A Note on Determining the Extent of the Water Raman Peak in Fluorescence Spectroscopy

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A method is proposed for automatically determining the upper and lower boundaries of the Raman scatter peak in fluorescence spectroscopy from empirical data. Accurate peak boundaries are needed to calculate accurate Raman peak areas, used for normalizing fluorescence signals to produce data in units that are comparable between instruments. Comparisons of Raman peak boundaries across nine individual instruments (FluoroMax 3 (FM3) fluorometers from HORIBA Jobin Yvon and Cary Eclipse (CE) fluorometers from Varian Inc.) at the excitation wavelength $\lambda_{ex} = 350$ nm reveal consistent results. At 350 nm excitation, the Raman peak was confined by the emission wavelengths of 382–418 nm, with boundaries determined for the FM3 fluorometers deviating by no more than 0.5 nm and 1.5 nm with respect to the start and end of the peak, and CE fluorometers deviating by up to 1.5 nm and 2 nm, respectively. Peak width was a function of fluorometer type and excitation wavelength. For the FM3 instruments, widths increased from approximately 30 nm at $\lambda_{ex} = 300$ nm to 40 nm at $\lambda_{ex} = 380$ nm, while for the CE instruments, peaks were approximately 5–8 nm narrower. Code for implementing the procedure in MATLAB, which allows for the adjustment of input parameters to compensate for noisy data, is provided in the Supplemental Material (available online).

Index Headings: **Fluorescence spectroscopy; Intensity standardization; Calibration; MATLAB program; Water Raman peak; Instrumentation.**

In fluorescence spectroscopy, the intensities of spectra collected on individual instruments are comparable after correcting for spectral bias[1] and calibrating to the signal of a fluorescent standard.[2–4] While quinine sulfate has traditionally been used as a calibration standard for natural organic matter fluorescence,[2,3] Lawaetz and Stedmon recently proposed calibrating signals to the Raman scatter peak at 350 nm excitation in a water blank.[5] The accuracy of this method depends in part upon accurately determining the area under the Raman peak, which requires an appropriate range for the integration of Raman peak signals to be specified.

The width of the Raman scatter peak depends upon instrument setup, including the resolution of the monochromators, and may therefore vary between instruments.[6] In order to specify an integration range valid across as broad a range of instruments and wavelengths as possible, Lawaetz and Stedmon[5] proposed defining the Raman peak width as peak position ± 1800 cm^{-1}. At a 350 nm excitation wavelength, this equates to a 58 nm wide scatter band spanning from 370 to 428 nm and peak widths of 28, 41, and 79 nm at 250, 300, and 400 nm excitation, respectively.

Reported here is an improved method for calculating the peak width directly from empirical data. Data used to test the methodology consist of emission scans of clean water samples collected from nine fluorometers as part of an interlaboratory comparison.[4] Each was one of two models currently in common use, the FluoroMax 3 (FM3, from HORIBA Jobin Yvon) and the Cary Eclipse (CE, from Varian Inc.). The FM3 is a highly sensitive photon-counting spectrofluorometer in which samples are irradiated by a continuous xenon arc lamp over a specified integration time, whereas the CE averages signals received by the detector over a series of discrete lamp pulses. The CE has single-grating excitation and emission monochromators blazed at 370 nm and 440 nm, whereas the excitation monochromator in the FM3 is typically blazed at 330 nm and the instrument may have single- or double-grating emission monochromators usually blazed at 500 nm. Double monochromators, when present, can largely eliminate secondary light scatter. Also, FM3 optics consist only of mirror and plane gratings rather than lenses and concave gratings as in the CE, resulting in better transmission efficiencies away from the blazed wavelength. As a result of such differences, Cary Eclipse measurements tend to be significantly noisier than FluoroMax measurements.

Raman scans at excitation wavelength $\lambda_{ex} = 350$ nm were collected using each instrument (five FM3 and four CE units) over emission wavelengths $\lambda_{em} = 365$–450 nm at intervals of 0.5 nm, using 5 nm slit widths on the excitation and emission monochromators. Scans at excitation wavelengths between $\lambda_{ex} = 280$–450 nm were also collected by seven of these instruments (four FM3 and three CE units) at emission intervals of 2 nm, which were then interpolated to 1 nm intervals. Scans were corrected for spectral bias using instrument-specific excitation and emission correction factors prior to examining Raman peaks, as previously described.[4]

