An Analysis of 8-Quinolinylboronic acid D-Fructose Complex using Fluorescence Spectroscopy

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Fall 2024, Chem 4190

ABSTRACT

This research was to investigate fluorescence of 8-Quinolinylboronic acid (8-QBA)/D-fructose complex at a pH of 8, in a *tris*(hydroxymethyl)aminomethane (TRIS) buffer.

The first three scans (a, b, and c) were run at an excitation wavelength of 350 nm to measure signal to noise ratio and observe the Rayleigh and Raman scattering. The water scan demonstrated a Rayleigh scattering was at 351 nm, with a Raman scattering at 396 nm.

The ratio of signal to noise, as determined from the air scan showed an S/N ratio of 349 comparing the noise to the signal intensity of the Rayleigh peak.

The difference in scattering, in water, between excitation and Raman wavenumber was 3351 cm^{-1}. This gave a relative error of 2.9% from excitation wavenumber, from a reported wavenumber for water of 3450 cm^{-1}. Further scans of water were performed at excitations of 300 nm and 350 nm. They yielded relative error in their Raman peak wavenumbers of 0.35%, 0.79, 1.65% versus the reported wavenumber of water's Raman peak at 3450 cm^{-1}, from the excitation wavenumber.

Scans were performed on two sets of three dilutions of D-fructose in 1-QBA, which found the peak binding fluorescence at a concentration of 0.0167M D-fructose/4.17μM 8-QBA. The scans found the maximum fluorescence intensity occurred at 417 nm. An excitation scan determined the maximum excitation wavelength was 313 nm.

The maximum excitation and emission wavelengths were set for a read intensity mode scan. A set of five serial dilutions of 8-QBA and D-fructose, with values of 5.0 μM down to 0.3125 μM 8-QBA in 10 mM D-fructose, at a pH of 8 were scanned in a read mode at with the 5 second read integration time. Those results were then plotted intensity versus concentration to get a linear plot. A linear regression showed the equation produced was $y = 148.36x + 84.858$ with an R^2 of 0.9995.

1.0 INTRODUCTION

Fluorescence is the, almost immediate, emission of light by a substance, after absorption of light. The light emitted is, usually, a longer wavelength than that of the absorbed light. Fluorescence has been known for some time, mentioned in the records of Aztecs and written about in the 1500's by Bernardino de Sahagún and by Nicolás Monardes.[1] A.E. Becquerel observed, in 1842, that

solar ultraviolet radiation caused calcium sulfide to emit light and was the first to state that the emitted light was a longer wavelength to that of the light triggering the fluorescence. The first paper published on fluorescence was by George Stokes. The paper was titled “Refrangibility”. George Stokes named the process fluorescence.[2]

As spectroscopy was more utilized, in the early 20^{th} century, fluorescence spectroscopy developed as a tool of chemists and other scientists. With advances in quantum mechanics, and importantly, quantum electronics, unraveled fluorescence and phosphorescence mechanisms. Absorption of a photon of sufficient energy, by a molecule, will cause an electron to jump to a higher energy molecular orbital. This electron is in a singlet state. Usually, the singlet electron energy dissipates through the process of internal conversion, or vibrational relaxation. When the electron has reached the lowest energy level of the excited state with no vibrational or collisional moiety that can accept the correct energy to change an electronic level, the excited state survives long enough to emit a photon. An electron dropping from an excited electronic level to the ground state can use the energy change to emit a photon. This process is usually called fluorescence. The difference between the excitation and emission wavelengths is referred to as the Stokes shift, and accounts for the energy lost due to vibrational relaxation. The emission wavelength accounts for the energy of the excited electron dropping to the ground state.

On rarer occasions, the excited electron can transition to a triplet state, changing spin orientations in the process. This transition is an inter-system crossing. It may still slowly return to a ground state molecular orbital through vibrational relaxation, but it can also emit a photon accounting for a larger block of energy, in a process referred to as phosphorescence. This process takes significantly longer than fluorescence. With fluorescence, the emission of photons by the fluorophore ends as soon as exposure to the excitation light ceases, on the order of 10^{-12} to 10^{-9} s. In phosphorescence, the time between electron excitation and photon emission can extend up into seconds. The excited triplet state lifetimes are between 10^{-4} to multiples of seconds.[3, 4]

Though fluorescence is not common, and most compounds absorb light at some wavelength, the linearity of fluorescence over a wide range allows for sensitive quantitative analyses of fluorescent and chemicals that can affect a fluorophore’s fluorescence. 8-QBA has a strong affinity for diols, such as in carbohydrates. 8-QBA alone shows minimal fluorescence at a pH above 5. At a pH of 8, most 8-QBA carbohydrates complexes show significant fluorescence. The complex of 8-QBA and fructose has a pronounced fluorescence. making that complex a good

subject for study. The fact that 8-QBA is soluble and doesn't have a harsh effect on biological tissues makes it a good candidate for measuring cell membrane glycosides.[5]

