



The Measurement of Sensitivity in Fluorescence Spectroscopy

Among instrumental techniques, fluorescence spectroscopy is recognized as one of the more sensitive. In fluorescence, the intensity of the emission of the sample is measured. The reason for the high sensitivity of fluorescence techniques is that the emission signal is measured above a low background level. This is inherently more sensitive than comparing two relatively large signals as in absorption spectroscopy. The sensitivity of fluorescence techniques is as much as 1000 times more sensitive than absorption spectroscopy.

A spectrofluorometer with high sensitivity is an asset for the researcher. It is obvious that an instrument with high sensitivity will be able to perform experiments with the fluorescing species at a low concentration. For analytical measurements, sensitivity determines the detection limit of a material. For research in life science, it may be important to work at concentration levels that are very low. This is almost always the case in biomedical research. A more subtle requirement is the need to measure *changes* in fluorescence during the course of an experiment. Even though the gross signal levels may be high, the changes in the signal may be quite small. The ability to reliably measure these small changes is directly dependent upon the sensitivity of the fluorometer.

The measurement of sensitivity has been discussed in the literature [1]. Unfortunately, there is little consensus on a standard procedure for determining this important capability. This paper discusses the measurement of sensitivity and details two methods that are applicable to any instrument.

The Spectrofluorometer

The sensitivity of fluorescence is dependent on both the fluorophore and the instrument. The response of a fluorophore will depend on the molar absorptivity and the quantum yield. These factors are, in general, beyond the control of the analyst.

The sensitivity of a spectrofluorometer depends on a number of factors. Instrumental contributions to sensitivity are described below.

1. Source intensity. In general, a brighter excitation source will result in brighter emission. The source for most fluorometers is a xenon arc, which has a high intensity between 200-900 nm, the spectral region where most fluorescence experiments are performed. While a high power arc lamp is good for highest intensity, a more important criteria is the brightness of the lamp. The brightness is a function of both the power and the size of the arc. Arc lamps for commercial fluorescence systems range from 75-450 watts.

2. Efficiency of the optical system. The light collection efficiency is a function of two factors. A high efficiency optical design is characterized by a low f number. For example, $f/2$ is more efficient, or faster, than $f/4.5$. It is imperative that the various optical components (e.g., source, monochromators, sample cuvette position, and detectors) are optically matched. A high speed design, however, is prone to high stray light. Also, the gratings, mirrors, and lenses that are incorporated into the spectrofluorometer all have associated optical losses and will decrease the intensity of the light passing through the system. The number of optical elements, the thickness of lenses, and the coatings on mirrors and lenses will affect the throughput.

3. Spectral bandpass of the monochromators. The bandpass of commercial fluorometers may be varied between 0.5-30 nm. Doubling the bandpass of a monochromator will increase the throughput of light by a

factor of four. Resolution, however, is worse at high bandpass. The bandpass may be adjusted by the analyst to balance sensitivity and resolution.

4. Efficiency of the detector. Both analog and photon counting methods of detection are used in commercial instruments. In general, photon counting is considered to be slightly more sensitive. Both methods are subject to degradation because of noise in the electronics.

It is the combination of the effects from all of these contributions that determine the sensitivity of the fluorescence spectrometer. It follows, then, that the most appropriate way to quantify the sensitivity of a spectrofluorometer is to measure a standard sample using the complete instrument system.

The Signal-to-Noise Ratio

The sensitivity of a spectrofluorometer is expressed as the ratio of the signal of a standard sample to the rms noise level [2]. Sensitivity has often been expressed as a signal level only, for example, a certain number of counts with no mention of the noise. This practice is discouraged. Sensitivity should *always* be specified as a signal-to-noise ratio (SNR).

The Ideal Sample for Signal-to-Noise Ratio Determination

The requirement for reproducible and consistent results in signal-to-noise ratio measurements between various instruments and laboratories places considerable emphasis on having available a stable and reproducible sample. A Standard Method has been published for measuring the limit of detection of quinine sulfate (2). Other workers have specified the signal level of 50 femtomolar fluorescein, a particularly strong fluorophore. A sample of such low concentration is not recommended because of the high probability of error in sample preparation and storage. Other fluorophores in a glass or plastic matrix have been used and proposed as possible standards. Most are difficult to prepare and not readily available.

For several years, fluorescence instrument manufacturers have used the Raman band of water for measuring the signal-to-noise ratio and have been quoting the use of this material in sensitivity specifications for their products. The Raman band of water is inherently reproducible and does not degrade with time. Water is convenient to obtain in a pure state, allowing interlaboratory comparisons to be made with high confidence levels. No preparation or dilution is required. The Raman band is a low-level signal, providing a good test for both the optics and the electronics of an instrumental system.

