Quantitative and qualitative analysis of fluorescent substances and binary mixtures by use of shifted excitation Raman difference spectroscopy

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ABSTRACT

Shifted Excitation Raman Difference Spectroscopy (SERDS) implemented with two wavelength-stabilized laser diodes with fixed wavelength separation is discussed as an effective method for dealing with the effects of fluorescence in Raman spectroscopic analysis. In this presentation we discuss the results of both qualitative and quantitative SERDS analysis of a variety of strongly fluorescing samples, including binary liquid mixtures. This application is enabled by the Volume Bragg Grating® (VBG®) technology, which allows manufacturing of compact low-cost high-power laser sources, suitable for extending the SERDS methodology to portable Raman spectrometers.

INTRODUCTION

Raman spectroscopy has been experiencing a period of growing interest for qualitative and quantitative analysis in a vast scope of applications pertinent to various industries, including pharmaceuticals [1-5], petrochemical [6-8] and law enforcement [9-13]. This growth is enabled by increase in the offering of affordable Raman instrumentation, some is portable and ruggedized for field use. Such increase in availability and portability is powered in turn by compact, high-performance wavelength-stabilized laser diodes. However, with widening of the scope of applications for Raman spectroscopy the challenge of sample fluorescence becomes more and more evident. Besides the natural fluorescence that many substances possess, there are issues of sample contamination with fluorescent compounds in the field that one must deal with in real life situations [9-10]. One example of that is fluorescent agents such as caffeine and flour regularly used cutting illicit street drugs [10].

To combat sample fluorescence laser excitation at longer wavelengths has long been used [14-16]. Indeed, if it were not for sample fluorescence nearly all Raman spectroscopy could be performed with the same short-wavelength laser, especially since blue and violet diode lasers are becoming more and more powerful and affordable. As it is right now, 785 nm and 830 nm laser excitation wavelengths have been an acceptable compromise for substances with weak short-IR fluorescence, and 1064 nm excitation has to be resorted to in order to deal with samples exhibiting stronger short-IR fluorescence.

Although the long-wavelength, typically1064 nm, Raman instruments have been considered the benchmark in dealing with fluorescent substances, dealing with that excitation wavelength presents a number of issues. First of all, as

is well known, the Raman signal diminishes as the 4^{th} power of laser wavelength, which means that it is ~ 3.4 times weaker with 1064 nm excitation as compared with commonly used 785 nm excitation. Generally it means that significantly longer collection times are necessary to collect as much Raman signal when using 1064 nm laser. Furthermore, the use of 1064 nm excitation laser requires using InGaAs detectors instead of silicon CCDs. In case of dispersive Raman systems this means InGaAs arrays, which have more than an order of magnitude higher thermal noise than that of the silicon CCDs. As a result, InGaAs detector arrays for dispersive Raman instruments must be cooled to achieve satisfactory noise levels. Although cooling the detector is not a problem for laboratory instruments, it presents an obvious issue for portable, battery-operated Raman instrumentation, as it means more power consumption, larger battery and shorter battery life, in addition to increase in cost and complexity of the instrument.

For these reasons the use of the Shifted Excitation Raman Difference Spectroscopy (SERDS) approach has long been considered attractive, effectively expanding the use of the conventional dispersive Raman instrumentation equipped with inexpensive and efficient CCD detectors to the classes of samples that exhibit fluorescence. Historically the SERDS method has been used in laboratories employing tunable-wavelength lasers. However, the use of this type of laser sources presents a significant challenge for portable Raman systems. Generally these lasers are much more costly than simple and efficient wavelength-stabilized laser diodes. But in addition to this there is an issue of the exact wavelength control over these lasers required to perform accurate qualitative and especially quantitative Raman analysis, as it is not fixed or stable, which requires constant active wavelength monitoring for accurate analysis.

For these reasons we have studied a comparatively much more simple and practical approach to performing SERDS analysis using two affordable wavelength-stabilized laser diodes operating at slightly offset wavelengths. The lasers are stabilized by use of the Volume Bragg Grating (VBG) technology and are very compact, efficient and inexpensive, making them well suited for portable battery-operated Raman instrumentation. The use of such fixed-offset SERDS method is compared here with the conventional Raman analysis employing baseline fitting and also a long-wavelength 1064 nm dispersive Raman system.

