Monograph

Introduction to Raman Spectroscopy
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Contents

Preface

Raman spectroscopy is an easy-to-use analytical technique that identifies liquids and solids within seconds. The sharp features of the Raman spectrum form a unique fingerprint for each IR-active substance. Thanks to this, every chemical structure can be distinguished in any matrix, even isomers and polymorphs.

Raman spectroscopy uses a small spot focus for analyzing as little as a few milligrams or microliters of sample. Systems that are equipped with raster sampling are capable of averaging over a larger sample area and can thereby overcome sample heterogeneities while maintaining high spectral resolution, and even map the individual components in a heterogeneous mixture. Raman spectroscopy requires no sample preparation and measurements can be done through most container materials. Because Raman spectroscopy does not require any reagents or special materials for analysis, it avoids waste.

Raman spectra stem from the inelastic scattering of light from energy exchange with molecular vibrations. Raman Spectroscopy is most often performed with near-infrared laser excitations of 785 nm or 1064 nm. The wavelengths are dictated by the availability of lasers for each unique wavelength. Near-infrared is chosen as it alleviates much of the fluorescence interference that is problematic at shorter wavelengths. Since these lasers are diode-based, the exact wavelength is not known and in fact they can be tuned with temperature. The American Society for Testing and Materials (ASTM) developed a set of standards for calibration that is now used world-wide to standardize Raman spectra.
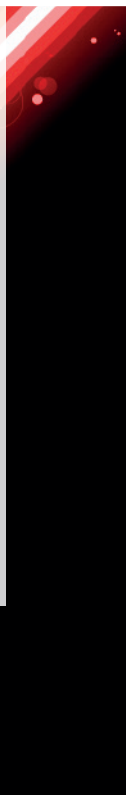
History

Raman spectroscopy has a long history as a research technique and, in fact, was one of the early methods for the determination of the structure of simple molecules. It was developed in the 1930s, but could only establish itself as a common analytical technique when lasers emerged in the 1960s. Its early days were marked by large monochromators and the era's – by today's standards – primitive lasers. Long acquisition times accompanied the bulky equipment. By advances in laser technology, these hurdles have since been overcome. However, while Raman spectroscopy was busy outgrowing its teething problems, the requirements of the market towards the technique developed as well. Speed, ease of use, flexibility, and reproducibility matter today more than ever – not only in benchtop systems but also in handheld Raman devices.

In the early 2000s, Raman spectroscopy quickly became a favorite as a material identification tool. As the number of handheld Raman spectrometers has grown, the markets too have grown. The ease of sampling that is characteristic of these instruments has expanded the use of handheld spectrometers to pharmaceutical and chemical analysis. The sharp spectral features inherent in Raman spectroscopy make it more amenable to a complex mixture analysis, and often less statistical analysis is required to model a system than in NIR spectroscopy.

Raman spectroscopy is also often considered a complementary method to NIR or MIR analysis. One simple way to compare is that Raman spectroscopy has the easy sampling capability of NIR, and the sharp spectral peaks as observed in MIR. The intensity of Raman scattering is related to the polarizability of the molecule and this often makes Raman spectroscopy the best technique for organic molecules. One of the «poor» Raman scatterers is water, making Raman spectroscopy is an excellent method of analysis for aqueous solutions.

You want to be sure

Raman Spectroscopy is now an accepted technique for pharmaceutical analysis and most handheld systems conform to 21 CFR Part 11 and USP standards. It is considered a robust technique for incoming quality control (QC) and for process monitoring. Moreover, Raman spectroscopy is also considered a Quality by Design (QbD) technique in the pharmaceutical industry. The small laser spot can locate APIs quickly in the presence of excipients and the modern handheld designs carry sufficient computing power for complex chemometric analysis and for large spectral libraries. The simple measuring process makes Raman spectroscopy a powerful method with minimal user training.

Theory – a first approach

When electromagnetic radiation interacts with matter several processes can occur that are useful for chemical analysis. Absorption of visible light is the basis of one of the most common forms of chemical analysis – which is photometry – and the quantitative relationship known as Beer's law

$$A = \varepsilon c l \quad \text{(Eq. 1)}$$

where A is the absorbance, ε is the molar attenuation coefficient, c is the molar concentration, and l is the pathlength. With accurate pathlengths this leads to a simple method to determine the concentration of an analyte. It is also possible that the absorbed radiation is reemitted at a longer wavelength after vibrational relaxation occurs. The emitted radiation is called fluorescence.

Fluorescence too is a powerful method for chemical analysis. It can be quite sensitive, being a zero-baseline technique. In other words, if no analyte is present the signal is zero. This is the converse of absorption spectroscopy where no analyte means all of the radiation hits the detector and the detector noise can be large. The equivalent of Beer's law for fluorescence is

$$F = K c \quad \text{(Eq. 2)}$$

where F is the intensity of the fluorescence, K is a constant, and c is the concentration. This simple linear equation holds well when absorbances are less than 0.05. Above that, the self-absorption of the fluorescence needs to be accounted for.

If the electromagnetic radiation is in the mid-infrared (MIR) region of approximately 2.5 µm to 24 µm then molecules are able to absorb the light through excitation of molecular vibrations. Non-linear molecules have $3N - 6$ vibrations and linear molecules have $3N - 5$ vibrations, where N is the number of atoms. The frequency ν of a molecular vibration can be complicated due to group vibrations, but in a simple diatomic molecule it can be calculated from Hooke's law

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad \text{(Eq. 3)}$$

where k is related to the bond strength between the atoms and μ is the reduced mass of the atoms. Since the bond strength and the reduced mass do not change during vibrations this equation predicts sharp spectral lines. MIR spectra indeed are composed of relatively sharp features, but they do have a line width due to their lifetime and rotational broadening. The sharp lines and, often characteristic frequencies for chemical groups, make MIR spectroscopy appealing for molecular identification. The interference from glass prohibits MIR cuvettes such as those used for UV/VIS absorption spectroscopy. This makes a tedious sample preparation necessary, thereby complicating sampling and quantification. Since the 1980s, ATR (attenuated total reflection) sampling systems have solved many of the sample preparation issues.

