

Comparable Raman Spectroscopy Across Different Instruments and Excitation Wavelengths

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November 2004

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ISSN 1744-0602

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We gratefully acknowledge the financial support of the UK Department of Trade and Industry (National Measurement System Policy Unit)

Approved on behalf of the Managing Director, NPL
by Martyn Sené, Head, Division for Quality of Life

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1. Introduction

1.1. Context

This report is a result of part of the work done under the project titled “Validation of Raman Spectroscopy for Quality Control in Pharmaceutical Applications” (also known as “RamanQC”) which was formulated in response to the Invitation to Tender (ITT) GBBK/C/019/00009, as part of the DTI’s first “Measurements for Biotechnology” programme. The project was led by the National Physical Laboratory and delivered by a broad consortium of interested parties including GlaxoSmithKline, Le Pevelen Scientific, Jasco UK and the University of Strathclyde.

For Raman spectroscopy to be widely applied to the analysis of biopharmaceuticals, it is important that the measurements obtained be comparable. However, at present it is clear that different results are likely to be observed when measurements are acquired using different instruments or different laser wavelengths. In this report, we look at the extent of this problem and propose solutions to the acquisition of comparable Raman data.

This section gives a general introduction to Raman spectroscopy. Section 2 presents a comparison of the different Raman technologies and compares their advantages and disadvantages for the analysis of sensitive biological samples. In Section 3, protocols are given to help users obtain comparable Raman data. Section 3.4 presents a number of case studies, and methods and materials are presented in section 5.

1.2. What is Raman spectroscopy?

When a photon is incident on a given material it may be either absorbed or scattered. There are two types of scattering: elastic, where the energy of the scattered light is unmodified, and inelastic, where the energy of the scattered light is changed. Raman spectroscopy is a measure of wavelength and intensity of the inelastically scattered light from a given material; a Raman spectrum gives information on the stretching and bending vibrational modes of a molecule [1].

In order to explain the Raman effect, one can adopt the quantum mechanical approach illustrated in **Figure 1.1**. This shows that frequency difference between scattered photons and incident photons occurs as a result of the coupling between the incident radiation and the quantised states of the scattering system (target material). Theoretically, incident photons can lose energy (Stokes shift) or gain energy (Anti-Stokes shift) by a vibrational quantum of the target molecule. The intensity of the anti-Stokes shift is generally less than the Stokes shift, as anti-Stokes scattering occurs from an excited state ($v=1$), which according to the Boltzmann distribution is less populated than the ground state ($v=0$). As a result, the more intense lines of the Stokes Raman spectrum are typically detected [2].

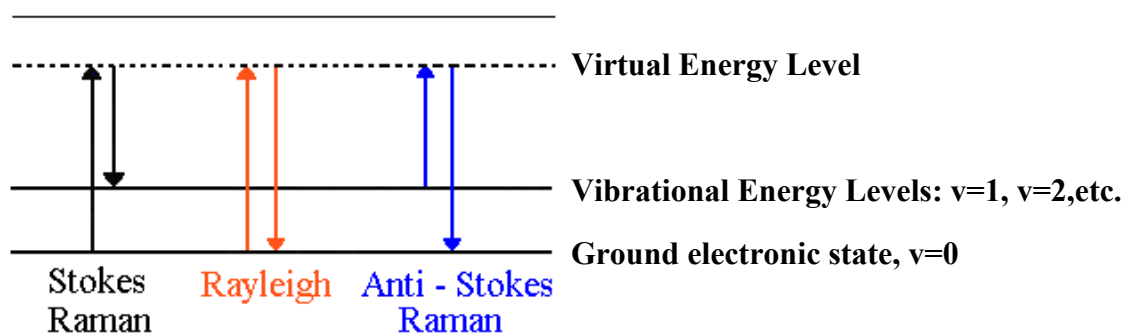


Figure 1.1. Origin of Rayleigh (elastic) and Raman (inelastic) scattering.

To a first approximation, a particular molecular species will give rise to specific vibrational bands at the same frequency regardless of the molecule in which they are found. However, the precise wavenumber of these bands is also dependent on inter- and intramolecular effects. This is observed in protein samples where vibrational bands from the molecular species forming the polypeptide chain are influenced by peptide bond angles and hydrogen bonding patterns.

1.3. Factors affecting the Raman scattering intensity

The *selection rule* in Raman spectroscopy governs whether a molecular vibration is Raman active or not. The selection rule states that for a molecule to be Raman active, the vibrations within a molecule must cause a net change in the polarisability of that molecule. A large change in the polarisability of a molecule will result in a large Raman signal. Polarisability measures the ease with which the electron cloud around a molecule can be distorted and therefore depends on how tightly the electrons are bound to the nuclei. Typically, strong Raman scatterers are moieties with distributed electron clouds, for example C=C double bonds [3]. In addition to the selection rule, experimental factors can also affect the Raman scattering intensity. The Raman intensity in photoelectrons, $S(e^-)$, can be described as:

$$S(e^-) = (P_D \beta D K) (A_D \Omega_D T Q) t$$

Equation 1

Where: P_D is the laser power density (in $\text{photon s}^{-1} \text{cm}^{-1}$), β is the differential Raman section (in $\text{cm}^2 \text{molecule}^{-1} \text{sr}^{-1}$), D is sample concentration (in molecules cm^{-3}), K is the path length (in cm), A_D is sample area monitored by the spectrometer (cm^2), Ω_D is the solid angle of collection of the spectrometer at the sample (in steradians), T is transmittance, Q is quantum efficiency of the detector (e^- per photon) and t is the observation time (in seconds).

It is clear to see that any change in the alignment of laser and collection optics can affect the parameters in this equation, with obvious consequence to the Raman signal. This underlines the importance of calibrating the instrument prior to making any Raman measurements, as well as the need for rigid mounts for the sample, laser optics and collection optics.

2. Raman spectroscopy: specialised vs. dispersive Raman systems

One of the chief difficulties in analysing protein samples with Raman spectroscopy is background fluorescence, especially where samples are in solution. Biological samples such as proteins are often fluorescent in the visible range. The absolute intensity of this fluorescence is small, but because of the sensitivity of Raman spectrometers, it presents a major problem to data interpretation [4]. Recent technological advances have been used to address this problem (see Figure 2.1).

