

- Sparrow, M.C., J.F. Jackovitz, C.H. Munro, W.F. Hug, and S.A. Asher, “A New 224nm Hollow Cathode UV Laser Raman Spectrometer”, *J. App. Spectroscopy*, Vol. 55, No. 1, Jan 2001.

A New 224 nm Hollow Cathode UV Laser-Raman Spectrometer

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Abstract

We developed a high throughput UV Raman spectrometer which utilizes a simple, inexpensive new 224.3 nm hollow cathode, quasi-CW laser. The 224.3 nm laser is useful for resonance Raman excitation of polycyclic hydrocarbons and for studies of protein aromatic amino acids. We demonstrate the utility of this excitation to study the environments of tyr and trp in horse heart Myoglobin. We compare these results to previous studies using 229 nm excitation from a frequency doubled Ar⁺⁺ lasers. In addition, we demonstrate that this 224.3 nm excitation can be used to detect sub ppm concentrations of aromatic molecules in aqueous solutions.

Introduction

UV resonance Raman spectroscopy (UVRS) is a powerful tool to examine molecular structure and dynamics. The high UVRS signal-to-noise ratios makes it possible to study trace analytes such as polycyclic aromatic hydrocarbons and to study dilute protein and peptide solutions. Its high spectral selectivity makes it ideal for studying aromatic amino acid environments in proteins. We recently demonstrated that UVRS excited within the * amide peptide absorption bands is the most powerful dilute solution methodology to determine protein secondary structure. It clearly is the most powerful methodology available for studying the first steps in protein folding and unfolding.

Although UVRS is a powerful spectroscopic technique, it has found rather slow acceptance due to the cost and complexity of previous laser sources, and because UVR spectrometers were not *clearly* commercially available. The first UV laser sources were low repetition rate nsec YAG lasers, which were frequency doubled to pump dye lasers, which were then frequency doubled and mixed to obtain tunable UV excitation¹. Alternative strategies utilized frequency tripled and quadrupled YAG lasers followed by Raman shifting. The duty cycle of these lasers were very small (10^{-8}). This resulted in high pulse energies and high incident fluences, which resulted in nonlinear optical phenomena, which complicated Raman spectral measurements. The use of frequency doubled, high repetition rate Excimer laser-pumped dye lasers increased the duty cycle 100-fold, but non-linear phenomena could still pose spectral measurement challenges. The development of the intracavity frequency doubled Ar⁺⁺ and Kr⁺⁺⁺ solved these problems since the lasers were CW^{2,3}. These lasers are quite reliable, but are expensive.

In the work here we describe a new 224.3 nm optimized UVR spectrometer, which utilizes a new, relatively inexpensive laser. This hollow cathode laser is small, has a relatively high electrical power efficiency and requires no water-cooling. We believe that this laser source presents revolutionary opportunities to develop portable inexpensive UVR spectrometers.

Experimental

Acetonitrile and sodium perchlorate were purchased from Fisher Scientific (Pittsburgh, PA). Horse heart Myoglobin (Mb) was purchased from Sigma. Dodecyldimethylamine was purchased from Akzo-Nobel and the benzoic acid (BA) was purchased from Aldrich. The Raman cross-sections of tyr and trp were calculated from previously determined values of the absolute Raman cross-sections of the 932 cm^{-1} band of perchlorate and the 918 cm^{-1} band of acetonitrile¹.

The Mb samples were circulated through the beam as a temperature controlled, free surface flow stream⁴. The use of a free surface avoids sample decomposition on window surfaces and interference from the broad silica Raman bands. Raman scattering was collected by a pair of plano-convex silica lenses and dispersed by the Raman spectrometer described below.

The dodecyldimethylamine and benzoic acid solutions were measured using a micro Raman spectrometer similar to that reported previously⁵, where an Olympus BX60 microscope was coupled to a Spex 1702 monochromator. Light was collected using a 36X Cassegrain objective, and the sample was illuminated by reflection of focused light by a small prism attached to the center of the objective.

Results and Discussion

Hollow Cathode HeAg Laser

Fig 1 illustrates the design of the HeAg 224.3 nm hollow cathode laser tube. The laser tube consists of a 40 cm x 3 mm inside diameter extruded pure silver transverse cathode, which is parallel to a brush-type anode. The plasma gain medium utilizes sputtered Ag in a mixture of helium and other noble gases. Lasing involves a $4d^9 5p^1 \rightarrow 4d^9 5s^1$ transition⁶.

The power supply provides negative, square wave pulses to the cathode at pulse widths variable from 30 to 500 μ sec. The corresponding instantaneous currents range from 5 A to 30 A. The laser can be operated in a single pulse mode, or at a variable repetition rate up to 300 Hz with a pulse width 100 μ sec. Although the laser can be designed for CW operation, we used a pulsed mode version to minimize the size and complexity of the laser tube and power supply. The average input power is less than 100 W. The peak output power at 224.3 nm is typically about 100 mW. For the 100 Hz repetition rate and the 100 μ sec pulse width typically used we obtain a 1% duty cycle, which results in an average output power at 224.3 nm of 1 mW. The laser can be operated at temperatures from -100 to 100 $^{\circ}$ C without the need for warm-up or preheating.

The laser frequency bandwidth is less than 3 GHz. The mode quality of the output beam was determined from the measured focused spot size using the following equation:

$$d = \frac{4m^2 \lambda f}{D}$$

where d is the measured spot size, f is the focal length of the focusing lens, D is the beam diameter and λ is the laser wavelength. The measured m^2 value is approximately 18.

Therefore these lasers can be focused to a ~ 5 micrometer spot with a NA=0.5 lens.