In addition to the fundamental vibrations of a molecule, it is possible to have overtones and combinations. These occur at near multiples of the fundamental vibrations at shorter, near-infrared (NIR), wavelengths (i.e., higher frequencies) between 0.77 μm and 2.5 μm. They are only near multiples as the perfect parabolic potential well becomes distorted at higher energies and this leads to inexact multiples of the fundamental vibrations. Furthermore, the fundamental vibrations with the highest frequency and the largest anharmonicity are those with a X–H stretch. For organic molecules, these tend to be C–H, O–H, N–H, and S–H. Because of hydrogen bonding and rapid exchange of labile protons, these bands tend to be broader. As these bands become broad and overlap, they become less useful for molecular identification. However, glass is transparent over this region of the spectrum and it is easy to construct sample cells of known pathlengths for quantitative analysis. The overlap of information often precludes a simple Beer's law analysis at one wavelength, but multicomponent analysis provides very accurate quantitative results. NIR spectroscopy is particularly useful when measuring water content.

Scattering of light

The above mentioned interactions all involve the absorption of light. The second way in which light interacts with matter is through scattering. In classical theory of light waves, scattering is much like the carrier wavelength in an FM radio; in Raman this is the laser excitation wavelength, and the sidebands are the energy of the molecular vibrations. These molecular vibrations occur through the formation of an induced dipole from the cloud of electrons around the molecule. Quantum mechanics recognizes that light is quantized into bundles called photons. It is easy to picture a particle of light (photon) hitting a molecule and bouncing off. When the photon strikes a molecule and bounces off without transferring energy, it is elastic scattering and is called Rayleigh scattering. About 1 in 1000 photons scatters through Rayleigh scattering and, as we will see, the relationship between the intensity of the scattering and the wavelength is through the fourth power; short wavelengths scatter much more than longer wavelengths (Figure 1).
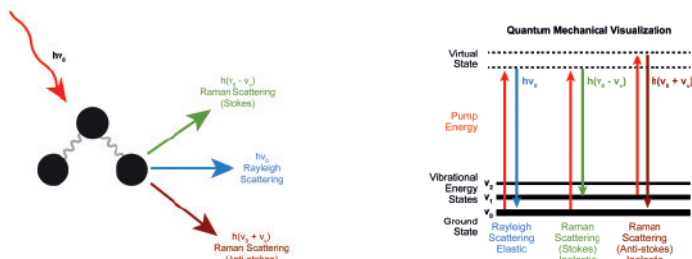

Figure 1 Illustration of Raman scattering and its electronic diagram

This explains why the sky is blue; when you look up, the light you see is the blue (short wavelength) part of the solar spectrum that is scattered to your eyes. It also explains the red sunsets which are the light headed directly to your eyes after the shorter blue wavelengths have been scattered away.

Raman scattering

While most scattering occurs in the elastic form, about 1 in 10^6 scattering processes will deposit energy into the molecule. In this process, the photon will lose energy. The complete theory of Raman scattering is very complex and intensities and frequencies are calculated only approximately with modelling programs. In spite of the complexity, it is useful to show how the spectra arise. We can start with the idea of an electric field oscillating at

$$E = E_O \, cos(2\pi v_l t) \quad \text{(Eq. 4)}$$

This describes a wave moving with a laser frequency of v_l and a maximum electric field of E_O at time t. Molecules have electrons moving rapidly around their atoms and in the bonds between atoms. These electrons are described as the electron cloud and they are subject to external electric fields. The more the electrons react to the electric field, the more polarizable the molecule is. As the electron cloud becomes distorted by the electric field of the light, an induced dipole moment, m, is formed:

$$m = \alpha E = \alpha E_O \, cos(2\pi v_l t) \quad \text{(Eq. 5)}$$

The variable that relates the induced dipole moment to the electric field is the polarizability, α. This variable is a measure of how easily a bond is deformed by the electric field of the light. One can often predict the relative Raman intensity of molecules from their chemical structure. Molecules with large π bonded systems, like aromatic compounds, are very polarizable and exhibit strong Raman intensities. Molecules with few electrons in their bonds, like an O–H or N–H, will exhibit weak Raman signals.

An important point to consider in Raman scattering is that it is the change in polarizability due to stretches of the bond that affects the strength of the signal. This is described by

$$\alpha = \alpha_{eq} + (r - r_{eq})\left(\frac{\partial \alpha}{\partial r}\right) \quad \text{(Eq. 6)}$$

where $\frac{\partial \alpha}{\partial r}$ describes how the polarizability changes with the internuclear radiation, r, as r changes from its equilibrium value r_{eq} during a molecular vibration in a bond with an equilibrium polarizability of a_{eq}. The key concept that leads to an equation for Raman scattering is that the vibration is a harmonic oscillator given by

$$r - r_{eq} = r_m \, cos(2\pi v_{vib} t) \quad \text{(Eq. 7)}$$

where r_m is the maximum length that the bond stretches during a vibration with a frequency of v_{vib}. We can now place this equation (Eq. 7) in the polarizability equation (Eq. 6) to get

$$a = a_{eq} + \left(\frac{\partial a}{\partial r}\right) r_m \, cos(2\pi v_{vib} t) \quad \text{(Eq. 8)}$$

when this is substituted into the equation for induced dipole we get

$$m = \alpha_{eq} E_O \, cos(2\pi v_l t) + \frac{E_O}{2} r_m \left(\frac{\partial \alpha}{\partial r}\right) cos \, [2\pi(v_l - v_{vib})] + \frac{E_O}{2} r_m \left(\frac{\partial \alpha}{\partial r}\right) cos \, [2\pi(v_l + v_{vib})] \quad \text{(Eq. 9)}$$

While this was derived from classical theory, this dipole moment can be used as an operator for the quantum mechanical description of Raman scattering.