The Raman band of water is not due to fluorescence but is a result of Raman scattering. The Raman band of water simulates fluorescence in that the emission occurs at a longer wavelength than the excitation. For water, the Raman band is always red-shifted 3382 cm^{-1} relative to the excitation.

The integration time and the bandpass of the monochromators must be clearly specified, since both will affect the measured signal-to-noise ratio. Although the intensity of the Raman band varies with the wavelength of excitation, this is easily controlled in a scanning fluorometer. The Raman band is generally measured at 397 nm with excitation at 350 nm.

Determining the Signal-to-Noise Ratio

In general, measurement of the signal from the Raman band of water is straightforward. In Figure 1, the signal at 397 nm is measured as the signal above the average of the background.

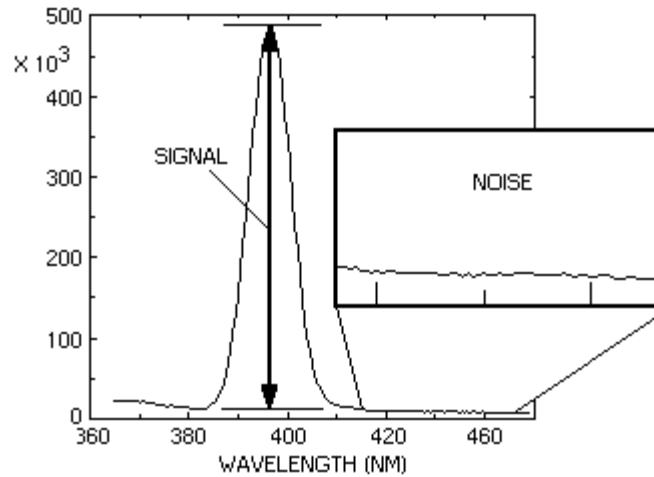


Figure 1. Emission scan of the Raman band of water [7].

Unfortunately, there is no accepted convention for measuring the noise. The noise produced in a fluorometer system can be measured in a number of different ways and the value obtained will have a large impact on the calculated signal-to-noise ratio. The greatest variation in measured values of signal-to-noise ratio can be attributed to the method of measuring the noise level.

There are two predominant measurement situations in which a fluorometer is used. The noise is measured slightly differently for these two cases.

1. Detection of weak fluorescence in the presence of background noise

This is the classical analytical experiment in which the challenge is to test the ability of the instrument to resolve a small signal from the background. The principal source of noise is the dark current from the photomultiplier tube and the associated electronics in the detection circuitry.

In ASTM Standard E 579 [2], the background signal and associated noise is measured in the absence of the fluorescent species of interest. A cuvette is placed into the sample holder containing the solvent only. The gain of the instrument is increased until the background noise can be readily measured and a recording made. Quinine sulfate is then added to the cuvette in a sufficient and known concentration that will allow the signal to be measured on the same scale. A determination of the average signal level is made.

This protocol is not suitable if the Raman band of water is used as a measure of sensitivity. The most common procedure for water is to set the excitation monochromator to 350 nm and scan the emission monochromator from 365 nm to 460 nm. The signal is measured at 397 nm and the noise is measured in a region where the signal is negligible, generally between 420-460 nm. A typical spectrum is shown in Figure 1.

The determination of the value for the noise level and how it is applied in the calculation of the signal-to-noise ratio has been discussed in a number of published standards [2,3]. If, for example, the instrument reports values to the user in the form of a strip chart or X-Y plot, the background noise will appear as a wavy line on the chart. The user visually determines the noise level by drawing a pair of lines through the extreme peak values of this noisy trace, thereby determining the peak-to-peak amplitude of the noise. This peak-to-peak value is then divided by 5 to convert it to an approximate rms (root mean square) value.

After obtaining the values for the signal and the rms noise, the signal-to-noise ratio may be calculated.

$$\text{SNR} = \text{signal level}/\text{rms noise level}$$

Measuring the peak-to-peak value of a noise plot is not a meaningful mathematical method of evaluation of a data set. The root mean square (rms) is the statistically correct method for measuring the noise in a data set [3,4,5]. The rms value for the noise in a given data set is identical to the standard deviation of that data set. It is tedious to calculate the rms value of a data set from chart paper. It is, however, quite convenient to measure the standard deviation, and thus the rms value, using commonly available data analysis software [6]. If a software package is not available, the rms value can be estimated by dividing the peak-to-peak noise by 5. This is an empirical approximation that is allowed only for convenience.

