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Photoprotection of riboflavin containing beverages

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Preface

This project was carried out at the Department of Physics at NTNU in Trondheim.

I would like to thank my supervisor Kalbe Razi Naqvi, Professor at the Department of Physics, for providing me with the opportunity to write my master thesis under his supervisement, as well as for his guidance, patience, valuable suggestions, constructive comments and most of all for teaching me more in the past five months than I could hope to learn. A special thank you is to Heng Li, postdoctoral fellow at the Department of Physics, for his help at the lab and for providing access to unpublished data.

I am also grateful to Thor Bernt Melø and Kristin Sæterbø for their help at the lab and with chemicals.

Abstract

Riboflavin, also known as vitamin B_2 and is one of the most easily absorbed nutrients, can be found in many different organisms. The most abundant source of riboflavin is milk and dairy products; however it is also present in meat, fish and certain types of vegetables and fruit. Riboflavin is an important part of a healthy diet in order to keep skin, eyes and nervous systems healthy. Some studies indicate that riboflavin plays a preventive role in cancer and cardiovascular diseases. [1, 2]

Milk is known to be extremely sensitive to light. Riboflavin is one of the factors responsible for the light-induced degradation of milk. In combination with light and oxygen, riboflavin may act as a photosensitizer. When vitamin B_2 absorbs blue-green light, an excited triplet state of riboflavin is generated through a process called intersystem crossing. Reactive oxygen species, such as singlet oxygen, is then formed by the reaction of excited riboflavin triplet with dissolved oxygen present in milk. Light exposure of milk can lead to off-flavour and damage of vitamins by reaction of singlet oxygen with amino acids and lipids in milk. Unfortunately, most of the packaging materials today do not protect milk from light completely. The formation of singlet oxygen can also be prevented by adding quenchers that are able to deactivate riboflavin triplets. Certain amino acids and carotenoids are well known flavin quenchers.

The purpose of this study was to investigate how well riboflavin triplets can be quenched by the amino acids cysteine, histidine, methionine, tyrosine and tryptophan. The quenching properties of hydrophilic carotenoid crocin were studied as well. Crocin has been under investigation of researches at the Departement of Physics at NTNU. Lumiflavin, which is one of the riboflavin's photodegradation products, was used instead of riboflavin. The former is more stable and has similar photochemical characteristics as the latter.

The quenching of lumiflavin triplets was studied by using laser flash photolysis. It involves irradiating the sample under investigation with a short-lived laser flash. The method was used to measure the kinetic decay rate of lumiflavin in an aqueous buffer with and without different concentrations of a quencher. The data were fitted to two different decay models. From pseudo-first-order rate constants, the quenching rate constants were determined for each amino acid and crocin. All amino acids and crocin used in this study showed a quenching effect on the lumiflavin triplets. Further, it was determined whether the fitting models are suitable for these kind of measurements by simulating the decay of lumiflavin with and without any quencher. More studies on the fitting models have to be done to be able to get reliable results.

Contents

1 Introduction

Riboflavin, also known as vitamin B_2, can be found amoung a variety of foods. It has been shown that riboflavin may act as a photosensitizer of several biological compounds. Milk is one of the main sources of riboflavin and is known to get a particular off-flavour, caused by exposure to the light. Riboflavin is responsible for these light-induced changes due to its sensitivity to light, producing high-reactive singlet oxygen. Besides affecting the sensory quality, light can cause undesirable photodamage on vitamins and aminoacids in riboflavin containing beverages. [3]

Photooxidation reactions in milk occur due to the ability of the riboflavin triplet to excite the ground state oxygen into the harmful singlet oxygen. Reactive singlet oxygen can oxidize and degrade particular amino acids, which result in off-flavors in milk. These amino acids contain sulphur, and its oxidation leads to the formation of dimethyl disulfide and methional. The latter ones are known to be the reason for the off-flavour in milk caused by sunlight. [3, 4]

The off-flavour in milk is most evident when exposed to wavelengths corresponding to the blue part of the visible electromagnetic spectrum [5]. To prevent the photodamage, packaging materials have been developed to block the light. It has been proven that blocking blue light below 500 nm and UV radiation significantly reduces the formation of undesired flavors in milk [4]. However, it does not eliminate it completely and prevents photooxidation of milk only during storage.

The solution to the problem of photodamage to milk is to use food additives, such as amino acids. The most commonly used are tryptophan and cysteine. They are used as a substrate for the riboflavin triplet and are able to accelerate the decay of the excited riboflavin triplets proportional to their concentration, and by doing so preventing the formation of the singlet oxygen. Other compounds, which are proven to quench singlet oxygen are carotenoids, but they are not easily dissolved, as most of them are hydrophobic. An example of a naturally occuring hydrophilic carotenoid is crocin [4].

1.1 Objectives

The aim of the present project is to study the quenching of lumiflavin triplets by the hydrophilic carotenoid crocin and the amino acids histidine, tyrosine, tryptophan, methionine and cysteine. In addition, it will be determined whether the crocin is a more efficient quencher than amino acids. In order to do so, the rate constants, k_{q}, of all quenchers will be found. The k_{q}-values of the amino acids are then to be compared

with the k_{q}-value of the crocin.

Another aim of this work is to find the best method for analyzing the data acquired from the measurements. To prove whether the results are reliable or not, the simulation of the data will be performed and analyzed in the same way as the real data. The results obtained from the simulation will be compared with results obtained from the measurement performed at the lab.

2 Background

2.1 Riboflavin

Riboflavin, also known as 7,8-dimethyl-10-ribityl-isoalloxazine, is a watersoluble component of the B_2 vitaminic complex. As mentioned earlier, it is present in a variety of foods, such as milk, dairy products and beer [2, 6]. The chemical structure of riboflavin can be seen in Figure 1.

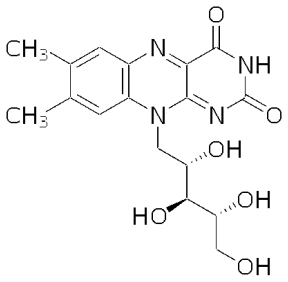


Figure 1: Chemical structure of riboflavin

The absorption spectrum of riboflavin is shown in Figure 2, and has peaks at 220, 265, 375 and 446 nm. It fluoresces yellow with λ_{max} around 520 nm. [1, 7]

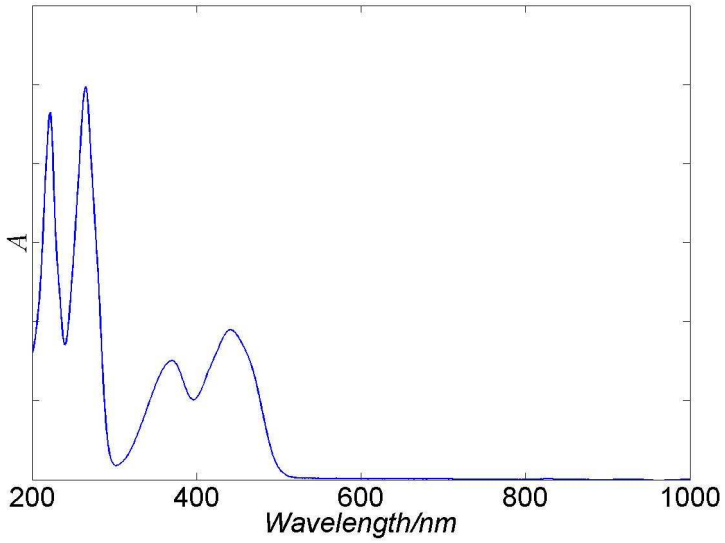


Figure 2: Absorption spectrum of riboflavin in aquaous solution at neutral pH [4].

2.1.1 Riboflavin as a photosensitizer

The oxygen molecule O_2 is one of the most important molecules for all living things, as long as it is in its ground state. However, the same oxygen molecule becomes very harmful when it is in its first electronically excited state. The ground state of the oxygen molecule is a triplet, $O_2^{\dagger 0}$, while the first two excited states are both singlets, $^1O_2^*$.