The first term describes elastic or Rayleigh scattering; the induced dipole from this term oscillates at the same frequency as the electric field of the incident laser light. The second term describes an induced dipole oscillating at the laser's electric field frequency minus that of the vibration's induced dipole. This represents the electric field losing energy into the vibration and it is the most common form of Raman scattering. When energy is lost by the electric field, the shift is called a Stokes shift. The third term anticipates a case where the vibration is already excited and the vibration's energy is imparted into the photon's energy. This type of inelastic scattering is called an anti-Stokes shift.

If we multiply the Stokes and anti-Stokes by Boltzmann's equation we can account for the relative populations of molecules in the ground and excited states and we find the temperature dependence of Raman scattering:

$$\frac{I_{Stokes}}{I_{anti-Stokes}} = \frac{(v_o - v_{vib})^4}{(v_o + v_{vib})^4} \, e^{\, h v_{vib}/kT} \quad \text{(Eq. 10)}$$

This relationship illustrates the ratios of Stokes to anti-Stokes as a function of the vibrational frequency and the sample's temperature. The ratio decays rapidly as the vibrational frequency increases and it also decreases as the temperature of the sample increases.

These are both easily rationalized; the Stokes intensity is related to the number of molecules in the sample that are in their ground state. As the vibrational frequency increases so does its energy $(E = hv)$, and the number of molecules in this excited state will decrease. They do not have enough energy at room temperature to support a large population. Likewise, as the temperature increases, more molecules will be in the excited state and the amount of anti-Stokes Raman scattered photons will increase.

The quantum mechanical expression for the polarizability is beyond the scope of this monograph. However, by partially deriving it, we can determine a few valuable features of Raman scattering, and hence one can find the quantum mechanical expression of the polarizability in the annex of this monograph.

Comparing spectroscopic methods

It should be understood that molecular vibrational spectroscopies only detect two or more atoms that have a bond (molecular bond) between them. Metals or metal ions are detected spectroscopically through atomic absorptions or emissions or through indirect methods that use metal ion sensitive molecular indicators.

Table 1 provides a few of the predictable properties of each spectroscopic method. With respect to chemical analysis, the last 3 rows are the most important. With the exception of MIR spectroscopy, all methods permit sampling in glass containers. Furthermore, once glass or silica is possible then fiber-optic probes become possible for convenient at-line or in-line sampling. It is also important to understand the role of solvents in sampling. The dependence of MIR on polar molecules makes it very sensitive to water as a solvent or even as an impurity. On the other hand, water has virtually no interference in UV/VIS, fluorescence, or Raman spectroscopy. It is strong in NIR spectroscopy, but because the overtones and combination modes are generally weaker than fundamental modes, water does not oversaturate the signal. In NIR water is often the analyte of interest.
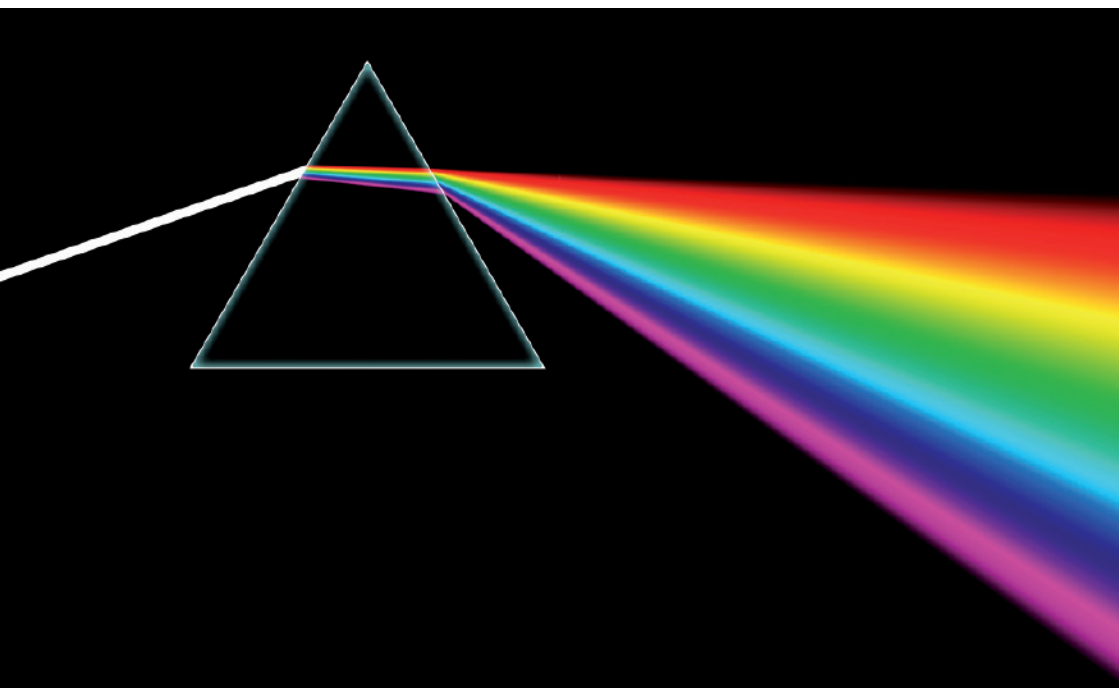
Table 1 Overview of common spectroscopic techniques

