

**MULTIVARIATE ANALYSIS
OF THE FLUORESCENCE DECAY
OF 3-HYDROXYFLAVONE
IN VARIOUS SOLVENTS**

by

Douglas Y. DeSario

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Chemistry with Distinction.

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ABSTRACT

The photokinetic parameters of the fluorescence decay of 3-hydroxyflavone were determined in six solvents, which were chosen for their widely differing polarities, dielectric constants, and hydrogen bonding capabilities. This was accomplished by the separation of three-dimensional time-resolved emission decay matrices through multivariate analysis. Particular attention was paid to the dual fluorescence of the main conformers of 3-hydroxyflavone – the normal and the tautomer – which forms the basis of the use of this molecule, and its derivatives, as fluorescent probes of solvent properties. The normal fluorescence was found to occur strongly only in solvents with the capability to hydrogen bond with the 3-hydroxyl group, thereby slowing the Excited State Intramolecular Proton Transfer (ESIPT) reaction that, unhindered, partially relaxes the normal excited state to the tautomer excited state. The photokinetic parameters that were calculated were the average excited state lifetimes, their initial intensities, and matrices of rate constants that quantified both the decay of each conformer of 3-hydroxyflavone in the excited state, and also the influences of each conformer on the others' fluorescence. These parameters, as well as the λ_{max} of the individual conformers, compared favorably to previous studies of this molecule.

Chapter 1

INTRODUCTION

1.1 The Significance of 3-Hydroxyflavone

3-Hydroxyflavone is one of the most fundamental members of the class of molecules known as the flavonoids. This group of natural and synthetic compounds – comprised of anywhere between 3000 and 6500 distinct molecules, depending on the author (Rijke) – is known for its importance in plant signaling and pathogenesis, as well as its antioxidant properties and antimicrobial activities (Rijke). Most importantly for the purposes of this project is the unusual dependence of the fluorescence of flavonoids, and 3-hydroxyflavone in particular, on solvent properties.

1.1.1 Usefulness as a Probe of Solvation Effects

Solvent properties that influence the solvation, conformation, or behavior of dissolved molecules can affect the solute in a number of observable ways. The mechanisms by which solvents act on solute molecules can be classified as either specific or nonspecific solvation effects. Specific solvation effects are the result of interactions such as hydrogen bonding; in other words, molecule-to-molecule attractions or repulsions. Models of specific solvation effects treat the solvent as a collection of individual molecules. In contrast, nonspecific solvation effects, such as dielectric constant, treat the solvent as a uniform continuum with which the solute molecules interact.

Both types of solvation effects can have a dramatic influence on the fluorescence spectrum and decay of 3-hydroxyflavone and its derivatives; for example, the observed emission maxima can be red- or blue-shifted depending on the solvent. Numerous attempts to discern the nature and mechanism of this influence have been made. The conclusions that have been reached revolve around the two main conformations of 3-hydroxyflavone, labeled the normal and the tautomer, which interconvert in the excited state through an excited state intramolecular proton transfer (ESIPT) reaction, depicted in Figure 1.1.

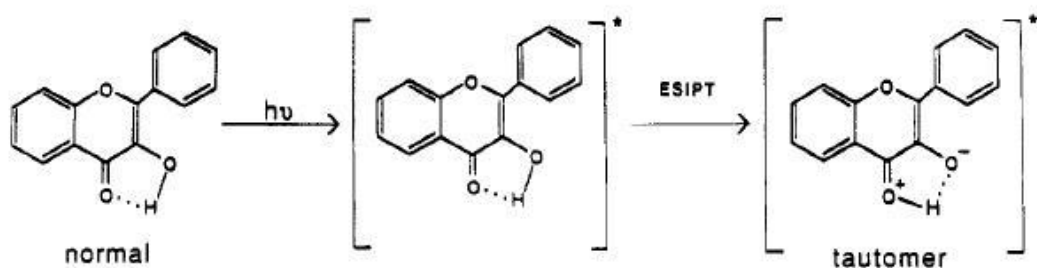


Figure 1.1 The ESIPT reaction of 3-hydroxyflavone (Brucker)

The unique electronics of 3-hydroxyflavone are what sets it apart as a fluorescence probe. The existence of multiple excited-state conformers is not in itself particularly remarkable; however, a look at an energy diagram of these conformers in the ground and excited state shows that, while the normal conformer possesses the lowest energy in the ground state, the tautomer is at a lower energy level in the excited state. This unusual occurrence is the key to what makes 3-HF such an effective probe

of solvent properties, as we will see shortly. A simplified energy diagram is depicted in Figure 1.2.

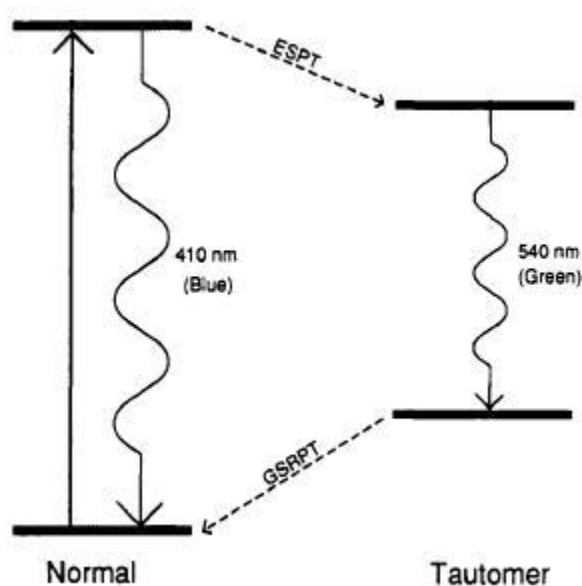


Figure 1.2 An energy diagram of the normal and the tautomer conformers of 3-hydroxyflavone
(modified from Schwartz)

As a result of this divide in the energy diagram, the fluorescence spectrum of 3-hydroxyflavone is split into two distinct bands. Due to the relative organization of the energy levels shown in the preceding image, the relaxation of the normal excited state (N^*) to the normal ground state (N) represents a greater loss of energy than the relaxation of the tautomer excited state (T^*) to the tautomer ground state (T). This means that a photon generated from the relaxation of the N^* state will be carrying more energy than a photon from the fluorescence of T^* , and therefore the wavelengths will be very different. On average, the fluorescence of the normal conformer is

observed at 410 nm, whereas the fluorescence of the tautomer results in a photon of roughly 540nm.

While interesting, this still does not answer the question of why 3-hydroxyflavone can be used as a probe of solvation effects. The answer is that the absorption maximum, the relative sizes of the two fluorescence peaks, as well as the wavelengths at which the two conformers most strongly fluoresce (λ_{max}), have all been found to be directly dependent on various solvent properties such as polarity, hydrogen bonding donor capability, and electronic polarizability (Klymchenko, Methods in Enzymology). In general, any interaction that slows down the conversion of the N* to the T*, through inhibition of the ESIPT reaction, will result in a greater amount of normal fluorescence, whereas the lack of such interactions will result in nearly no normal fluorescence, as ESIPT is far faster ($\sim 10^{-13}$ s) than fluorescence ($\sim 10^{-7}$ s). The dependence is both quantifiable and sensitive – and in many cases has been found to be linear – which means that these fluorescence characteristics can be used as indicators of solvent properties. Thus far, only these four parameters - ν_{abs} , ν_{N^*} , ν_{T^*} , and $I_{\text{N}^*}/I_{\text{T}^*}$ - have been used to quantify solvation effects, and little attention has been paid to the fluorescence decay of 3-hydroxyflavone and its dependence on the solvent. Therefore, an analysis of the fluorescence decay of the molecule could discover additional methods for utilizing 3-HF probes beyond those that have already been reported, by adding photokinetics to the list of factors capable of being used to quantify a solvent or microenvironment. More practically, knowledge of the photokinetics of the molecule would be useful to facilitate descriptions of its behavior in the excited state in various environments, information that can be put to use in a variety of areas.

1.1.2 Potential Application in Organic Solar Cells

As stated above, flavonoids are natural products present in plants as, among other things, radical scavengers and anti-oxidizing agents. They serve some of the same purposes in human diets as well (Rijke). This has led some to consider the possibility of incorporating some type of flavonoid into new types of organic solar cells, which, among other problems, have experienced difficulty coping with the issue of photodegradation. Plants have adapted to a life in the sun by producing a wide variety of radical scavengers to protect themselves, so it comes as little surprise that organic solar cells would require the same sort of protection. While at the time of this writing, the primary interest in 3-hydroxyflavone and its derivatives is its use as a fluorescent probe, its potential application to future organic photovoltaics should not be dismissed, and any further detailed knowledge into the excited state conformations and electronics of this class of molecules would be beneficial to the genesis of such research.

1.2 Traditional Fluorescence Decay Analysis Methodologies

The most widely used methods of fluorescence decay analysis are currently based around Global Analysis. First described by Knutson, Global Analysis determines a set of monoexponential decays which are added together in varying proportions - through the manipulation of the pre-exponential factors - to construct the decays of the individual components that make up a fluorescence decay profile. There are also a number of other methods in use for analyzing time resolved emission spectra, such as Spectral Reconstruction (Das) or TRANES (Periasamy), which differ from Global Analysis in that they break down the spectra into a series of wavelength intervals, instead of treating the entire spectrum as a whole. Despite this difference,

both of these approaches rely on iteratively fitting the fluorescence decays to a series of monoexponential factors.

There are a number of issues with these traditional approaches. First of all, to simplify the system, some previous research describes methods to trap the excited states of 3HF by, for example, freezing the solution to 77K (Strandjord, 1983). This method is not generally employed in recent work, but there have been efforts to synthesize derivatives of 3HF that exclude certain interactions, simplifying the fluorescence (Klymchenko, 2003) and making the key features easier to isolate.

Additionally, analyzing the spectral decays as small slices of the overall spectrum decaying independently of each other, as TRANES and Spectral Reconstruction do, is based on a large, and often incorrect, assumption, namely that such slices are independent in the first place. Particularly in instances of complicated decays comprised of multiple components that are directly interacting and interconverting, such as in the case of 3HF, it is often found that different aspects of the fluorescence decay influence each other. If the decay is analyzed as a series of discrete chunks, rather than as the entire spectrum decaying together, it is possible to come to incorrect conclusions about the rates of decays of various components. At the very least, a large quantity of information describing how the molecule behaves in the excited state will be lost by dividing the analysis in this manner.

Lastly, iterative fits to a multiexponential function with more than three monoexponential factors have been mathematically proven to be unreliable and prone to error (Istratov, Lanczos). The reason for this is that it is difficult or impossible to resolve more than three or four components using models based on iteratively fitting experimental data to a series of exponential functions, as three monoexponential

decays fit almost any decay profile within experimental error to a high degree of accuracy. This results in the loss of details of the fluorescence, and indeed the inaccurate determination or elimination of entire components. Because all of the methodologies discussed so far use iterative fitting, this is the main improvement that multivariate analysis brings to fluorescence decay analysis. An example of the ability of a three component multiexponential decay to describe a five component system is given in Appendix A.