Riboflavin may act as a sensitizer. Under light, riboflavin may be excited to the first excited state, ^1S, and then undergo intersystem crossing, ISC, to a triplet state, $S^\dagger$, Equation 1. Under suitable conditions, the triplet of the sensitizer may lead to the foramtion of the singlet oxygen, Equation 2. The last one will then undergo a chemical reaction. [8, 9]

$$S_0 \rightarrow (^1S) \overset{ISC}{\rightarrow} (^3S) \tag{1}$$

$$S^\dagger + (O_2^{\dagger 0}) \rightarrow S_0 + (^1O_2^*) \tag{2}$$

The sensitization event only occurs if the condition $E(S^\dagger) > E(^1O^*)$ is satisfied, where E is the electronic energy of the species within the parentheses, and if the energy spins on both sides of Equation 2 are equal.

The riboflavin triplets may decay to the ground state with the rate constant k_1. However, the formation of a radical cation ($S^{\bullet +}$) and a radical anion ($S^{\bullet -}$) by annihilation is also possible with the rate constant k_2, Equations 3 and 4. [4]

$$S^\dagger \overset{k_1}{\rightarrow} S_0 \tag{3}$$

$$S^\dagger + S^\dagger \overset{k_2}{\rightarrow} (S^{\bullet +}) + (S^{\bullet -}) \tag{4}$$

When there is no substrate for riboflavin present, the rate equation for the reactions above can be given as Equations 5 and 6. [10]

$$\frac{d[S^\dagger]}{dt} = -k_1[S^\dagger] - k_2[S^\dagger]^2 \tag{5}$$

$$[S^\dagger] = \frac{k_1[S^\dagger]_0 e^{(-k_1 t)}}{k_1 + k_2[S^\dagger]_0[1 - e^{(-k_1 t)}]} \tag{6}$$

The processes described above can be illustrated by the Jablonski-type diagram for flavins, shown in Figure 3.

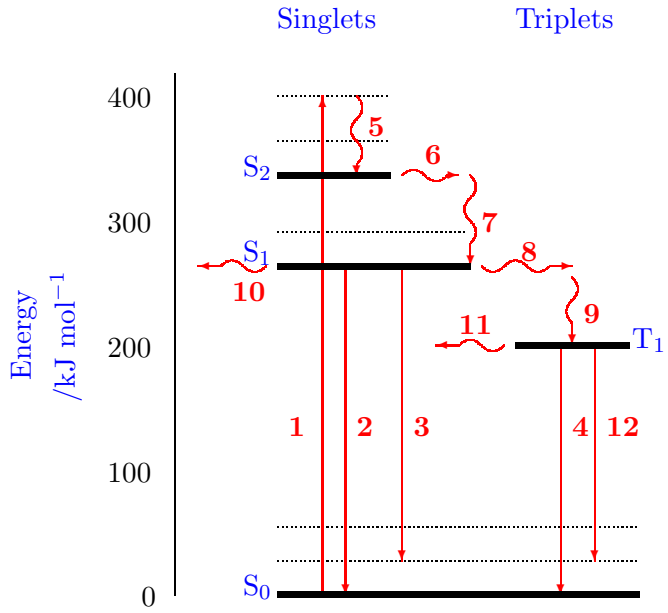


Figure 3: Jablonski-type diagram for flavins showing electronic (solid horizontal lines) and vibrational (dashed horizontal lines) energies above the ground state S_0. The S_1, S_2 and T_1 represent first excited singlet, second excited singlet and first triplet excited states, respectively. The processes involved are: 1 = absorption, 2 and 3 = fluorescence, 4 = phosphorescence, 5,7 and 9 = non-radiative decay, 6,10 and 11 = internal conversion, 8 and 12 = inter-system crossing. [7, 4]

2.2 Lumiflavin

Lumiflavin is a product of the photolysis of riboflavin and is known as 7,8,10-trimethyl isoalloxazine. The molecular formula of lumiflavin is $C_{13}H_{12}N_4O_2$ and the chemical structure can be seen in Figure 4.

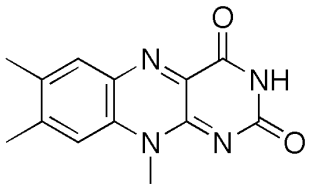


Figure 4: Chemical structure of lumiflavin

The photochemical characteristics of lumiflavin are similiar to those of riboflavin. Also lumiflavin produces singlet oxygen under illumination by light. However lumiflavin is more stable due to slower photodegradation under the same light intensity [4]. Thus

lumiflavin was used instead of riboflavin.

2.3 Amino acids

Amino acids are small molecules which consist of a carboxylic group, an amine group and a side-chain group. The last one is different for each amino acid. Amino acids are the building blocks in proteins and play an important role in nutrition. There are 20 standard amino acids, which can exist in either of two isomers, D or L amino acids. These are the mirror images of each other. Amino acids used in this study were L-histidine, L-tyrosine, L-tryptophan, L-cysteine and L-methionine. Their chemical structures can be seen in Figure 5.

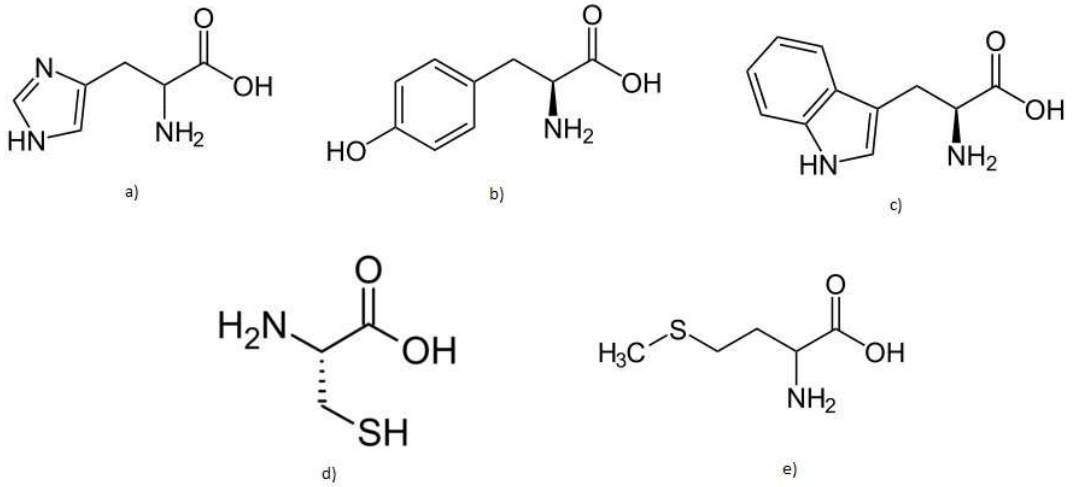


Figure 5: Chemical structures of aminoacids used in this study: a) L-histidine, b) L-tyrosine, c) L-tryptophan, d) L-cysteine and e) L-methionine.

2.4 Carotenoids

Carotenoids are conjugated polyene pigments, produced by algae, bacteria and plants, and are often yellow, orange or red. Carotenoids occur in insects, birds and animals, and there are about 40 carotenoids present in food. Among them are lycopene, α-carotene and β-carotene the most abundant. Besides the most known function as vitamin A precursors, carotenoids posess many other important biological properties. They play an important role in cancer protection, by increasing cell communication and acting as immune enhancers. However, carotenoids have recieved most attention

due to their antioxidant properties, by being able to deactivate reactive molecules such as free radicals and toxic forms of oxygen, which cause damage in living organisms.[8]

The best documentated antioxidant property of carotenoids is quenching of the singlet oxygen,$^1O_2^*$. Crocin has been studied at the Department of Physics at the NTNU in recent years.