Spectroscopic Method	UV/VIS	Fluorescence	MIR	NIR	Raman
Range	190–1000 nm	200–1000 nm	200–4000 cm^{-1}	4000–12500 cm^{-1}	0–4000 cm^{-1}
Source	deuterium/ tungsten halogen	laser	globar tungsten	globar tungsten	laser
Molecular source	electronic transitions	electronic transitions	vibrational transitions	vibrational overtone & combination transitions	vibrational transitions
Involved bonds	σ and π bonds	σ and π bonds	polar bonds such as C=O, C–O, C–F (favors polar bonds)	H-containing bonds such as O–H, C–H, N–H, S–H	homonu-clear bonds (favors polarizable bonds)
Signal	absorption	emission	absorption	absorption	scattered light
Quantification	log I_0/I ~ conc. (Lambert-Beer law)	intensity ~ conc. x K	log I_0/I ~ conc. (Lambert-Beer law)	log I_0/I ~ conc. (Lambert-Beer law)	intensity ~ conc.
Calibration	atomic emis-sion lines	atomic emission lines	Connes' law	standard materials	standard materials
Sampling	glass, or fused quartz cuvettes, fiber-optical probes	glass or fused quartz cuvettes, fiber-optical probes	salt plates, Nujol mulls, ATR sampling	Fiber-optical probes, glass containers	glass vials, plastic containers, direct fiber-optical probes
Selectivity	poor	poor	excellent, uses library matching	fair, requires chemometric models	excellent, uses library matching
Sensitivity	medium	high	medium	medium	medium – low. high with SERS

Selectivity is important both for material identification and quantification in the presence of other materials. The number of molecular sources for UV/VIS and fluorescence spectroscopy is limited to electronic states within the analyte. This number of possibilities is much more limited than the number of possible vibrations. Conjugation of unsaturated bonds and inclusion of metals can create

more possibilities, but in general it is difficult to precisely identify a material from UV/VIS or fluorescence spectroscopy. This problem is exacerbated by the width of the absorption peaks in these electronic spectroscopies. The width comes from the vibrational and rotational modes associated with the bands. Each electronic transition has vibrational and rotational transitions associated with it that lead to what are called rovibronic states. These cause the broadening of the bands. As one moves to vibrational spectroscopy the broadening is much less, only associated with rotational states that become very closely spaced as the molecule increases in size.

Selectivity is also important for quantification. If the material's spectrum overlaps with the solvent's spectrum or with other species in the sample it requires more complex models with large training sets to create a quantitative model. This leads to chemometric methods that employ multivariate tools to quantify materials on the basis of complex overlapped spectra.

Sensitivity is generally expressed as a detection limit. The absorption methods suffer from the large background signal that is present when the amount of analyte is low. In other words, they require detection of a small signal change in the presence of a large signal. However, the strong absorbance of some molecules, in particular with extended π-bonds, can

be detected at nanomolar levels without too much difficulty (10 to 100 µmol/L is more typical). ATR MIR systems too can achieve limits of detection in the range of 100 µmol/L; NIR systems can reach the same range with sufficient pathlength.

Fluorescence spectroscopy is much more sensitive than absorption methods. This is in large part due to its zero baseline. The background for fluorescence spectroscopy is in principle zero, though in reality there are small noise sources in detectors which never allow a perfectly zero baseline. Single molecule detection with fluorescence spectroscopy has been achieved. More common, however, are nanomolar levels of detection. The limit to fluorescence spectroscopy is the small number of molecules which exhibit strong fluorescence. Fluorescence spectroscopy is most often performed on a small number of strongly fluorescent tags that are used to detect the analyte indirectly.

Raman spectroscopy has a reputation for possessing poor sensitivity. It is true that the strength of Raman spectroscopy is its high selectivity due to narrow bandwidths and its easy sampling. A modern Raman spectrometer detects most organic molecules and small anions down to 1 mmol/L or about 10 times higher than absorption methods. Powders or other solid samples can be detected with quite low mass sensitivity due to the use of tightly focused laser beams. Raman

microscopes can easily detect less than 1 mg of a solid material and quality systems have monolayer sensitivity for coatings. There are two exceptions to this limit: resonance Raman scattering (RRS) and surface-enhanced Raman scattering (SERS). RRS occurs when the laser excitation matches an absorption band of the analyte. This can produce detection limits as much as 1000 times lower than standard Raman spectrometers, however it is often plagued by fluorescence. SERS will be explained in detail later in this monograph. It is an enhancement of Raman scattering that occurs at specially prepared silver or gold surfaces. The enhancement from SERS can be as high as 10^8 and it too has been shown to produce single molecule detection in many cases.

Table 2 Overview between the various Raman and IR active bands with relative signal intensities.

Functional group	Chemical	Formula	Reference Values [cm⁻¹]
Alkane	n-hexane n-heptane	C_6H_{14} C_7H_{16}	CH_2 in-phase twist: 1305–1295 C–C skeletal stretch (n-alkane): 900–800
Branched alkane	2,2,4-trimethylpentane	C_8H_{18}	C–C skeletal stretch (branched alkane): 1175–1165; 1170–1140; 1060–1040; 950–900
Cycloalkane	cyclohexane	C_6H_{12}	cyclohexane ring breathing: 802
Haloalkane	trichloromethane	$CHCl_3$	C–Cl stretch: 760–740; 675–655; 635–630; 615–605
Alkene	1-hexene	C_6H_{12}	monoalkyl C=C stretch: 1650–1638
Alkyne	3-hexyne	C_6H_{10}	disubstituted C≡C stretch: 2237–2230
Aromatic	benzene toluene	C_6H_6 C_7H_8	unsubstituted aromatic ring breathing: 992 monosubstituted aromatic ring breathing: 1010–990
Alcohol	ethanol	C_2H_5OH	in-phase CCO stretch: 900–800
Aldehyde	acetaldehyde	C_2H_4O	alkyl aldehyde C=O stretch: 1740–1725
Ketone	acetone	$(CH_3)_2CO$	alkyl ketone C=O stretch: 1720–1712
Ether	methyl tert-butyl ether	$(CH_3)_3COCH_3$	dymmetrical COC stretch: 1720–1712
Carboxylic acid	acetic acid	CH_3COOH	dimer C=O stretch: 1687–1625
Ester	ethyl acetate	$CH_3COOCH_2CH_3$	O-C=O in-plane deformation (acetate ester): 644–634
Amine	triethylamine	$(C_2H_5)_3N$	tribustituted amine C–N stretch: 1250–1000; 833–740
Amide	dimethylacetamide	$CH_3CON(CH_3)_2$	tertiary amide C–N stretch: 750–700
Nitrile	acetonitrile	CH_3CN	C≡N stretch: 2250–2230

Raman instrumentation

Throughout history, Raman spectrometers have undergone several conceptual changes. Especially, the needs for bright excitation sources and sensitive detectors grew constantly.