Fig. 2 shows the Raman spectrometer. The laser light is simultaneously steered and frequency filtered by a pair of Pellin-Broca prisms. This is especially necessary for the HeAg laser due to its intense, non-lasing plasma lines. These prisms also separate the frequency-doubled output from the fundamental output of the Ar⁺⁺ laser.

Fig 2 also shows the Czerny-Turner spectrograph, which uses a second stage to reimage the dispersed light in order to reduce the stray light on the CCD detector. The mirrors were dielectrically coated to have a 40% reflectivity at 224.3 nm and > 95% reflectivity for light at 229.4 nm (a 1000 cm⁻¹ Raman shift). These 5 mirrors and the grating (50 % efficiency) are expected to give throughputs of ~0.5% at 224.3 nm and ~39 % at 229.4 nm.

We used a solar-blind intensified CCD detector made by Roper Scientific (IMAX-1024x256). This solar-blind intensifier has a very low equivalent background illumination which improves the spectral signal to noise ratios. In addition, we tested numerous CCD detectors (backthinned as well as coated with fluorophores) and found that the intensified CCD gave much higher signal to noise ratios. This occurred even when the technical specifications indicated that the unintensified detectors should give better signal-to-noise ratios.

The 228.9, 237 and 244 nm laser lines of s and p polarizations were used to measure the spectrograph throughput efficiency. The ~1 mW laser beams were directed through the spectrograph entrance slit and the transmitted power was measured at the detector image plane. The values for s, p polarized light are shown in Fig. 3. The effective monochromator efficiency is the average of that for s and p polarizations. We empirically found that the expression

$$T(\nu) = A * \frac{e^{N + M}}{e^{N + M} + B} + (D + C) \quad (1)$$

effectively models throughput at 224.3 nm, 228.9 nm, 238.2 nm, and 244 nm. $T(\nu)$ is the percent transmission of the monochromator at the Raman shifted frequency (cm^{-1}), where $A=0.3213$, $B=61.3024$, $C=0.007251$, $D=0.00002613$, $N=0.007471$ and $M=-0.5264$ are fitting constants for p polarization. The fit constants for s polarization are $A=-9.6841$, $B=12692.3261$, $C=9.8035$, $D=-0.0008987$, $N=-0.004572$, $M=12.7368$. The measured 224.3 nm Raman spectra were spectrometer efficiency corrected by using the relationship:

$$I_{corr}(\nu) = \frac{I_o(\nu)}{T(\nu)} \quad (2)$$

where $I_{corr}(\nu)$ is the corrected intensity, $I_o(\nu)$ is the observed intensity and $T(\nu)$ is the calculated frequency dependent throughput of the monochromator.

Fig. 4 illustrates the Rayleigh rejection performance of the spectrometer using neat acetonitrile in a quartz cell, a calcite crystal and a block of Teflon. Raman bands as close as 200 cm^{-1} from the Rayleigh line are easily observed for non-turbid samples. The spectrum of calcite shows the strong Raman band at 280 cm^{-1} . The spectrum of Teflon shows that, although highly scattering samples show significant Rayleigh scattering at low frequencies, Raman spectra can be easily measured down to less than 400 cm^{-1} from the exciting line.

Horse Heart Myoglobin Studies

Fig. 5 and 6 show 224.3 and 229 nm excited UV Raman spectra of horse heart Mb at pH 7.1 and 2.1. The samples contain 5 % by volume acetonitrile, which served as an internal standard. Acetonitrile was used for these Mb studies because the typical internal standards such as perchlorate and sulfate increase the solution ionic strength which alters the low pH Mb structure. A careful comparison of CD spectra and UVRS in the presence and absence of 5 % by volume acetonitrile demonstrates that acetonitrile does not alter the low pH Mb structure.

The 224.3 and 229 nm UVRS are very similar and show Raman bands from only the tyr and trp aromatic amino acids; horse heart Mb has two tyr and two trp. The observed Raman bands result from symmetric in plane tyr and trp ring vibrations. Trp gives rise to the 753 cm^{-1} W18 band, the 879 cm^{-1} W17 band, the 1006 cm^{-1} W16 band, the 1353 cm^{-1} W7 band and the 1553 cm^{-1} W3 band. Tyr gives rise to the 1172 cm^{-1} Y9_a and the 1611 cm^{-1} Y8_a band. Only the tyr and trp electronic transitions are in resonance at the 224 and 229 nm excitation wavelengths. The 918 cm^{-1} band derives from the acetonitrile internal standard.

The 229 nm pH 7.1 Mb spectra are similar to those of Chi et al⁷, except that the trp band relative intensities are increased by ~20 % compared to those of the tyr. Recent studies in our lab have demonstrated selective bleaching of the Mb trp Raman spectra if the sample flow rate is insufficient to remove photodegraded protein from the illuminated volume.

The 229 trp band Raman cross-sections decrease more than 3-fold as the pH decreases from neutral pH 7.1 to pH 2.1. This decrease results from the acid denaturation of

the protein, unfolding of the A helix, and exposure of the trp (attached to the A helix) to the aqueous solution. A much smaller decrease occurs for the tyr Y8_a band, because little exposure of the tyr occurs upon the acid denaturation.

The pH 7.1 224.3 nm Mb spectrum appears very similar to that at 229 nm. The major difference is that the W18 and W16 cross sections are decreased by ~25 % while the W3 cross section is increased ~25 % in the 224.3 compared to the 229 nm excitation. The Y8_a tyr cross sections are similar. The 224.3 nm pH 2.1 UVRR also shows a ~3-fold decrease in the relative intensities of the trp bands compared to the tyr. Thus, 224.3 nm excitation can be used to monitor trp and tyr environments in proteins and peptides.