2. Detection of a small change in intensity of a large fluorescence signal

In certain experiments, the researcher is looking in a time-based mode for a small change in the level of a large signal. This situation occurs, for example, in the measurement of polarization, quenching studies, temperature effects on fluorescence, and in ratio fluorescence using probes such as Fura-2. In this situation, the fluorometer must have excellent stability and low noise to detect very small changes in the intensity of the signals. Here we are concerned with such factors as lamp stability, short and long term drift of the detector, electronics, and optics, and, most important, the quantum noise in the signal itself. The low level, random, dark noise from the detector and electronics is less important, since the contribution of the dark noise is very low in comparison to the noise contributed by the other sources.

Under these conditions, system noise is determined using the Raman band of water by measuring the stability and noise over a short period of time. Water is placed into a cuvette and a suitable signal obtained with excitation at 350 nm and emission at 397 nm. The instrument is allowed to accumulate data in a time-based mode and a recording is made of the signal level over a period of several minutes. This recording is analyzed for stability and noise and it is these variations in the signal level which constitute a lower limit of detectability for this type of experiment. The assumption is that the sample is inherently stable and does not photolyze or change in any way during the course of the test.

The signal-to-noise ratio for this type of experiment is expressed in the same manner (equation 1) as discussed earlier. Because of the additional contributions to the noise, the SNR is generally lower than when measured on a weak fluorescence signal.

Procedures for Determining the Signal-to-Noise Ratio of Spectrofluorometers

1. Detecting a weak fluorescence signal over background noise

A very clean quartz cuvette is filled with pure, distilled water. The water must be free of contaminants such as particulates which would scatter the light and hydrocarbons which may be fluorescent. It must also be free of any bubbles, as these will cause excessive scatter. The usual instrument settings are as follows:

Excitation wavelength: 350 nm
Emission scan range: 365-460 nm
Spectral bandwidth (ex, em): 5 nm
Filter time constant: 1 sec
Wavelength step size: 0.25 nm
Smoothing: None
Gain: As required to obtain an appropriate signal

A research grade instrument will produce a scan similar to Figure 1 which clearly shows the Raman signal at 397 nm well above the background noise. Inspection of the data shows a Raman signal with an amplitude of approximately 500,000 counts and, zooming in on the background, a noise level with a peak-to-peak variation of less than 1000 counts (see Figure 2). Using Equation 1,

$$\text{SNR} = 490,000 / (980 / 5) = 2,499$$

The sloping base line in Figure 2 is due to low level fluorescence or scattering and is generally not indicative of the noise in the fluorometer. The noise measurement should exclude the sloping background.

For a more rigorous correction for the sloping base line, a least squares method may be used to calculate the slope and intercept of the base line, then subtract each calculated point from the corresponding observed point [3]. Alternatively, the data may be smoothed using a suitable algorithm. The smoothed curve is then subtracted from the unprocessed data.

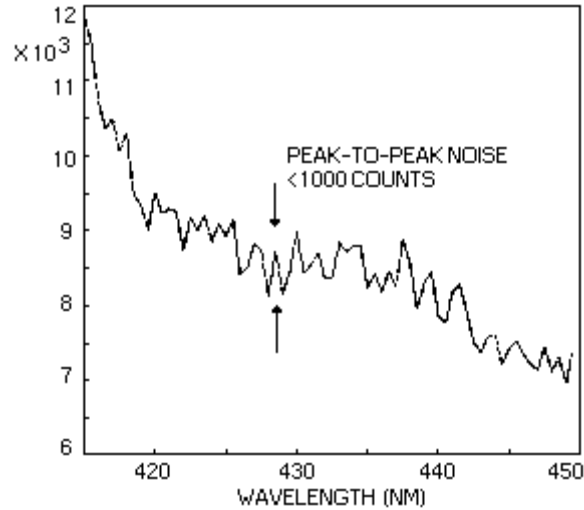


Figure 2. Expansion of the noise region of the emission scan of the water Raman.