The very first observation of Raman scattering was performed in 1927 by C. V. Raman using a 7″ telescope focused on the sun as excitation source. Within a year he had replaced this source with the narrow line from a mercury lamp and photographic plates were used as the detector. This simple method was very popular and was used to determine the structures of many simple molecules. In the 1960s, when the first lasers became available, Raman spectroscopy became a popular spectroscopic technique for a wide range of chemical analyses.

Current Raman spectroscopy owes its efficient designs and low cost to the telecommunication industry. In the early 1970s, the rapid development of diode lasers, optical switching and filter technology, and fiber optics gave the Raman manufacturers the tool to change the industry from one of large research instruments to small practical devices. What were once instruments in the size of automobiles have now been transformed into handheld battery operated material analyzers.

Raman system components

Much has changed since C. V. Raman focused the sun rays with a telescope onto a sample. The configurations of modern spectrometers have transitioned with advances from several fields. Up until the 1980s, most Raman spectrometers collected the scattered laser light with a lens in 90 degree geometry (right angle collection). Intense wavelengths close to the laser line, due to Rayleigh scattering, are filtered out while the rest of the light is focused on a dispersion device and distributed onto a detector.
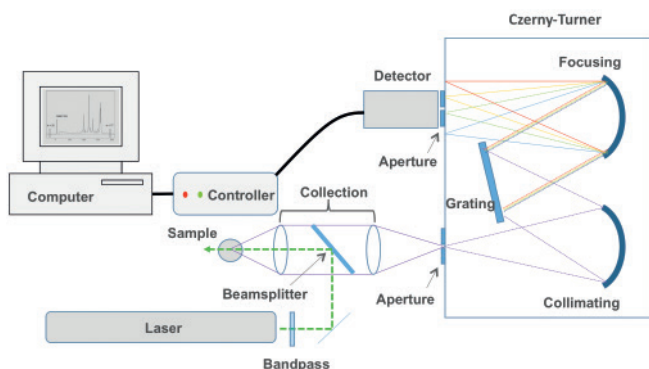

Figure 2 Classical Czerny-Turner Raman system

While effective, these sampling configurations were unwieldy. Most modern collection systems now incorporate normalized or 180 degree collection. Figure 2 illustrates a traditional Czerny-Turner design with 180 degree collection; however, most modern systems have departed from this configuration since the light is focused off-axis by the mirrors and creates astigmatism.

180 degree scattering could not be accomplished without advances in beam directional and filtering components. Beamsplitters (dichroic filters), as the name implies, redirect a portion of the light beam while allowing the remainder to continue on a straight path. Dichroic beamsplitters are made from multiple layers of different dielectric materials. As the term dichroic implies they transmit efficiently one range of wavelengths and reflect efficiently another range of wavelengths. Advances in this technology arose from innovations in epifluorescence microscope systems. The result is a mirror that can reflect light shorter than a particular «cutoff» wavelength and transmit light longer than that wavelength.

These optical components contribute several features to modern Raman instrumentation. The Czerny-Turner design removed the laser line intensity with its large size. Often these systems were 1 meter focal length. The introduction of dielectric filters to remove the laser excitation immediately caused Raman instrument designers to create much smaller devices.

The fundamental component of a Raman spectrometer is the laser. With progressive technological development, the large size of the first Raman spectrometers – due to the size of the laser – shrunk. In the beginning, it was not uncommon that the laser was a separate module. The telecommmunication industry which produced the technology for the telecom market also gave Raman spectroscopy miniaturized diode lasers that operate for days off of batteries.

The diode lasers in contemporary Raman systems differ from those found in optical storage devices or laser pointers. An important part of a laser is the optical cavity that stores large optical powers and «leaks» a small percentage to be used for spectroscopic measurements. The small size of the diode laser causes it to have multiple laser frequencies called modes at which it emits laser light. The large gain curves of the semiconductor materials further permit the mode-hopping of diode lasers. This is detrimental to Raman spectroscopy which requires a single frequency. This problem has been solved by using stabilized or cooled lasers. All modern diode laser Raman systems use stabilized lasers to produce a well-defined laser excitation.

The spectrometer design of present-day Raman systems differs from the Czerny-Turner design. The Czerny-Turner off-axis design produces astigmatisms that reduce the spectral resolution of the spectrum.

This was first addressed with toroidal mirrors which correct the Czerny-Turner astigmatism. In the early 1990s Raman manufacturers started designing an integrated Raman system that eliminated the laser module and were tuned to one set of optical filters. This eliminated the need for mirrors, reflecting a range of wavelengths, by which the use of achromatic lenses pushed through. Once lenses were introduced, several on-axis spectroscopic designs became common in Raman systems. Current systems use two lenses and a reflection or transmission grating to disperse the light and bring it back in focus on the focal plane array detectors.

Historically Raman spectroscopy evolved from photographic plates to collect the Raman spectrum to sensitive photomultiplier tubes to electronically record the spectrum with a monochromator to the return of the photographic plate in the form of the Charge Coupled Device (CCD) detector. CCDs create electronic wells that separate the electrons and holes created by photoelectric events. The CCDs are so efficient at the separation that the wells can collect photoelectrons for minutes with only mild cooling. The great advantage of the CCD over the photomultiplier is that a complete Raman spectrum can be collected in a single acquisition.