1.3 Benefits of Multivariate Data Analysis

In contrast to the traditional methods outlined above, a multivariate analysis determines spectra and decays by taking the entire three-dimensional fluorescence decay spectrum – what will be referred to as an Emission Decay Matrix – into consideration. By doing so, it avoids the assumptions of traditional methodologies; instead of fitting the data to a set model, multivariate analysis allows the data to construct the model. This allows multivariate analysis to detect components that traditional methodologies would overlook, due to the complexity of the multiexponential decay, provided the fundamental decays of the components are significantly different from each other. The matrix algebra that is central to the multivariate techniques used in this research will be discussed in detail in Data Analysis, section 2.3.

1.4 Objective of Research

The objective of this thesis project was to perform a multivariate photokinetic analysis on the fluorescence decays of 3-hydroxyflavone in various solvents, in order to determine the species-associated spectra and decays of the various

conformers of the molecule, and to note their dependence on solvation effects. The species-associated decays were then used to determine such descriptive values as the average lifetimes of the different excited states. A secondary objective was to look for any additional components contributing to the fluorescence of 3HF that had not been previously described by other methods.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

3-Hydroxyflavone was used as received (99%, Aldrich Chemical Company, Milwaukee, WI). Solvents used were of the highest purity available. Stock solutions were prepared in cyclohexane (certified ACS spectranalyzed, Fisher Scientific, Pittsburgh, PA), acetonitrile (HPLC grade, Fisher Scientific), methanol (HPLC grade, Fisher Scientific), DMSO (ACS certified, Fisher Scientific), cyclohexanol (ReagentPlus, 99%, Sigma Aldrich), and toluene (ACS certified, Fisher Scientific), using sonication and gentle heating to aid in dissolution when necessary. Stock solutions were diluted to 5.0 μM for all trials. 1.0 μM Perylene (99.5%+ sublimed, Adlrich Chemical Company, Milwaukee, WI) was used as a standard in ethanol (ACS/USP grade, 200 proof, Pharmco, Brookfield, CT). All stock solutions were prepared fresh and used within a week, all sample solutions were prepared and used the same day.

2.2 Instrumentation

The source used is a mode-locked Ti:sapphire laser (Mira 900-F, Coherent, Santa Clara, CA) pumped by a 10 W Nd:YVO₄ frequency doubled laser (Millenia X, Spectra Physics, Mountain View, CA). A beam of radiation exits the cavity pulses at a rate of 80 MHz, or once every 12.5 ns, regulated by a frequency

stabilization system (Mira Synchro-Lock, Coherent, Santa Clara, CA). This is too fast to observe the entire emission decay, so the repetition rate is reduced to 5 MHz – one every 200 ns – by a cavity dumper (Model 9200, Coherent, Santa Clara, CA). To synchronize the excitation pulse to the detector, a beam splitter deflects a small fraction of the beam of radiation to a triggering unit (OCF-401-1, Becker & Hickl GmbH, Berlin, Germany). The wavelength of the excitation beam was then halved to 363 nm using a harmonic generator (TP-1B fs Tripler, Uniwave Technology, Chatsworth, CA). The strength of this beam was measured at 20 mW. A Soliel-Babinet compensator (RC-10, Optics for Research, Caldwell, NF) sets the polarization of the excitation beam to vertical. The sample fluorescence is collected 90° to the excitation beam, through a filter chosen to remove 90%+ of all photons below 380 nm, to eliminate the majority of the scattered excitation photons from the response. The sample fluorescence was not collected through a polarizer, as previous works indicate that anisotropic effects on the fluorescence intensity of free 3-Hydroxyflavone in solution are negligible (Chaudhuri, Guharay). The sample emission is spatially dispersed according to wavelength by a grating (150 groove/mm, blazed at 450 nm) spectrograph (HR320, Jobin Yvon Horiba, Metuchen, NJ). The dispersed sample emission is collected by a microchannel plate image intensifier (High Rate Intensifier, Kentech Instruments Ltd., South Moreton, U.K.), connected to the previously mentioned optical triggering unit and coupled to a CCD (charge-coupled device) camera unit (Picostar HR 12, Lavision GmbH, Goettingen, Germany). All images were acquired within a period of 30 ns, in 0.1 ns intervals, under the control of a picoseconds Delay Module (DEL-150, Becker and Hickl GmbH, Berlin, Germany), and stored on the same computer that also controls the data acquisition system.

UV/Vis absorbance spectra of 3-Hydroxyflavone in various solvents were acquired using a diode array spectrophotometer (Hewlett-Packard/Agilent Technologies, Model 8452A, Palo Alto, CA).

2.2.1 Data Acquisition

The three dimensional – wavelength versus “laboratory z-axis” versus photon intensity – images collected by the CCD at each time interval are integrated along the z-axis to obtain emission spectra at each time slice. These spectra are then collected and stored together in a single two dimensional 1376x300 matrix, which is then analyzed according to the procedure described in section 2.3. All image manipulation and data analysis routines described below were developed in house in the MATLAB programming environment (The Mathworks, Natick, MA). All samples were agitated with a stir bar during data collection to prevent photobleaching.

2.3 Data Analysis

The aforementioned 1376x300 matrix is a convenient format of storing large time-resolved emission decay matrices (TR-EDM). The range of wavelengths observed varies slightly among the replicate measurements. Data are acquired at intervals of roughly 0.3 nm. Each wavelength slice is then stored in a single row element in the matrix. Conversely, each time slice of 0.1 ns is stored in a unique column. The value of the matrix at any row i and column j is the response: the summed intensity of light collected by the CCD at that particular wavelength and time. Each TR-EDM, labeled matrix $\mathbf{D}$, is a product of the species-associated spectra and decays $\mathbf{X}$ and $\mathbf{Y}$, with a noise matrix $\mathbf{E}$ superimposed:

$$\mathbf{D} = \mathbf{XY}^T + \mathbf{E} \quad 2.1$$

Multivariate analysis is a method of splitting the matrix $\mathbf{D}$ into $\mathbf{X}$ and $\mathbf{Y}$, in addition to determining the photokinetic parameters – average lifetimes, decay rates, etc – that describe the decays of each component in $\mathbf{Y}$.

2.3.1 Determination of the Number of Components

The first task is to determine the number of important components, N , that contribute to the overall TR-EDM. In other words, how many individual shapes are needed to define the entire shape of the spectral information present in $\mathbf{D}$ adequately? To answer this question, it is necessary to factor the matrix into a series of column and row factors. The singular value decomposition (SVD) was chosen as the method to perform this task, as it determines the smallest set of column and row factors that describe the majority of the variance of the data matrix. These column and row factors are ordered in terms of decreasing significance; the first singular vectors describe the largest fraction of the variance of the matrix, and hence the largest portion of the spectral response. The SVD converts any matrix to the product of three orthogonal factors:

$$\mathbf{D} = \sum_{p=1}^P \sigma_{pp} \mathbf{u}_{\cdot p} \mathbf{v}'_{\cdot p} = \mathbf{U} \mathbf{\Sigma} \mathbf{V} \quad 2.2$$

where P is the smaller of the two dimensions of $\mathbf{D}$, the matrix rank, in this case 300. The number of factors in $\mathbf{U}$, $\mathbf{\Sigma}$, and $\mathbf{V}$ used to completely describe $\mathbf{D}$ is therefore equal to P . However, we are interested in only describing the major spectral features of $\mathbf{D}$, which are mostly contained in the first N singular vectors determined through SVD.

In order to determine N – which is called the pseudorank – it is necessary to measure the relative importance of each of these 300 factors, and determine how many are required to describe the sample emission in $\mathbf{D}$. For this analysis, this was

accomplished in two ways: by comparison of the magnitudes of the factors in $\mathbf{\Sigma}$, the matrix that stores the intensity data of $\mathbf{D}$, as well as a comparison of the frequency content of each component in $\mathbf{U}$. The assumption made is that spectral components will be both larger and have much lower frequency content than noise components, which are defined by being random and small. Multiplying the first N components of $\mathbf{U}$, $\mathbf{\Sigma}$, and $\mathbf{V}$ together in the manner of equation 2.2 outputs a new $\mathbf{D}$ matrix composed primarily of spectral information, removing the majority of the contribution of the noise matrix $\mathbf{E}$ to $\mathbf{D}$.

Note that $\mathbf{U}$ and $\mathbf{V}$ contain spectral and temporal information, but they do not directly represent the species-associated spectra and decays of the components. It is possible for an experienced researcher to look at $\mathbf{U}$ and $\mathbf{V}$ and infer useful information directly from their plots; however, as they are not easily interpreted, and because they are not directly used to determine the photokinetic parameters of the fluorescence decays, these plots are contained in Appendix B.

2.3.2 Factorization by Photokinetic Matrix Decomposition

There are an infinite number of basis sets that can be used to reconstruct any given matrix, of which $\mathbf{U}$, $\mathbf{\Sigma}$, and $\mathbf{V}$ only represent one such set. While these three factors are very useful in quickly determining the number of components present in a TR-EDM and reducing the amount of high frequency noise present in $\mathbf{D}$, the information they contain is more often than not difficult to interpret directly, and they rarely describe the true fluorescence spectra of the sample components. It is therefore beneficial to extract more informative basis sets for the continued analysis of $\mathbf{D}$.

The decomposition, called Photokinetic Matrix Decomposition, was developed in-house specifically to

factor first order TR-EDMs into combinations of dynamically linked spectra and fundamental (monoexponential) decays:

$$\mathbf{D} \approx \sum_{n=1}^N \gamma_{nn} \mathbf{s}_{\bullet n} \mathbf{z}'_{\bullet n} = \mathbf{S} \mathbf{\Gamma} \mathbf{Z}^T \quad 2.3$$

where the columns of $\mathbf{S}$ and $\mathbf{Z}$ are photokinetically informative factors of the columns and rows of $\mathbf{D}$, respectively, and $\mathbf{\Gamma}$ is a diagonal matrix of relative intensity factors. (Rowe)

The matrix factors acquired using this decomposition, $\mathbf{S}$ and $\mathbf{Z}$, respectively, are more easily interpreted because they are physically relevant. Each column of $\mathbf{S}$ is a combination of spectra that decays at the rates defined by the corresponding monoexponential factor (given in the column of $\mathbf{Z}$). The columns of $\mathbf{S}$ have been called the “decay-associated spectra”. The components of $\mathbf{S}$ are distinguished not by what is fluorescing, as the component (species-associated) spectra are, but by the rate at which the fluorescence of that particular component is decaying. The columns of $\mathbf{Z}$, which are called fundamental decays, are the monoexponential decays that the components of $\mathbf{S}$ are following. These are not component spectra and decays yet, because more than one process, column of $\mathbf{Z}$, may impact the decay of an individual sample component. For example, a component of $\mathbf{S}$ may have spectral contributions from two excited states with opposite signs if one excited state is decaying while the other is being produced at the rate defined by the corresponding column of $\mathbf{Z}$. Consequently, this component of $\mathbf{Z}$ will contribute to the decays of both excited states. It should be noted that, unlike $\mathbf{U}$ and $\mathbf{V}$, $\mathbf{S}$ and $\mathbf{Z}$ are correlated, not orthonormal.