Trace Studies of Aromatic Species

Previous UVRS studies of aromatic polycyclic hydrocarbons demonstrated low ppt detection limits for these species (Reference). Fig.7 shows the concentration dependence of the 224.3 nm solutions of benzoate in a 2.4 mM solution of dodecyldimethylamine. The spectra of the highest concentration BA solution (2.4 mM) shows bands only from the benzoate ring and from water. The aliphatic amine has no resonance transitions and does contribute to the RRS. The BA spectrum shows a series of ring vibrations. The intensities decrease as the BA concentration decreases. The UVRS of 1.5ppm (1.2×10^{-5} M) benzoate-dodecyldimethylamine solution is shown in figure 8 with the solvent subtracted spectrum in the inset. The 1600 cm^{-1} band from the benzoate is still clearly visible. We estimate that under our conditions where we used 4 min spectral accumulation times that we have a detection limit of ~100 ppb ($\sim 1 \times 10^{-6}$ M) by using the $\sim 1600 \text{ cm}^{-1}$ BA band for detection. Longer accumulation times would give rise to lower detection limits.

Conclusions

The development of the HeAg laser for UV Raman excitation at 224.3 nm provides an economical and convenient excitation source for deep UV Resonance Raman spectroscopy. It allows us to further explore the properties of biological molecules by means that were not previously possible. The development of a higher throughput monochromator, coupled with a solar-blind intensified CCD detector provides greater sensitivity without the use of external rejection filters. This in turn gives us the ability to examine sensitive biological samples with a minimum amount of excitation power while still retaining a very good level of signal to noise.

We have shown that the 224.3 nm HeAg laser can be used for studying the trp and tyr solvent environment in proteins and peptides. It is also useful for detection of very dilute solutions (<ppm) of resonant enhanced analytes such as benzoic acid. Our results indicate detection limits of 100ppb or lower are capable with this system. We have also demonstrated the use of acetonitrile as a suitable alternative to perchlorate for use as an internal standard for protein and peptide studies.

Acknowledgements

We are pleased to acknowledge financial support from NIH grant 2 R01 GM30741-16A1 and Bechtel Bettis Inc.

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Figure Captions

Figure 1. Hollow Cathode laser tube.

Figure 2. Schematic diagram of the Raman Spectrometer. The HeAg laser provides 224.3nm excitation. The frequency doubled Ar laser provides 228.9, 238.7 and 244 nm excitation wavelengths. Pellin-Broca prisms are used for beam steering and elimination of plasma lines.

Figure 3. Calculated efficiencies of the monochromator relative to 224.3 nm excitation. The efficiency curve for randomly polarized light was used for correcting spectra acquired with the HeAg laser.

Figure 4. 224.3 nm excited Raman spectra of Teflon block, Calcite and neat Acetonitrile. Teflon and Calcite spectra: power = 0.2 mW. Integration time = 20 seconds. Acetonitrile spectrum: Power= 0.3 mW, Integration Time = 10 sec. Slit Width = 150 mm

Figure 5. 224.3 nm UV Raman spectra of Horse Heart Myoglobin vs. pH. Spectra have been normalized to the 918cm⁻¹ CH₃CN band intensity.

Figure 6. 228.9 nm UV Raman spectra of Horse Heart Myoglobin vs. pH. Spectra have been normalized to the 918cm⁻¹ CH₃CN band intensity.

Figure 7. Log-log of the 1600 cm^{-1} band intensity versus benzoate concentration.

Figure 8. 224.3 nm UV Resonance Raman spectrum of 1.5ppm ($1.2 \times 10^{-5}\text{ M}$) dodecyldimethylamine-benzoate solution. Laser power = 0.5mW. Integration time = 4 min. Inset: solvent subtracted spectrum of 1.5 ppm dimethyldodecylamine-benzoate solution showing 1600cm^{-1} band.

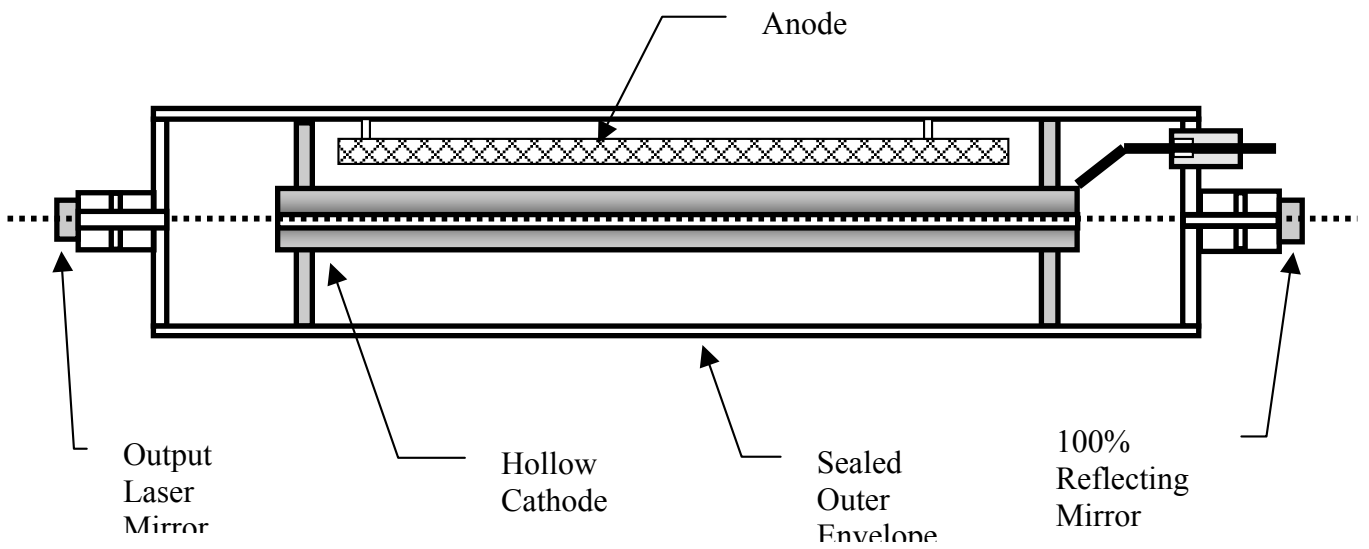


Figure 1.