The hypothesis posed in this research was that the fluorescence exhibited by 8-QBA/fructose would produce a linear fluorescence range over which concentration could be determined. This paper will determine the signal to noise ratio of the fluorometer, the approximate saturation of 8-QBA with D-fructose, and determine the equation for a linear range of fluorescence for 8-QBA/D-fructose complex. 8-QBA can be used to measure fructose. It's ability to complex, then fluoresce at pH ranges close to that of biological organisms, makes it a good measurement tool for carbohydrates in a biological setting.

2.0 EXPERIMENTAL

2.1 Chemicals

The following chemicals were used as standards for the experiments described in this paper. D-Fructose, ≥99%, Sigma Chemical Co., PO box 14508, ST Louis, MO 63178. CAS# 57-48-7; *tris*(hydroxymethyl)aminomethane (TRIS) buffer, >99%, Fisher Chemical, 300 Industry Drive, Pittsburgh, PA, United States, 15275, CAS# 77-86-1; 8-Quinolinylboronic acid (8-QBA), technical grade, Sigma Chemical Co., PO box 14508, ST Louis, MO 63178. CAS# 86-58-8; and Deionized water: >99.9% pure, Georgia State University, Science Annex, Atlanta, GA, CAS# 7732-18-5.

2.2 Equipment

The equipment used included the model LS 55 Fluorescence spectrometer by PerkinElmer, serial number 108683.

2.3 Experimental Conditions

Experimental conditions include a scan rate of 150 nm/minute, performed at room temperature (20° C) and at approximately 1 bar.

2.4 Procedures

The first set of procedures are of air and water, assessing noise, Rayleigh, and Raman emissions, using the PerkinElmer LS55 spectrometer. The scan of air is to determine noise levels. It will include six scans:

Table 1

ID	Sample	Scan Mode	Excitation λ (nm)	Gain	Emission λ (nm)	SBW (nm)
a	air	emission	350	high	300-500	10
b	water	emission	350	high	300-500	10
c	water	emission	350	low	300-500	10
d	water	emission	300	high	250-450	10
e	water	emission	350	high	370-470	10
f	water	emission	300	high	320-420	10

Sample *a* is for noise determination, *b* was run at high sensitivity for Rayleigh and Raman scattering, *c* was run at low sensitivity for Rayleigh and Raman scattering, and for determining the ratio of Rayleigh and Raman scattering peak heights, and to yield the fluorometer's sensitivity gain factor. Sample *d* shows Rayleigh peak measurements at different excitation and emitted wavelengths, *e* is a Raman only scattering spectrum at 350 nm excitation wavelength, and *f* is a Raman only scattering spectrum at 300 nm excitation wavelength.

2.41 FL Scan

For fluorescence emission tests, solutions of TRIS buffer (pH 8), 8-QDA, and D-fructose required preparation. 100 mL of 10 mM TRIS buffer was furnished. Stock solutions of 8-QBA and D-fructose stock solutions of 50 μM 80QBA in 10 mM TRIS buffer, and stock 0.1 M fructose in 0.01 M TRIS buffer were supplied. Another stock solution of 50 mM D-fructose in 10 mM TRIS buffer was supplied.

To get optimal wavelengths for emission and excitation, the emission scan method was set, then the slit width was set to 10 nm. The excitation monochromator was set to 310 nm and emission monochromator was set from 330 to 600 nm. A scan of 8-QBA was taken, then 0.1 M drops (assumed 0.05 mL) of fructose added and mixed, then scanned. These proceed using the same sample, so that scans of a sample having 0, 5, 10, and 15 drops of 0.1 M fructose were performed. Table 2 shows these dilutions and the subsequent concentrations.

Table 2 Shows the dilution of fructose in an 8-QBA solution, each sample having an integral of 5 drops of 0.1 M D-fructose.

Drops of 0.1 mM fructose	mL vol added to fructose	total volume	8-QBA (μM)	fructose (M)
0	0.00	2.50	5.00	0.0000
5	0.25	2.75	4.55	0.0091
10	0.50	3.00	4.17	0.0167
15	0.75	3.25	3.85	0.0231

The number of peaks and wavelengths were measured, along with the I_f (emission intensity) for the complex maximum, and correlating that with the concentration of fructose. This was designed to find the concentration of the binding saturation of 8-QBA/fructose (C_{fru}).