It may at first be difficult to recognize why PMD improves upon methods utilizing iterative fit optimizations, particularly without a detailed look at the mathematics of the algorithm. The reason is that PMD is built around the “self-similarity” of monoexponential decays. The area under an exponential curve in an

interval under which the value of y decreases by an amount Δy is equal to any other interval in which the value of y also decreases by the same Δy . This property allows PMD to construct and solve a generalized eigenvalue problem, the solutions to which are the rates of the fundamental decays. These solutions are unique, as are those found through SVD, which distinguishes the vectors found through PMD from the infinite number of possible basis vectors. PMD therefore avoids standard optimization techniques revolving around nonlinear regression of exponential curves, and can properly separate a high-order multiexponential decay into its fundamental monoexponential decays.

2.3.3 Determination of Species-Associated Spectra

The conversion from the decay-associated spectra to the species-associated spectra is a matter of inferring how the decay-associated spectra interact by making a few basic assumptions about the behavior of the species-associated spectra. These interactions between the components of $\mathbf{S}$ are reflected in the transformation matrix $\hat{\Gamma}$ and its inverse in the equations:

$$\hat{\mathbf{X}} = \mathbf{S}\hat{\Gamma} \quad 2.4$$

$$\hat{\mathbf{Y}} = \mathbf{Z}\hat{\Gamma}^{-1} \quad 2.5$$

where $\hat{\mathbf{X}}$ and $\hat{\mathbf{Y}}$ are the experimental estimates of $\mathbf{X}$ and $\mathbf{Y}$. The elements of $\hat{\Gamma}$ are varied following the criteria below to approximate $\mathbf{X}$ and $\mathbf{Y}$.

First of all, unlike $\mathbf{S}$ and $\mathbf{Z}$, $\mathbf{X}$ and $\mathbf{Y}$ should not be below zero at any point, except for residual measurement error. A component displaying negative intensity makes no physical sense; therefore, it is necessary to combine the spectra in such a manner that all components are equal to or above zero at all wavelengths and times.

As already mentioned, spectra are generally considered to have low frequency content, so any combinations that drastically increases the frequency content of a smooth component is avoided if possible. Lastly, individual component spectra are usually unimodal. If a spectrum is seen to fluoresce in two distinct bands, or if it shows shoulders or other deformities, it is possible that it contains elements of other components that still need to be removed. These criteria are applied, with their relative order of importance equivalent to the order that they are described above, and $\hat{\Pi}$ is altered until satisfactory values for $\hat{\mathbf{X}}$ and $\hat{\mathbf{Y}}$ have been determined.

In the final step of the analysis, the photokinetic rates, the average lifetimes, and the initial intensities of the components of $\hat{\mathbf{Y}}$ are determined using the following equations:

$$\hat{\mathbf{W}} = \hat{\Pi}^{-1} \quad 2.6$$

$$\mathbf{K} = \hat{\mathbf{W}}\mathbf{\Lambda}\hat{\mathbf{W}}^+ \quad 2.7$$

$$\mathbf{y}_0 = \hat{\mathbf{W}}\boldsymbol{\gamma} \quad 2.8$$

where $\hat{\mathbf{W}}$ is the transfer (rate) eigenvector matrix, $\mathbf{\Lambda}$ is the transfer (rate) eigenvalue matrix, $\boldsymbol{\gamma}$ is a vector of intensity factors of the components, $\mathbf{K}$ is the transfer matrix containing the photokinetic rates, and $\mathbf{y}_0$ is a vector containing the initial intensities of the components. The average lifetimes of the components are found by taking the inverse of the diagonal elements of $\mathbf{K}$. The diagonals of the photokinetic rate matrix $\mathbf{K}$ contain the rates of the individual components. The off-diagonals contain the interaction terms, which represent how each component affects the rate of decay of the other components. More rigorously, if the rate of decay of the intensity of the fluorescence follows the following equation:

$$\frac{d\hat{\mathbf{Y}}}{dt} = \mathbf{K}\hat{\mathbf{Y}} \quad 2.9$$

then the rate of decay of the components of the species-associated spectra at time j is determined through the operation:

$$\frac{d}{dt} \begin{bmatrix} \hat{\mathbf{y}}_{1j} \\ \hat{\mathbf{y}}_{2j} \end{bmatrix} = \begin{bmatrix} k_{11} & k_{12} \\ k_{21} & k_{22} \end{bmatrix} \begin{bmatrix} \hat{\mathbf{y}}_{1j} \\ \hat{\mathbf{y}}_{2j} \end{bmatrix} = \begin{bmatrix} k_{11}\hat{\mathbf{y}}_{1j} + k_{12}\hat{\mathbf{y}}_{2j} \\ k_{21}\hat{\mathbf{y}}_{1j} + k_{22}\hat{\mathbf{y}}_{2j} \end{bmatrix} \quad 2.10$$

This means that the rate of decay of each component is not simply equal to the rate constant of a single component multiplied by the concentration, but that it is dependent on all other components as well. The degree to which the decay profile of each component depends on the others, and the direction of each influence, is contained in the $\mathbf{K}$ matrix. Negative values represent downward trends; any interaction terms that are negative can be interpreted as instances where one component is speeding up the decay of another. Conversely, if an interaction term is positive, that means that one conformer is slowing down or inhibiting the decay of another. The photokinetic rates and average lifetimes are most accurate and easily interpreted method of comparison between this analysis and all previous work on the fluorescence decay of 3-hydroxyflavone.

Chapter 3

RESULTS AND DISCUSSION

3.1 UV/Vis Absorbance

The first tests that were performed in this project were UV/Vis absorbance measurements of 3-hydroxyflavone, to check the wavelengths at which the molecule would fluoresce most readily and strongly. The UV/Vis absorbance data are shown in Figure 3.1 for 3-hydroxyflavone in a number of solvents. The molecule was found to have an absorbance peak of around 345 nm, in accordance with all previous works.

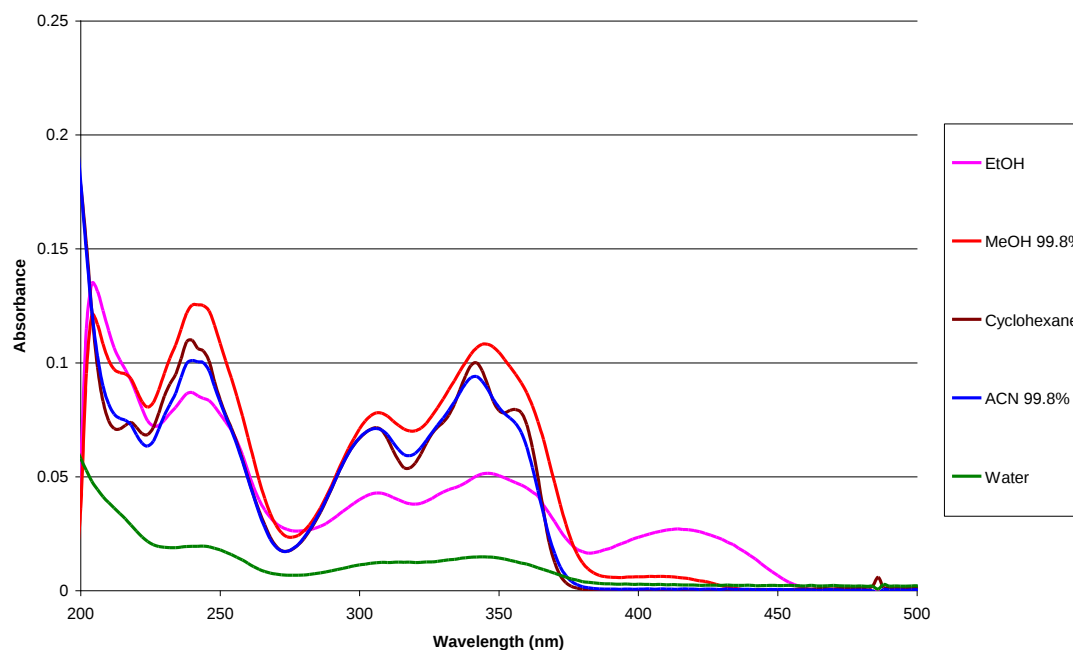


Figure 3.1 Representative UV/Vis spectra of 5×10^{-6} M 3-hydroxyflavone

3.2 Choice of Excitation Wavelength

Though the above data infer that 3-hydroxyflavone is more excited by photons of around 350 nm, due to the design of the instrument, adjustments to the excitation wavelength used were necessary. The reflectivity of the optics used in the Mira laser limits the tunable region of the laser, which is broken up into a number of discrete regions. The maximum intensity of the laser is greatest in the center of these regions, and drops precipitously towards the edges. The only way for this instrument to reach an excitation wavelength of 350 nm is to set the Ti:sapphire to 700 nm, and then double the frequency of the light using the harmonic generator. Unfortunately, 700 nm

is directly on the lower edge of one of the tunable regions of the laser, which means that the closer the excitation pulse gets to the optimal value of 350 nm, the weaker the pulse becomes. This trade-off resulted in settling on an excitation wavelength of around 363 nm, but as previous studies and the absorbance data shown in Figure 3.1 show, 3-HF absorbs only a small fraction of this wavelength compared to 350 nm.

3.2.1 Calibration of the Spectrograph

Before and after every solvent's set of trials, the excitation wavelength of the laser was checked using the common fluorescence probe perylene. This was to ensure that any change in the position of the grating spectrograph was accounted for in the data. The spectrograph can be adjusted to collect at widely different wavelengths; because of this, the individual pixels collected by the CCD do not have a fixed wavelength value. The conversion from pixels to wavelength must be performed for each EDM, using the twin peaks of perylene as a wavelength standard. Below is an example of a fluorescence spectrum of perylene.

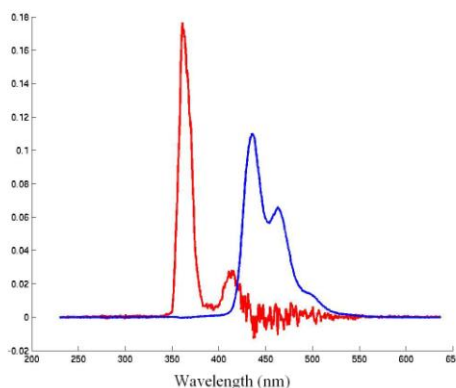


Figure 3.2 Normalized fluorescence spectrum of 1 μM perylene in ethanol

These spectra are separated in a much simpler manner than the 3-HF trials, as there is no need to dissect its decay, and the spectra are generally uncomplicated. The red line is the excitation pulse, along with a bit of Raman scattering, and the blue line is the fluorescence spectrum of perylene. The λ_{max} values of perylene in ethanol are well known, so it was possible to use the distance between them and their absolute values in pixels to fit this spectrum to the proper wavelength scale. Perylene is also used to check the optics and decay acquisition for any obvious errors before beginning data collection.