To calculate the boundaries of the Raman peak for each scan, the gradient of the scatter signal ($dS/d\lambda_{em}$) was calculated from consecutive differences at each data point using the MATLAB function gradient.m, then smoothed using a 5-point moving average (Fig. 1, left panel). The peak was assumed to begin at the first defined-length sequence of positive gradients within a specified distance from the center of the peak and to conclude at the last defined-length sequence of negative gradients (Fig. 1, center panel). Defining an upper and lower range limitation is necessary to exclude peaks occurring outside the potential range for primary Raman scatter, due to, e.g., secondary scatter, Rayleigh scatter, or background fluorescence. A range limitation of ± 1800 cm^{-1} from the peak center, consistent with Lawaetz and Stedmon,[5] was applied by default, equating to maximum allowed peak widths of 28 to 79 nm at excitation wavelengths between 250 and 400

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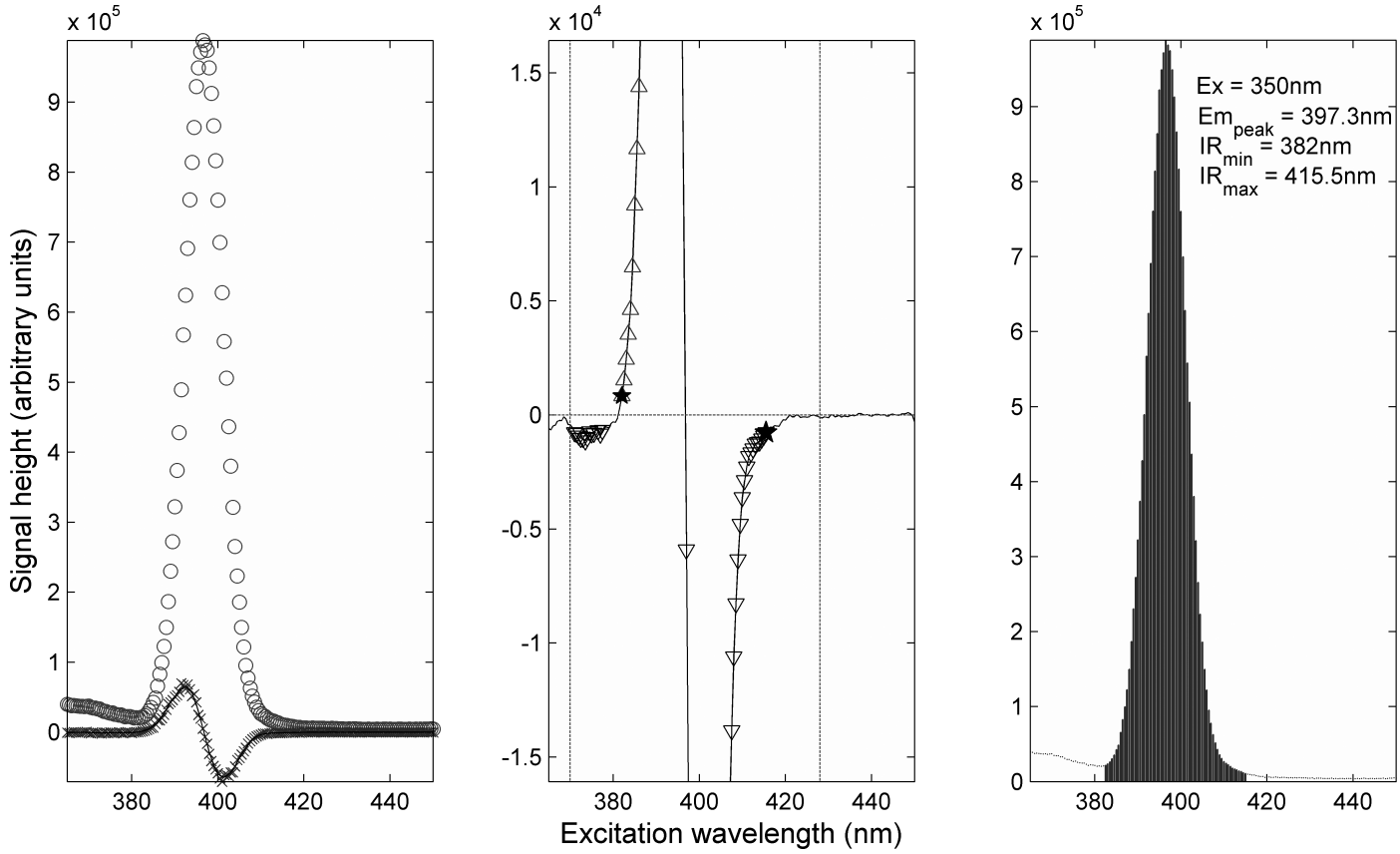