EXPERIMENTAL

The SERDS experiments were performed using a dual-laser SERDS laser source, LS-2, produced by PD-LD, Inc. The lasers operated at the 784.5 nm and 785.5 nm wavelengths. The wavelength separation was selected to correspond to the approximate line width of the Raman lines of the substances under study. Note that the exact wavelength separation is not significant for the accuracy and practicality of SERDS, however, wavelength separation that is much smaller than the width of the Raman bands would result in increase in noise in SERDS spectra.

The LS-2 laser source delivers the output of either one of the lasers to the output port via a fiber-optic switch. A fiber-optic cable was attached to the output port of the LS-2 and then coupled into a bench-top Raman system. The system delivered the laser light to the sample and then collected the Raman signal in the back-reflection geometry, together with fluorescence and the Rayleigh scattering. The notch filters installed in the optical system suppressed the Rayleigh scattering and the collected signal was delivered to the input port of the spectrometer via the collection fiber in the probe.

For the experiments with 785 nm excitation we used a laboratory F/3 bench top spectrometer with 30 cm focal length and 600 groves per millimeter diffraction grating. The spectrometer had and slit and resolution. The detector was a cooled CCD with 1024x256 pixel array from Princeton Instruments. For SERDS analysis the spectra were collected sequentially with laser #1 and then with laser #2. The lasers ran continuously during the course of the experiment to assure best stability of the output power. The wavelengths of the lasers were stable to < 5 pm over the course of the day. The samples under study were either in liquid or solid form. Majority of the samples (usually liquids) were placed in small glass vials and illuminated through the bottom of the vial.

A series of experiments were conducted to quantitatively evaluate the performance of SERDS versus more conventional methods in situations where high amount of fluorescence obscures the Raman signal. The conventional methods usually involve baseline fitting for removing the relatively smooth spectrum of the fluorescence. In order to compare the performance of these methods with SERDS we have selected a simple substance with strong fluorescent background. The model substance was dark rum, whose Raman spectrum is essentially that of pure ethanol. We have used two types or rum that produced moderate and strong fluorescence background, respectively. The spectra were then treated according to the analysis methods under study (baseline fitting and SERDS), after which the coefficient of determination (R2) was computed for identification of the pure ethanol in the spectrum. The R2 was used as the effective figure of merit for this study.

Besides the quantitative analysis described above, we have analyzed an emerald specimen as an example of qualitative comparison of various Raman techniques using a naturally fluorescent mineral. As it happens, emerald fluoresces even at the 1064 nm excitation wavelength, and so its analysis is challenging for both systems. The results of these tests are discussed below in detail.

In addition to the two methods mentioned above, we have used long-wavelength Raman analysis with 1064 nm laser excitation as a benchmark comparison. Long-wavelength Raman has long been considered the preferred method for studying of highly fluorescent compounds, so it was rather interesting to evaluate it side by side with SERDS performed at 785 nm. We have used Bayspec Agility 1064 nm dispersive Raman system for these experiments. The data were collected for samples of pure ethanol and the two types of rum, which were then processed by the same methodology to compute the R2 coefficient. The InGaAs sensor in the system is cooled to approximately -20C to reduce thermal noise.

All the experiments were performed at the same power on the sample (450 mW) for both the 785 nm and the 1064 nm systems. For better comparison the spectral resolution of the Spex spectrometer was also adjusted by opening of the slit width to match that of the 1064 nm Agility system (approximately 18 cm^{-1}). The exposure time was always selected to keep the maximum of the signal just below the saturation level of the detector array. Therefore, when large