3.3 Number of Separable Components in TR-EDM

The number of separable components present in the time-resolved emission decay matrices of 3-hydroxyflavone in each of the six investigated solvents were determined using SVD, as described above. The plots used to determine the numbers of components in each EDM are shown in Appendix B. Note the frequency content histogram plots in each group of four figures. The point at which the frequency content dramatically jumps is taken to be the boundary between components containing mostly signal versus noise. The numbers of components are collected in Table 3.1.

Table 3.1 Number of components in the EDM of 3-HF

Solvent	Cyclohexane	Toluene	DMSO	CAN	Cyclohexanol	Methanol
N	2	2	2	2	3	3

Note that the TR-EDMs for each solvent include an excitation peak caused by scattering off the cuvette and the solvent, and in ideal cases the excitation peak is a

unique component in the EDM. It was included in Table 3.1 because the excitation peak was found to be included with some part of the fluorescence spectrum in many solvents. The hydrogen-bonding solvents clearly lead to more fluorescence components than the other solvents, including what amounts to an additional component in the fluorescence. Methanol actually showed 4 components originally, but after the analysis it became clear that the third component was a background noise component, and it has been omitted from all subsequent figures.

3.4 Species-Associated Spectra and Decays

Plots of the decay-associated spectra, **S** and **Z**, can be found in Appendix C. While they are important for the overall analysis, and the information they contain concerning the fundamental decays of the components is at times more useful and physically relevant than the species-associated spectra, they can be difficult to interpret visually when one is more accustomed to viewing species-associated spectra. The plots of the species-associated spectra and decays – $\hat{\mathbf{X}}$ and $\hat{\mathbf{Y}}$ – for each solvent are given in Figures 3.3 and 3.4. In all of the following figures, the different colors represent the spectral components; in sequential order, they are blue, green, red, and cyan. The third component in methanol was omitted from the plot of its fluorescence spectrum as it was pure noise and obscured the important features of the figure. It was left in the decay profile to provide further evidence of the lack of spectral information in this component; it is almost a perfect baseline. Note that in all solvents, when assigning the identities of fluorescence components, all prior work supports the normal fluorescence as, on average, 410 nm, while the tautomer fluorescence is at roughly 540 nm. There is as well a third common form of the molecules, the anion, whose formation will be

discussed shortly, which tends to fluoresce between the normal and tautomer, around 450 nm.

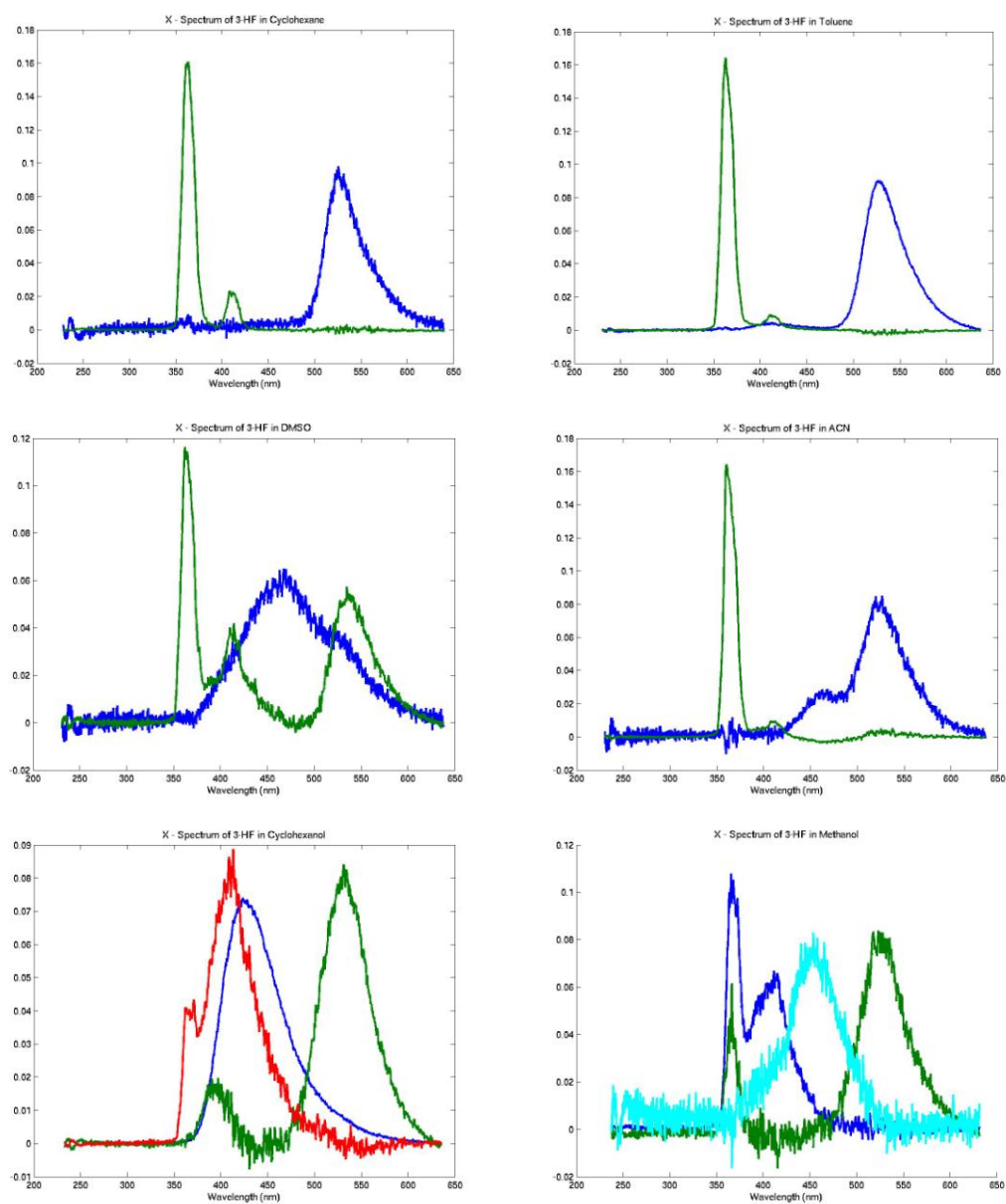


Figure 3.3 Normalized fluorescence spectra of $5 \cdot 10^{-6}$ M 3-HF in various solvents. Order of solvents from top left: cyclohexane, toluene, DMSO, ACN, cyclohexanol, methanol

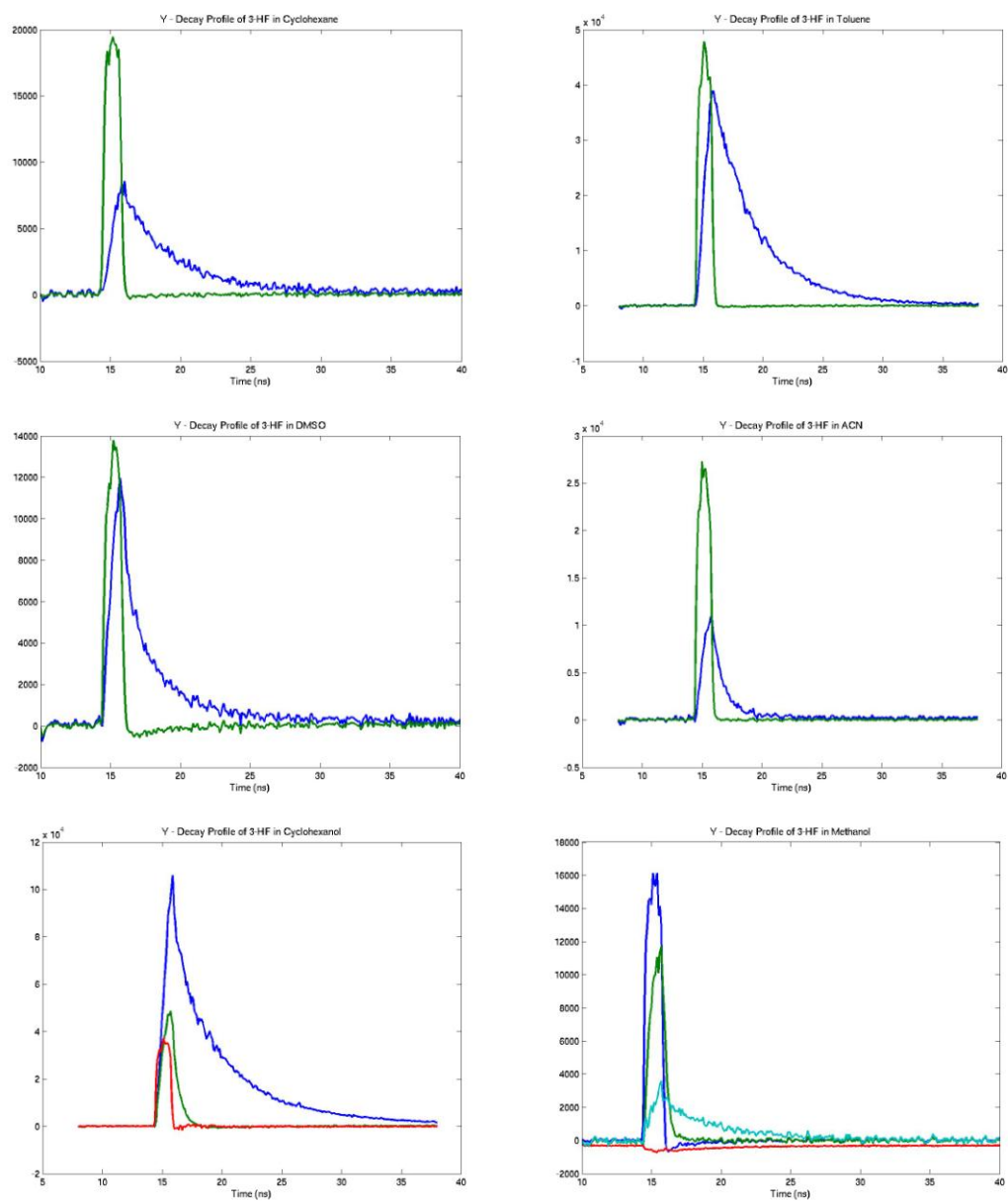


Figure 3.4 Decay profiles of 5×10^{-6} M 3-HF in various solvents. Order of solvents from top left: cyclohexane, toluene, DMSO, ACN, cyclohexanol, methanol

Clearly, the nonpolar, non-hydrogen bonding solvents (cyclohexane and toluene) have the simplest fluorescence spectra and decays of the solvents investigated. There is a clear distinction between the large tautomer band – expected from previous analyses of 3-HF – and the second component, which includes a sharp excitation peak and what could be either a small Raman scattering peak or a tiny fluorescence attributed to the normal conformer. For the remainder of the paper, it will be assumed that there is some normal conformer in the second component of these two solvents. This assumption is made because there is no physical process directly inhibiting the fluorescence of the normal conformer, only that it is much slower than the ESIPT reaction in nonpolar, non-hydrogen bonding solvents. Therefore, there should be a small amount of normal fluorescence present; whether or not it would be too small to be detectable is a different question entirely. Note how the decay profile of the second component drops vertically after the maximum, with no sign of any exponential contribution. This suggests that either there is no detectable normal conformer fluorescence in the second component, or that any excited state normal conformer that is present decays so quickly that it is inseparable from the excitation pulse.