Once the peak binding saturation for 8-QBA/fructose the emission wavelength (λ_{em}) of maximum fluorescence intensity (I_f) from the previous scans. The excitation monochromator was set to a range from 280 to 350. Scans were run to get a gain that was good maximum that was not off scale.

2.42 FL Read

A solution of 10 mL 8-QBA/fructose complex solution was placed in a 10 mL volumetric flask, with a concentration of fructose at the approximate Cf_{ru} determined earlier, and a concentration of 5 μM 8-QBA. The 1 mL of 0.1 M stock fructose solution and 1 mL of 50 μM 8-QBA were added to a 10 mL volumetric flask, and then the flask was filled to the mark with 10 mM TRIS buffer containing fructose in a concentration of 50 mM and mixed. Serial dilutions of this were done within the cuvette. These dilutions were prepared as shown in Table 4.

The excitation wavelength and emission wavelength were set to the optimal values previously determined. The TRIS buffer was used to get a background. Each of the five dilutions were scanned with the next dilution then prepared from the cuvette solution. The background was subtracted by automation.

The peak intensities were read, then plotted vs concentrations. The concentrations and intensities were used to do a linear regression, demonstrating the linearity of the concentration vs fluorescence intensity. All data was saved to files.

3.0 RESULTS AND DISCUSSION

3.1 Rayleigh and Raman Scattering

Four scans of an air scan, run in an emission mode. The slit width was initially set to 10 nm, progressively going to progressively smaller slit widths, to get a sharp peak with a peak that is not off scale. The fourth scan got a good, high gain, scan at a slit width of 3 nm. This was done at an excitation wavelength of 350 nm.

The Rayleigh peak showed clearly at 351 nm, with possible, very small, Raman peaks at 396 and 427 nm. The first Raman peak was about 700 times less intense than the Rayleigh, and the second Raman peak about 108 times less intense. The wavelength shifts for the Raman scattering were 45 and 76 nm, respectively. The first Raman peak could possibly be water in the air, given it's showing at approximately the reported energy of the water's Raman scattering of 3450 cm^{-1}, due to the vibrational stretching of water.[6] However, the 427 nm peak did not correspond to either the N^2 or O^2 gas Raman peak offsets, so this is an unlikely interpretation.

The air scan, *a*, was also used to determine the noise of the scans. The average intensity, excluding peaks, was 0.122, with a maximum intensity of 2.709. The largest peak had a value of 901.588. Correcting these to the average 'zero' value of 0.122, yielded 901.466 and 2.587 for the maximum signal and noise, respectively. Using the signal to noise ratio (S/N) formula of the signal divided by the noise yielded an S/N ratio of 348.8.

The second and third scans (*b* and *c*) were of deionized water, with emissions in the wavelength range between 300 and 500 nm, using an excitation wavelength of 350 nm. The difference in the scan settings between the two was that the b scan was performed at a high gain, with a slit width of 3 nm, and the c scan at a low gain and a slit width of 10 nm. Some of this information is shown in Table 3. Figure 1 shows the scan.

Table 3 This table shows the excitation, Rayleigh, Raman wavelengths, and the associated full width half maximum (FWHM) and gain of the scans of deionized water.

Scan	λ_{exe} (nm)	$\lambda_{Rayleigh}$ (nm)	Peak Height	FWHM	λ_{Raman} (nm)	Peak Height	FWHM	Gain
b	350	351	564.5	4.5	396.5	19.4	4.5	high
c	350	351	174.5	13.5	-		-	low

Both gave strong Rayleigh peaks at 351 nm, with only scan b yielding a discernible Raman peak. That peak was at 396.5. This is a difference of 45.5 nm. For scan *b*, the Rayleigh peak was at an energy of 28490 cm^{-1} and it's Raman peak 25221 cm^{-1}, showing an energy difference of 3269 cm^{-1}. This is a difference of 3351 cm^{-1} from the excitation energy, which is a 2.9% relative error from water's 3450 cm^{-1}. From the measured Rayleigh wavenumber, it is a relative error of 5.2%. Scan *c* had no discernable Raman peak. The drop in energy of a Raman peak is expected given the scattering is inelastic, with some photon energy being converted into vibrational energy.