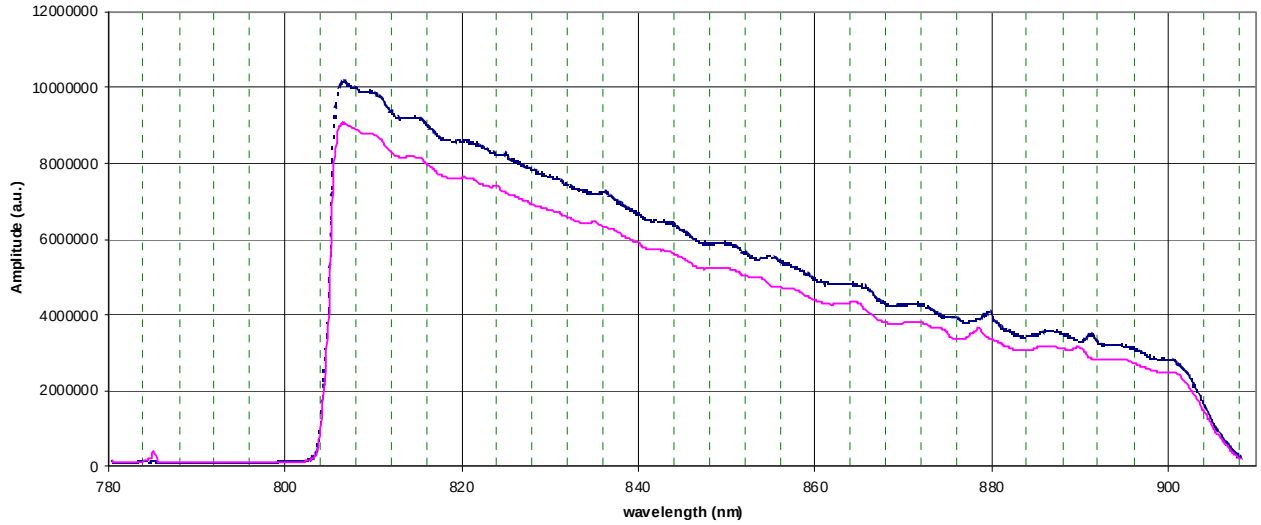

Figure 1. Raman spectra of a binary mixture of ethanol and methanol in the presence of large amount of fluorescence from rhodamine 6G dye. The spectra shown in this figure were collected at the two slightly offset laser wavelengths.

amount of fluorescence was collected the exposure time was shortened accordingly. When performing analysis of highly fluorescent samples, we have employed signal averaging to study the effect it has on the increase of the signal to noise ratio. Note that due to the shortening of the exposure time when fluorescence is present there can be a proportionately larger number of averages performed within the same measurement time. Furthermore, due to smaller Raman scattering cross-section at longer wavelength, the measurement times for the 1064 nm system are typically longer than those for the 785 nm systems (approximately by factor of 5 for the systems employed in this study). Therefore, an interesting comparison between the 785 nm and the1064 nm excitation wavelength is the R2 achieved within the comparable total collection times.

All collected spectra were treated according to the same procedure. Once the Raman spectrum of the sample was obtained and saved, a spectrum of the background was collected using the same measurement parameters as those used on the sample of interest. That included the exposure time and the number of averages performed. The background spectrum was then subtracted from the sample spectrum. The resultant data were corrected by the combined spectral response of the system, which included the transmission of the laser suppression filters and the relative pixel response of the CCD. Therefore, all the spectra used in this study were free from the stationary noise (e.g. pixel noise of the detector). For computing of the R2 we used spectra that were centered (i.e. with zero mean) and normalized (i.e. with unitary scalar product).