The polar aprotic solvents are next: acetonitrile and dimethyl sulfoxide. As has been reported elsewhere (Protti), 3-hydroxyflavone in DMSO readily converts to the anionic form of the molecule, as shown in Figure 3.5.

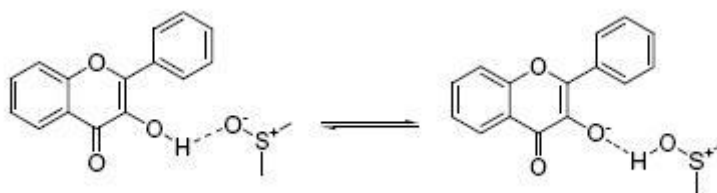


Figure 3.5 The formation of the anion of 3-hydroxyflavone in DMSO (Protti)

This leads to the broad fluorescence band located between the normal and tautomer, which are both visible in the spectrum but relegated to the second component, along with the excitation. This can be seen clearly in the decay profile of DMSO; the profile of the second component dips below the x-axis and then rises to zero following an exponential curve, indicating that there are fluorescing conformers in that component. It is not possible to enforce the non-negativity criterion on the second component of the DMSO decay, because it clearly includes multiple components. Sign values of calculated components are arbitrary until the final calculation of the species-associated spectra and decays, which means that when an individual fluorescence component contains multiple “true” components, it is possible that one or more of them will have opposite signs. If these components were separated, they would be flipped over the wavelength axis, but since they are grouped together, this is impossible. Therefore, the decay profile of DMSO is allowed to have negative components, with the understanding that this is a reflection of the incomplete separation of fluorescence components.

In contrast, the final acetonitrile has a much simpler spectrum than DMSO, appearing similar to toluene with the addition of a small anionic peak. This difference between the two otherwise similar (polar aprotic) solvents arises from the

fact that DMSO is both highly polar and a strong hydrogen bond accepting solvent, but acetonitrile is simply a highly polar molecule. It can be concluded that hydrogen bonding capability is far more influential than polarity on the fluorescence of 3-hydroxyflavone, particularly when looking at the intensity of the normal fluorescence.

Lastly, we come to the polar, protic, hydrogen bond donating solvents: cyclohexanol and methanol. The first component of cyclohexanol most likely is a result of the normal conformer, as it appears to be too blue-shifted to be a result of the anion, but the possibility exists for it to contain the anion as well. Judging from the work of Protti, the spectrum should contain a contribution from the anion. As shown in Figure 3.6, methanol aids the formation of the anionic conformer of 3-hydroxyflavone similarly to DMSO, and there is no obvious reason why this same reaction should not take place in other alcohols, including cyclohexanol.

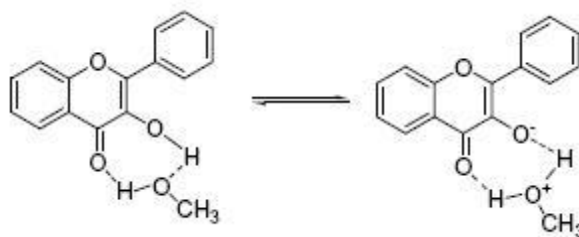


Figure 3.6 The formation of the anion of 3-hydroxyflavone in methanol (Protti)

It appears that the normal conformer has a strong presence in all three components, which could be a result of the viscosity of cyclohexanol. This shifting behavior results in the interpretation of the fluorescence of the normal conformer as

having a pseudorank greater than 1, which means that it is spread across multiple components. Note the similarity, too, between the spectrum of methanol and that of DMSO, save the fact that in methanol, the contributions of the normal and tautomer species were successfully separated.

The excitation wavelengths and values of λ_{max} for each conformer of 3-hydroxyflavone are given in Table 3.2

Table 3.2 Values of λ_{ex} and λ_{max} for each conformer of 3-HF

Solvent	Cyclohexane	Toluene	DMSO	ACN	Cyclohexanol	Methanol
λ_{ex} (nm)	361	363	362	361	365	365
λ_{max} N*	410*	413	410	410*	393, 410, 425*	414
λ_{max} T*	526	527	536	523	532	526
λ_{max} A*	N/A	N/A	468	463	425*	454

In the above table, N* represents the excited state of the normal conformer, T* is the tautomer, and A* is the anion. In instances where the existence of a particular conformer in a solution is in doubt, or if the identity of a peak cannot be definitively determined, an asterisk (*) is used to denote uncertainty in the λ_{max} . Table 3.3 summarizes the contributions of each conformer to each component of $\hat{\mathbf{X}}$ and $\hat{\mathbf{Y}}$, based on the expected shapes and positions of the fluorescence of each conformer. The separations are rarely ideal. An “X” indicates a definite presence of a conformer in a given component, whereas a “~” indicates a possible presence, cast in doubt because of the small size of the peak, or its location at a position that is not immediately indicative of one component or another.

Table 3.5 Initial intensities of individual components

Solvent	Cyclohexane	Toluene	DMSO	ACN	Cyclohexanol	Methanol
1	7.62±0.06	36.2±1.1	10.9±0.9	9.76±2.37	90.3	15.6
2	18.6±0.5	47.7±0.5	13.2±3.2	27.0±7.4	46.8	11.4
3	N/A	N/A	N/A	N/A	36.2	6.06

Table 3.6 Average lifetimes of individual components

Solvent	Cyclohexane	Toluene	DMSO	ACN	Cyclohexanol	Methanol
1 (ns)	3.27±0.41	3.48±0.27	1.49±0.11	0.90±0.16	5.46	0.19
2 (ns)	0.55±0.09	0.14±0.02	0.46±0.11	0.17±0.03	0.52	0.36
3 (ns)	N/A	N/A	N/A	N/A	0.12	0.63

The largest magnitudes of interaction terms occur in the polar protic solvents, indicating again that these solvents contribute the greatest complexity to the fluorescence of 3-hydroxyflavone. Errors in above tables were determined by repeating the analysis with each of the three replicates obtained in each solvent, and finding the standard deviations of each parameter. The errors in cyclohexanol and methanol were not able to be determined in this manner, either because each individual replicate alone did not result in the same distribution of components, making a comparison between them difficult or impossible, or because without the noise-reducing benefit of averaging, the individual components were too noisy to be effectively analyzed. The reported errors represent the maximum error of these measurements, as using this technique loses the benefits of averaging. A more sophisticated error analysis, such as a Monte Carlo approach, would obtain more accurate estimates of error, and most likely would be usable with all of the solvents.

3.5 Comparisons of TR-EDM Characteristics to Previous Studies

To determine how well this analytical method conforms to the extensive body of literature that has been gathered on 3-hydroxyflavone, it is instructive to compare some basic characteristics of the spectra. The λ_{max} is the most familiar and easily obtained characteristic of a fluorescence spectrum, and as it is one of the parameters currently used to distinguish solvent parameters when employing 3-HF as a fluorescence probe, it is of particular relevance. Table 4.1 gives a comparison between the λ_{max} values obtained for each conformer of 3-HF in each investigated solvent, along with any comparable literature value.

Table 4.1 Comparisons of λ_{max}

Solvent	Conformer	Multivariate Analysis (nm)	Literature Value (nm)
Cyclohexane	Normal	410	
	Tautomer	526	
Toluene	Normal	413	
	Tautomer	527	
DMSO	Normal	410	400 ^a
	Tautomer	536	534 ^a
	Anion	468	529 ^a
ACN	Normal	410	405 ^b
	Tautomer	523	530 ^b
	Anion	463	
Cyclohexanol	Normal	393 – 425	
	Tautomer	532	
	Anion	425	
Methanol	Normal	414	406 ^c
	Tautomer	526	529 ^c
	Anion	454	480 ^a

^aProtti ^bBrucker ^cStrandjord, 1985

There is excellent agreement between the experimental tautomer fluorescence maxima values and the values found in the literature. The normal fluorescence maxima determined through this analysis were all slightly red-shifted, compared to known values. The values for the emission maximum wavelength of the anion that were found do not match at all with what was found in this analysis, though this could be a reflection of the sensitivity of the anion to experimental conditions, including a vastly different λ_{ex} of 430 nm used in the work of Protti. Values for the nonpolar solvents could not be found for normal laboratory temperatures and pressures, ostensibly because the lack of normal fluorescence hurts the performance of 3-HF as a solvent probe, but the tautomer was determined to have a λ_{max} of 525 nm in methylcyclohexane cooled to 77 K (McMorrow). This fits perfectly with the values obtained for cyclohexane and toluene.

The parameter that is most often discussed in time-resolved studies is the average lifetime. Lifetimes of quickly fluorescing components are notoriously difficult to measure; a number of different methods have been employed to do so in the literature, but there is still a paucity of information to be found. Nevertheless, comparisons between average lifetimes determined in this analysis and those found in the literature are given in Table 4.2. Any cell containing an inequality symbol indicates that the conformer in question could not be separated from other conformers in the EDM, or is present in more than one component, and so the average lifetime cannot be known with greater certainty. The majority of components were not fully determined as a result of this uncertainty.

Table 4.2 Comparisons of average lifetimes

Solvent	Conformer	Multivariate Analysis (ns)	Literature Value (ns)
Cyclohexane	Normal	< 0.548	0.85 ^a
	Tautomer	3.272	
Toluene	Normal	< 0.14	
	Tautomer	3.48	
DMSO	Normal	< 0.457	
	Tautomer	< 0.457	
	Anion	1.490	
ACN	Normal	< 0.169	
	Tautomer	< 0.904	
	Anion	< 0.904	
Cyclohexanol	Normal	< 5.463	
	Tautomer	< 0.521	
	Anion	< 5.463	
Methanol	Normal	< 0.187	
	Tautomer	< 0.356	
	Anion	0.629	

^aBrucker ^bStrandjord, 1985 ^cSchwartz

In addition, decay lifetimes were found for 3-HF in formamide (normal: 0.037 ns, tautomer: 0.675 ns, anion: 0.675 ns) (Parthenopoulos), methylcyclohexane (tautomer: 3.9 ns) (Schwartz), hexanol (tautomer: 0.655 ns) (Strandjord, 1985) and egg yolk phosphatidylcholine liposomes (tautomer: 1.074 ns hydrophilic, 2.82 ns hydrophobic; anion: 0.792 ns hydrophilic, 5.34 ns hydrophobic) (Chaudhuri). While not directly relevant to the solvents analyzed in this project, these numbers serve to illustrate that the average lifetimes shown in Table 4.2 are at least reasonable. By all metrics, multivariate analysis resulted in values comparable to those found in the literature.