It is interesting to compare the SNR calculated above with the result based on the true rms value of the noise. The standard deviation for the data shown in Figure 2 was measured with commercial data analysis software [6c]. The sampling rate of the computer system was set to 1 sample/second to approximately duplicate the 1-second time constant used to acquire the data. A correction was made for the sloping baseline. The standard deviation was 243 counts, resulting in a SNR of:

$$\text{SNR} = 490,000/243 = 2,016$$

2. Detecting a small change in the intensity of a large signal

The instrument is run in the time-based mode to record the fluorescence intensity as a function of time. A clean quartz cuvette is filled with pure distilled water and the instrument set as follows:

Excitation wavelength: 350 nm
 Emission wavelength: 397 nm
 Spectral bandwidth (ex, em): 5 nm
 Filter time constant: 1 sec
 Smoothing: None
 Gain: As required to obtain an appropriate signal

A recording is made of the water Raman signal for a period of several minutes. After zooming in on the noise, the trace will appear similar to Figure 3. The peak-to-peak variations on the signal can be readily determined and the calculation made using equation 1. The absolute intensity of the Raman signal is determined by subtracting the average value of any background which may be present.

$$\text{SNR} = 1,000,000/(6,100/5) = 819$$

The rms value of the noise calculated as the standard deviation of the data using data analysis software was 1198. The resulting SNR is 804.

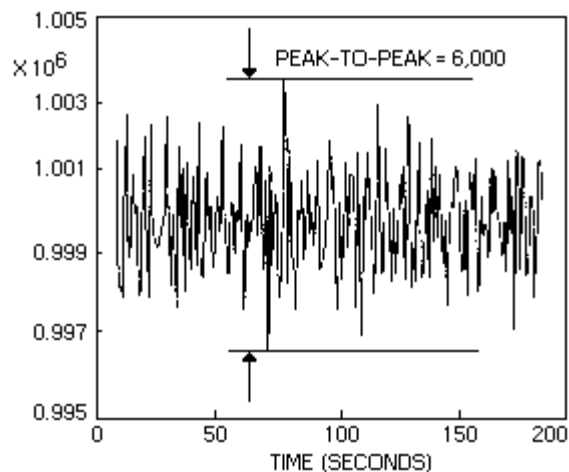


Figure 3. Time-based measurement of the Raman band of water [7].

Variations in Method of SNR Calculation

The primary reason for uncertainty in the calculated SNR is the noise level. In the first example, the noise was 196 counts when estimated from the peak-to-peak values and 243 counts when measured as the standard deviation. The resulting SNR's were 2499 and 2016, respectively. Based on this example, it is safe to assume that a difference in signal-to-noise ratios of as much as 25% between two instruments is not significant, providing that instrument parameters and all other mathematical manipulations are identical.

In some cases, manufacturers and experimentalists may vary the procedure and/or the method of calculating the signal-to-noise ratio. This may be done because of certain limitations of the instrument, differing wavelength requirements, or other necessary experimental variations. It is important to note the impact of these variables on the final result.

It has become common practice to multiply the noise value by 3, as shown in equation 2.

$$\text{SNR} = \text{signal level}/\text{rms noise level} \times 3$$

Eq. 2

The use of the factor of 3 arises from ASTM E 579 for the determination of the limit of detection of quinine sulfate. The limit of detection is defined as the concentration that will give a signal that is 3 times the rms value of the noise. Since this is a deviation from the classical definition of the signal-to-noise ratio, which is clearly defined in ASTM E 579, the use of the factor of 3 should be made explicit.

The instrument time constant may be increased to 2 or 5 seconds, reducing the noise level and improving the calculated SNR. The data should not be smoothed or filtered by either analog or digital means. The goal of the exercise is to test the inherent ability of the spectrofluorometer to gather data.

The optical bandpass, a function of slit width, may be increased beyond 5 nm, which will improve the SNR dramatically. Doubling the bandpass of both monochromators may increase the signal by as much as a factor of 16 (2^4)! Once again, the response of the noise is more complex. In the case of a weak fluorescence signal, changing the bandpass will have little effect on the noise because it is due primarily to the dark count of the detector. If small changes in a large fluorescence signal are being measured, the noise will increase as the square root of the signal intensity. The SNR will, therefore, increase as the bandpass increases, but with a square root dependence on the signal [4].

Conclusion

The signal-to-noise ratio of the Raman band of water is the most appropriate method to quantitate the sensitivity of a spectrofluorometer. The procedure for measuring sensitivity should reflect the application for which the instrument is intended. If sensitivity is an important specification to the user, then the signal-to-noise ratio of the Raman band of water should be measured using identical procedures and calculations when comparing different instruments. Caution should be exercised in comparing SNR's measured at different bandpasses.

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6. For example: (a) SigmaPlot, Jandel Scientific. (b) LabCalc or GRAMS 386, Galactic Software. (c) PSI-Plot, Poly Software Intl. Other data analysis products are also available.

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