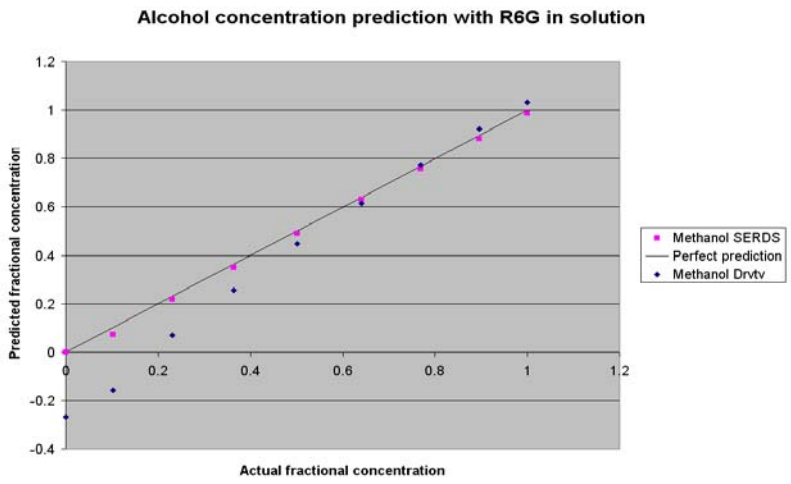

Figure 2. Prediction of the concentration of methanol in binary mixture with ethanol with large amount of fluorescence from R6G dye for SERDS and numerical differentiation methods.

When performing the analysis of binary mixtures, both SERDS spectra and the Raman spectra processed by conventional methods were compared with the library spectra for identification. Note that in no case there was any *a priori* knowledge used for either identification or quantitative Raman analysis. Once identified, the spectra were also processed for quantitative determination of the relative concentration of the two major identified constituents, as will be discussed below in more details.

RESULTS

Due to the fact that SERDS spectra are automatically free from any background, have zero integral over the spectral window and nearly perfect derivative shape they are extremely convenient for performing automated Raman analysis, as they turn out to be nearly orthogonal for majority of the substances of interest to Raman analysis. In order to illustrate this point we performed quantitative Raman analysis on binary mixtures of methanol, ethanol, isopropanol and acetone in the presence of a strong fluorescence background. In this experiment the obtained SERDS spectrum was compared with library SERDS spectra, the components of the mixtures were identified and then relative concentration of the two most likely components in the mixture was determined. No multivariate calibration methods (e.g. partial least square

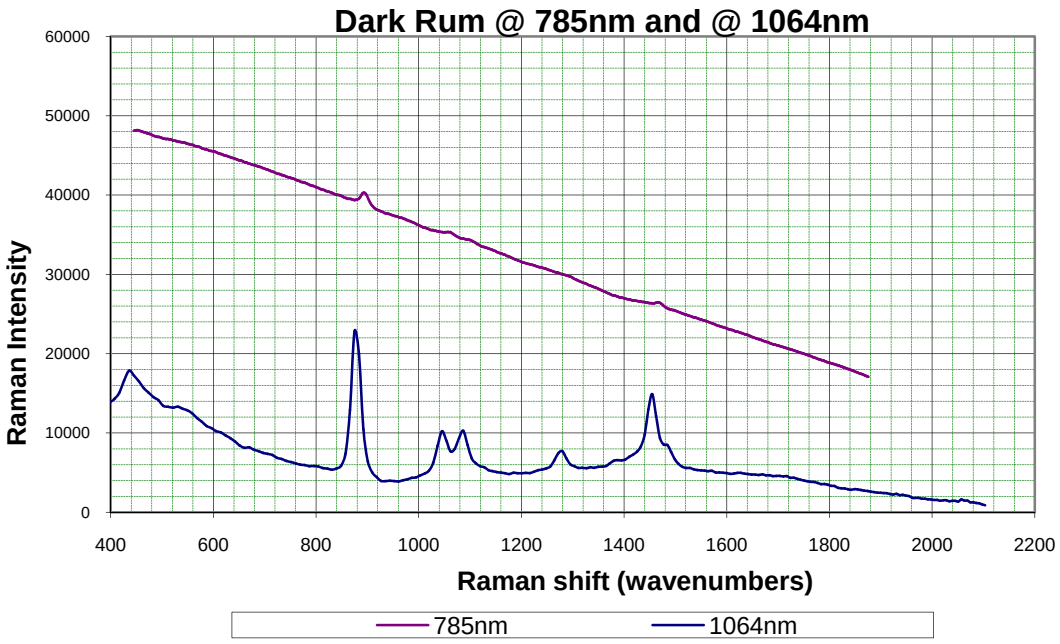

Figure 3. Raman spectra of dark rum collected at 785 nm (0.15 sec exposure time, 100 averages) and 1064 nm (15 sec exposure time, 1 average).

regression, PLSR) were employed, i.e. the system was not trained on the known mixtures – rather all samples were treated as completely unknown.