Chapter 4

CONCLUSIONS

4.1 Final Conclusions and Future Work

The method used in this project achieved the stated goal of separating the fluorescence components of 3-hydroxyflavone while considering the entire spectrum, including interaction terms between components. The mathematics are sound and do much to avoid some of the inadequacies of current approaches to time-resolved spectroscopy that have been expounded. It is my opinion that the mixing of components would to a great extent be solved by utilizing an excitation wavelength more suited to the molecule. The intensities of the fluorescence of the various components are simply too small, and the decay times too rapid, for the conformers to be separated by the mathematics into individual components. In this instance, the benefit of this technique also acts as its handicap: the assumption-less, self-modeling approach ensures that the results obtained are free from any specific model, but simultaneously puts a wall around the results obtained, making it impossible for the researcher to go in of his own accord and alter a spectrum by splitting a single component into two obviously distinct conformers. For this reason, the initial data collection parameters are vital to the success of the entire analysis. Poor conditions will not simply result in larger error; they will result in inseparable components and indeterminable photokinetic factors, as we have seen here. It may very well be the case that the components of 3-hydroxyflavone would be inseparable regardless of any

changes made to the data collection, since PMD utilizes the fundamental decays to separate individual components and the decays of some components are fast enough to be currently grouped in with the excitation pulse, but until data are collected using a better excitation wavelength it will be difficult to know for sure.

Future work on this topic, if undertaken, would certainly involve a thorough examination of all the nearby wavelengths accessible by the instrument, and the maximum incident intensities at each of these wavelengths. This, coupled with a full excitation spectrum of 3-hydroxyflavone in these solvents, would hopefully reveal a set of conditions that would lead to much greater fluorescence intensities than those observed in this work, most likely in the range of 330 to 355 nm. A more successful approach would be to replace the optics of the laser with a set that has a higher reflectivity in the desired wavelength region, but this would require a significant investment of time and money for uncertain gains.

It is possible that increasing the concentration of 3-HF in solution would lead to increased intensities, but there are two major issues with that approach. 3-hydroxyflavone is not a particularly solvable molecule in most of these solvents, particularly the polar ones. Water (and deuterated water) had originally been identified as a solvent to investigate, but the molecule's resistance to solvation in water proved too much to work with. Secondly, when conducting fluorescence measurements on solutions of increasing concentration, one runs into the problem of inner filter effects, which are diminished responses due to the attenuation of the excitation pulse by a concentrated solution of absorbent molecules. Lastly, a better filter that selectively removes the majority of the excitation scattering from the response would greatly

increase the sensitivity of the analysis to the fast decays of the normal tautomer, which were often inseparable from the excitation.

It is my hope that at some point in the future, a method of utilizing the photokinetic parameters of the fluorescence decay of 3-hydroxyflavone as indicators of solvent and microenvironment properties will be determined. While too few solvents were investigated in this research to come close to a model, even under ideal circumstances with perfect separation and resolution, it is clear that there are definite patterns in the rates of decay of the fluorescence components in various solvents, particularly with regards to the sensitivity of the normal conformer to hydrogen bonding effects. This would expand the list of useful parameters originally given in the Introduction - ν_{abs} , ν_{N^*} , ν_{T^*} , and $I_{\text{N}^*}/I_{\text{T}^*}$ - to include information from the time domain as well, increasing the usable information content of such spectra by an entire dimension.

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Appendix A

FITTING A FIVE COMPONENT DECAY TO THREE COMPONENTS

It was stated in the main text that it has been proven that a five component multiexponential decay curve can be fit within experimental accuracy to a three component system. While a full proof of this statement is beyond the scope of this work, and is available in the references, it is potentially more instructive for those most likely to consult this work – in short, chemists – to see an example firsthand. In this example, a group of researchers are attempting to iteratively fit a multiexponential decay (that I have defined to have five components) to a model. It is assumed for the sake of brevity that they have already ruled out the possibility of mono- and biexponential decays, and are now in the process of determining the optimal three-component exponential decay that fits the data.

A1 An Ideal Multiexponential Decay

First, an ideal hypothetical five component multiexponential decay with no added noise is created.

$$y_0 = 0.9e^{-0.5x} + 0.7e^{-0.9x} + 0.5e^{-0.1x} + 0.3e^{-0.3x} + 0.6e^{-0.8x} \quad A1$$

This equation is not intended to model any particular behavior; it is merely a set of numbers chosen at random with the intent of producing a decay that looks somewhat similar to a fluorescence decay profile. The decay is plotted as a solid blue line in Figure A1, and the individual components are plotted as red circles.

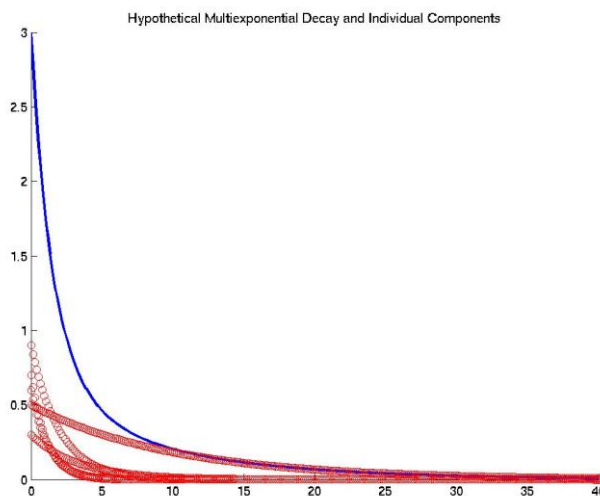


Figure A1 Hypothetical multiexponential decay and its individual components

These y values were fit using a Nelder-Mead simplex algorithm, which optimizes a fit by minimizing the sum of the residuals, to a three component exponential decay.

$$y_{\text{reg}} = 0.3535e^{-0.881x} + 0.5272e^{-0.2199x} + 2.1101e^{-0.7148x} \quad \text{A2}$$

Clearly, this equation bears no resemblance to A1. If this were an analysis of a true fluorescence decay, the estimates for the decay lifetimes and rates would be far off. Now it is necessary to assess how well the decay has been fit. A cursory look at the decay in Figure A2 is promising. The black line is the three component decay, the blue lines are the individual components, and the open red circles trace the path of the original five component decay.

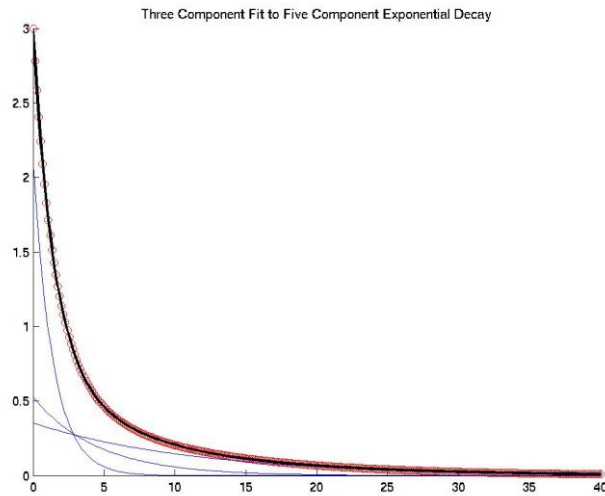


Figure A2 A three component fit to a five component exponential decay

The black line looks like it goes directly through all of the red circles, but a better indicator of the strength of the regression is the magnitude of the residuals. If residuals are normally distributed about zero and show no trend or correlation to x , it is assumed that they are truly random, which infers that the model adequately describes the data. A graph of the residuals as a function of x and a histogram of the distribution of residual values are shown in Figure A3.

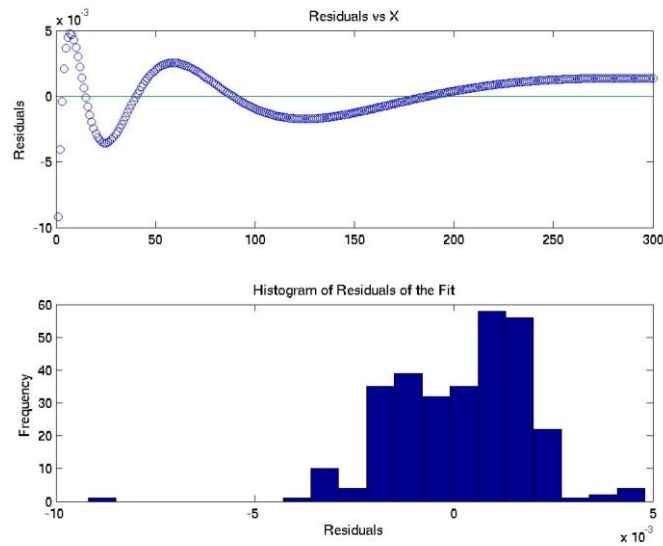


Figure A3 Plot and histogram of residuals of the fit

The scale of the residuals is on the order of 10^{-3} , and the r^2 value is 0.9999. These values are quite small, indicating a regression that is a close approximation of the original function, but there is a distinct pattern to the regression. This sinusoidal pattern is common to Nelder-Mead simplex fits, and while it implies that the residuals do not contain pure noise, it will be seen in the next section that this discrepancy is not significant.

A2 A Multiexponential Decay with Added Noise

Now to generate a multiexponential decay that more closely models data that one is likely to acquire in a real experiment, which is to say one with noise. A new data series was generated by taking the original function in equation A1 and adding a random component with a normal distribution about zero and a variance of 0.05.

$$y_{\text{err}} = y_o + N(0,0.05) \quad \text{A3}$$

This equation generates a decay profile similar to that in Figure A1, but with a small error component, certainly within what one would expect as experimental error.