2.4.1 Hydrophilic carotenoid crocin

Crocin (**C**) is a natural hydrophilic carotenoid. It has several medical properties, and is found in saffron and some plants. The chemical structure of **C** is shown in Figure 6. Due its sugar side chains, **C** is highly soluble in water. [4]

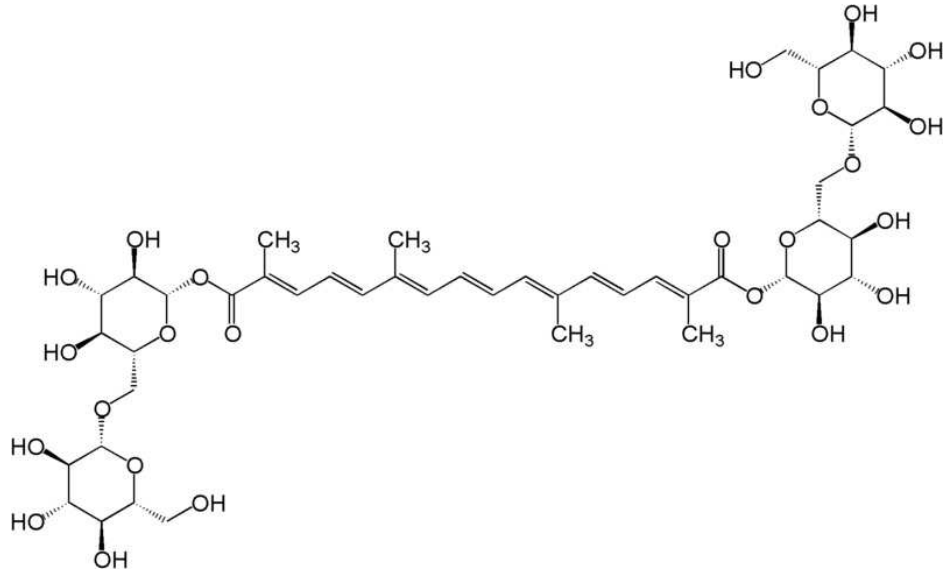


Figure 6: Chemical structure of crocin

The absorption spectrum of **C** can be seen in Figure 7, with the absorption maximum at 443 nm.

2.5 Quenching of triplet riboflavin by amino acids and C

As mentioned earlier, the $^1O_2^*$ becomes very harmful to all photosynthetic organisms once it has been formed, damaging almost each organic molecule it encounters. Besides a couple of exceptions, the latter one is an undesirable process. One of the ways of preventing this process is by converting $^1O_2^*$ into $O_2^{\dagger 0}$, before any damage happens. Another way is to avoid the formation of $^1O_2^*$ itself, which is found to be a better solution. [9]

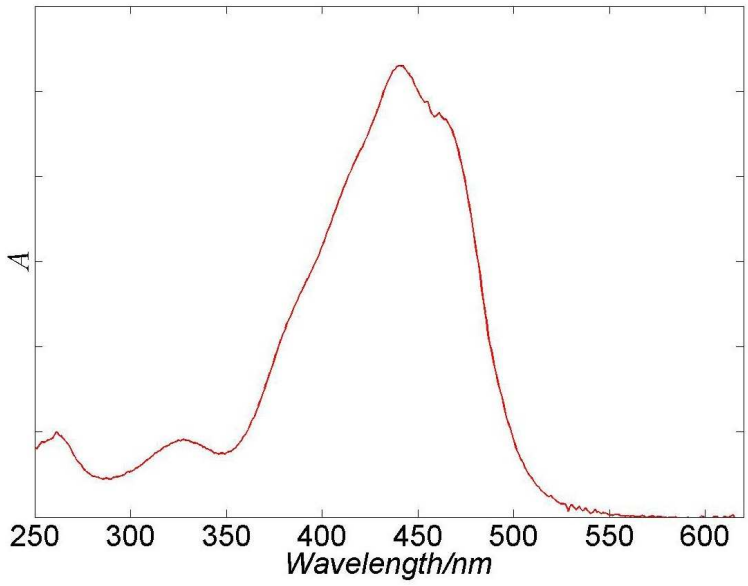


Figure 7: Absorption spectrum of **C** in aquaous solution with neutral pH [11].

Certain amino acids have been proven to have a quenching effect on the riboflavin triplet by electron transfer. Carotenoid **C** has been investigated at the Deprtement of Chemistry at the NTNU in recent years. It has been found that **C** has the property of quenching the triplet states of riboflavin by electron transfer and by energy transfer.

If a sensitizer, $S^{\dagger}$, meets an excited singlet molecule $^1Q^0$, a process of quenching of the former by the latter will occur if $E(S^{\dagger}) > E(Q^{\dagger})$, as it can be seen in Equation 7 [9].

$$S^{\dagger} +^1 Q^0 \rightarrow ^1S^0 + Q^{\dagger} \tag{7}$$

However, if the triplet energy of the $Q^{\dagger}$ happens to be higher than that of $^1O_2^*$, the former one may act as an sensitizer as well. In order to prevent the formation of the $^1O_2^*$, there is a need for an acceptor which cannot itself sensitize $^1O_2^*$. For this purpose both carotenoids and amino acids are perfectly suited for preventing the formation of the $^1O^*$ due to their low triplet states. Any $^1O_2^*$ that is formed will be neutralized when it meets a $^1Q^0$. This will happen through a physical process in which the harmful reagent $^1O_2^*$ is disarmed and the product $Q^{\dagger}$ is further reverted to $^1Q^0$ by converting its electronic energy into heat. This process is shown in Equation 8 [9].

$$^1O_2^* +^1 Q^0 \rightarrow O_2^{\dagger 0} + Q^{\dagger} \tag{8}$$

Equations 3, 4 and 7 give the rate equations for the decay of the triplet lumiflavin

when a quencher is present, which are given by equations 9 and 10.

$$\frac{d[\mathrm{S}^\dagger]}{dt} = -k_1[\mathrm{S}^\dagger] - k_2[\mathrm{S}^\dagger]^2 - k_\mathrm{q}[\mathrm{Q}][\mathrm{S}^\dagger] \tag{9}$$

$$[\mathrm{S}^\dagger] = \frac{(k_1 + k_\mathrm{q}[\mathrm{Q}])[\mathrm{S}^\dagger]_0 e^{(-(k_1+k_\mathrm{q}[\mathrm{Q}])t)}}{(k_1 + k_\mathrm{q}[\mathrm{Q}]) + k_2[\mathrm{S}^\dagger]_0[1 - e^{(-(k_1+k_\mathrm{q}[\mathrm{Q}])t)}]} \tag{10}$$

where k_1 is the first-order rate constant of triplet quenching by phosphorescence and inter-system crossing. In addition there may be some contribution from other quenchers present, such as an impurity or oxygen. k_2 is the second-order rate constant of triplet quenching by the triplet-triplet annihilation process and k_q is the second-order rate constant of a quencher.

3 Materials and methods

3.1 Materials

Lumiflavin, **C** and the amino acids histidine, tyrosine, tryptophan, cysteine and methionine were purchased from Sigma-Aldrich. The L-form of the amino acids was chosen due its dominance in a human body.

All of the abovementioned chemicals were dissolved in a phosphate buffer of 20 mM with pH 7.2. The buffer was made by mixing 5.6 ml 0.2 M NaH_2PO_4 and 14.4 ml 0.2 M Na_2HPO_4. This was followed by dilution to 200 ml with distilled water. All experiments were performed by using this buffer.

Prior to each photolysis experiment the samples were deoxygenated with argon gas in cuvettes closed by rubber septa for 10 minutes. A flow of argon gas was maintained during the measurements. The measurements were performed at 295 K.

3.2 Instrumentation

3.2.1 Absorption spectrophotometer

In order to perform absorption spectroscopy a Shimadzu UV-visible scanning absorption spectrophotometer of type UV-1601PC was used.

3.2.2 Laser flash photolysis

The technique used to study the characteristics of excited states and associated photoproducts, involves irradiation of the sample with a short laser pulse and measurement of ΔA, the resulting change in the absorbance of the sample. order to investigate kinetics of the intermediate products of the lumiflavin formed by the laser pulse, the decay of the absorption at the excitation wavelength of 442 nm was studied as a function of time t, the time elapsed since the firing of the laser [12].

