_Q is the corresponding rate of the quenching process. A distinction is made between two quenching processes, dynamic and static fluorescence quenching. In the case of dynamic quenching, the energy of the excited fluorophore is transferred to the quencher by a collision and is therefore also referred to as collision quenching. The following equation describes the process of dynamic quenching:

$$F^* + Q \overset{k_Q}{\rightarrow} F + Q^*$$

In contrast, in static fluorescence quenching the fluorophore and the quencher form a complex which does not fluoresce or fluoresces less. This is illustrated by the following equation

$$F + Q \rightleftharpoons FQ$$

A quenching process can be quantified with the help of the Stern-Volmer equation. It describes the fluorescence of a dye as a function of the concentration of a quencher [Q]. It is given as

$$\frac{F_0}{F} = \frac{\Phi_0}{\Phi} = 1 + k_{SV} * [Q]$$

Here F is the fluorescence intensity with and F_0 without quencher, and Φ is the quantum yield. with and Φ_0 without quencher. The Stern-Volmer constant k_{SV} determines the slope of the straight line.

2 Implementation

First, the calibration and test solutions are prepared from the stock solutions and later measured on the fluorescence spectrometer.

2.1 Production of the master solutions

The following stock solutions are produced in the laboratory on the 4th floor,

- 250 ml of a 0.5 M H_2SO_4 solution (M = 98.08 g/mol, ρ = 1.84 g/cm^3)
- Approx. 50 ml quinine solution witch a concentration of 0.1 g/l (sensible weight!)

2.2 Production of the calibration solutions

To prepare the calibration solutions, 10 ml of the 0.5M H2SO4 solution is added using a volumetric pipette and then add the corresponding volume of the quinine stock solution. In order to avoid a systematic error, the actual quinine concentration should be calculated and used in the experimental protocol for evaluation.

	V desired [ml]	c desired [mg/l]	Addition of quinine solution [µl]	V total [ml]	c real [mg/l]
1	10	0.05			
2	10	0.1			
3	10	0.15			
4	10	0.2			
5	10	0.3			
6	10	0.4			
7	10	0.5			
8	10	0.6			
9	10	0.7			
10	10	0.8			
11	10	0.9			
12	10	1			

2.3 Production of the test solutions

To avoid the *inner filter* effect, a diluted sample solution (1:199) must be prepared before the measurement. This dilution must be taken into account in the later evaluation.

- 10 ml of a dilution of tonic water in 0.5 M H2SO4 solution
- 10 ml of a dilution of bitter lemon in 0.5M H2SO4 solution.

Carbonic acid in tonic water / bitter lemon can lead to pipetting errors and should therefore be removed by repeated shaking.

2.4 Optimisation of the measurement parameters for quinine

In order to obtain meaningful measurements, the relevant emission range and the optimal excitation wavelength must first be determined.

1. An overview fluorescence spectrum of a quinine-containing solution is to be recorded. For this purpose, a radiation wavelength of 300 nm is selected and the emission is measured in a wavelength range from 200 to 800 nm. Explain the signals that occur and determine the relevant emission range.
2. In order to find the optimal excitation wavelength, an excitation spectrum is measured. To do this, the excitation wavelength is varied from 275 to 375 nm. Determine the optimum.

2.5 Measurement on the fluorescence spectrometer

Now the created calibration and test solutions are to be measured on the spectrometer with the optimised measurement parameters. It should be noted that residues from previous samples in the cuvette can distort the measurement. Therefore, rinsing is recommended before each measurement. The sample to be measured should be used for rinsing in order to minimise the error.

1. In order to carry out a background correction later, a blank measurement of the pure 0.5M H2SO4 is first carried out.
2. A fluorescence spectrum is then measured for each calibration solution
3. A fluorescence spectrum is measured for the diluted solutions of tonic water / bitter lemon
4. In order to determine the reproducibility of the spectrometer, multiple measurements of individual samples are to be carried out. These can be used later in the discussion of errors.
5. A few µl of a saturated NaCl solution should be added to a quinine-containing sample. What changes can be observed?

Topics of the colloquium

The theoretical foundations section of this script does not claim to be complete, therefore external sources should also be used for preparation. The following topics may be part of the colloquium:

- Construction of an absorption or fluorescence spectrometer
- Lambert-Beer law
- Jablon'ski term scheme
- Kasha rule
- Franck-Condon principle
- Stokes shift
- Inner filter effect
- Fluorescence quantum yield
- Fluorescence quenching
- Temperature dependence of static / dynamic fluorescence quenching