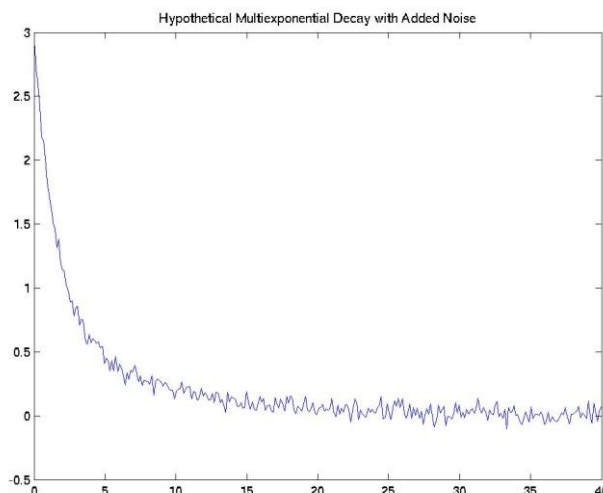


Figure A4 Hypothetical multiexponential decay with added noise

Again, a Nelder-Mead simplex was used to find the optimal three component regression. The equation obtained was exactly the same as the original regression, which makes sense if the added noise is truly normally distributed.

$$y_{\text{reg,err}} = y_{\text{reg}} \quad \text{A4}$$

Figure A5 is analogous to Figure A2; the regression is the black line, the blue lines are the individual components of the regression, and the red circles are the individual data points of y_{err} .

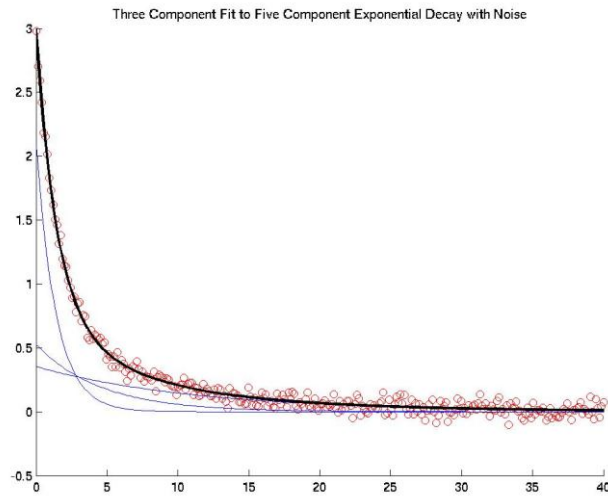


Figure A5 A three component fit to a five component decay with added noise

The same residual plots are shown in Figure A6. These plots show that the random noise completely dominates the residuals; the residuals show no trend or correlation to x , and they are normally distributed in the histogram.

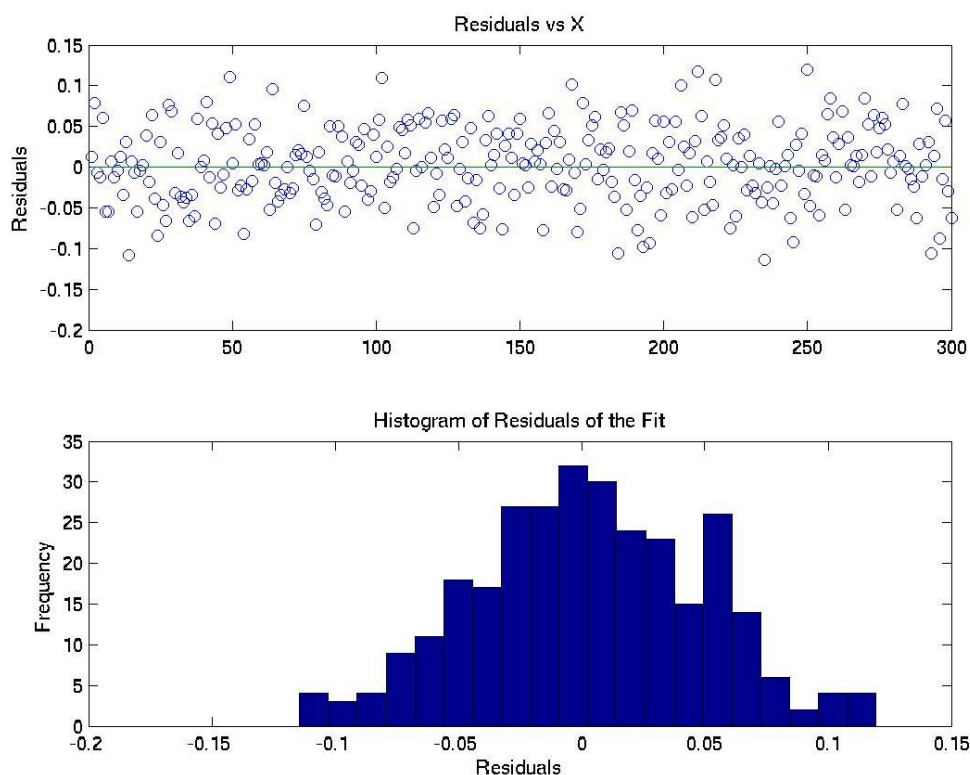


Figure A6 Plot and histogram of residuals of the fit to a noisy decay

The r^2 value for this regression is 0.9899. When the “true” values of the multiexponential decay – by which I mean, the equation used to generate the original decay profile, y_0 – were used as a regression to its own noisy multiexponential decay y_{err} , the fit also had an r^2 value of 0.9899. The r^2 is what most chemists are used to looking at when determining quality of fit, so the problem with this method is immediately evident: there is no obvious way to determine the difference in quality of fit between the three component and the five component models when the data is noisy. Even in ideal circumstances, the three component system can approach a perfect fit to the higher order decay; no chemist would reject a model with an r^2 value greater

than 0.9999 in favor of one with more components. For these reasons, iterative fits to multiexponential systems are inherently untrustworthy for multicomponent systems. Not only can the answers be completely wrong, but it is often not even possible to know if you could be wrong.

Appendix B

SINGULAR VALUE DECOMPOSITION PLOTS

The following plots were used to determine the number of components present in the fluorescence of 3-hydroxyflavone in each of six distinct solvents. The top two figures are of $\mathbf{U}$ and $\mathbf{V}$, and the bottom two show the magnitudes of the components of $\mathbf{\Sigma}$, and the frequency content of the first 50 components of $\mathbf{U}$. The use of these plots is described in section 2.3.1.