FIG. 1. Identification of the boundaries to the water Raman scatter peak: (**Left**) The gradient of the Raman scatter peak (circles) is calculated at each data point (crosses) and smoothed (bold line), (**center**) positive (up-pointing triangles) and negative (down-pointing triangles) gradients are identified, then the first and last sequences of positive and negative gradients occurring within a specified distance (1800 cm^{-1}) from the peak maximum (confined by the dashed vertical lines) are designated the beginning and end of the peaks (stars). (**Right**) The shaded area shows the integration range for the Raman peak determined by this method.

nm. A tolerance level of 1% of the maximum gradient was specified, indicating the threshold below which a gradient was considered indistinguishable from zero, while the sequence length was set to 8 data points (see below). Increasing sequence length and tolerance level reduces the opportunity for noisy data to be identified as part of a peak, which reduces the width of the peak. Sequence length also depends upon wavelength resolution, such that longer sequences may be preferable for data measured at shorter emission wavelength intervals.

The method was further used to determine the effect of varying the excitation wavelength upon the width of the water Raman scatter peak. Default settings (1% tolerance, sequence = 8) were used for all instruments. Table I shows the median extent of the Raman scatter peak at excitation wavelengths between 280 nm and 440 nm. Peak widths depended upon the fluorometer model, with FM3 peaks on average approximately

TABLE I. Position of the water Raman scatter peak as a function of excitation wavelength (λ_{ex} in nm) for four FluoroMax 3 (Horiba J Y) and three Cary Eclipse (Varian Inc.) fluorometers. The table shows the median emission (λ_{em}) wavelengths in nanometers corresponding to the beginning (lower), middle (center), and end (upper) of the peak.

		Fluoromax 3		Cary Eclipse	
λ_{ex}	$\lambda_{ex\ center}$	$\lambda_{em\ lower}$	$\lambda_{em\ upper}$	$\lambda_{em\ lower}$	$\lambda_{em\ upper}$
280	309.5	300	325	–	–
300	334.1	320	350.5	322	346
320	359.1	345	376.5	345	374
340	384.4	369	403.5	371	403
350	393.7	382	417.5	383	417
360	410.2	394	431	396	431
380	436.4	419.5	459.5	420	457
400	463.0	445	488	446	480
420	490.0	471	517	472	514
440	517.4	497	548	497	540

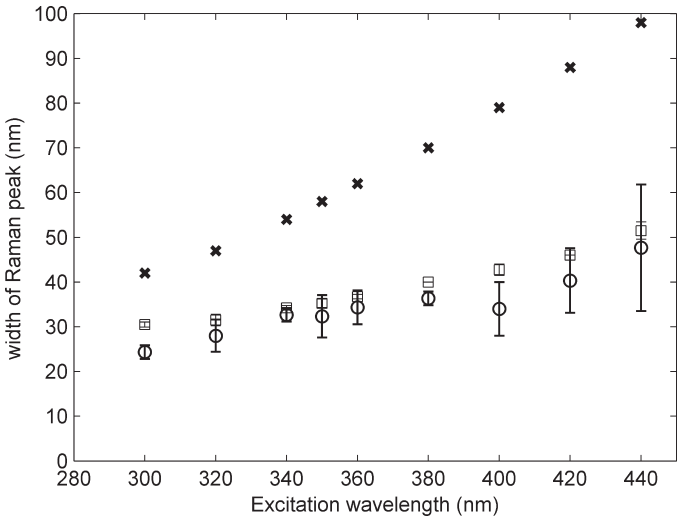

FIG. 2. Empirically determined Raman scatter peak width (mean ± SD) as a function of excitation wavelength for three Cary Eclipse (circles) and four FluoroMax 3 (squares) fluorometers. Widths of peaks defined as 1800 cm^{-1} from the peak center are depicted for comparison (crosses).

TABLE II. Identification of the boundaries to the water Raman scatter peak at $\lambda_{ex} = 350$ nm, showing the effect of varying the sequence length. For six FluoroMax 3 instruments (FM1–6) and three Cary Eclipses (CE1–3), the table shows the median position of the lower and upper peak boundaries and the deviations (in nm) from the median boundaries by each fluorometer. Results for the default sequence length of 8 data points are given in bold.