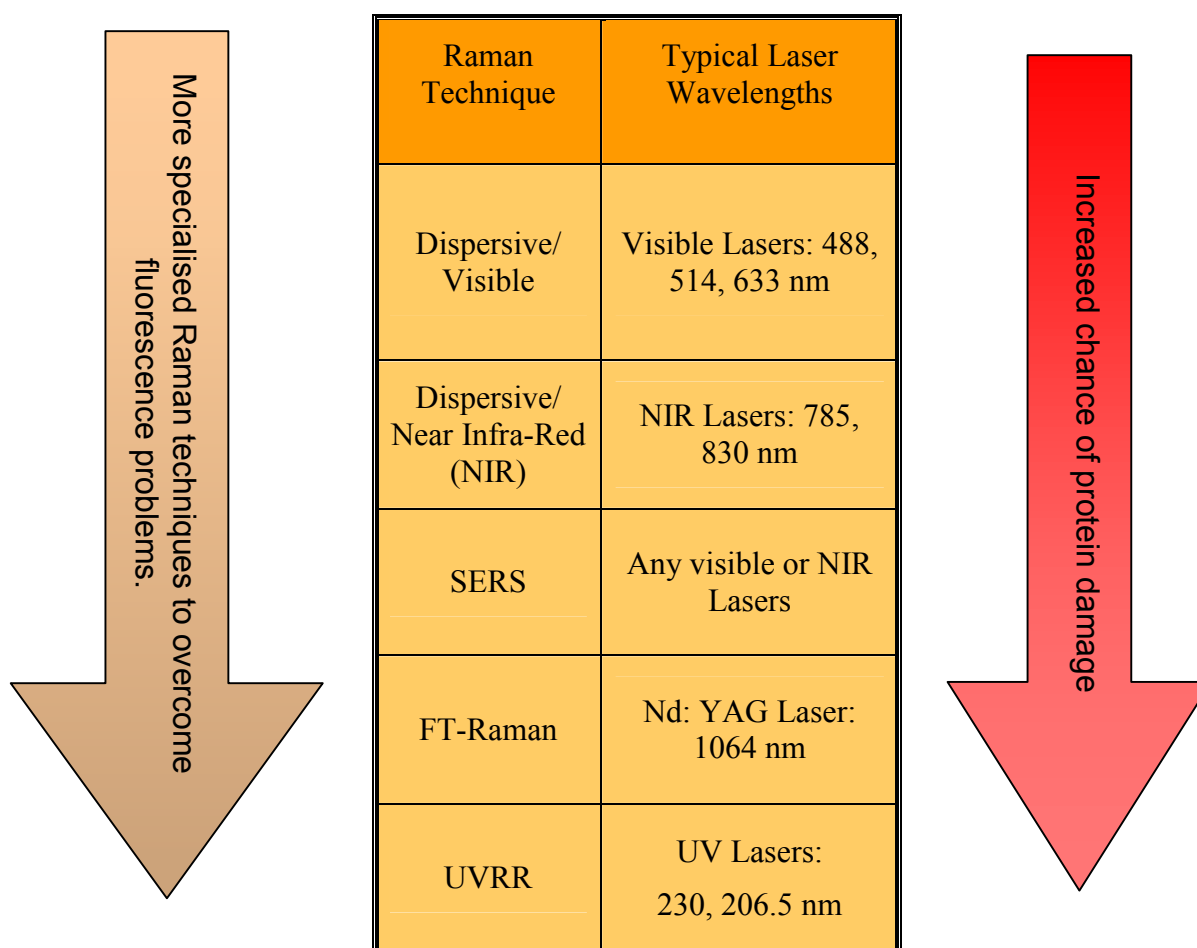


Figure 2.1. *Technological Advances in Raman spectroscopy*

Difficulties in measuring fluorescent samples can be overcome by using a variety of specialised Raman techniques such as FT- (Fourier Transform)-Raman, SERS (Surface Enhanced Raman Spectroscopy) and UVR (Ultra-violet Resonance Raman)). However, each of these techniques has advantages and disadvantages. We review each of the main Raman techniques in the following sections.

2.1. FT-Raman

FT Raman spectroscopy systems have been available since 1987. Commercial systems use a Nd:YAG laser (1064 nm) with a near infrared interferometer coupled to either a liquid nitrogen cooled germanium (Ge) or indium gallium arsenide (InGaAs) detector [5]. When it was introduced, FT-Raman had many advantages over the

dispersive Raman systems then available. In particular, it has provided a useful means of recording the Raman spectra of biological samples, especially when fluorescence interference is a problem. [6, 7] [8].

Although FT-Raman is often employed as a way of avoiding fluorescence, there are limitations to this technique:

- a) the requirement for a high laser intensity, which can be disruptive to the samples
- b) the aqueous solution often used in bio-measurement absorbs significant amounts of the Near IR radiation used in FT-Raman and this affects the relative intensities of bands [5] [7]. In addition, the 1064 nm laser beam is invisible but quite hazardous to the eyes and therefore laser safety with this type of laser can be an issue.

2.2. *Resonance Raman*

Resonance Raman (RR) is of particular interest in the analysis of biomolecules containing a chromophore or group of chromophores. Resonance Raman enhancement permits the isolation of the Raman bands belonging to these chromophores and structural units located close to them. The vibrational motions of the chromophore(s) of interest may be enhanced up to 10^6 fold, whereas other modes remain weak. Certain vibrational transitions are thus selectively enhanced, and as a result higher order transitions, not normally visible in conventional Raman scattering, can be detected by Resonance Raman. This effect gives rise to new bands and additional information; such signal enhancement also results in the requirement of much lower analyte concentrations. Since many molecules absorb in the ultraviolet, intense UV lasers are commonly used for RR spectroscopy. Like FT-Raman, RR spectroscopy is prone to induce sample lability and photo or thermal decomposition. At present, the high cost of lasers and optics for this spectral region has been a barrier to wide uptake of this technology and so use of this technique has been limited to a small number of specialists [9-12] .

2.3. *Surface Enhanced Raman Spectroscopy*

Surface Enhanced Raman Spectroscopy (SERS) depends on an enhancement effect that arises from an interaction between an adsorbed analyte and a roughened metal surface. There are two main theories on the mechanism involved in producing the SERS effect, “electromagnetic enhancement” and “chemical enhancement.” The predominant view is that both occur and that they operate multiplicatively. “Electromagnetic enhancement” occurs when an incident photon falls on the roughened surface and excitation of the electronic plasmon resonance of the metal occurs. The result of the excitation is a large increase in the electric field. The analyte, which is in close proximity to the metal surface, is bathed in this amplified electric field resulting in the enhancement of the Raman scattering. “Chemical enhancement” also known as “charge transfer,” describes the resonance enhancement from new resonant intermediate states created by adsorbate-substrate bonding, i.e. the adsorbate molecular orbitals are broadened into resonance by interaction with the conduction electrons of the metal. However, this only operates on the first layer of adsorbates [13] [14, 15]. In addition, the proximity of the metal surface also results in a dramatic quenching of fluorescence. The combination of these effects is to greatly increase the intensity of the Raman scattered light relative to the fluorescence.