Laboratory Raman spectroscopy
Laboratory Raman spectrometers provide greater versatility than handheld Raman systems. They may feature microscope attachments, confocal sampling, chemical imaging, sampling automation, and multiple wavelength, spectral range, and resolution options. The size and power requirements of these instruments preclude field operation. However, they adapt well for R&D labs and for on-, at-, and in-line analysis with proper sampling attachments.

Raman microscopes
Raman microscopes provide two valuable features: imaging and mapping. All Raman instruments provide a tightly focused beam at the sample. The brightness of the beam makes it nearly impossible to see its exact location on the sample and eye-safety also requires glassware to block out the wavelength of the laser excitation. A Raman microscope uses filters and digital cameras to permit the laser beam to be observed on the sample. This also means that small micron size samples can be located and examined.

Mapping is also a popular application for Raman microscopy. Many natural samples like biological or botanical samples and synthetic materials like pharmaceuticals or plastics are heterogeneous. A Raman microscope with a motorized stage can acquire Raman spectra over points on a surface and create a Raman map that identifies materials or changes across the sample.

Confocal Raman microscopy

One way to consider a Raman micro-scope is an imaging system where the sample image is focused through the aperture of the spectrograph. The spec-trograph aperture can take on a new meaning and it is called the confocal aperture. This permits the spectrograph-microscope combination to also pro-duce spatial maps in the z-direction to produce depth profiling.

Pure imaging microscopy uses a large beam of light to illuminate an area of the sample. Raman microscopy uses a tightly focused laser beam to illuminate a small, usually ~1 micron, spot on the sample. The actual spot size is deter-mined by many factors, but it can be made to be diffraction limited, i.e., limi-ted by nothing but the Abbe diffraction limit. This focused spot is the beam waist of laser beam that expands rapidly away from the focus. The spectrograph (con-focal) aperture acts as a spatial filter that removes the signal from areas other than the beam waist (Figure 3).
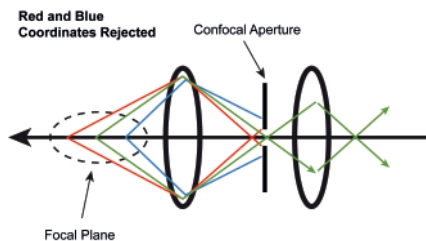

Red and Blue Coordinates Rejected   Confocal Aperture

Focal Plane

Figure 3 Ray tracing diagram illustrating the spatial filtering of depths in confocal spectro-scopy

The result is that only the signal from the depth in the sample at which the beam waist is located is collected. This lets the system adjust the depth viewed in the sample by controlling the z-axis of the microscope stage. If this is combined with x-y translation of the stage one can pro-duce a 3-dimensional map of a sample. This has produced many applications such as measuring the thickness and identity of multilayer semiconductor and polymer films and coatings, dispersion of active ingredients in tablets, capsules, and cardio stents, capturing the space distribution of carbon nanotubes inter-twined within semiconductors, analysis of various organelles in eukaryotic mem-branes and cells, and characterization of bacterial cell structures.

Raman chemical imaging/mapping

A light microscope provides information about the physical shape, color, and to-pographical structure of a material, but does not provide details on the chemical makeup of the surface. Several types of analytical spectroscopy tools can be in-terfaced with a light microscope (MIR, NIR, UV/VIS, and Raman) to provide spa-tially resolved chemical information. A complete spectrum is obtained at each pixel of the image, and then interrogated to generate false color images based on the material composition, phase, crystal-linity, and strain of a material.

Each Raman spectrum is associated with automated stage movements. Stage movement pattern can be point-to-point, grid, or synced with a particle size identification algorithm. Figure 4 displays a green leaf, each half of which was dipped in a solution of sodium nitrate or coated with sulfur powder. A light microscope image can only display a green leaf, but the Raman chemical image will display the chemical contamination with the sulfur powder and sodium nitrate solution. Chemical imaging can also be combined with depth profiling to show a three dimensional chemical image of the sample.

Compact Raman microscopes have been gaining traction because of their low cost, implementation in mobile labs, and reduced work areas.

Benchtop Raman systems
While handheld Raman systems have to be, primarily, light-weighted, safe, cost-effective, and easy to use (little to no additional training for the end user), benchtop Raman systems have to fulfill different requirements.

For instance, benchtop Raman spectroscopy systems offer the opportunity of coupling with numerous types of sampling interfaces, and because of their size they offer higher resolution and sensitivity compared to handheld Raman systems. With the higher sensitivity of benchtop Raman systems, it is also possible to quantify substances down to low concentrations, while handheld Raman systems are often used to fulfill identification and qualification needs.

Some systems acquire spectra using raster scan techniques which can prevent laser damage to the sample and, at the same time, provide a comprehensive image of heterogeneous samples as it averages the spectra of different sample points.
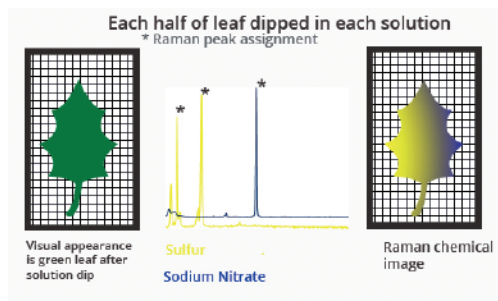

Figure 4 An example of Raman chemical imaging. Left, the light microscopy image of the sample and on the right the chemical image produced by selection of different Raman bands (middle).

Handheld and portable Raman analyzer

The telecommunications boom created diode lasers with a low power consumption and small, low-cost optical components, and the digital camera market provided sensitive low-cost detectors. The combination of these developments have made it possible to build Raman systems that can be held in one hand.

The market for handheld Raman spectrometers started out with systems for the identification of unknown materials. The systems were used to identify «white powder» threats, and also unknown materials for hazardous material (hazmat) squads. More recently these handheld systems have found applications ranging from geology and archeology to the pharmaceutical industry.