	Lower bound of emission peak				Upper bound of emission peak			
Sequence length	4	6	**8**	10	4	6	**8**	10
Median (nm)	382.5	382.5	**382.5**	382.5	417.5	417.5	**417**	417
Deviation (nm) from median:								
FM_1	−0.5	−0.5	**−0.5**	−0.5	0	0	**0.5**	0.5
FM_2	0	0	**0**	0	0	0	**0.5**	0.5
FM_3	0	0	**0**	0	−1	−1	**−0.5**	−0.5
FM_4	−0.5	−0.5	**−0.5**	−0.5	−2	−2	**−1.5**	−1.5
FM_5	−0.5	−0.5	**−0.5**	−0.5	−0.5	−0.5	**0**	0
FM_6	0	0	**0**	0	0.5	0.5	**1**	1
CE_1	−0.5	−0.5	**−0.5**	−0.5	10	−2.5	**−2**	−2
CE_2	0.5	0.5	**0.5**	0.5	5.5	5.5	**0**	0
CE_3	1.5	1.5	**1.5**	1.5	1	1	**1.5**	1.5

TABLE III. Percent difference in Raman area calculated using defined peak boundaries (1800 cm^{-1} either side of the maximum) relative to empirically determined Raman boundaries.

	Excitation wavelength (nm)								
	300	320	340	350	360	380	400	420	440
FM_2	2.0	2.2	2.9	3.3	3.1	4.1	4.6	5.7	8.2
FM_3	0.7	1.5	2.3	2.8	2.7	3.7	4.3	5.4	10.6
FM_4	1.3	1.5	2.2	2.8	3.3	4.0	4.6	5.4	9.5
FM_6	1.1	1.6	2.1	2.4	2.6	3.2	4.0	4.7	6.4
CE_1	1.0	3.5	5.2	2.3	5.4	33.0	137.2	114.1	190.9
CE_2	2.3	3.8	4.7	5.4	3.3	9.2	50.6	54.0	104.0
CE_3	6.3	20.3	4.2	−1.3	4.4	0.8	18.1	3.8	10.6

5 to 8 nm wider than CE peaks across a broad excitation wavelength range (Fig. 2). No data are available for $\lambda_{ex} = 280$ nm for the CE instruments because the peak started below the measurement range. Within each fluorometer type, peak widths increased approximately linearly with increasing excitation wavelength. Variation in peak widths between FM3 instruments was very small (at most 1 nm), while greater variability was observed between CE instruments.

The method was applied to emission scans at $\lambda_{ex} = 350$ nm excitation produced by the nine instruments (FM1–6 and CE1–3) to determine the upper and lower boundaries of the Raman scatter peak (Table II). Results are shown for sequence lengths ranging between 4 and 10 data points. Increasing the sequence length had little impact upon the median range of the Raman peak, which was bounded below by 382.5 nm and above by 417.5 nm, and had no effect upon the determination of peak onset for any fluorometer. However, it did reduce variability at the end of the peak for two CE instruments. For the FM3s, sequence lengths between 4 and 10 at a tolerance of 1% produced good results. For the CE instruments, longer sequence lengths (8 or more) reduced variability in peak widths between instruments. At a sequence length of 8, increasing the tolerance level from 1 to 3% decreased the peak width for all instruments by 5 to 6.5 nm. While increasing the tolerance level may be useful for very noisy scans in certain situations, a tolerance level set too high will result in underestimation of the peak width. In this study a default tolerance level of 1% produced good results for all scans.

It is informative to examine the effect of using the empirically calculated peak boundaries ("empirical" method) compared with defining them as ± 1800 cm^{-1} from the peak center ("defined" method) when calculating Raman areas. Raman area is calculated by numerical integration of the area under the peak, minus the baseline area, which can be approximated by a trapezoid.[4] The baseline is calculated from the signal at the start and end of the peak; consequently, differences in the peak boundaries affect both the calculation of the integral under the peak and the area within the baseline trapezoid. While methods have been proposed that do not include baseline subtraction,[5] baseline subtraction can improve the accuracy of Raman area determination when background signal is present (see, for example, the scan in Fig. 1).

Raman areas were calculated at a range of excitation wavelengths using the boundaries determined according to the two methods for scans from seven instruments. The percent difference between the areas (A) was calculated according to Eq. 1:

$$\text{Difference } (\%) = 100 \times (A_{\text{Empirical}} - A_{\text{Defined}})/A_{\text{Empirical}} \quad (1)$$