SERS provides both rich spectroscopic information and high sensitivity, so that it is ideally suited for trace analysis (in the micromolar range). One feature of SERS is that the signal is extremely sensitive to any change in adsorbate orientation at the metal surface [16-18]. This can be viewed as both an advantage and a disadvantage. The advantage is that surface orientation information can be obtained. The disadvantage is that the spectral bands are extremely sensitive to any change in adsorbate orientation at the metal surface, hence obtaining a reproducible SERS signal can be difficult.

2.4. Dispersive Raman

Clearly, there are a number of limitations when the specialised Raman techniques described above are employed, most significantly a tendency for damage to the sample to occur due to high illumination intensities. Such problems are minimised when a “conventional” dispersive Raman system is employed. The use of a single-grating spectrograph in modern dispersive Raman systems gives better sensitivity and efficiency in the collection of the Raman signal when compared with the 1064 nm laser of an FT-Raman instrument because the scattering efficiency shows a $1/\lambda^4$ wavelength dependency [19, 20]. As a result, a much lower laser power and shorter accumulation time can be used, with benefits for light sensitive samples. The details of such an instrument set up are shown in **Figure 2.2**.

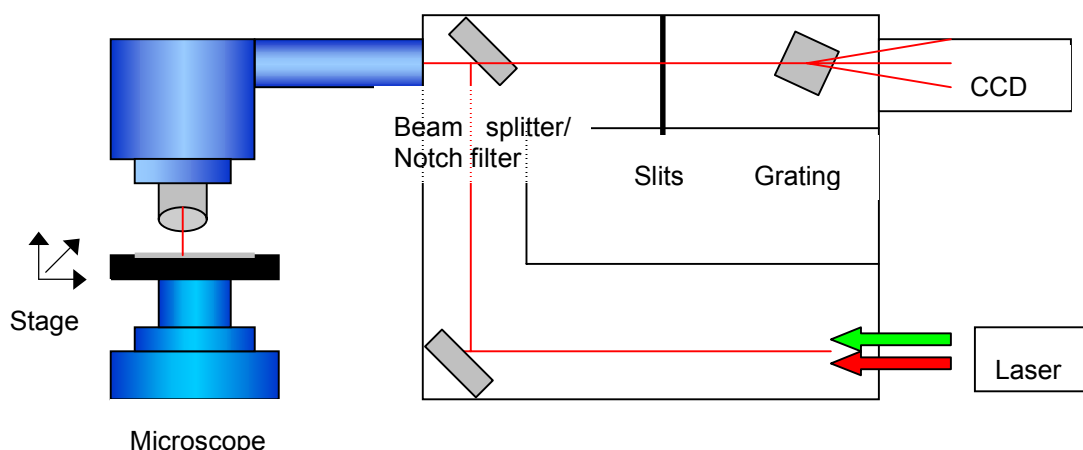


Figure 2.2. Schematic of a dispersive Raman system

An important component in the system is the presence of a notch filter that blocks the laser line as well as unwanted scattered light and provides high transmission of the collected scattered light before reaching the detector. This provides a strong signal and reduces noise, eliminating the need for multiple filters for spectral noise reduction.

Although fluorescence can be a problem in conventional dispersive Raman, this can be minimised by selecting a laser wavelength that causes minimal fluorescence. This can generally be achieved by using a red or near-infra-red wavelength, although the fluorescence characteristics are sample dependent. The caveat with this is that, as noted previously, the intensity of the Raman scattered light falls off very steeply with increasing wavelength. Therefore it is ideal to have access to multiple laser lines, either within a system or across multiple instruments. As discussed below, this raises further issues of spectral comparability.

3. Practical Guide

This section recommends practical procedures that can be used to obtain comparable Raman data of proteins. Further information can also be obtained from the methods and materials (section 5) and case studies (section 3.4).

3.1. *Instrument optimisation with silicon*

Silicon has a narrow well resolved vibrational band centred at 520 cm^{-1} and is often employed to calibrate the Raman instrument prior to sample measurement. Before its use, it is necessary to clean the silicon sample. This can be achieved by sonicating the silicon sample in various solvents: 5 minutes in acetone, 5 minutes in ethanol and 5 minutes in de-ionised water. It was then dried under N_2 and then mounted onto a glass slide and stored until use. Any subsequent cleaning of the silicon can be achieved by wiping the surface with a lens wipe soaked in methanol.

To optimise the Raman instrument, the silicon slide is placed on a sample stage of the microscope and focussed using suitable objective. The surface of silicon is inhomogeneous in nature and defects in the silicon can affect the magnitude of the Raman signal, so it is advisable to focus on the same spot of the silicon for each measurement to be made. Once focussed, the laser beam is allowed through the microscope to the surface of the silicon and a video imager can be used to evaluate the laser alignment. The laser beam should ideally be centred on the video image or crosshairs. After appropriate laser alignment, the silicon signal is acquired, as per manufacturer's instructions. The signal-to-noise ratio should then be optimised. For example, on the Renishaw instrument the area of the CCD detector used to acquire the signal should be minimised; also the microscope is enclosed to reduce stray light.

The signal from the silicon should result in a peak centred at 520 cm^{-1} . Any deviation from this value can be corrected using an appropriate 'offset' function, available with most modern Raman instruments. After such correction procedures, a final silicon spectrum should be recorded.

3.2. *Using a standard to correct Raman shifts*

In order to evaluate the suitability of a standard for Raman shift corrections, Raman spectra (typically with six replicates) of the chosen standard (usually a chemical calibrant such as cyclohexane) should be obtained. The mean peak positions should be calculated and plotted against expected values (usually obtained from the literature). Ideally, there should be a number of peaks distributed across the spectral range of interest. A linear plot indicates that any correction is not frequency dependent, and that the spectra can be corrected by a simple shift along the wavenumber axis. The required correction can then be obtained from the intercept or from a mean correction value (see **Figure 4.2**).

This correction step is only necessary to make data more comparable between instruments. A case study of the use of cyclohexane as a standard is given in section 4.1. The cyclohexane spectrum should be acquired after taking each Raman spectrum of a given sample. There are seven main peaks in the cyclohexane spectrum that are used to act as frequency standards. The correction procedure involves calculating the difference between expected (literature value) and experimental of the frequency values from the seven main peaks; the mean correction value can be evaluated from

this or from a linear fit as described above. The x - (wavenumber) axis of a Raman spectrum can then be corrected using this mean correction value [21].