In order to compare the results obtained by SERDS with what conventional Raman analysis would predict, the fluorescence background was removed via numerical differentiation and then similar analysis was carried out as in the case of SERDS. The Raman spectra were collected on a series of solutions with different concentration of methanol in the presence of ethanol. All solutions contained large amount of R6G dye to simulate strong interference from fluorescence. The raw Raman spectra of one of these solutions are shown in Fig. 1. Figure 2 shows the predicted concentrations of methanol in the mixtures obtained via SERDS and the conventional method of numerical differentiation. As one can see, even though both methods give reasonably accurate prediction of the methanol concentration when it is present in large proportions, the picture is quite different when lower proportion of the methanol is present in the mixture. The conventional method starts loosing accuracy at about 20 – 25% concentration and then fails completely at concentrations lower than 10%. SERDS, on the other hand, predicts accurate concentrations even at 5% methanol content in the mixture. Note that in these experiments the ratio of Raman signal to fluorescence background was about 1:200, so that Raman peaks were barely observable over the fluorescence.

In order to illustrate the power of SERDS method for this type of application we analyzed strongly fluorescent Jamaican dark rum, which is often used a benchmark in Raman analysis of fluorescent samples. Fig. 3 shows a raw Raman spectrum of the dark rum collected with 785 nm and the 1064 nm excitation. The spectra are shown before any baseline correction has been performed. As it can be observed, the amount of fluorescence in the dark rum obscures the Raman signal nearly completely, when the 785 nm excitation is used. As expected, the 1064 nm excitation produces much smaller amount of fluorescence, although fluorescence is still prominent even in that wavelength region.

For comparison, Fig. 4 shows SERDS spectra of the dark rum obtained with different number of averages versus the SERDS spectrum of ethanol. The spectra are vertically offset for clarity. The improvement in signal to noise

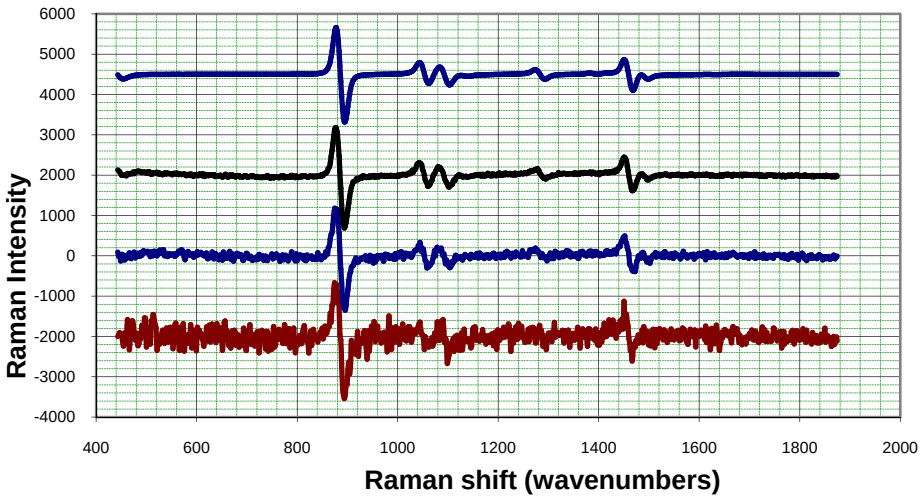

Figure 4. SERDS spectra of dark rum collected at 785 nm with different number of averages. The spectra shown are (bottom to top): rum with 1, 10 and 100 averages, pure ethanol.

with the increase in the number of averages is quite apparent, whereas the total acquisition time is still similar to that of the 1064 nm system even when 100 averages are performed.

For a visual comparison of SERDS with the baseline fitting method, in Fig. 5 we are showing baseline-corrected conventional Raman spectra obtained in the identical conditions as the SERDS spectra above. The Raman spectrum of pure ethanol is also shown for comparison.