For the Fluoromax instruments, percent difference in Raman areas ranged from 0.7 to 2% at $\lambda_{ex} = 300$ nm, increasing to 6–11% at 440 nm (Table III). For the CE instruments, percent differences were more variable and typically higher overall, reflecting the presence of noisy data adjacent to the Raman peak, particularly at higher excitation wavelengths. The reason for the discrepancy of 20.3% for CE_3 at $\lambda_{ex} = 320$ nm is illustrated in Fig. 3. In this case, defining an overly broad peak width for a scan with significant fluorescence signals adjacent to the Raman peak caused both the area under the peak and the baseline area to be over-estimated, with the result that the Raman area was ultimately under-estimated by around 20%. If the baseline were not subtracted when calculating the Raman

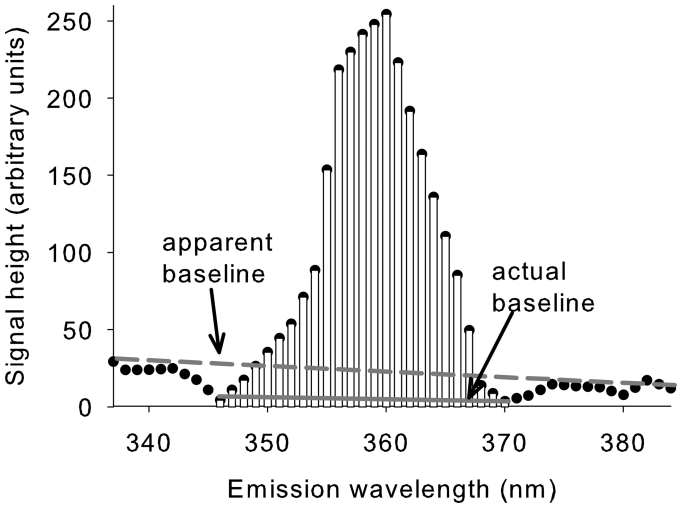

FIG. 3. Raman scan from CE_3 at $\lambda_{ex} = 320$ nm. Using the defined integration range (λ_{em}= 337–384 nm corresponding to peak maximum ± 1800 cm^{-1}), the Raman area is determined using each point depicted in the figure. Using the empirical integration range (λ_{em} = 347–370 nm), Raman area is calculated only from data within the shaded region.

area, the Raman area would instead have been over-estimated by 13%. Full results for Raman area comparisons for which baseline subtraction was omitted are provided in the supplemental materials (Table S1). These results are generally consistent with Table III except that Raman area is consistently over-estimated using this approach. Also, improvements due to the empirical method were typically slightly wider (e.g., at 350 nm excitation, absolute percent differences were 3–4% for FM3 and 4–9% for CE scans).

The method described in this study can be implemented automatically using the MATLAB function "RamanIntegrationRange.m" provided in the Supplemental Materials to this paper and in the FDOMcorr toolbox for MATLAB (http://www.models.life.ku.dk/FDOMcorr). In default mode, the function takes a matrix of scans and a vector of corresponding excitation wavelengths and calculates the upper and lower limits to the Raman peaks. The user may specify tolerance level, sequence length, and half-width (range limitation), or else these will be calculated using the default settings of 1%, 8, and 1800 cm^{-1}, respectively. While the default settings are likely to be satisfactory in most situations, the user is able to optimize these settings for the particular data set at hand using the visualization tools provided, which produce plots similar to those in Fig. 1.

The widths of the peaks calculated empirically according to the empirical method described here are as much as three times lower than would be estimated using a fixed wavenumber distance from the peak maximum. Specifying peak widths that are too broad increases the likelihood that noise and scatter phenomena unrelated to the primary Raman peak are included in the calculation of Raman areas, reducing the accuracy of measurements calibrated in Raman Units. The results of this study indicate that the accuracy of Raman area calculation can be improved by precisely determining the peak boundaries, with the degree of improvement depending on factors including instrumentation and the method used for determining Raman area. To improve the intercomparability of fluorescence intensities between studies, it has been recommended that Raman area calibration be performed using an excitation wavelength of 350 nm.[4,5] While for scans obtained at 350 nm with sensitive instrumentation the improvements in the accuracy of Raman areas due to the method presented here are likely to be modest (below 4%), greater improvements are anticipated for less sensitive instruments and for scans obtained at higher excitation wavelengths.

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SUPPLEMENTAL MATERIAL

Supplemental material mentioned in this paper, including one table and one MATLAB script, are available in the online version of the journal, available at www.s-a-s.org.

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Rapid Analyses of Tiny Amounts of Powder Samples Using Transversely Excited Atmospheric CO_2 Laser-Induced Helium Gas Plasma with the Aid of High-Vacuum Silicon Grease as a Binder on a Metal Subtarget

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Rapid quantitative analyses of powder samples available in tiny amounts have successfully been conducted by utilizing a transversely excited

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