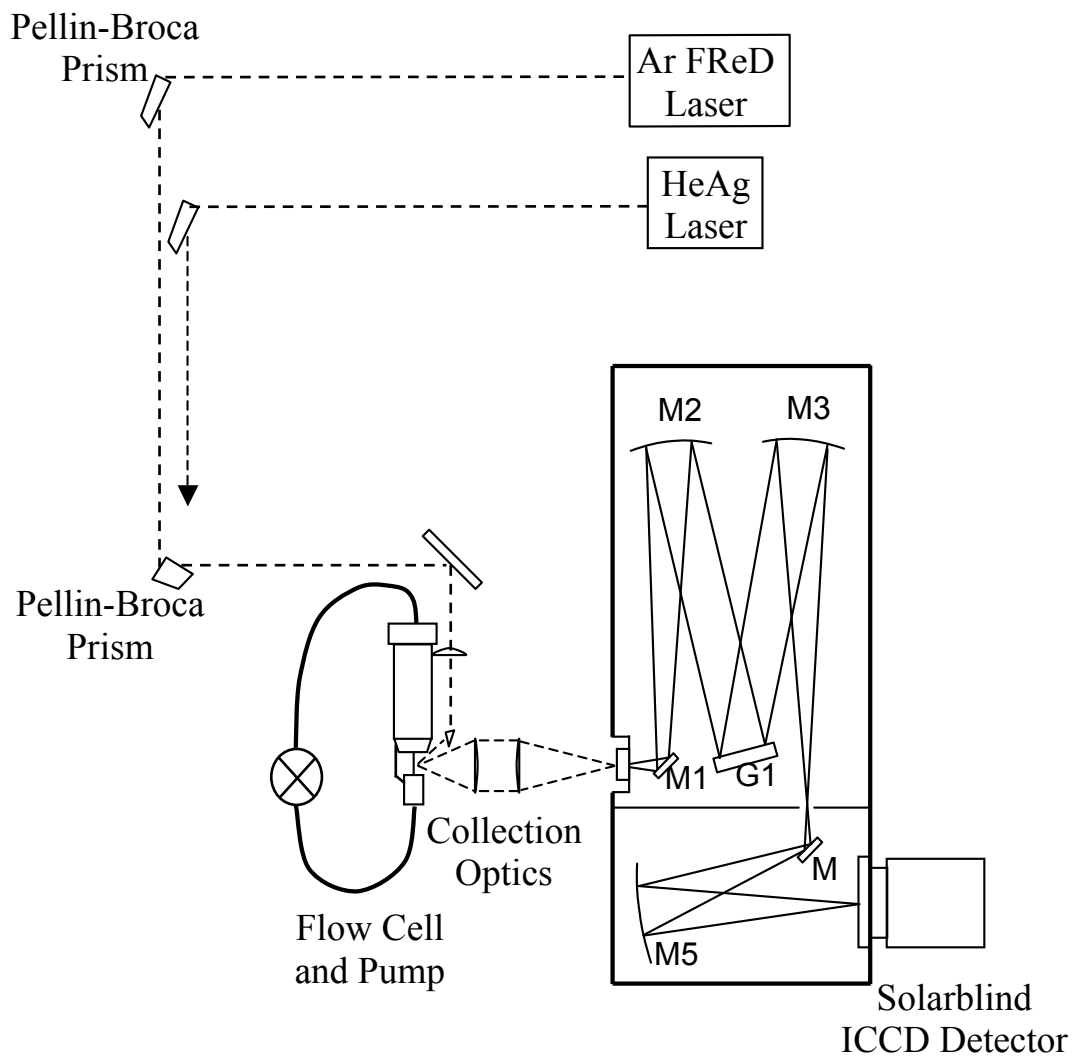


Figure 2

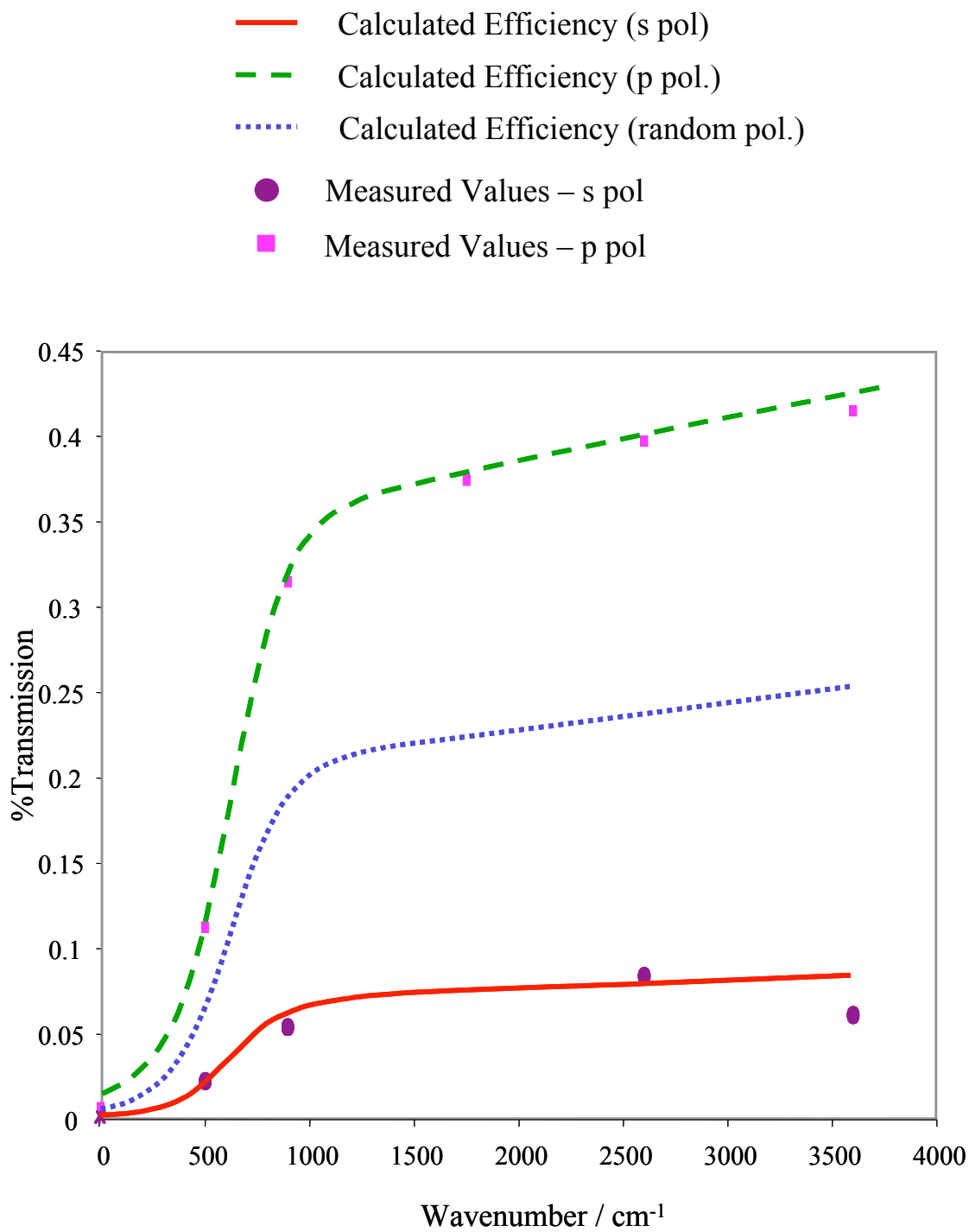