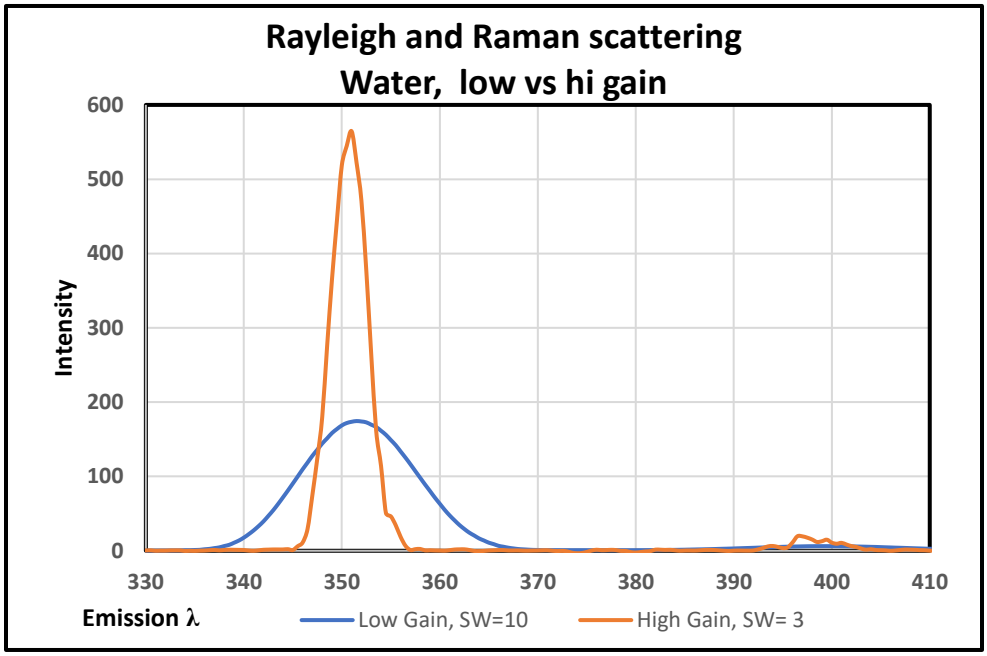

Figure 1 The excitation wavelength was 300 nm, the emissions in the range between 300 and 500 nm, scan b is the high gain, with a 3 nm slit width, scan c being at low gain with 10 nm slit width.

The Rayleigh peak should be at the excitation wavelength, given it is an elastic scattering. If we took the Rayleigh peak was measured at 350 nm, the energy would be 28571 cm^{-1} and the predicted Raman energy would be 25121 cm^{-1} which corresponds to 398 nm. The measured vs predicted (at a Rayleigh peak at 350 nm) was a difference of 1.6 nm. The measured vs the "predicted from measured" (at a Rayleigh peak at 351 nm) was a difference of 2.9 nm.

The Rayleigh peak heights were 564.5 and 174.5, high and low gain scans, respectively. This shows a ratio of 3.23 between the high and low gain. The FWHM of the *b* Rayleigh peak was small at 4.5 nm, but the *c* FWHM was 13.5 nm, three times higher. The *c* scan was run at a much higher slit width of 10 nm, vs the *b* scan run at 3 nm. The FWHM of the *b* Rayleigh and Raman peaks were the same. The width of the *c* peak seems to have been influenced by the higher slit width, which was 3.33 times larger than the *b* scan.

The heights of Rayleigh and Raman peaks, in *b*, were 564.5 and 19.4, respectively. The Rayleigh peak was about 29 times higher than the Raman peak. When the areas under the respective peaks were calculated and the ratio computed, they also came out at about 29 times greater for the Rayleigh peak.

With scan *d*, the excitation wavelength was set to 300 nm, the gain to high, and the emission wavelengths from 250 to 450 nm. This was run at both a slit width of 10 and then 5 nm. A strong peak occurred at 300 nm, Rayleigh scattering peak. Unlike the *b* and *c* scans, the Rayleigh peak here was exactly at the excitation wavelength. The Rayleigh wavenumber was 33333 cm^{-1} with a Raman wavenumber of 29795 cm^{-1} with a difference of 3438 cm^{-1}. Using the reported 3450 cm^{-1} for the stretching vibrational mode of water, the relative error was 0.35%.

With scan *e*, the excitation wavelength was set to 350, the emission scan range to 370 to 470, with a slit width first at 10 nm, then dropped to 5 nm. This produced a Raman peak at 398.5 nm. This shift was 48.5 nm. In terms of energy the Raman peak was at 35094 cm^{-1} from an excitation of 28571 cm^{-1}, with a difference of 3477 cm^{-1} which is only 27 cm^{-1} from the reported 3450 cm^{-1} for the stretching vibrational mode of water. This was a relative error of 0.79%.

With scan f, the excitation wavelength was set to 300, the emission range was set to 320 to 420 nm, and a slit width 10 nm. A moderately broad peak was observed at 334 nm. This may be a Raman scattering peak. If so, this shift is 34 nm. In terms of energy the Raman peak was at 29940 cm^{-1} from an excitation of 33333 cm^{-1}, with a difference of 3393 cm^{-1} which is 57 cm^{-1} from the reported 3450 cm^{-1} for the stretching vibrational mode of water. This is a relative error of 1.65%.