The design of handheld Raman instruments is often very similar to laboratory systems. The main difference is the instrument size. Handheld Raman instruments offer a lightweight alternative to benchtop Raman systems, capable of fast analysis to mainly cover identification questions in the field.

Handheld Raman instruments such as the Mira M-1, offer fast Raman spectroscopic analysis anywhere, e.g., in the warehouse, in the process, in QC, in the field, and in the laboratory. With their small size and their robustness, handheld Raman instruments brought Raman spectroscopy to a new level.

Raman process analyzers

Process Raman analyzers can operate in-line, on-line and at-line. By definition, in-line systems directly interface with the process stream, on-line systems measure a by-pass, and at-line systems rest on a bench or cart and the operator brings the sample to the device.

Process systems are designed to operate in harsh environments and are equipped with enclosures designed to meet the demands that arise from this. Devices have an International Protection Rating (IP code), which is noted as «IP» followed by two digits and an optional number. It classifies the degrees of protection against the intrusion of solid objects, dust, accidental contact, and water in electrical housings. IP67 is a popular rating for handheld Raman systems that establishes the device housing as protection from dust ingress and damage by immersion in water up to one meter depth. Process housings typically do not need to meet these requirements. Manufacturers follow on the National Electrical Manufacturers Association (NEMA) guidelines for hazardous locations. A popular enclosure requirement for hazardous areas is a NEMA 4X rating that protects personnel against access to hazardous parts and provides a degree of protection of enclosed equipment from windblown dust, ingress of water, and additional protection against corrosion. These enclosures can be cooled to prevent the inside environment from heating up.

Special techniques

Orbital-Raster-Scan

One of the characteristics of dispersive spectroscopy is the inverse relationship between light collection and resolution: the more light you collect, i.e., the larger the interrogation spot, the lower the resolution. The interrogation spot size is also known as the etendue of the system. With conventional spectroscopic instruments, a large interrogation area (large laser spot) would require a large aperture in the spectrometer to efficiently collect the entire laser light. Increasing the aperture has a negative effect on the valuable information about the material spread over the detector resulting in poor spectral resolution. This is illustrated in Figure 5. For pure samples a tightly focused laser beam and small spectrograph aperture may be sufficient. However, when the sample is heterogeneous or sensitive to the high laser power in a tightly focused beam, it is necessary to expand the beam size or reduce the laser power.

Patterned rastering technologies, e.g., the ORS (Orbital-Raster-Scan) technology, can overcome the etendue loss while still interrogating a large sample area.

The advantage of ORS for the analysis of heterogeneous samples is illustrated in Figure 6. A conventional spectrometer with a tightly focused beam may produce high resolution, but it is inferior when analyzing heterogeneous substances because it cannot spatially target all components of the mixture, or it can miss them completey, resulting in zero or low signal. A conventional system requires a large aperture to capture all sample components. This, however, results in a loss of resolution. ORS increases the interrogation area on the sample while maintaining high spectral resolution.
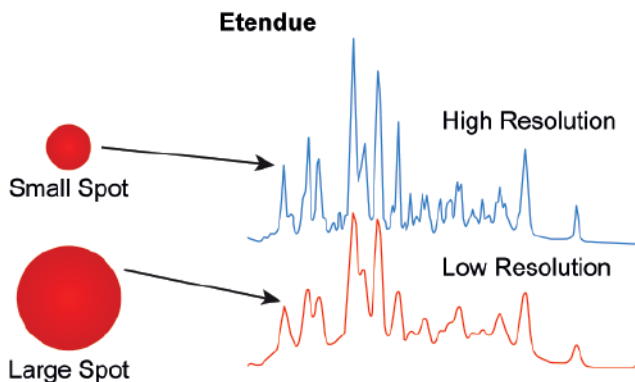
Figure 5 Example of etendue. Top: A small excitation spot imaged onto the entrance aperture of a spectrograph produces high spectral resolution. Bottom: A large excitation spot imaged onto the entrance aperture produces poor resolution.

Raster scanning's three main advantages:

1. Higher laser powers can be employed, because the laser spot does not rest on the same location. Even nanoparticles in suspensions can be susceptible to sample burning.
2. The rastered beam collects data at several locations of the sample providing a more reproducible result.
3. It enables a high throughput, high signal-to-noise, high resolution measurement system that can cover a large area without losing resolution.

Figure 7 and 8 demonstrate the benefits from ORS when analyzing pharmaceuticals. Pharmaceuticals are mixtures of excipients and active pharmaceutical ingredients (APIs) in carefully controlled proportions. Effervescent cold medicines, for example, contain three different APIs: aspirin to relieve pain, chlorpheniramine maleate as an antihistamine, and phenylephrine bitartrate as a decongestant. With the small beam diameter of most Raman systems and the small particle size of the APIs, analyzing the APIs is difficult. Several spectra have to be gathered at different points on the sample, even for homogeneous samples.

By using ORS and the larger interrogation area, the spectrometers can capture the APIs in a single analysis.
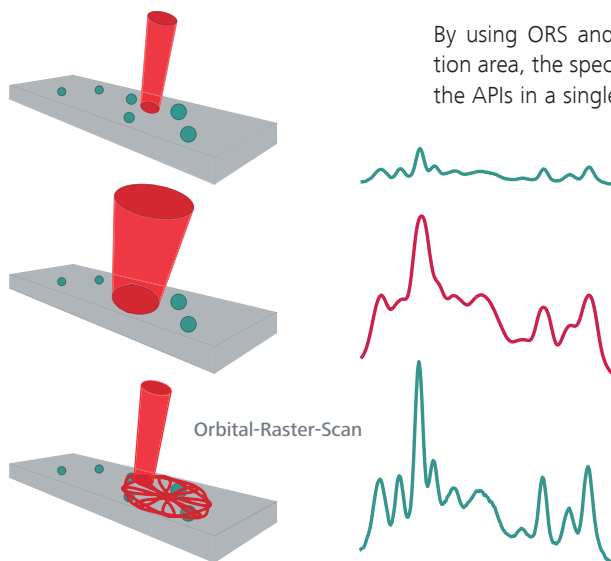