3.3. *Intensity corrections*

Published Raman spectra are rarely corrected for variations in spectrometer sensitivity across the Raman spectrum, which often leads to severe distortion of relative peak intensities, which is a barrier to the application of searchable libraries. The issue of intensity corrections is not so problematic if comparing spectra run on a given modern instrument. Ideally, correcting the data against an internal standard can do this. For example, past workers [22] have identified the phenylalanine band in the protein spectra (at 1004 cm^{-1}) as a possible candidate. This involved normalising the data against this band. The phenylalanine band was chosen because it is not sensitive to conformation or microenvironment and therefore can be used as an internal standard to normalise the Raman spectrum of proteins. This type of normalisation is primarily to correct for concentration differences and should work for comparisons of different samples of the same protein. In the past, intensity corrections were problematic when comparing from one instrument to another. In contrast to frequency corrections, intensity data is more difficult to correct because spectrometers vary in transmission efficiency and detector quantum efficiency with wavelength [2]. Until recently, the main problem was not in finding the procedure for such an intensity correction but in finding suitable certified artefacts for such corrections. Recently, NIST (National Institute of Standards and Technology) has announced the commercial availability of reference material suitable for Raman spectrometers employing 785, 532 and 1064 nm laser lines¹. It is hoped that eventually, such a series of calibrants will provide the basis for developing system independent Raman libraries.

3.4. *Acquisition of protein Raman data*

The correct volume of sample should be introduced into the sample cell using a pre-cleaned glass pipette. It is important not to introduce any gas bubbles into the cell or contaminate the sample in any way, as this will affect the measurement. Following the sample introduction, the exterior of the cell should be rinsed and dried under nitrogen.

Before mounting the cell into the sample stage, it is important to find the optimum focus of the system. This is achieved by using a cell containing cyclohexane, which gives a much more intense signal than that of protein samples. The cyclohexane cell should be placed in the sample stage of the microscope and then the objective lens brought close to the cell surface. A spectrum should be acquired under a continuous cycle (e.g. with a 1 s detector time, single accumulation), whilst the moving the lens slowly away from the cyclohexane cell. The focusing is considered optimal at the point when the largest signal is observed. The cyclohexane cell is then carefully removed and replaced by the protein cell. Following acquisition of the protein spectrum, a background Raman spectrum of buffer or water should be acquired under the same experimental conditions and parameters. A subtracted spectrum is then generated (protein cell signal minus background cell signal).

¹ NIST SRMs 2243 for 488 nm and 514.5nm excitation; 2241 for 785 nm excitation and 2242 for 532 nm excitation. See <https://srms.nist.gov/> for more information. These reference materials actually employ fluorescence to calibrate the sensitivity of Raman instruments across a range of frequencies.

4. Case studies

4.1. Case study 1: Cyclohexane as a Raman shift standard

A typical cyclohexane spectrum is shown in **Figure 4.1** below. As expected, the spectrum has sharp, narrow peaks across the spectral range of interest i.e. 300 – 3000 cm^{-1} . To evaluate the suitability of cyclohexane as a Raman shift standard, six Raman cyclohexane replicates were obtained. The mean Raman shifts for each peak and its standard deviation are shown in **Table 4.2**. The standard deviation for all the peaks is less than 1 cm^{-1} and compares well with the values obtained from a similar study conducted by McCreery *et al.* ([2]). The experimental peak positions in **Figure 4.1** were plotted against the expected values from the literature [22] and this shows a linear relationship with the line of the plot passing close to the origin (**Figure 4.2**).

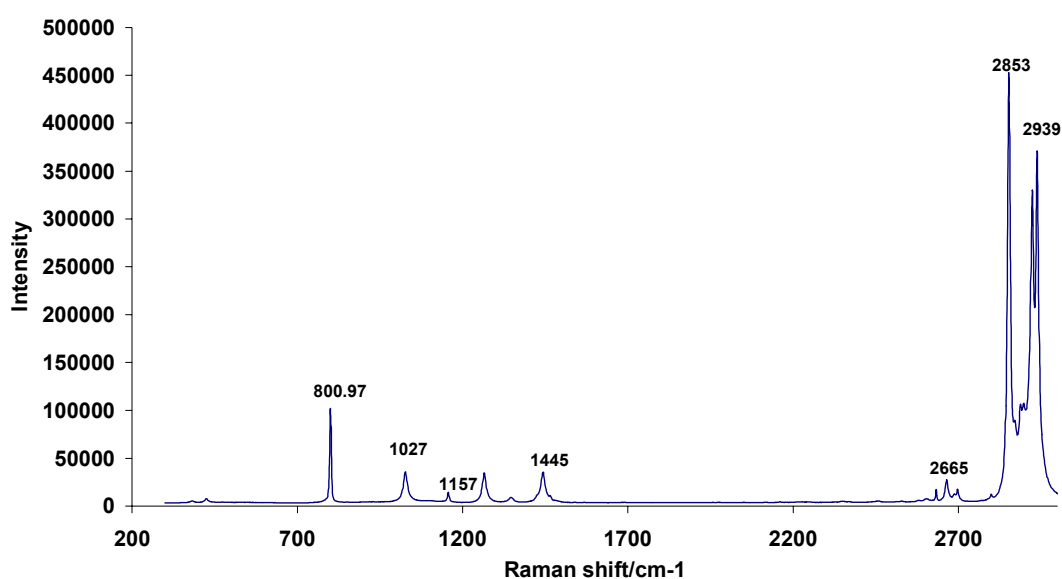


Figure 4.1. Typical cyclohexane spectrum.

Wavelength (nm)	514
Laser power (mW)	20
Objective magnification	20
Accumulations	1
Detection time (s)	1
Sample holder	cavity slide with glass covering
Spectral resolution (cm^{-1})	4
Gain	High
Binning	1
Focus	0%
Cosmic Ray removal	off

Table 4.1. Experimental conditions used in Figure 4.1.

Literature value (cm^{-1})	Mean observed value (cm^{-1} ; $n = 6$)	Standard Deviation (cm^{-1})	Mean intensity (arbitrary units)
801.3	800.97	0.00	73008
1028.3	1028.13	0.57	26752
1157.6	1157.83	0.57	11199
1444.4	1444.66	0.58	25606
2664.4	2665.10	0.00	21400
2853.9	2852.55	0.61	341211
2939.3	2938.05	0.61	282786

Table 4.2. Mean Raman shifts of the 7 major cyclohexane peaks.