As was mentioned earlier, in order to perform quantitative comparison of the two methods we have selected the coefficient of determination, the R^2, as the figure of merit for each approach. Table 1 compares the R^2 values for the ethanol in the dark rum obtained via baseline fitting, SERDS and long-wavelength Raman. The values are shown for different number of averages.

Table 1. Coefficient of determination (R^2) for ethanol in dark rum for various methods of Raman analysis. Note that in case of the 1064 nm excitation, the R^2 computed with full spectrum (starting at 200 cm^{-1} case [a]) is different than that computed with truncated spectrum (starting at 400 cm^{-1} case [b]). Note that total acquisition time for 785 nm with 100 averages is the same as that for1 average for the 1064 nm system.

# of averages	785 nm Raman	SERDS at 785 nm	1064 nm Raman
1	0.246	0.569	0.765[a] or 0.9[b]
10	0.281	0.683	
100	0.287	0.892	

As is evident from these results, the SERDS method offers distinct advantages in certain situations. Its advantage in comparison with the baseline fitting method is primarily in accurate elimination of the fluorescence, whereas the latter method allows only approximate removal of the fluorescent background and only in the situations where it is varying very slowly. Even in the examples considered here, where the fluorescence has no identifiable spectral features, the residual left after the baseline removal deteriorates the R^2 significantly.

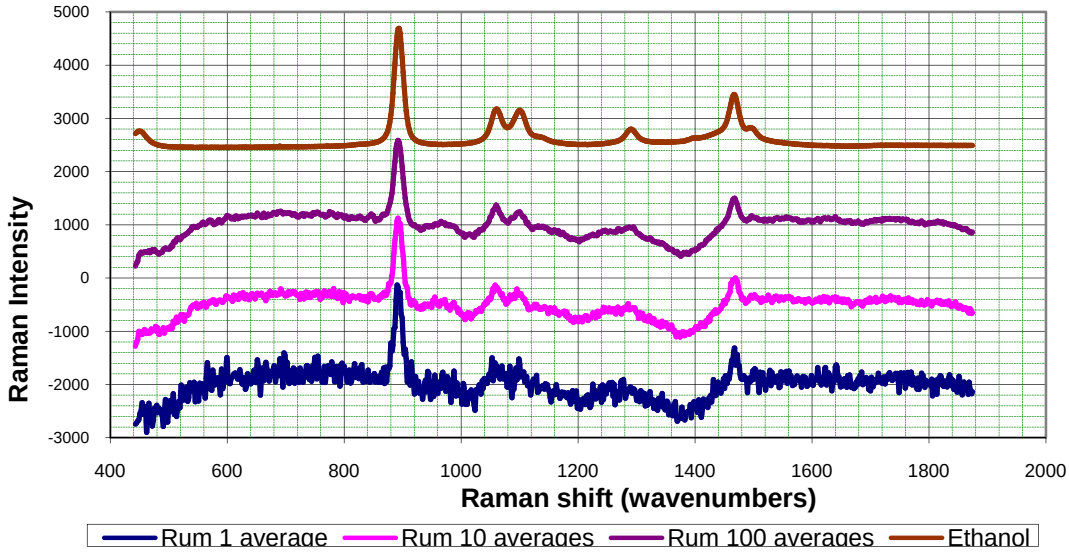

Figure 5. Raman spectra of dark rum collected at 785 nm with baseline removed by polynomial fitting. The spectra shown are (bottom to top): rum with 1, 10 and 100 averages, ethanol.

Fig. 6 shows Raman spectra of emerald collected at 785 nm and 1064 nm. As is evident from this result, the amount of fluorescence is not reduced by moving to the 1064 nm excitation. The SERDS method, on the other hand, produces very clear and identifiable spectrum, as is shown in the same figure.

DISCUSSION

As is evident from these results, the SERDS method offers distinct advantages in certain situations. Its advantage in comparison with the baseline fitting method is primarily in accurate elimination of the fluorescence, whereas the latter method allows only approximate removal of the fluorescent background and only in the situations where it is varying very slowly. Even in the examples considered here, where the fluorescence has no identifiable spectral features, the residual left after the baseline removal deteriorates the R^2 significantly.