Laser flash photolysis method was applied, by using the pulsed laser and an optical parametric oscillator (OPO). The OPO-combination (EKSPLA model NT342A-SH-10Hz) consists of a neodynium-doped yttrium aluminium garnet (Nd:YAG) solid state laser (model NL301G/TH), an OPO (model PG122) with UV extension and non-linear crystals. The output wavelength of the laser is 1064 nm with a frequency of 10 Hz. The pulse duration of the laser is 6 ns and covers the output wavelength range of 210-2300 nm. In addition, a xenon arc lamp, that is pulsed for a short time μs, two monochromators, a digital oscilloscope and a photomultiplier tube are used. The setup can be seen in Figure 8. The sample is located between the first monochromator, MC1

and the second monochromator, MC2. The second monochromator is used as a filter, while the photomultiplier tube is used in order to measure the intensity of the light leaving the exit slit of the monochromator [4, 12].

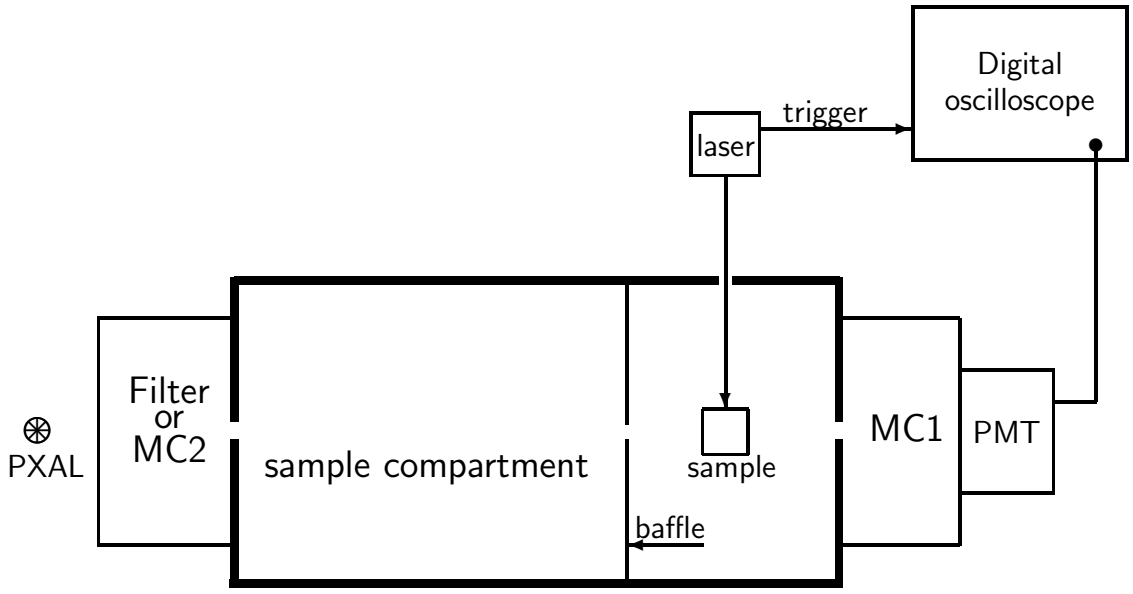


Figure 8: Setup for laser flash photolysis. PXAL = pulsed xenon arc lamp, MC = monochromator, PMT = photomultiplier [12]

The change in absorbance at a particular wavelength λ is calculated by using Equation 11, where I_0 and I are the intensities of the beam transmitted through a sample unexposed and exposed to the laser pulse, respectively.

$$\Delta A(t) = \lg[I_0(t)/I(t)] \tag{11}$$

The absorbance was calculated from the signals. Initially, the ΔA was calculated for lumiflavin only. Same measurements and calculations of the lumiflavin triplet decay in terms of absorbance were performed for different concentrations of each amino acid. At last, the ΔA was calculated for lumiflavin added **C**. For each concentration of the quencher the measurement of the signal was performed three times. An average of these was then calculated and fitted to the decay models, followed by determination of the quenching rate constants.

3.3 Data analysis

3.3.1 Fitting the data

As mentioned earlier, both several amino acids and hydrophilic carotenoid **C** has the ability to quench the triplet states of lumiflavin. The triplet decay of riboflavin follows mixed exponential decay in the presence of a quencher, when its concentration is low, Equations 9 and 10. However, Equations 9 and 10 can be simplified to the simple first-order rate equation 12. Here, the triplet-triplet annihilation process becomes negligible with $k_2 = 0$ [3, 13]. This kind of decay is called single exponential decay.

$$[\mathrm{S}^{\dagger}] = [\mathrm{S}^{\dagger}]e^{(-(k_1+k_\mathrm{q}[\mathrm{Q}])t)} \tag{12}$$

Models for mixed and single exponential decays, Equations 9, 10 and 12, were used to fit the data. These equations had to be rewritten in terms of absorbances. The relationship between the concetration of a sensitizer and its absorbance is given in equation 13. Here A is the absorbance and ϵ is the extinction coefficient of the lumiflavin triplet and l is the path length, which equals to the width of the quvette of 1 cm. The extinction coefficient of the lumiflavin triplet at 296 nm is known to be 1.47 $\times 10^4$ M^{-1} cm^{-1} [14].

$$A = [\mathrm{S}^{\dagger}]\epsilon l \tag{13}$$

The model for mixed exponential decay in terms of absorbance can be seen in equation 14. Here, A_m is the absorbance of lumiflavin at 296 nm, while A_{m0} is the absorbance at $t = 0$.

$$A_\mathrm{m} = \frac{(k_1 + k_\mathrm{q}[\mathrm{Q}])A_{\mathrm{m0}}e^{(-(k_1+k_\mathrm{q}[\mathrm{Q}])t)}}{(k_1 + k_\mathrm{q}[\mathrm{Q}]) + \frac{k_2}{\epsilon}A_{\mathrm{m0}}[1 - e^{(-(k_1+k_\mathrm{q}[\mathrm{Q}])t)}]} \tag{14}$$

The model for single exponential decay in terms of absorbance is given in equation 15. Here, A_s is the absorbance of lumiflavin at 296 nm, while A_{s0} is the absorbance at $t = 0$.

$$A_\mathrm{s} = A_{\mathrm{s0}}e^{-(k_1+k_\mathrm{q}[\mathrm{Q}])t} \tag{15}$$

The decay of the triplet state can be accelerated by the presence of certain quenchers with decay rates proportional to their concentrations, equation 9. From the pseudo-first-order decay kinetics, the second-order rate quenching constants, k_q, can be determined from the pseudo-first-order rate constants, k_1', for different concentrations of the

quenchers, equation 16, where [Q] is the concentration of the quencher and k_1 is the first-order rate constant, corresponding to the lumiflavin triplet lifetime in the absence of quencher [3].

$$k_1' = k_1 + k_q[Q] \tag{16}$$

Numerical solutions to the differential equations described above, were obtained by using MATLAB, where the function *lsqnonlin* was used to find the best fit. Excel was then used to plot the first-order rate constants against the concentration of the quenchers to determine the second-order rate constants of the quenchers.

3.3.2 Delayed fluorescence

To be sure that the measurements performed by laser flash photolysis were reliable, the same measurements were performed by Heng Li, using delayed fluorescence. It is a non-collisional energy transfer process that has a fluorescence characteristic emission spectrum, but a much longer lifetime than normal fluorescence. Delayed fluorescence can be described as a very weak light emitted by samples which has been illuminated. Delayed fluorescence are divided into P-type, E-type, Recombination and Triplet Excitation. E-type, which was used in this study, occurs when a molecule in the lowest triplet state is elevated, by thermal activation, to the first excited singlet state. Then it returns to the ground state by emission of the photon [15].