Obtained from six spectral replicates (spectral fingerprints are similar to those in Figure 4.1).

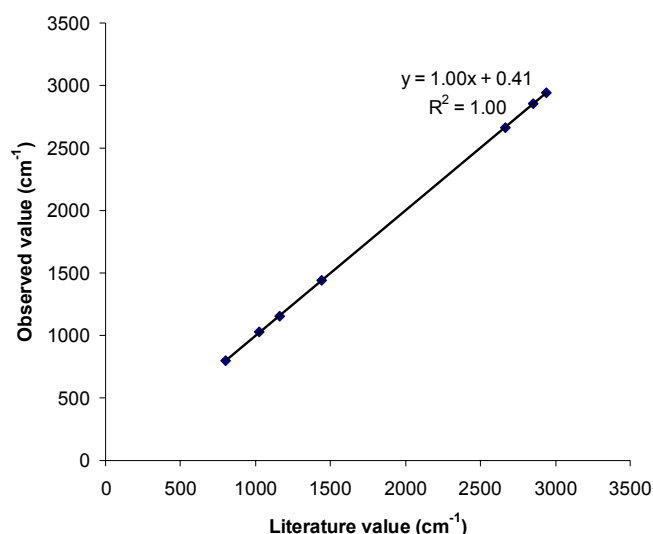


Figure 4.2. Plot of literature vs. experimental mean Raman shift.

Values obtained are listed in Table 4.2. Note the linearity of the fit (indicated by an R^2 value close to 1) and the non-zero intercept, indicating the correction required.

4.2. Case study 2: The need for Raman shift corrections

The importance of Raman shift corrections was evaluated by a comparative study between different instruments (two Renishaws, one Dilor) and operators in 3 different laboratories. Each laboratory was instructed to optimise their instrument according to the manufacturer's instructions and to obtain the Raman spectrum of solid BSA (bovine serum albumin) from a common source, using their chosen experimental parameters. The data obtained from the study are shown in **Figure 4.3**. For comparative purposes, Lab 2 data was scaled by a factor of 6, whereas Lab 3 data was scaled up by a factor of 2.2

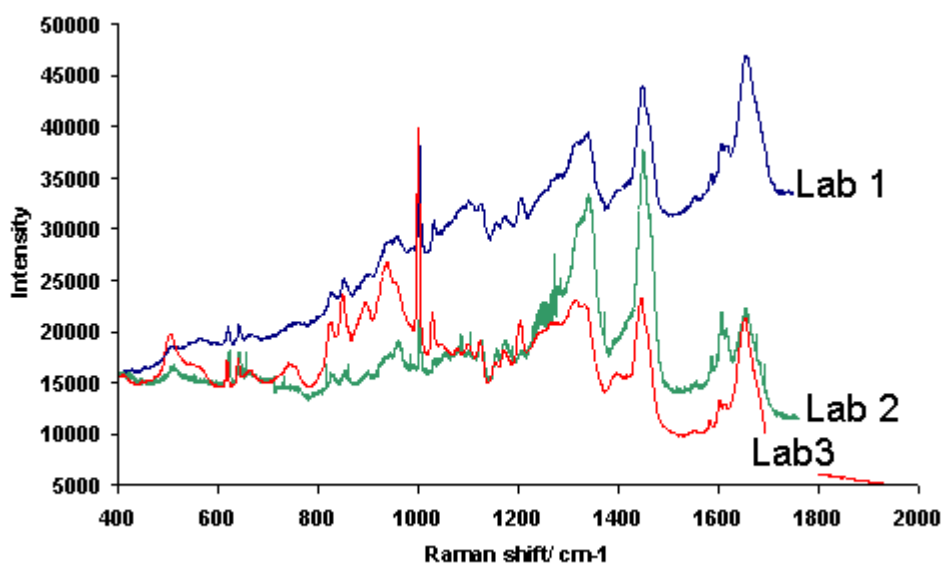


Figure 4.3. Raman spectrum of BSA solid obtained by three different laboratories.

The results from the three laboratories should give the same Raman spectral fingerprints i.e. with the Raman peaks in the same positions. It must be remembered that our prime interest is in the peak positions, and not their intensities.

Visual examination of the results indicates that all three spectra have similar spectral fingerprints. However, upon closer examination, there are slight differences in the relative Raman shift values (**Figure 4.4**). The results are summarised in **Table 4.3**. Assuming that data from Lab 1 is accurate, then the mean Raman shift corrections required to make the other two instruments more comparable can be calculated. Results show that Lab 1 and Lab 2 gave similar values, with the Lab 2 requiring a mean Raman shift correction value of only 0.25 cm^{-1} . Data from Lab 3, however, gave the largest difference, with a mean Raman shift correction of 2.75 cm^{-1} .

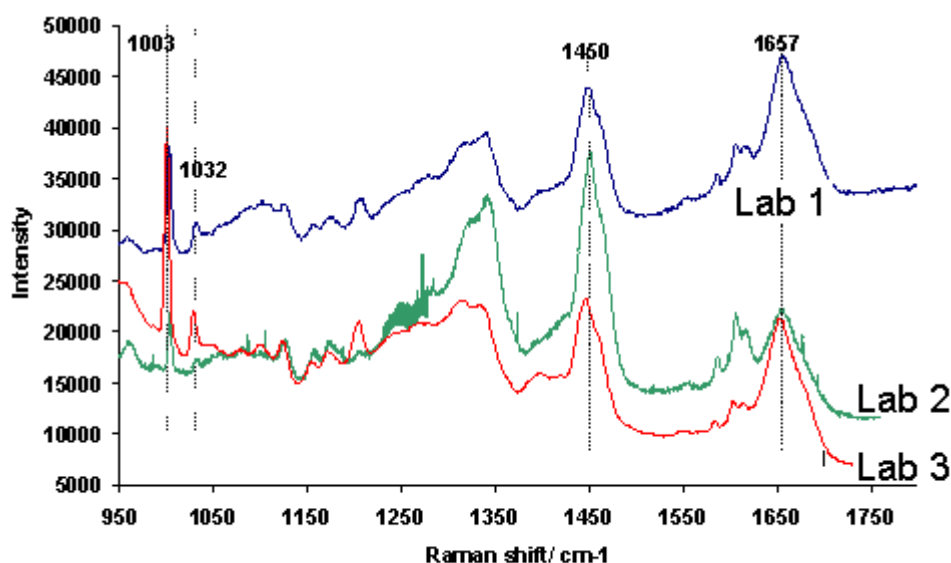


Figure 4.4. A closer look at the spectrum in Figure 4.3.