The setting details for these scans can be found in Table S-1, in the supplemental section.

3.2 Fluorescence Scans

The fluorometer excitation monochromators to 300 nm, the slit widths for both the excitation and emission monochromators to 10 nM each, and the emission monochromators wavelength range from 330 to 600. Three scans were made, starting with 2.5 mL of 5 μM 8-QBA solution with 5 drops of (0.05 mL) of 0.1 M D-fructose, mixed, and scanned. Then with each successive scan adding 5 drops (0.05 mL) of 0.1 M D-fructose and mixing, prior to the scan. An emission peak was found at wavelength of 417 nm, for 8-QBA and 8-QBA/fructose complex.

The scans indicate the binding saturation peaks at 10 drops (0.0167 M D-fructose with 4.55 μM 8-QBA, at a pH of 8) with a peak emission at 417 nm.

The slit widths for excitation and emission were set to 5 nm. The cuvette was cleaned and a new sample of 5 µM 8-QBA solution was added (2.5 mL). The above set of fructose additions and scans were made. Details of these dilutions can be found in Table 2, excluding the 8-QBA only scan. The scan plots can be seen in figure 2, and in the supplemental section, figure S-1 (first scan).

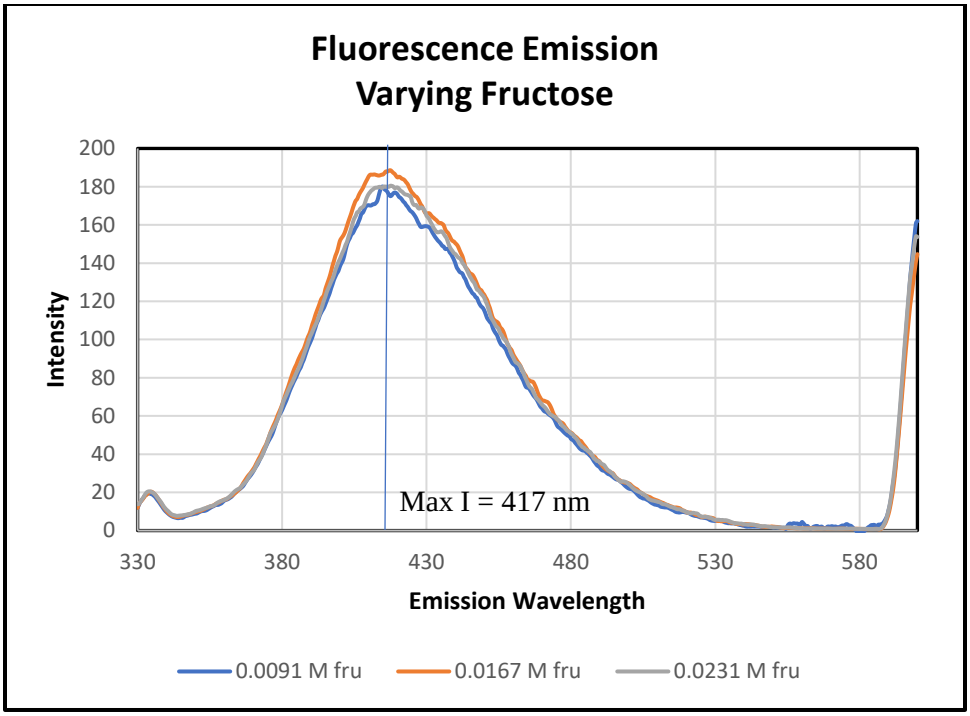

Figure 2 Scan of three concentrations of D-fructose in 8-QBA.

The peak emission occurs at a concentration of 0.0167M D-fructose and 4.17µM 8-QBA.

The fluorometer is set to an excitation scan method, the emission monochromator to 417 nm (optimal emission wavelength), the excitation monochromator to the range of 300 to 400 nm, and a scan speed of 150 nm/minute. Then a scan of the fructose/8-QBA complex was run. This can be seen in Figure 3.