Figure 3

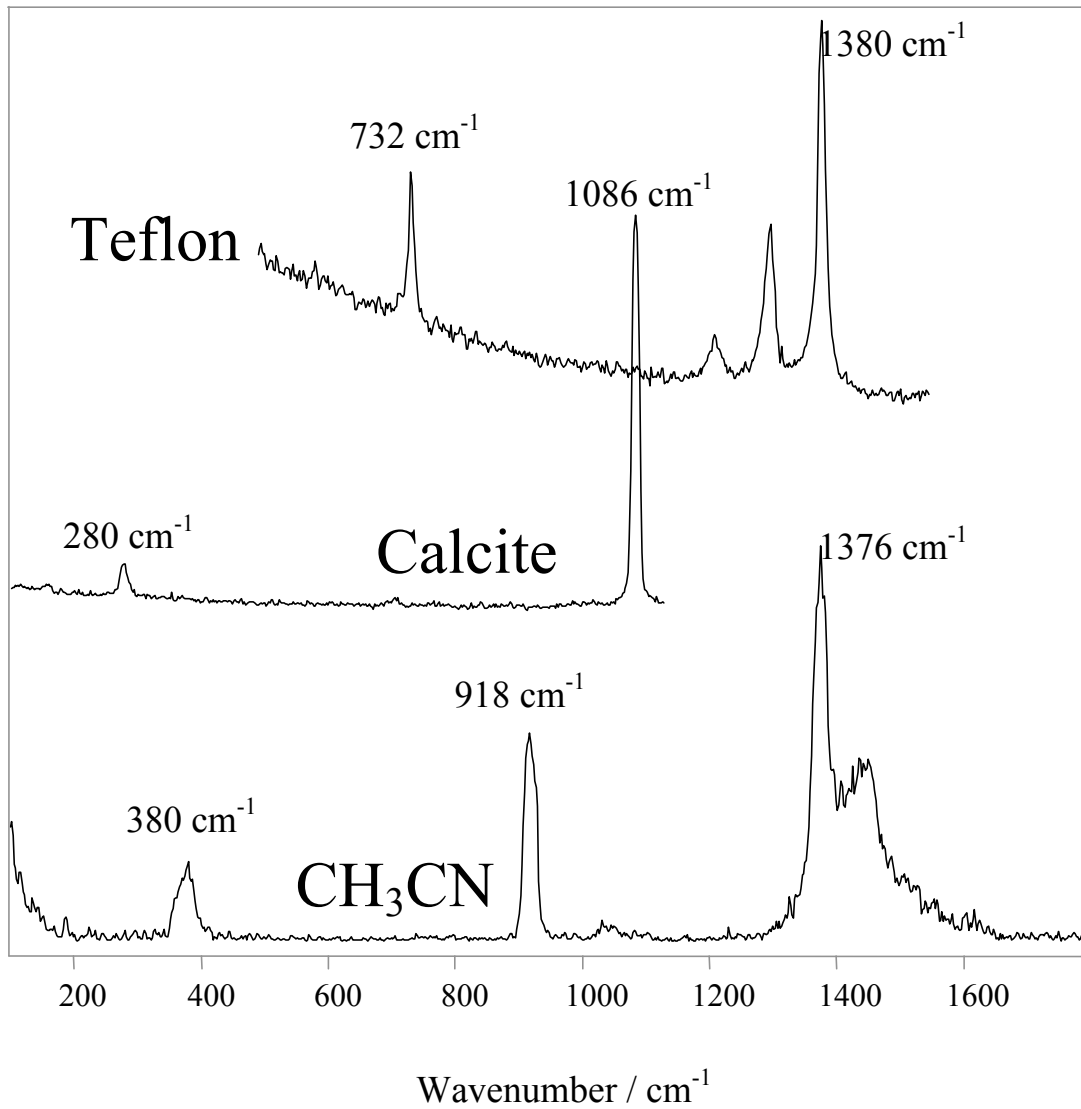


Figure 4.