	Peak number	Lab 1	Lab 2	Lab 3
Peak Raman shift (cm^{-1})	1	1657	1657	1653
	2	1450	1450	1448
	3	1032	1031	1035
	4	1003	1003	1001
Mean Raman shift corrections (cm^{-1})		0	0.25	2.75
Standard Deviation (cm^{-1})		0	0.50	0.96

Table 4.3. Raman shift values of the four peaks identified in Figure 4.3.

This small study emphasises the need for corrections of Raman shift data for inter-laboratory comparisons.

In a further study, the application of the recommended correction procedure using cyclohexane as standard was evaluated. This study involved measuring ibuprofen in solution with two different operators in laboratories referred to as Lab 1 and Lab 2. Ibuprofen was chosen because of its narrow and well resolved Raman peaks with 514 nm laser illumination, and low background fluorescence. Both laboratories followed

the optimisation protocol previously outlined and the resultant corrected spectra are shown in **Figure 4.5**. The mean Raman shift correction value made to each of the replicate is tabulated, along with their corresponding standard deviation (**Table 4.5**). The data clearly demonstrate the need for Raman shift corrections to achieve comparable data sets, Lab 2 having exceptionally high correction values of more than 4 cm^{-1} ; this has been attributed to a grating motor fault at the time of the experiment. The standard deviations values for both Lab 1 and Lab 2 are less than 0.5 cm^{-1} .

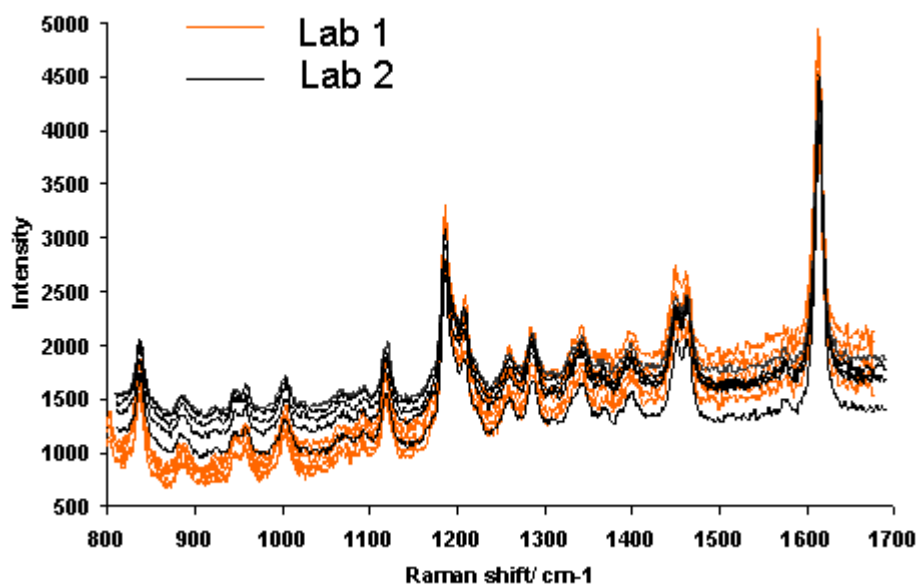


Figure 4.5. Comparison of spectra of saturated ibuprofen in water.

Spectra are five-fold replicates obtained from Lab 1 and Lab 2. The spectra have all been background subtracted and frequency corrected.

	Lab 1	Lab 2
Wavelength (nm)	514	
Laser power (mW)	20 (10% power)	15 mW (100% power)
Objective Magnification	20	
Accumulations	5	
Detection time (s)	10	
Sample holder	Cavity slides with glass wafer as cover	
Spectral resolution (cm ⁻¹)	4	
Centre wavenumber (cm ⁻¹)	1250	
Binning	1	
Gain	High	
Focus	0	
Cosmic Ray Removal	off	

Table 4.4. Experimental parameters used to collect data in Figure 4.5.

Laboratory	Replicate number	Mean Raman shift correction (cm ⁻¹)	Standard deviation (cm ⁻¹)
1	1	0.38	0.342
	2	0.246	0.331
	3	0.122	0.363
	4	0.186	0.297
	5	0.138	0.338
2	1	4.475	0.386
	2	4.475	0.275
	3	4.2	0.365
	4	4.05	0.387
	5	4.15	0.42

Table 4.5. Summary of Raman shift correction values made to each of the spectral replicates in Figure 4.5.

4.3. Case study 3: Choice of excitation wavelengths

One of the main drawbacks of using Raman spectroscopy in the analysis of biological samples is the background fluorescence signal. This is less problematic in solid samples as the fluorescence can be reduced by photobleaching; however, in solution this is less efficient due to diffusion of molecule in and out of the focal volume. This is clearly observed in the Raman spectra of α -glycoprotein when in solution (**Figure 4.6**). For comparative purposes, the solid signal has been scaled up by a factor of 4

and it is apparent that when the protein is in solution the large broad fluorescence background masks the Raman scatter. Similar results were obtained using BSA (Bovine Serum Albumin), HSA (Human Serum Albumin) and a biopharmaceutical vaccine supplied by NIBSC (National Institute for Biological Standards and Control).

