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Measuring the Sensitivity of a Fluorescence Spectrometer

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Key Words

- Fluorescence
- Raman Water Test
- Sensitivity
- Signal-to-Noise

Introduction

New applications in many areas of life science, materials, quality control and the development of novel fluorescence probes, particularly those based on expressed proteins have greatly increased the sensitivity requirements of fluorescence spectroscopy. While much of the sensitivity is directly related to the molar absorptivity and quantum yields of the molecules being analyzed, instrument performance is also critical to detecting low concentrations of fluorophores or small changes in larger signals.

In this technical note, we will describe a robust method for measuring the sensitivity of a fluorescence spectrometer and discuss the key parameters used to determine sensitivity.

Background

Because it is commonly available in most laboratories and does not require tedious sample preparation, most instrument manufacturers use the Raman emission spectrum from ultra-pure water to determine the sensitivity of their spectrofluorometer. To eliminate variations created by differing instrument parameters, the signal-to-noise ratio (SNR) of the intensity of the Raman peak compared to the noise present in a signal-free region is often used as a metric for reporting sensitivity, and thus instrument performance. However, there are different methods and techniques used to evaluate the SNR of an instrument.

Theoretically, the SNR can be calculated exclusively from the Raman peak signal fluctuations using the variation in the peak intensity over time, or a time scan analysis. However, the dark noise is not the major cause of signal variation, therefore making this method unreliable for determining SNR in low signal measurements.

The most accurate method of measuring SNR is to ratio the peak Raman line intensity to the average intensity of a signal free “baseline” region. This method introduces a few alternatives for quantifying the signal and the noise.

The value of the intensity of the signal can be evaluated either by a single intensity data point extracted from scanning data or by averaging the peak intensity using a time scan.

Likewise, there are two methods for measuring the noise value of the SNR. The first method based on measuring the fluctuations in a spectral region known to be free from signal. These random variations in the intensity values form the basis for calculating either the peak-to-peak noise (Max-Min) or the root mean square (RMS) noise (standard deviation) of the spectral response over a defined wavelength region. It is important with either measurement to have a statistically significant number of points to insure a representative noise value. One problem with this measurement is the uncertainty about residual signal remaining in the region assumed to be signal free. A baseline correction is frequently applied to remove any residual signal originating from broadband fluorescence or scattering.

Alternatively, the noise value can be determined from a time scan analysis. In this method, the noise is measured as a function of time at a single wavelength. As discussed above either the peak-to-peak variation or the RMS variation is calculated using a large number of points from the resulting time scan.

For measuring the SNR ratio, the Thermo Scientific Lumina fluorescence spectrometer uses a hybrid method where the value of the signal is determined by a scan of the Raman line and a time scan is used to evaluate the noise floor and to determine the noise value. From these two measurements, the SNR is determined.

Experimental

The spectra presented were acquired from ultra-pure water standard in a flame-sealed 10 mm pathlength quartz cuvette (Starna® Scientific) using a Lumina™ fluorescence spectrometer. Emission spectra from 370–460 nm were acquired using an excitation wavelength of 350 nm. Both the Excitation and Emission slits were set to 10 nm and five 100 ms measurements at each data point were averaged to produce a spectrum with 0.5 second “integration” time and 1.0 nm data interval.

Measurement data were exported to a spreadsheet program to calculate the standard deviation and to perform further statistical analysis. Both the peak-to-peak and root mean square noise calculations can be made using the spectral region around 450 nm. A baseline correction is applied to remove any residual signal from broad fluorescence features or scattering.

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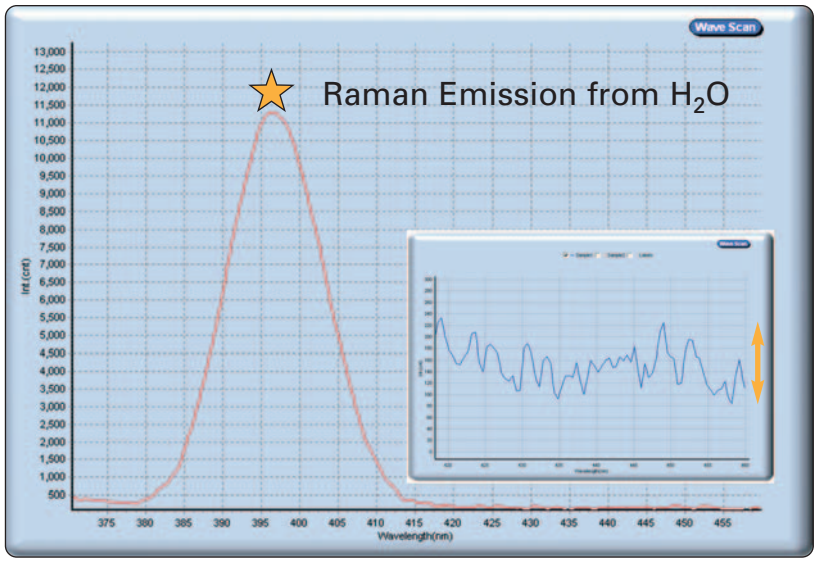

Figure 1: The Raman emission spectrum of water with an expansion of the "signal free" region