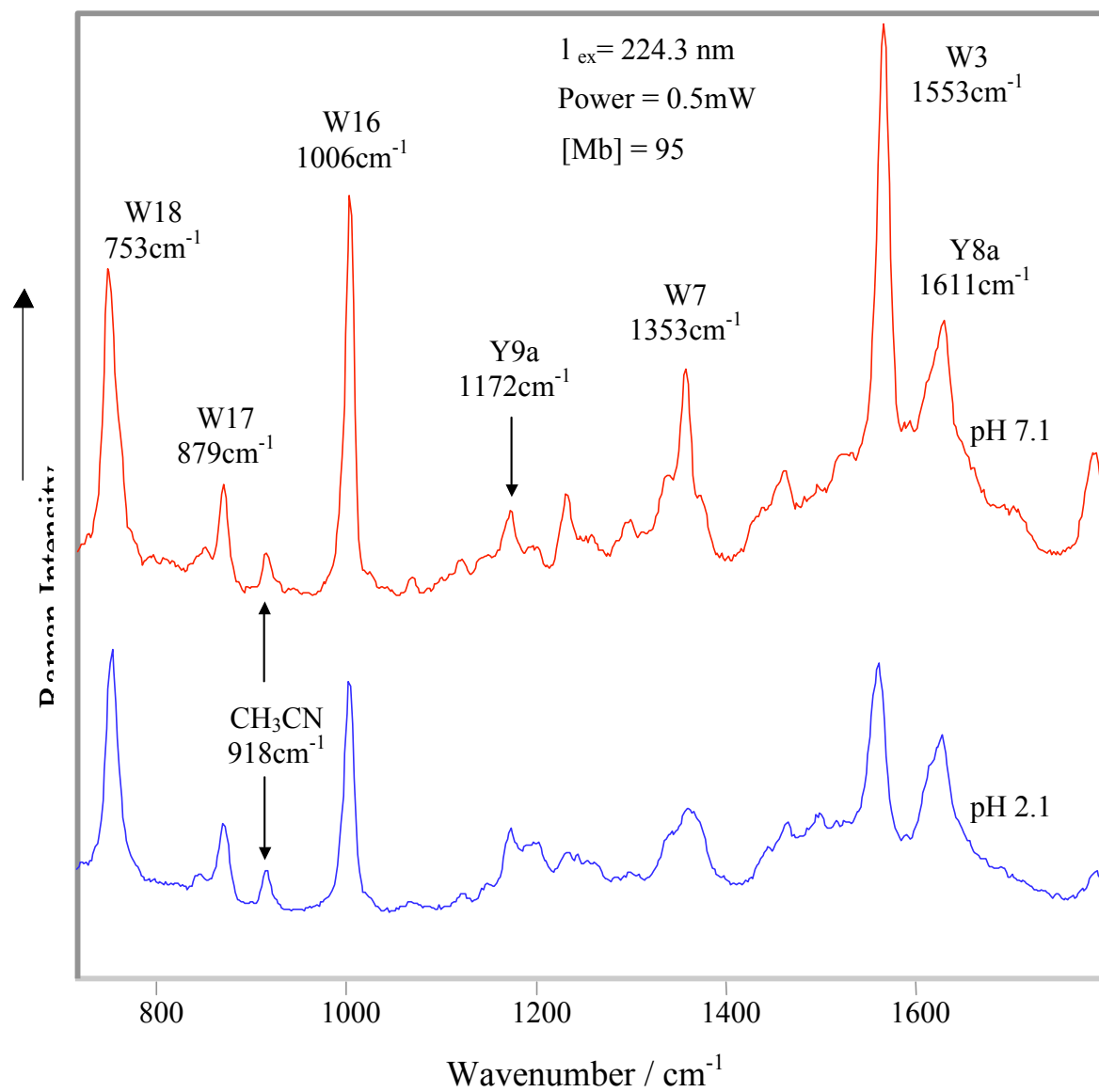


Figure 5