In comparison with 785 nm excitation, the 1064 nm Raman system usually produces much smaller amount of fluorescence, as is the case for the dark rum. For that reason it has traditionally been used as the benchmark in Raman analysis. However, as the side-by-side comparison with SERDS in similar experimental conditions shows, SERDS can produce very comparable in quality analysis. In the case under study, the advantages of stronger Raman signal and the better detector enjoyed by the 785 nm system resulted in nearly the same R^2 for the highly fluorescent rum in about the same total acquisition time. Furthermore, as the example of Raman analysis of a specimen of emerald demonstrates, the long-wavelength excitation is not the ideal solution for all cases, because some of the substances will exhibit fluorescence even with 1064 nm excitation.

CONCLUSIONS

We have shown that SERDS analysis can be easily and successfully carried out using two VBG-stabilized laser diodes operating at two slightly offset fixed wavelengths. The method has been shown to give accurate measurement of the concentration of a minor constituent in a binary liquid mixture of alcohols at 5% of the minor component in the presence of strong fluorescence background that exceeded the Raman signal by approximately 200:1 ratio. Any conventional Raman analysis carried out in the same conditions with the same excitation wavelength fails at approximately 3 times

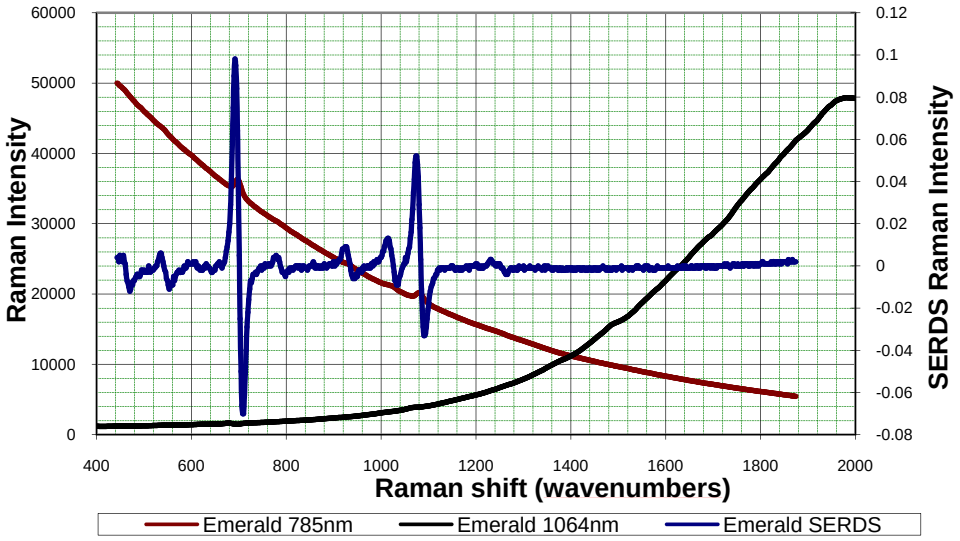

Figure 6. Raman spectra of emerald collected at 785 nm and 1064 nm (left axis). The left axis shows SERDS spectrum of emerald obtained with 785 nm excitation.

larger concentration of the same component in the mixture. The analysis was performed without any *a prior* knowledge about the substances in the sample, i.e. no prior training was performed on the system using any of the multivariate analysis methods (e.g. PLSR).

Overall, the advantages of higher resolution, larger number of pixels, better thermal noise and lower cost of the silicon CCDs make the 785 nm Raman systems very attractive for the field use. Therefore, it is quite clear that the availability of SERDS method made possible by the use of the VBG technology offers clear advantages for many situations where fluorescence is an impediment to Raman analysis. Although SERDS systems with 785 nm excitation will not replace the long-wavelength Raman analysis in all cases, it needs to be remembered that for some substances the 1064 nm systems will not be the right choice without SERDS either.

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