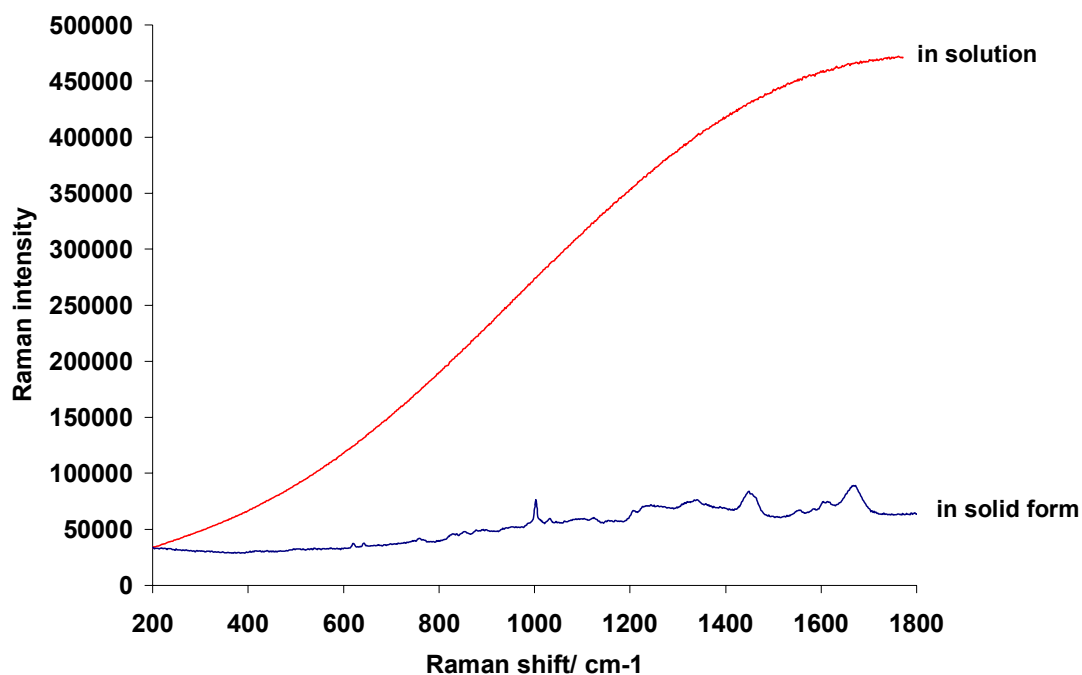


Figure 4.6. α -glycoprotein in solid and in solution.

Parameter		Solid	Solution
Laser	Wavelength (nm)	514	
	Power (mW)	30	8
Objective magnification		20	
Accumulations		10	5
Detector time (s)		10	
Spectral range (cm-1)		200-2000	200-4000
Mode		Continuous	
Gain		High	
Binning		1	
Focus (%)		0	
Cosmic Ray removal		Off	
Sample		Solid	100 mg·ml ⁻¹ in phosphate buffer (100 mM, pH = 7.0)
Slide		On a glass slide	Cavity slide; glass cover slip
Spectral resolution (cm-1)		4	
Gain		High	
Binning		1	
Focus		0%	
Cosmic Ray Removal		Off	

Table 4.6. Experimental conditions for Figure 4.6.

If the source of fluorescence is not linked directly to the protein or is loosely associated, then it should be possible to reduce or remove the fluorescence by protein purification methods. However, purification of HSA by size exclusion chromatography had little effect on the overall fluorescence (**Figure 4.7**). This indicates that such fluorescing impurities are either attached to the protein or that the fluorescence is intrinsic to the protein itself.

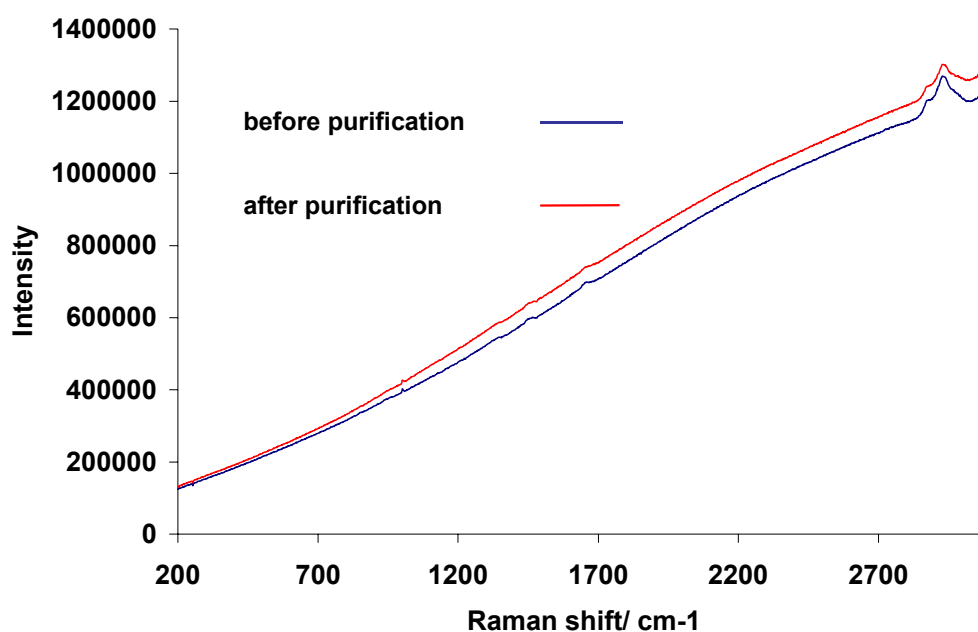


Figure 4.7. Raman spectrum of HSA in solution: before and after protein purification.

Wavelength (nm)	514
Laser power (mW)	30
Objective magnification	20
Accumulations	20
Detection time (s)	10
Sample holder	cavity slide with glass cover slip
Spectral resolution (cm ⁻¹)	4
Gain	High
Binning	1
Focus	0%
Cosmic Ray removal	off

Table 4.7. Experimental parameters for Figure 4.7.

The protein concentration was 100 mg·mL⁻¹ in phosphate buffer (100 mM, pH = 7.0)

A common way to overcome fluorescence is to use a different laser wavelength. **Figure 4.8** shows the Raman spectrum of HSA sample in solution obtained under 3 different wavelengths (514 nm, 633nm and 785 nm). These results are compared with the spectral fingerprint of solid HSA obtained at 514nm. For the purpose of

comparison the data were appropriately re-scaled². These data clearly show that the fluorescence background slightly improved by changing the excitation wavelength from 514nm to 633nm, then markedly improved by using an excitation at 785 nm. In addition, the Raman spectral fingerprint measured at 785 nm compares well with the solid HSA obtained at 514nm.