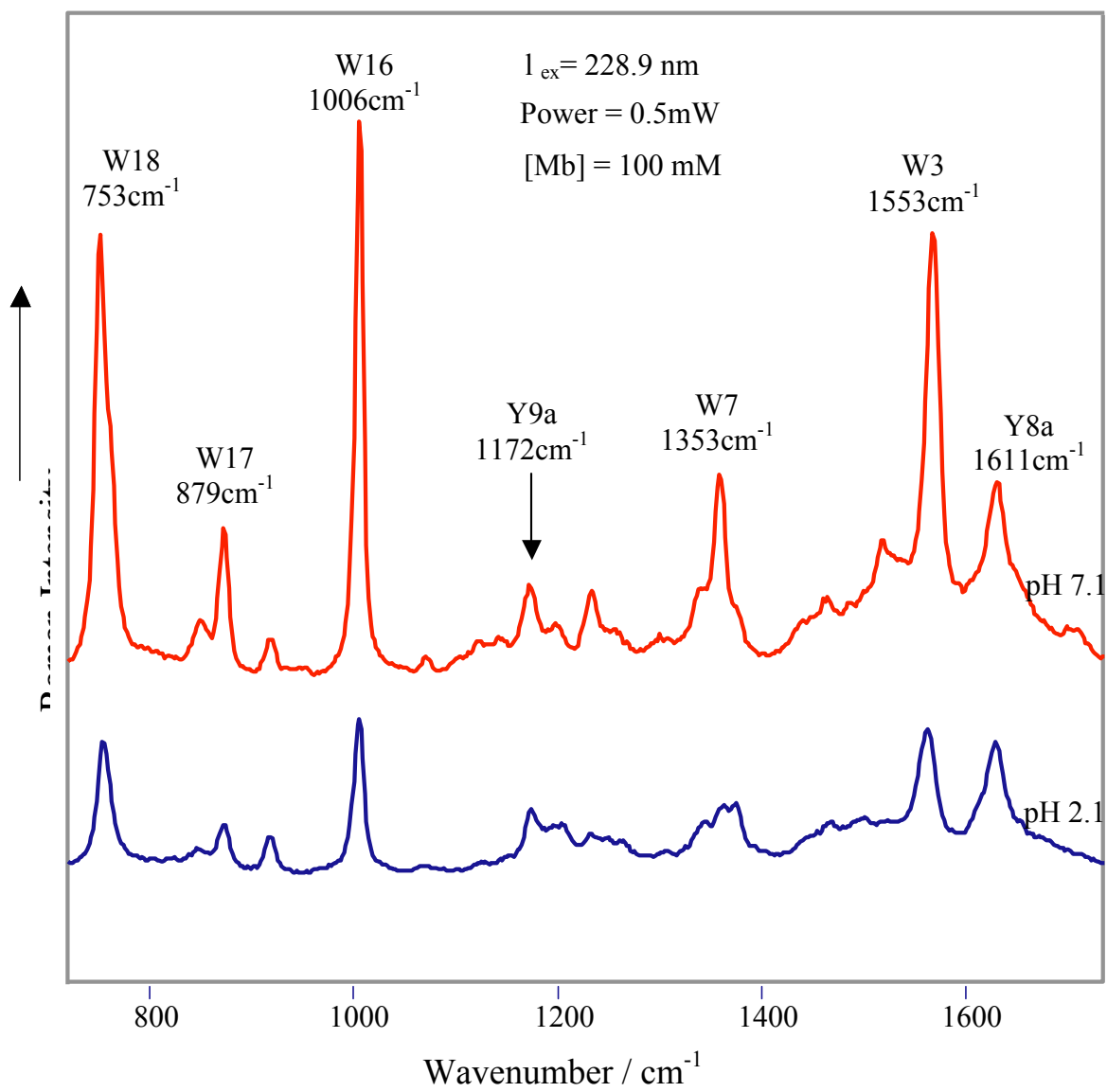


Figure 6

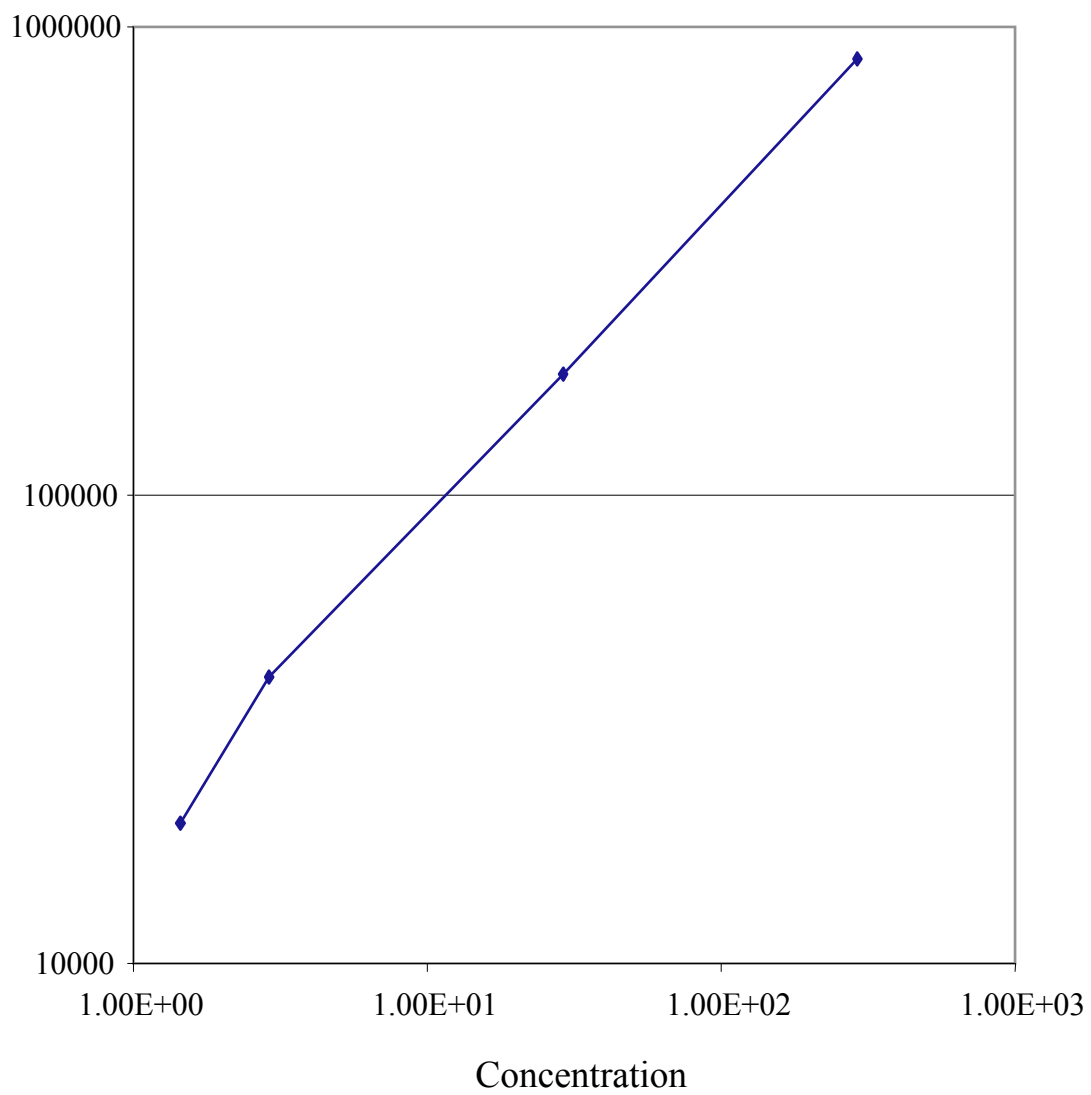