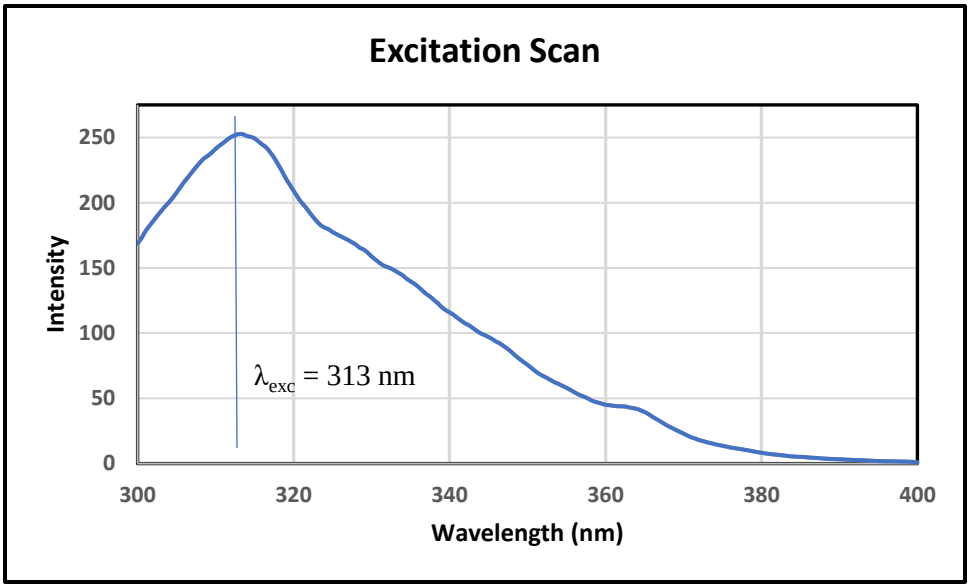

Figure 3

The excitation scan demonstrated that the peak excitation wavelength (λ_{exc}) was 313 nm. The emission peak wavelength (λ_{emi}) was 417 nm. This was only one nm different for λ_{exc} and exactly the same for the λ_{emi} , as mentioned in the literature.[7,8]

The Stokes shift was from 313 nm to 417 nm, 104 nm or 7968 cm^{-1} (31949-23981 cm^{-1}). For each photon absorbed resulting in the emission of a fluorescence photon, 7968 cm^{-1} of thermal energy was converted to vibration via internal conversion, prior to the fluorescence photon emission. 7968 cm^{-1} corresponds to $1.58x10^{-19}$ J.

3.3 Fluorescence Read

A solution was prepared using 1 ml of 50 µM 8-QBA and 1 mL 0.1 M D-fructose, in a 10 mL volumetric flask, then adding 10 mM TRIS buffer (containing 50 mM fructose) to the line. That was used as the initial solution, then making 1:1 dilutions, starting with that, using 50 mM D-fructose /10 mM TRIS buffer solution. This produced concentrations of 8-QBA/D-fructose of 5 µM 8-QBA/fructose complex. This was used to start successive 1:1 dilutions using the previous dilution and the previously mentioned TRIS buffer/fructose solution. This produced concentrations of 8-QBA/D-fructose of 8-QBA/fructose complex in concentrations of 5.000, 2.500, 1.250, 0.0625, and 0.3125 µM 8-QBA with 10 mM of TRIS buffer and 50 mM of D-fructose. Table 4 shows the materials and amounts used, and the concentrations produced.

Table 4 Starting stock of 5 µM 8-QBA, and 50 mM fructose,
Dilution solution was 10.0 mM of TRIS buffer and 50 mM fructose.

Stock (mL)	Previous dilution (mL)	TRIS Buffer w/ fructose (mL)	Concentration 8-QBA complex (µM)
10			5.0000
	1	1	2.5000
	1	1	1.2500
	1	1	0.6250
	1	1	0.3125

The fluorometer was set to an excitation wavelength of 313 nm, an emission wavelength of 417 nm, as determined in the previous runs. A slit width of 7/7 (excitation/emission) was set and the mode was set to read intensity. A background scan of the TRIS buffer gave too high a reading for a background, so the background (BG) reading was set to 30.39. It was hypothesized that the TRIS buffer was contaminated. That background value is automatically subtracted from the fluorometer intensity readings.

Scans were run for each of the dilutions (0.3125, 0.6250, 1.250, 2.500, and 5.000 µM 8-QBA in 50 mM fructose and TRIS buffer). The readings can be found in table 5.

Table 5 8-QBA-fructose complex concentration

Conc µM	Intensity
5.0000	821.538
2.5000	465.619
1.2500	273.275
0.6250	176.136
0.3125	124.927

Plotting these max intensities (I_f) vs the concentrations yielded a linear plot, with an R^2 value of 0.9995. An equation was produced via linear regression. The equation was $y = 148.36x + 84.858$ and plot are shown in Figure 4.