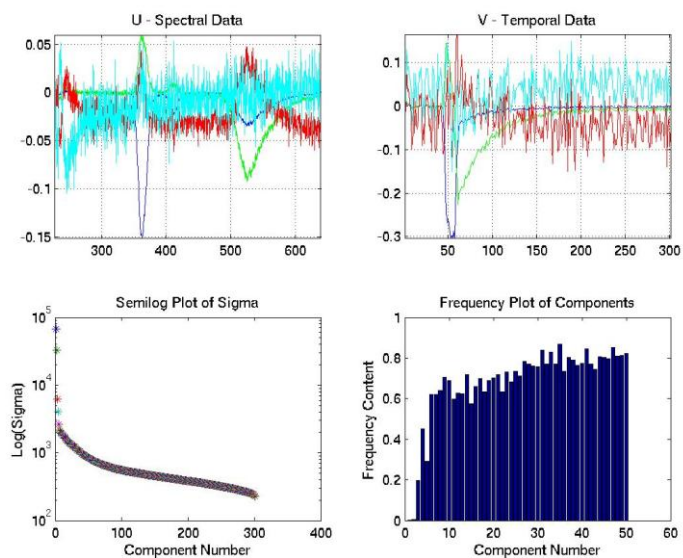


Figure B1 Determining the number of components in cyclohexane

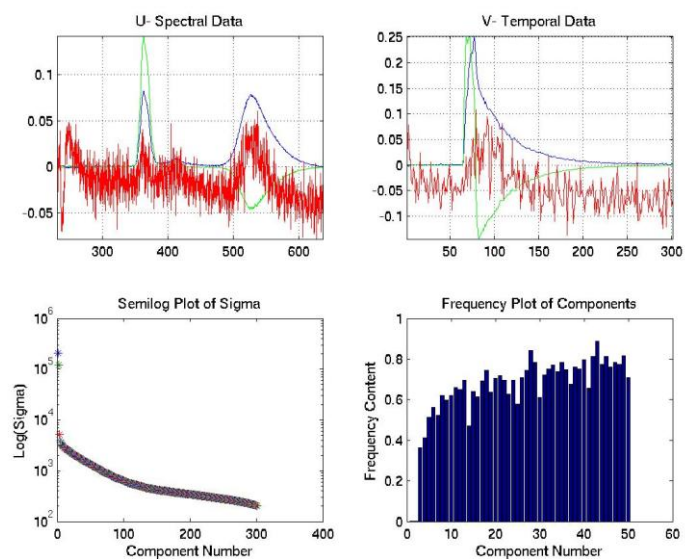


Figure B2 Determining the number of components in toluene

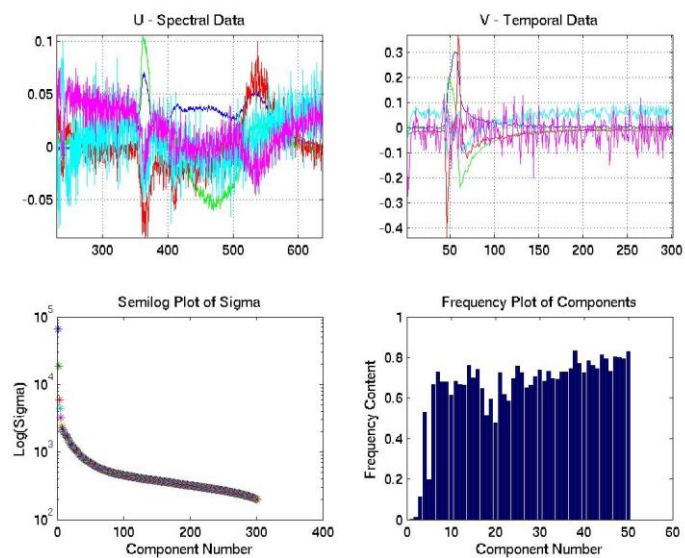


Figure B3 Determining the number of components in DMSO

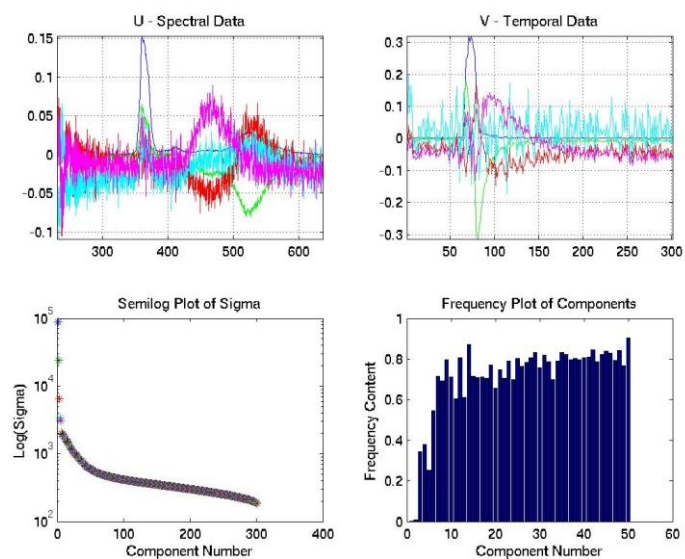


Figure B4 Determining the number of components in acetonitrile

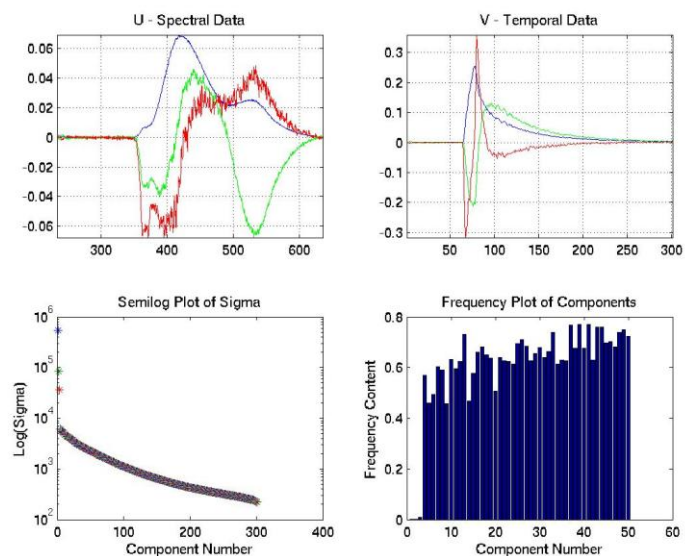


Figure B5 Determining the number of components in cyclohexanol

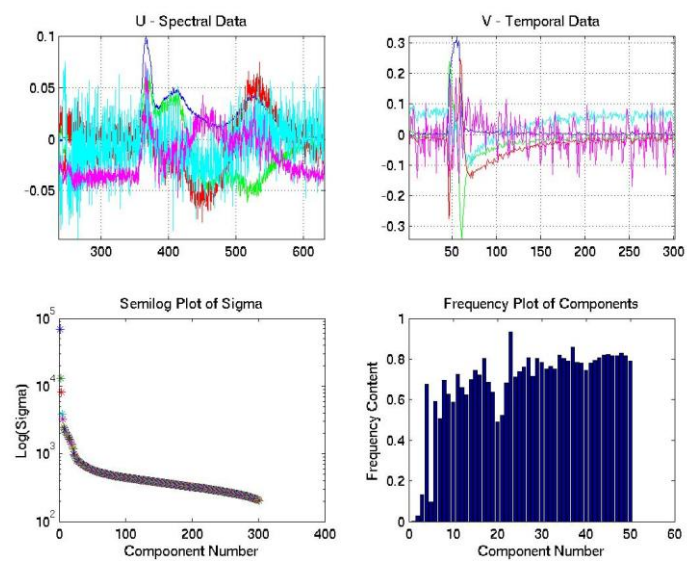


Figure B6 Determining the number of components in methanol

Appendix C

PHOTOKINETIC FACTOR PLOTS

This appendix contains the decay-associated spectra and the fundamental decays of 3-hydroxyflavone in the six investigated solvents. In each figure, the top plot is **S**, the decay-associated spectrum, and the bottom is **Z**, which shows the most significant fundamental decays present in each TR-EDM. A description of how these plots are used is given in section 2.3.2.

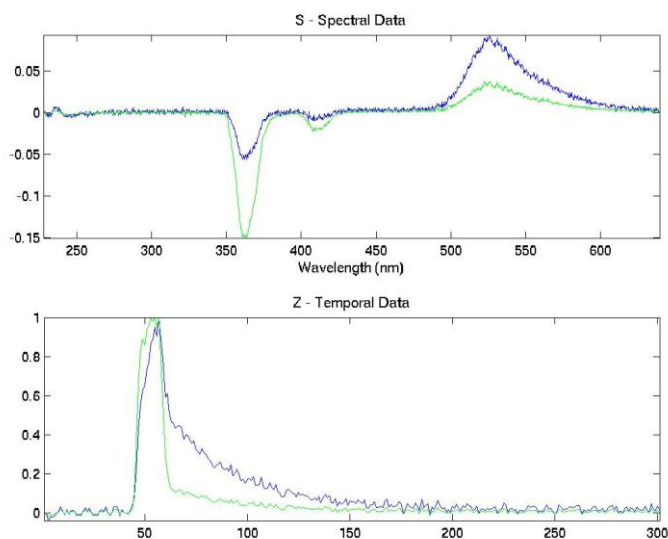


Figure C1 Photokinetic factors of 3-HF in cyclohexane

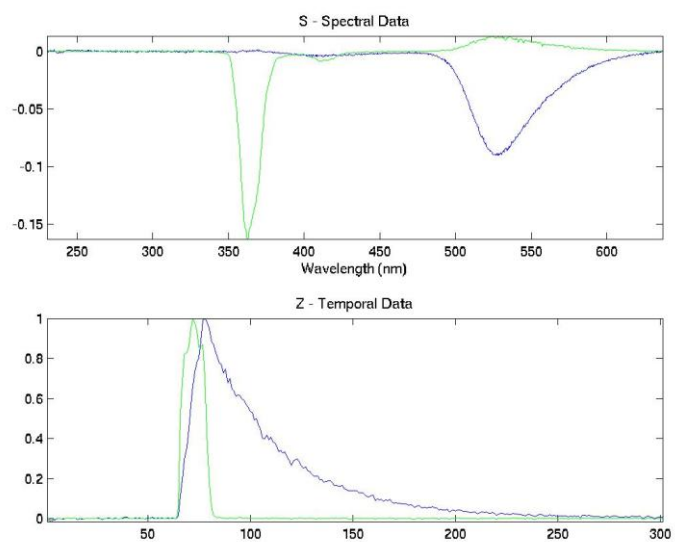


Figure C2 Photokinetic factors of 3-HF in toluene

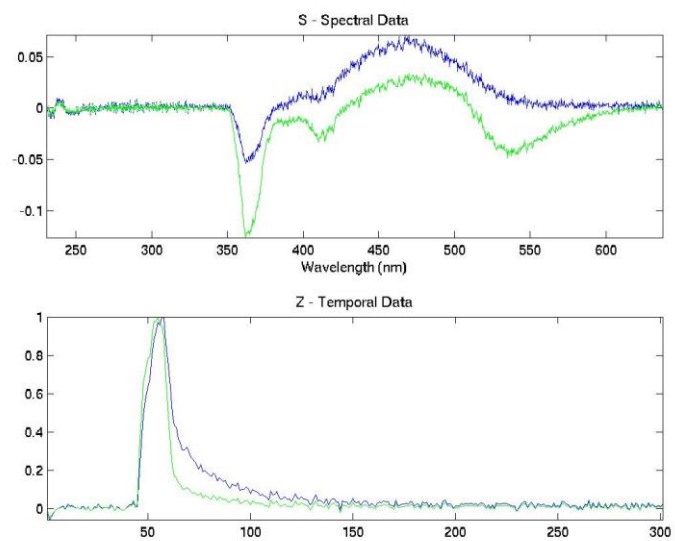


Figure C3 Photokinetic factors of 3-HF in DMSO

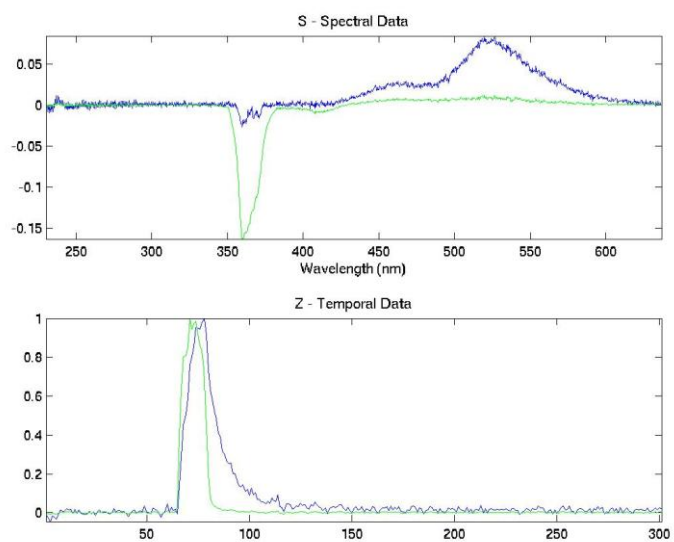


Figure C4 Photokinetic factors of 3-HF in acetonitrile

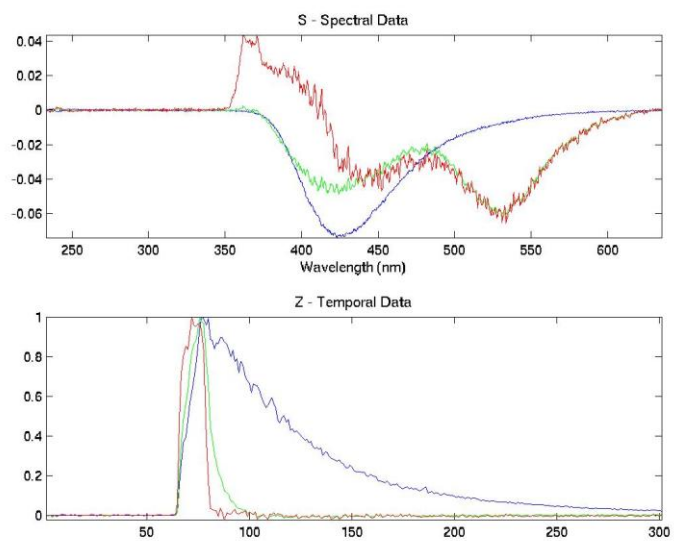


Figure C5 Photokinetic factors of 3-HF in cyclohexanol

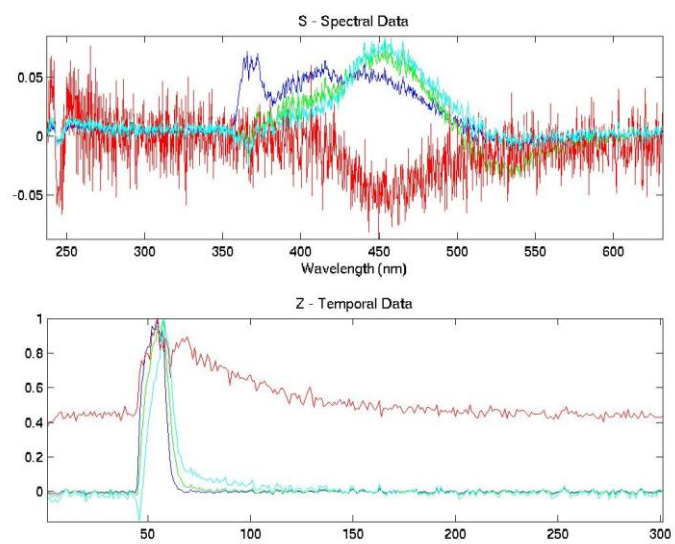


Figure C6 Photokinetic factors of 3-HF in methanol