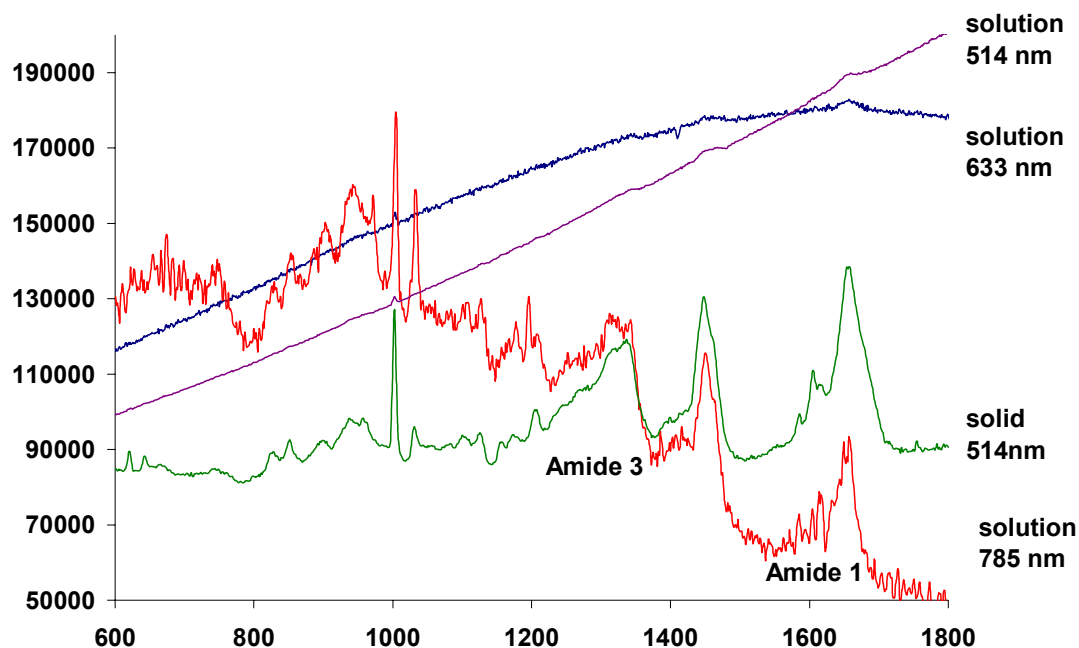


Figure 4.8. Effect of laser wavelength on fluorescence background in spectra of HSA. Spectra of HSA in solution obtained at: 514 nm, 633 nm and 785 nm are compared to a solid HSA spectrum obtained at 514 nm. Experimental conditions are given in **Table 4.8**.

² Solid HSA at 514 nm scaling factor = 0.6 and baseline shifted by 175,000 counts; solution HSA at 514 nm scaling factor = 0.2 and baseline shifted by 50000 counts; solution HSA at 785 nm scaling factor = 125 and baseline shifted by 25000 counts.

State	Solid		Solution	
Wavelength (nm)	514	514	633	785
Laser power (mW)	30	30	15	300
Objective magnification	20	20	50	50
Accumulations	20	20	20	5
Detection time (s)	10	10	10	60
Protein conc. (mg·ml ⁻¹)	solid	100	100	50
Sample holder	glass slide	cavity slide	cavity slide	quartz cell
Instrument	Renishaw ³			JY-Horiba

Table 4.8. *Experimental conditions for Figure 4.8.*

³ Other options for the Renishaw instrument were as shown previously.

5. Methods and Materials

5.1. *Materials*

All proteins were from Sigma-Aldrich (97 – 99% purity). Cyclohexane was 99% (minimum) purity from VWR International. Silicon was obtained from Goodfellow.

Cavity slides and glass cover slips were from VWR international. The Quartz Suprasil cell and Hellmanex 2 were supplied by Hellma UK.

Solutions were made up in ultrapure de-ionised water (18.2 M Ω).

5.2. *Instrument*

The main instrument used in this study was a Renishaw RM 1000 Raman system with 514 nm and 633 nm lasers. This is a dispersive Raman instrument with a spectral resolution of 4 cm^{-1} . The power of the laser excitation chosen for a particular experiment varied depending on factors such as the inherent strength of the Raman signal, the level of the background fluorescence, the maximum time between measurements, and the signal-to-noise required to produce adequate data. Typical values for protein samples employed in this study ranged between 10 mW and 40 mW. In general, it is advisable to use laser power no higher than required.

5.3. *General cleaning protocol*

All equipment used to prepare, extract and fill the sample containers was thoroughly cleaned and dried to avoid contamination, which could lead to erroneous signals. The cleaning step involves rinsing the items with acetone, then ethanol, followed by de-ionised water, and finally drying under a nitrogen flow. The sample containers used were either two well cavity slides or synthetic quartz cells. The cavity slide consisted of 2 polished round depressions on a standard glass slide (of size 76 x 25 mm, 1.25 mm thick), each depression is 15 mm in diameter and approximately 1 mm deep; a glass cover slip can be placed on top of the depression after sample introduction in order to prevent evaporative buffer loss. The quartz cell is cylindrical and made from Quartz Suprasil with 1 mm path length. Although preliminary studies showed no difference in the Raman signal collected between the two types of cell, the quartz cell was preferred for ease of use. The main disadvantage of the quartz cell is that it is not disposable and therefore requires thorough cleaning and maintenance. The cleaning step involved rinsing with detergent, such as 2% Hellmanex, followed by rinsing with de-ionised water and then drying with nitrogen gas.

5.4. *Protein sample preparation*

A disadvantage of Raman spectroscopy for protein analysis is the need for high concentrations of the analyte i.e. $> 20 \text{ mg}\cdot\text{ml}^{-1}$. In order to minimise problems during sample preparation, such as the formation of large aggregates and the introduction of air bubbles into the solution, the protein samples were prepared as follows.

Prior to weighing the protein, the protein bottle (stored in fridge) was allowed to stand at room temperature for 30 minutes before opening the lid (this step was necessary as most proteins are hygroscopic in nature). Protein samples were weighed straight into a pre-cleaned glass sample vial. After weighing, the correct volume of water (or buffer) was added using a clean glass pipette, in order to make up the desired concentration.

Solutions were allowed to stand for 30 minutes to allow protein to dissolve, whilst shaking the vial gently from time to time. Before use, the sample was checked under a fibre optic lamp to ensure that there were no visible particulates present in the solution. Prepared samples were used on the day of the experiment and any remaining samples were discarded.

6. Summary and conclusions

Academic institutes and instrument developers have promoted Raman spectroscopy as a method for biopharmaceutical analysis. Raman is known to give valuable chemical and structural information; however, there are certain limitations of the technique that need to be addressed. A specific problem associated with biological samples is high background fluorescence, especially when the protein is dissolved in solution. There are some specialised Raman techniques that can be employed to avoid the problems of fluorescence, for example, FT-Raman, UV Raman and SERS; however, such specialised techniques can introduce other artefacts resulting from sample denaturation during data acquisition. Dispersive Raman systems with their visible and NIR lasers therefore remain the instrument of choice for general biological measurement and the problems of fluorescence can be minimised by the appropriate choice of excitation wavelength.

Another problem highlighted by the inter-laboratory studies is that of correcting the Raman shift to achieve comparable spectral data. This study has demonstrated that Raman shift corrections can be reliably achieved using chemical calibrants such as cyclohexane. However, intensity corrections are more problematic and this is mainly attributed to the lack of suitable photostable artefacts available on the market. The recent release of appropriate reference materials by NIST should go some way towards rectifying this.

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