In order to avoid the triplet-triplet annihilation process, concentration of the lumiflavin triplet used was quite low with an absorbance of 0.03. This allowed us to use the single exponential decay model to fit the data. Otherwise, the data acquired from this method was handled and analyzed in the same way as the data acquired from the laser flash photolysis. Here the rate constants were determined by fitting the data with Excel. However, the rate constants were determined twice, first by fitting the data with the whole kinetic trace and then by fitting the same data with the tail decay trace. By using the latter part of the decay to fit the data, more accurate results were obtained, because the concentration of the lumiflavin triplet is even lower in this case.

3.3.3 Simulation method

To find out whether the fitting models are suitable for the current study and hence reliable, the results were also obtained by simulating the signal. The signal was simulated in MATLAB in terms of absorbance and time by using the mixed decay model. The decay was then fitted to the single exponential decay model. Simulation of signal was

performed for each concentration of each quencher as used in the laser flash photolysis method. The same procedure for obtaining the first- and second-order rate constants as for the flash photolysis method followed in Excel. The pseudo-first-order rate constants obtained by simulating the data by using mixed kinetics model were plotted against the quencher's concentration. In the same plot, the pseudo-first-order rate constants derived from fitting the simulated data to the single exponential decay model were plotted against the concentration of the quencher as well. The slope of the former plot was then compared to the slope of the latter. These steps were done for all quenchers.

4 Results

4.1 Kinetic measurements

4.1.1 Single exponential decay model

The decay of the triplet state of lumiflavin with and without quencher contribution was fitted to the single exponential decay model. The quenching rate constants of crocin and amino acids were found by varying their concentrations. Figure 9 shows absorbance time profile for triplet-excited lumiflavin observed at 296 nm in the presence and absence of different concentrations of crocin in anaerobic aqueous buffer at pH 7.2. Here the data were fitted to the single exponential decay model. It is clear that the decay of the triplet state of lumiflavin was accelerated by the presence of the crocin.

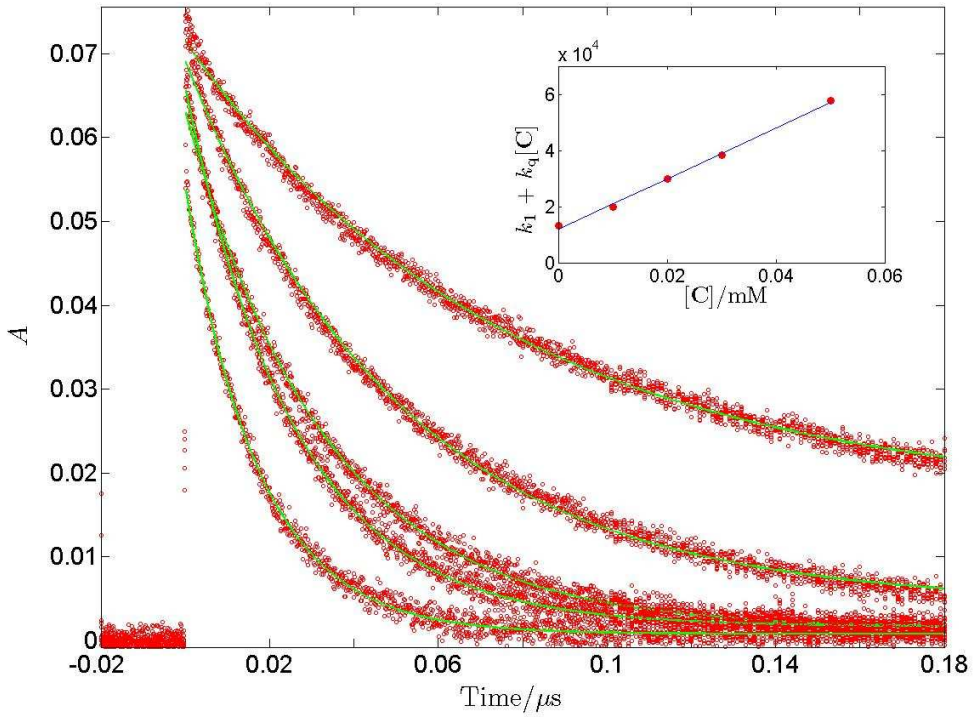


Figure 9: Absorbance time profile for lumiflavin without (curve on the top) and with different concentrations of crocin (curve at the bottom for highest concentration) as quencher, observed at 296 nm. The green lines are the fits of each curve to the single decay model. Insert: Dependence for the observed decay rate constant for triplet lumiflavin on the crocin concentration in M.

From the exponential decays, pseudo-first-order rate constants were calculated and found to be linearly dependent on the quencher concentration, as can be seen in the inset of Figure 9 for crocin in aqueous buffer at pH 7. This was observed for the other quenchers in this study as well. From such plots the second-order rate constants were derived for crocin and amino acids and are given in Table 1.

Quencher	k_q (M^{-1} cm^{-1})
Crocin	$9.00 \pm 0.28 \times 10^8$
Cysteine	$9.82 \pm 1.46 \times 10^7$
Histidine	$7.93 \pm 0.20 \times 10^7$
Methionine	$4.52 \pm 2.10 \times 10^7$
Tryptophan	$1.79 \pm 0.17 \times 10^9$
Tyrosine	$1.25 \pm 0.09 \times 10^9$

Table 1: Rate constants with standard deviation from laser flash photolysis for different quenchers using the single exponential decay model for fitting the data

The single exponential decay model suggests that amino acids tyrosine and tryptophan are the most efficient quenchers among the other in this study with the highest quenching constants. Amino acids cysteine, histidine and methionine are the least efficient due to lowest second-order rate constants. The quenching property of the carotenoid crocin seems to be moderate, with the quenching constant in the middle of between the lowest and the highest rate constants of the quenchers. The distribution of the errors indicates that the fitting of the data to the single exponential decay model is quite good.

4.1.2 Mixed decay model

The decay of lumiflavin triplet with and without quenchers was also fitted to the mixed decay model. The absorbance time profile for triplet-excited lumiflavin observed at 296 nm in the presence and absence of different concentrations of crocin in anaerobic aqueous buffer at pH 7.2 can be also seen in Figure 10.

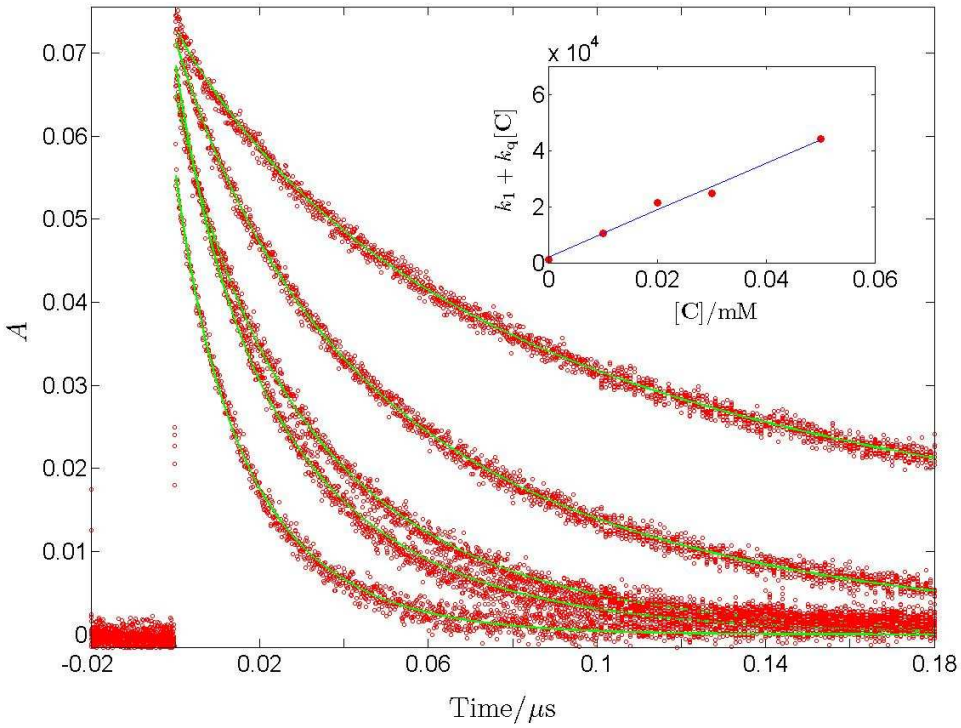


Figure 10: Absorbance time profile for lumiflavin without (curve on the top) and with different concentrations of crocin (curve at the bottom for highest concentration) as quencher, observed at 296 nm. The green lines are the fits of each curve to the mixed decay model. Insert: Dependence for the observed decay rate constant for triplet lumiflavin on the crocin concentration in M.