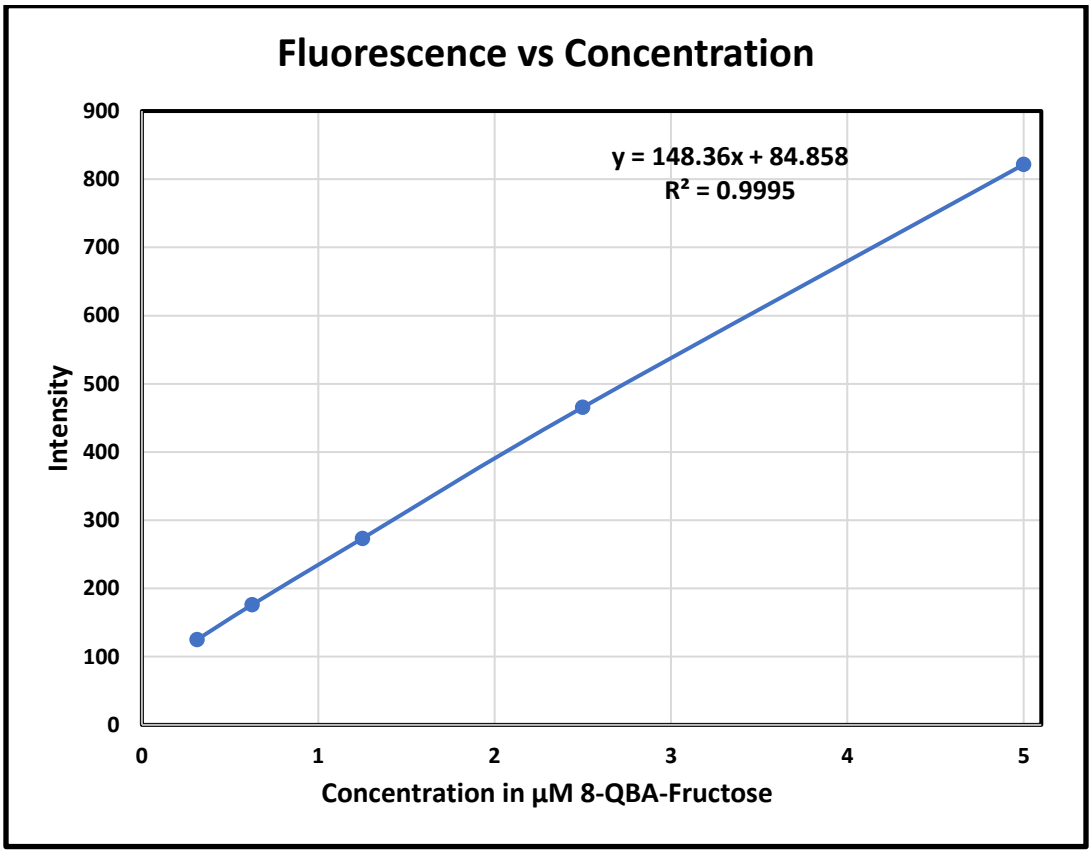

Figure 4 Fluorescence versus concentration of 8-QBA/fructose complex.

The points of the graph conformed to the line such that points deviating from the line could not be discerned visually. This was a demonstration of how concentration of fluorophores can be determined via fluoroscopy.

The ability to quantify D-fructose and other carbohydrates forms a basis for analyzing biological tissues to quantify carbohydrates. With 8-QBA complexing with the carbohydrate diols, increasing fluorescence in the process by a factor of about 40, it can be used to quantify glycoproteins and other cell surface carbohydrates, in vivo.

4.0 CONCLUSIONS

The first three scans (*a*, *b*, and *c*) were run at an excitation wavelength of 350 nm. The initial scan (a) demonstrated that the Rayleigh scattering of air was at 351 nm, with possible corresponding Raman scattering at 396 and 427 nm. The first Raman peak was barely detectable, being about 700 times shorter, the second, being about 107 times shorter.

The ratio of signal to noise, as determined from the air scan showed an S/N ratio of 349, comparing the noise to the signal intensity of the Rayleigh peak.

The Rayleigh scattering of water read at 351 nm, with corresponding Raman scattering at 396.5 nm, for water, when run at high gain. The Rayleigh peak height was about 49.7 times the height of the Raman peak. A quick check of peak areas was close to this measurement as well. The difference in scattering between the Rayleigh and Raman wavenumber was 3269 cm^{-1} (from excitation wavenumber 3351 cm^{-1}). Assuming this was due to water vibrational stretching, which should be about 3450 cm^{-1}, that gives a relative error of 5.2% (2.9% from excitation wavenumber).

Further scans of water were performed at excitations of 300 nm and 350 nm. They yielded relative error in their Raman peak wavenumbers of 0.35%, 0.79, 1.65% versus the reported wavenumber of water's Raman peak at 3450 cm^{-1}, from the excitation wavenumber.