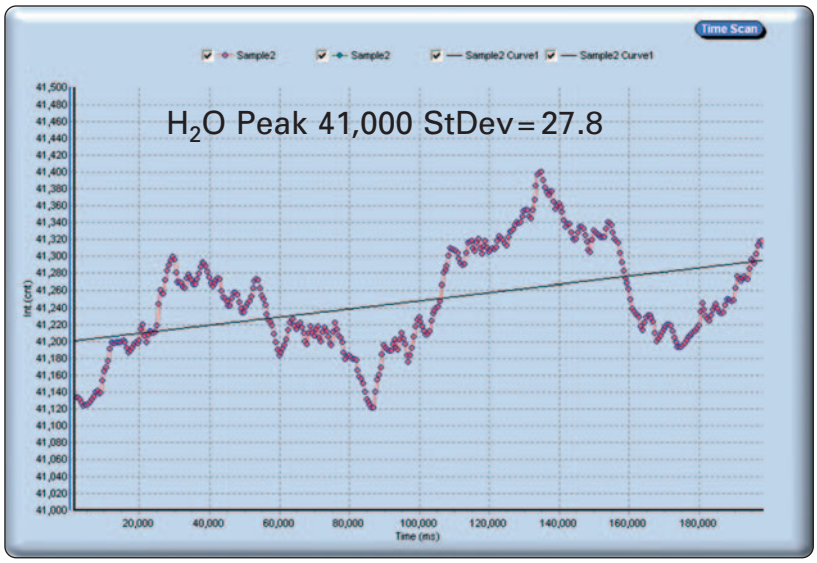

Figure 2: Plot of the Raman water intensity at 397 nm as a function of time

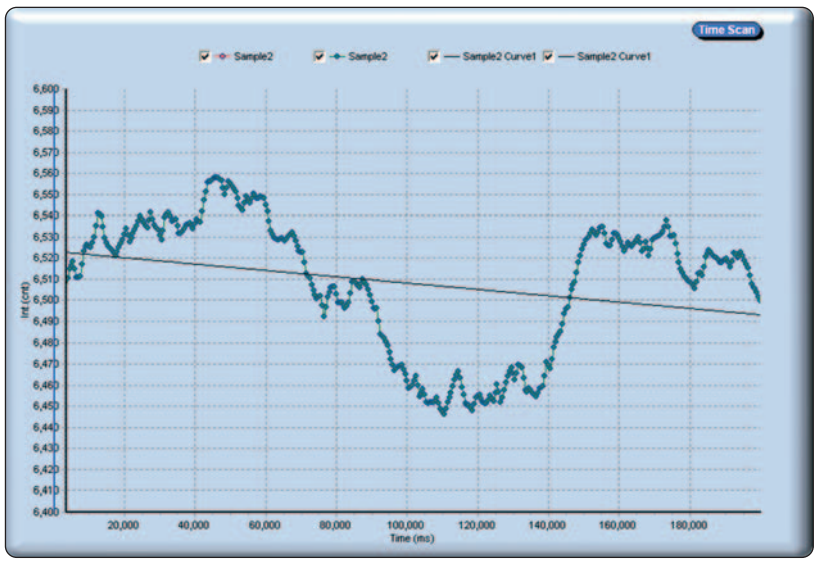

Figure 3: Plot of the baseline intensity at 450 nm with the water sample

Results and Discussion

A spectrum of the Raman scattering from the ultra-pure water sample is shown in Figure 1 with the spectral region used for the noise calculations expanded in the inset.

In most of the measurements, the noise increases as the intensity increases and some spectroscopists prefer determining the value of the signal by examining the variation in the peak intensity over time. Even with the weak Raman signal from water the intensity is large enough that the dark noise is not the major cause of signal variation. The fluctuations in the intensity of the peak maximum are determined using a time scan with excitation at 350 nm and measuring at 397 nm. Figure 2 shows the plot of intensity at 397 nm obtained every half second over 3 minutes. The standard deviation in the intensity is around 28 counts with an intensity of 41,000 counts. If the noise is taken as the standard deviation inside the peak signal, the SNR would be approximately 1,500:1. However, since this method does not take into account fluctuations at low intensities, it is not preferred for measuring the SNR.

An estimate of the noise floor can be calculated from a time scan of a region with no signal (450 nm). A 500 ms integration time was used with the excitation and emission slits set to 10 nm. Figure 3 shows the plot of the intensity at 450 nm acquired with a half second measurement time. The standard deviation for the intensity over several minutes is reported and is a factor of three lower than the variation in the intensity at the peak maximum. Since there is truly no "signal" present in this measurement, using the standard deviation of this measurement to calculate SNR would be meaningless.

Lumina Signal-to-Noise Calculations

An automatic validation routine is included with Thermo Scientific Luminous fluorescence software and facilitates a consistent and accurate measurement of the sensitivity and SNR. The software first determines the signal at 397 nm from a scan of the Raman water sample and then automatically performs a time scan to determine the value of the noise at 450 nm. Five measurements with a 100 ms integration time are averaged to produce a 0.5 second total measurement time for each point. A zone noise calculation is determined over ten second intervals to eliminate baseline drift caused by scattering or weak background fluorescence before the peak-to-peak and root mean square SNR computations are performed. Figure 4 shows the results displayed by the software for this validation procedure.

In this example, a SNR for the water Raman sample is almost 9,700:1 RMS and the peak to peak value is over 2800:1.

Conclusion

The combination of highly efficient monochromators, magnesium fluoride over coated optics, an efficient PMT detector, and state-of-the-art single processing electronics has resulted in an instrument with a very high signal-to-noise ratio that provides exceptional performance with weakly fluorescing samples. The automated performance verification routines included in the Luminous software provide a straightforward and simple way to verify that the Lumina fluorescence spectrometer is performing consistently at or above factory specifications.

Figure 4: Results of automatic SNR calculation

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