However, here the data were fitted to the mixed exponential decay model. From the exponential decays, pseudo-first-order rate constants were calculated, although with no linear dependence on the quencher concentration except for crocin and cysteine. The fitting for crocin can be seen in Figure 10. The fitting for other quenchers was quite poor and the obtained second-order rate constants, if any, were not in agreement with the results from any other methods. The quenching rate constants for crocin and amino acids according to the mixed kinetics are given in Table 2.

Quencher	k_q (M^{-1} cm^{-1})
Crocin	$8.39 \pm 0.57 \times 10^8$
Cysteine	$1.09 \pm 0.34 \times 10^8$
Histidine	$8.72 \pm 12.2 \times 10^6$
Methionine	$9.69 \pm 0.28 \times 10^6$
Tryptophan	$3.90 \pm 3.28 \times 10^7$

Table 2: Rate constants with standard deviation from laser flash photolysis for different quenchers using the mixed exponential decay model for fitting the data

The results indicate that the model of mixed kinetics is not a suitable fitting model in this case.

4.2 Delayed fluorescence measurements

The quenching rate constants for crocin and amino acids were determined by measuring the decay of triplet-excited lumiflavin by delayed fluorescence. The data acquired by using this method were fitted to the single exponential decay model with the whole trace and with the tail trace. The lumiflavin used for this kind of measurements had an absorbance of 0.03. The final rate constants for the different quenchers were calculated with an error and the results for the tail traice are given in Table 3. It should be noticed that the results obtained by delayed fluorescence are not final. No measurements were performed for methionine by this method.

Qencher k_q (M^{-1} cm^{-1})	
Crocin	$9.64 \pm 0.41 \times 10^8$
Cysteine	$9.74 \pm 0.67 \times 10^7$
Histidine	$6.66 \pm 0.38 \times 10^7$
Tryptophan	$1.30 \pm 0.04 \times 10^9$
Tyrosine	$1.08 \pm 0.05 \times 10^9$

Table 3: Rate constants with standard deviation from the delayed fluorescence for different quenchers using the single exponential decay model for fitting the data

According to the results obtained from the delayed fluorescence measurements, amino acids tyrosine and tryptophan are the most efficient quenchers of the lumiflavin triplet due to the highest quenching rate constants. Also this method confirms that amino acids cysteine and histidine are the least effective quenchers with the lowest second-order rate constants. Once again, carotenoid crocin lies in the between other species when it comes to the quenching properties.

4.3 Simulated measurements

At last the quenching rate constants were obtained by simulating the decay of lumiflavin triplet in MATLAB, using mixed kintecs model. The data acquired from the simulation were written in terms of absorbance and fitted to the single exponential decay model. The pseudo-first- and second-order rate constants were then determined in the same way as in the previous subsections of this chapter. The results are given in Table 4.

Quencher	k_q (M^{-1} cm^{-1})
Crocin	$7.34 \pm 0.62 \times 10^8$
Cysteine	$3.96 \pm 0.25 \times 10^7$
Histidine	$5.98 \pm 0.41 \times 10^7$
Methionine	$1.46 \pm 0.21 \times 10^7$
Tryptophan	$1.61 \pm 0.07 \times 10^9$
Tyrosine	$1.09 \pm 0.06 \times 10^9$

Table 4: Rate constants with standard deviation from the simulated measurements for different quenchers using single exponential decay model for fitting the data

The second-order rate constants obtained by this method are some smaller than those obtained from the real measurements, where the data were fitted to the single exponential decay. The plot of the pseudo-first-order rate constants obtained by simulating the data by using mixed kinetics model deviated slightly from the pseudo-first-order rate constants derived from fitting the simulated data to the single exponential decay model. However, despite the deviation in the slopes, the second-order rate constants derived from the pseudo-first-order rate constants from both plots were almost equal. This can be seen in Figure 11 for crocin.

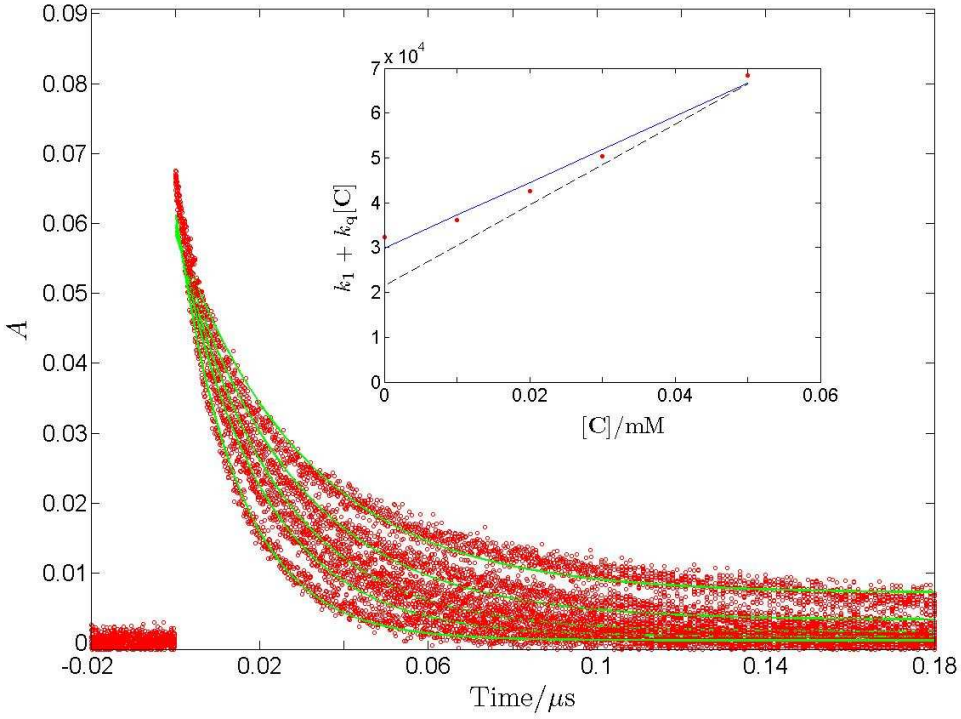


Figure 11: Simulated absorbance time profile for lumiflavin without (curve on the top) and with different concentrations of crocin (curve at the bottom for highest concentration) as quencher fitted to a single exponential decay. The green lines are the fits of each curve to the mixed decay model. Insert: Dependence for the decay rate constant for triplet lumiflavin on the crocin concentration in M, derived from simulating the decay (dashed line) and by fitting the simulated data to the single exponential decay(blue line).

5 Discussion

5.1 The quenching properties of the amino acids

The results of the current study showed the amino acids cysteine, histidine, methionine, tryptophan and tyrosine all exhibit the quenching properties when it comes to quenching of lumiflavin triplet. The obtained results indicate that tyrosine and tryptophan are the most efficient quenchers with highest rate constants. This is also supported by the fact that the quenching rate constants of tyrosine and tryptophan are higher than the rate constant for the bimolecular reaction between the oxygen and the riboflavin triplet state, which is 9.8 $\times 10^8$ $M^{-1}s^{-1}$ [3]. On the other hand, histidine and methionine were less efficient with the lowest quenching rate constants. The quenching properties of crocin appeared to be between of the lowest and highest rate constants of the amino acids.