Figure 7

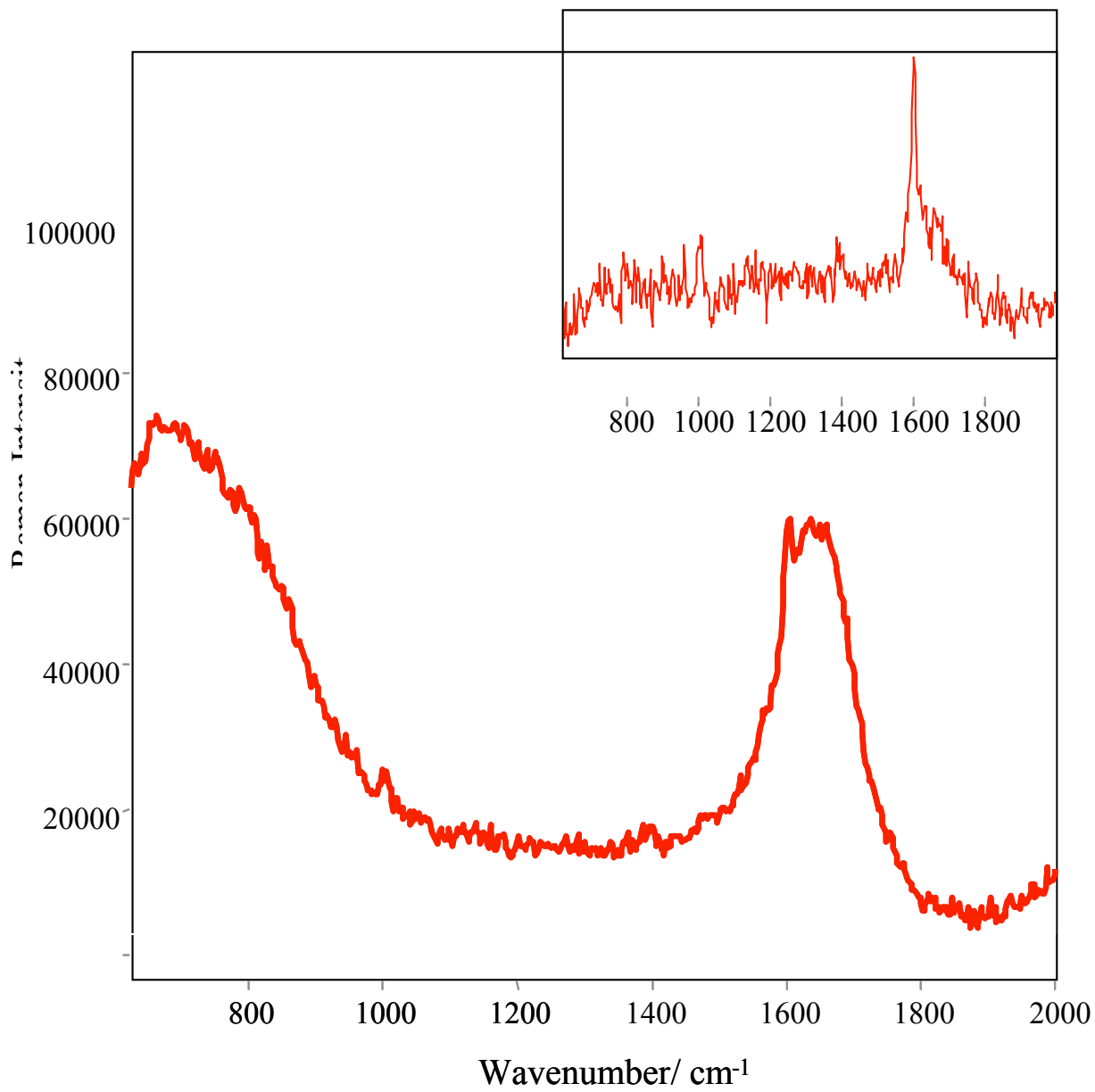


Figure 8