Scans were performed on two sets of three dilutions of D-fructose in 1-QBA, which found the peak binding fluorescence at a concentration of 0.0167M D-fructose/4.17μM 8-QBA. The scans found the maximum fluorescence intensity occurred at 417 nm. An excitation scan determined the maximum excitation wavelength was 313 nm.

The maximum excitation and emission wavelengths were set in the respective monochromators and the slit width set to 5 nm for both monochromators. The mode was set to emission read mode with a 5 second read integration period. A background scan of TRIS buffer showed too high a value to be valid so a value of 30.39 was set for the background value. The slit width seemed a bit too low, so was increased to 7 nm in both emission and excitation monochromators.

A set of five serial dilutions of 8-QBA and D-fructose, with values of 5.0 μM down to 0.3125 μM 8-QBA in 10 mM D-fructose, at a pH of 8 was prepared. They were scanned in a read mode at with the 5 second read integration time. Those results were then plotted intensity versus concentration to get a linear plot, supporting the initial hypothesis posed by this research. A linear regression showed the equation produced was $y = 148.36x + 84.858$ with an R^2 of 0.9995.

5.0 REFERENCES

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6.0 SUPPLEMENTAL INFORMATION

6.1 Formulas

Equation 1: $y = 148.36x + 84.858$

Equation of the line for the set five 8-QBA/D-Fructose concentrations vs intensities

6.2 Significant Figures

Significant figures used were the significant figures of the raw data plus one, for all calculation results.

6.3 Raw Data

Available on request.

6.4 Tables, Graphs, and Plots

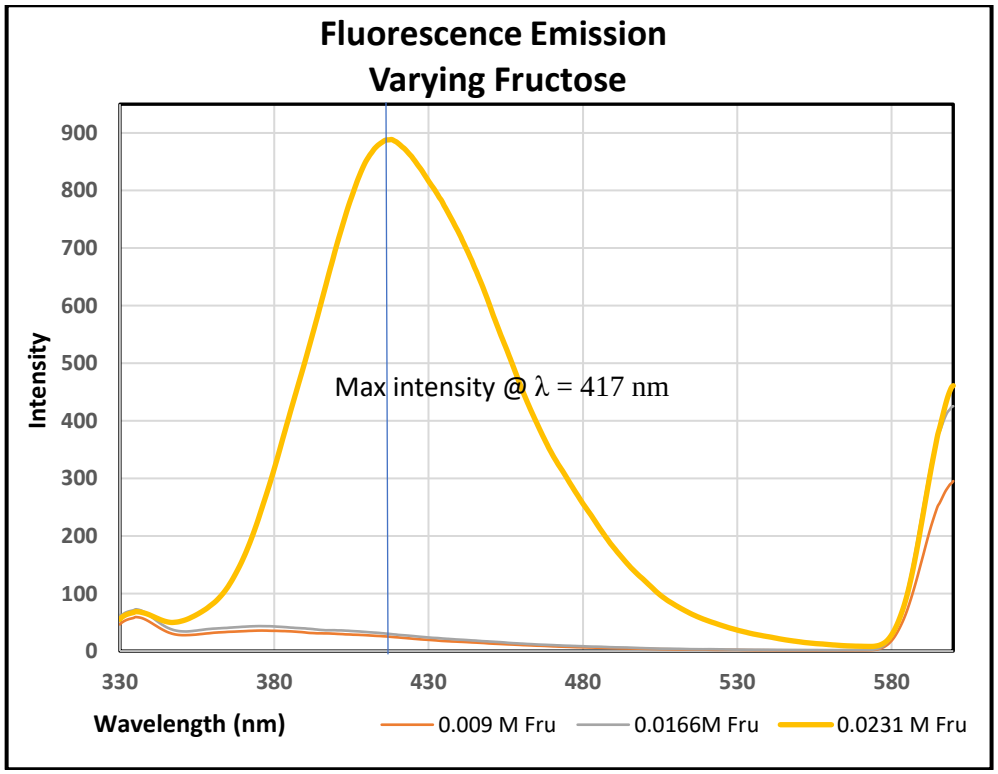

Figure S-1 The initial scans of 8-QBA and D-Fructose. The concentrations in legend are of D-fructose.

Table S-1

ID	Sample	Scan Mode	Excitation λ (nm)	Gain	Emission λ (nm)	SBW (nm)
a	air	emission	350	high	300-500	3
b	water	emission	350	high	300-500	3
c	water	emission	350	low	300-500	10
d	water	emission	300	high	250-450	5
e	water	emission	350	high	370-470	5
f	water	emission	300	high	320-420	10

The scans of water at various excitation and emission ranges, along with the gain and slit width of the scan.