In a work performed by Cardoso and co-writers [3], the reaction between the triplet excited state of riboflavin and amino acids was studied in an aqueous solution in pH range from 4 to 9, using laser flash photolysis. The results obtained by Cardoso and co-authors are presented in the table 5. The most of the second-order rate constants determined in our study are in good agreement with the results obtained by Cardoso and co-workers, especially for tyrosine and tryptophan. The rate constant for cysteine differed most. The reason for the deviations can be the fact that the concentration of the ground state of the lumiflavin was lower than in the experiment by Cardoso and co-writers, leading to a lower contribution of the triplet-triplet annihilation.

Amino acid	k_q (M^{-1} s^{-1})
Cysteine	$2.16 \pm 0.03 \times 10^7$
Histidine	$5.22 \pm 0.13 \times 10^7$
Methionine	$6.36 \pm 0.29 \times 10^7$
Tryptophan	$1.75 \pm 0.09 \times 10^9$
Tyrosine	$1.40 \pm 0.10 \times 10^9$

Table 5: Second-order rate constants with standard deviation for quenching of riboflavin triplet state by amino acids, at pH = 6.4 and N_2-saturated solutions [3].

Also the obtained rate constants for tyrosine, tryptophan and methionine agrees quite well with the results reported from other studies [16, 17].

Another study performed by Huvaere and Skibsted [18] confirmed the quenching properties of amino acids tryptophan and histidine. Mechanisms of flavin-mediated photooxidation of the above mentioned amino acids were investigated for aqueous solu-

tions. The rate constants for tryptophan and histidine were determined by laser flash photolysis here as well, and were calculated to be 2.70 $\times 10^9$ $M^{-1}s^{-1}$ and 2.00 $\times 10^8$ $M^{-1}s^{-1}$, respectively [18]. The results for tryptophan are in agreement with this study, whereas the rate constant for histidine shows to be a factor of 10 higher than in the current study.

The results for methionine and cysteine are not in agreement with the results obtained in a study performed by Drössler. Here, the fluorescence quenching of riboflavin in aqueous solution by methionine and cysteine was performed. The quenching rate constants of those amino acids at pH $\approx$ 7 were 2.24 $\times 10^9$ $M^{-1}s^{-1}$ and 5.26 $\times 10^8$ $M^{-1}s^{-1}$, respectively [19].

One of the reasons for the deviation of the results may be the use of different time scales in different studies. Most of the experiments, which the obtained results were compared to, used shorter time scale than the current study did. A short time scale may not give the best fitting, hence not completely reliable results.

5.1.1 Amino acids compared to other quenchers

In a study performed by Huvaere and co-authors [20], the triplet-excited riboflavin was found by laser flash photolysis to be quenched by polyunsaturated fatty acid methyl esters in for methyl linoleate tert-butanol/water and for methyl linoleate in acetonitrile/water with rate constants of 3.00 $\times 10^5$ $M^{-1}s^{-1}$ and 3.10 $\times 10^6$ $M^{-1}s^{-1}$, respectively. The study concluded that triplet-excited flavins are preferably quenched by amino acids and proteins. This is because the latter ones have second-order rate constants larger than unsaturated lipids, often with a factor up to 10^4. The different quenching mechanisms are the reason for such kind of large variations. The previous studies show that lipids react by hydrogen atom transfer, while amino acids react by electron transfer, which is a faster reaction than the first one [20].

The quenching of triplet-excited flavin mononucleotide was also recorded for phenols in aqueous buffer mixed with acetonitrille. The second-order rate constant was determined by laser flash photolysis to be 1.60 $\pm 0.5 \times 10^9 M^{-1}s^{-1}$ at pH = 7.0 [21].

5.2 The quenching properties of crocin

In this study carotenoid crocin clearly demonstrated quenching of the triplet excited state of lumiflavin. According to the results from all the measuring methods in this study, crocin is an effective quencher with high quenching rate constants. However, it is not the most efficient among the amino acids tested in this experiment. The quenching

rate constant of crocin is approximately a factor of 10 less than the rate constants of tyrosine and tryptophan. A similar study on the quenching of the riboflavin triplet by crocin was performed by Cardoso and co-workers. However, in their case crocin didn't show any quenching effect in the triplet state of riboflavin, neither did β -carotene nor lycopene. They concluded that all carotenoids perhaps reduced the formation of the triplet-excited riboflavin through an inner-filter effect. In addition carotenoids are efficient quenchers of singlet oxygen, but mainly via the energy transfer in the excited state [22].

Li and co-writers reported another carotenoid, β-carotene, as a very efficient quencher with a quenching rate constant of $5.00 \times 10^9 \mathrm{M}^{-1}\mathrm{s}^{-1}$, and suggested that as the number of double bounds of carotenoids increases, the quenching rate constant of carotenoid increases [23].

5.3 The fitting models

In order to find best suitable concentrations of the quenchers, the kinetic measurements were performed several times for each quencher. Besides the time spent on measurements at the lab, much of the time was spent on figuring out the best way to analyze the data. Different models for fitting the data had to be tried out before the results could be obtained. The models used in this study for fitting the lumiflavin triplet decay with and without quencher were single exponential decay and mixed kinetics. The results show that the fitting of the data to the mixed kinetics was good at low concentrations of the quencher or when it was absent. At higher concentrations of a quencher the fitting to the mixed kinetics was poor and clearly wrong results in the form of negative second-order rate constants. This indicates that mixed kinetics are not suitable at high concentrations of quenchers.

The fitting of the data to a single exponential decay showed to be better at both low and high concentrations of a quencher, but especially at high concentrations. The results for the second-order rate constants came out to be reasonable and in a good agreement with the results obtained by others.

In order to be sure one can neglect the second-order triplet-triplet annihilation process and hence fit the data to the single exponential decay, the concentration of the lumiflavin triplet has to be very low, as it was in this study. However, the triplet-triplet interaction predominates even at relatively low triplet state concentrations. In order to reduce this contribution to such an extent that it may become negligible, requires concentration of the triplet often to be below the detection limit of a conventional laser

flash photolysis setup [13]. The concentration of the lumiflavin triplet used in this experiment has been very low, and a first-order process can then be assumed instead of mixed decay kinetics. However, Vaish and Tollin observed that the lumiflavin triplet did not decay by a single exponential decay except at high concentrations of lumiflavin. At lower concentrations of lumiflavin, the decay followed mixed kinetics [24].

The second-order rate constants derived using the single exponential decay model are strongly supported by the results obtained from the delayed fluorescence method. In this method even lower concentration of the lumiflavin triplet was used. Thus it can be concluded that the single exponential decay model is suitable for fitting the data obtained from the laser flash photolysis when operating with the concentrations of lumiflavin and quenchers in the range used in this experiment.

More than four different concentrations of a quencher would have given more points on the plot of pseudo-first-order rate constants. This would lead to more accurate and reliable second-order rate constants.

5.4 Analysis of the simulated measurements

Although it is known that the triplet-excited lumiflavin follows mixed kinetics, the single exponential decay model was preferred for fitting of the data in this study as described in the previous section. Though the latter model was wrong to use in our study, the results obtained from the simulated decay of lumiflavin were in good agreement with results obtained by delayed fluorescence. This indicates that the single exponential decay model is suitable for fitting the data when working with the range of concentrations used in this study.

5.5 Quenching of lumiflavin triplet by the ground state

In addition to quenching of the lumiflavin triplets by triplet-triplet interaction and by quencher, the quenching may also occur by the ground state. An experiment by Naman and Tegner indicates an efficient quenching of the lumiflavin triplet state by its ground state, Equation 17, where ^{3}S is the triplet state of lumiflavin, G is its ground state and k_3 is the second-order rate constant of triplet quenching by the ground state G [25].

$$^3\mathrm{S} + \mathrm{G} \xrightarrow{k_3} \mathrm{G} + \mathrm{G} \tag{17}$$

Taking into consideration quenching by the ground state G, the rate equation for the decay of the triplet lumiflavin, equation 9, is now written as equation 18

$$\frac{d[^3\mathrm{S}]}{dt} = -k_1[^3\mathrm{S}] - k_2[^3\mathrm{S}]^2 - k_3[\mathrm{G}][^3\mathrm{S}] - k_\mathrm{q}[\mathrm{Q}][^3\mathrm{S}] \tag{18}$$

The evidence of triplet quenching by the ground state was also found in the analysis of lumiflavin triplet-state decay kinetics by Vaish and Tollin. According to their results, the value of the triplet-ground state quenching constant k_3 was quite large. They concluded that the triplet quenching would be quite efficient in those flavoproteins in which two flavin molecules are bound close to each other [24].

The evidences of quenching by the ground state described above were found when the current study was nearing the end and after all measurements were done. Thus, the quenching of the lumiflavin triplet by the ground state was not taken into account in this work. However, delayed fluorscence was run by Heng Li in order to study the contribution of the quenching rate constant k_3 to the lumiflavin triplet decay. The results indicated that $k_3[\mathrm{G}]$ was rather small when a quencher was present and may perhaps be negligible when the quencher concentration is high. The values of k_3 obtained from different studies are summarized in Table 6 along with values for k_1 and k_2. The fact that the rate constants differ from each other, indicates that the absolute correct values are not easy to determine. However, it is now known in what range the values of different rate constant should be.

Author	$k_1(\mathrm{s})^{-1}$	$k_2(\mathrm{M}^{-1}\mathrm{s}^{-1})$	$k_3(\mathrm{M}^{-1}\mathrm{s}^{-1})$
Yoshimura and Kato [26]	$1.5 \pm 0.1 \times 10^3$	not indicated	$7 \pm 0.5 \times 10^8$
Yoshimura and Ohno [27]	$1.4 \pm 0.1 \times 10^3$	$4.5 \pm 1 \times 10^9$	$< 2\times 10^7$
Yoshimura and Fritz [28]	$1.5 \pm 0.1 \times 10^3$	not indicated	$1.2 \pm 0.2 \times 10^8$
Vaish and Tollin [24]	0.67×10^3	8.9×10^8	3.7×10^8
Naman and Tegner [25]	1.46×10^3	$1.8 \pm 0.4 \times 10^9$	2.4×10^8

Table 6: The decay rate constants, some of them with standard deviation, of lumiflavin triplet from different studies, where k_1, k_2 and k_3 are first-order rate constant, rate constant for triplet-triplet quenching process and rate constant for quenching of the triplet by the ground state, respectively.

6 Conclusion

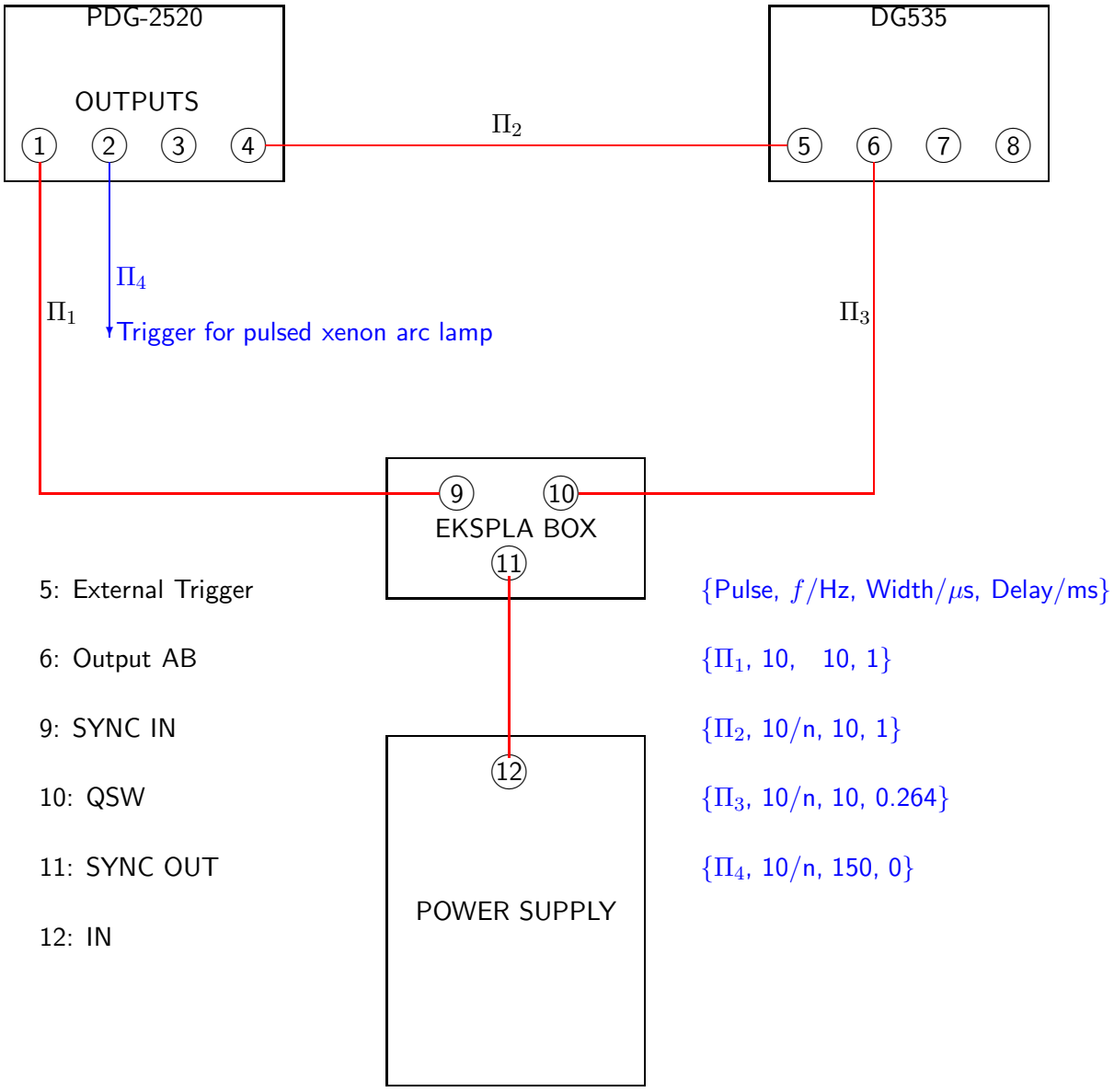
The decay of the lumiflavin triplet with and without a quencher was measured by laser flash photolysis. The decay was found to be accelerated in presence of different amino acids and **C** with decay rates proportional to their concentrations. The decay of the triplet was fitted to both single and mixed exponential decay models in the presence of chosen quenchers. The pseudo-first-order rate constants were obtained from the single and mixed decay kinetics monitored at 296 nm. The second-order, quenching, constants were then determined from the pseudo-first order rate constants for different amino acids and **C** with varying concentrations.

From the results, a broad range of rate constants can be seen for different amino acids and **C**. The obtained rate constants agrees quite well with the results reported from other studies. The amino acids tyrosine and tryptophan came out to be the most efficient quenchers with highest rate constant. This is supported by results from other studies. On the other hand, low second-order rate constants of amino acids histidine and methionine indicate that those are the least effective quenchers, which is supported by results from others as well. Carotenoid **C** seems to be an intermediate quencher, with a quenching rate constant between the lowest and the highest rate constants for the amino acids studied in this work. More measurements have to be done in order to be able to conclude properly concerning the quenching properties of **C**.

The analysis of the lumiflavin triplet decay showed that neither of single exponential decay nor mixed decay suits perfectly for fitting the data. However, the former model came out to give the best fit and reliable results. After performing the simulation method, it may be concluded that the single exponential decay model can be used for fitting the decay of the triplet with a quencher, whose concetration is within the concentration range used in this study.

Further studies should be carried out in order to be able to evaluate the decay rate constants of lumiflavin triplet by different pathways, as well as quenching rate constants of different quenchers.

A Appendix 1: Pulse sequence for single-wavelength kinetics



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