Orbital-Raster-Scan

Figure 6 Dispersive spectrometers use a tightly focused beam (top), resulting in a high spectral resolution, but components in heterogeneous samples can be missed completely. Simple broadening of the beam would result in a loss of spectral resolution (center). The ORS technique (bottom) scans a larger sample area and is therefore more likely to capture dispersed sample components. Meanwhile, it maintains the high spectral resolution that is required for analyte identification.
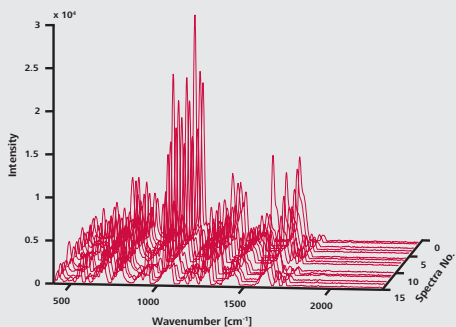
Figure 7 The 15 Raman spectra shown here were recorded at random locations on a single sample without ORS. Although peaks are observed at the same positions, intensities vary.
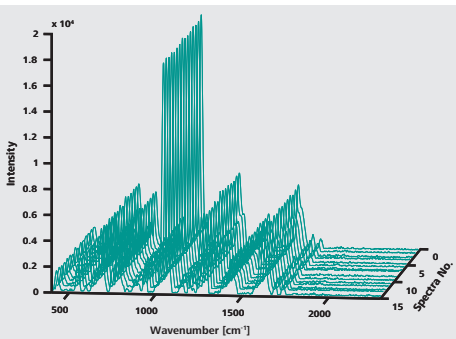

Figure 8 Like in figure 7, the 15 spectra shown here were measured at random locations on a single sample. However, in this measurement, ORS was used sampling an area of 3 mm diameter. The spectra are visibly congruent.

Surface-Enhanced Raman Scattering (SERS)

Surface-Enhanced Raman Scattering (SERS) is an interesting nanomaterial method to enhance Raman signals. Under proper conditions, the Raman signals from materials adsorbed to prepared nanomaterial surfaces are enhanced by a factor >10[6]. This enhancement turns the medium-sensitivity Raman technique into a trace technique capable of detecting and identifying single molecules.

Theory of SERS

The electromagnetic explanation for SERS can be found in textbooks on electrostatics. If one looks at the equation for the electric field surrounding a dielectric sphere, it is a simple relationship between the dielectric constants of the sphere and the surrounding materials. This is illustrated in Figure 9.

To explain this phenomenon a little more we should first ask «why electrostatics?». SERS is only observed when the nanoparticle is smaller than the wavelength of the laser excitation. For example, SERS nanoparticles are often < 100 nm in diameter and the laser wavelengths maybe 785 nm. This means to a first approximation the electric field of the light appears «static».

Another unusual feature of SERS is that it is limited to a small group of metals. The alkali metals have been shown to exhibit SERS, but their reactivity is far too high to be practical. Even in the Group 1B metals, copper is too reactive under most conditions. That leaves silver and gold as the most common SERS materials. The reason SERS is limited to these materials is their dielectric constant – which is not really a constant since it changes with the wavelength of the light. Silver and gold both have negative dielectric constants at visible wavelengths. If you look at the equation in Figure 9 you can see that when the real

dielectric constant of the metal (ε_r) is -2 and the dielectric constant of the surrounding (ε_0) is 1 then the denominator becomes small. In fact, if the imaginary part of the metal's dielectric constant is 0 then the electric field outside (E_{out}) of the particle would become infinite. The unique feature of silver and gold is that they have a full d orbital (d^{10}) and a single s^1 electron. This shielded electron is called a «free electron» and has the properties to produce a small imaginary dielectric constant and a negative real dielectric constant. The result is large electric fields around the particles relative to the small incident field of the laser excitation source (E_0).

The final part of the SERS theory is the relationship between Raman intensities and the electric field of the light. Raman scattering is linearly related to the intensity of the laser excitation and the laser intensity is proportional to the square of the electric field of the light. With relation to Figure 9 this means that the enhanced electrical field outside the particle increases the Raman scattering by a power of 2. The SERS effect also gains enhancement from the induced dipole of the molecule. During the Raman process the electric field associated with this dipole will also be enhanced by a power of 2. This means that the overall SERS enhancement scales as the 4th power of the field surrounding the nanoparticle.

This is a simplistic view of the SERS effect. The particle's shape can strongly affect the enhancement, the size can dampen or shift the enhancement to longer wavelengths, and the junction between particles is believed to also be a site of extra enhancement.
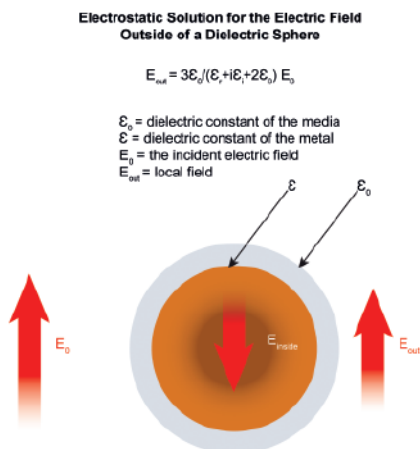
Electrostatic Solution for the Electric Field Outside of a Dielectric Sphere

$$E_{out} = 3\varepsilon_0/(\varepsilon_r + i\varepsilon_i + 2\varepsilon_0)\,E_0$$

ε_0 = dielectric constant of the media
ε = dielectric constant of the metal
E_0 = the incident electric field
E_{out} = local field

Figure 9 Illustration of the electromagnetic effect responsible for SERS. The essential concept is that the E_{out} becomes the field that generates E_{inside} and that creates a plasmon resonance within the particle.
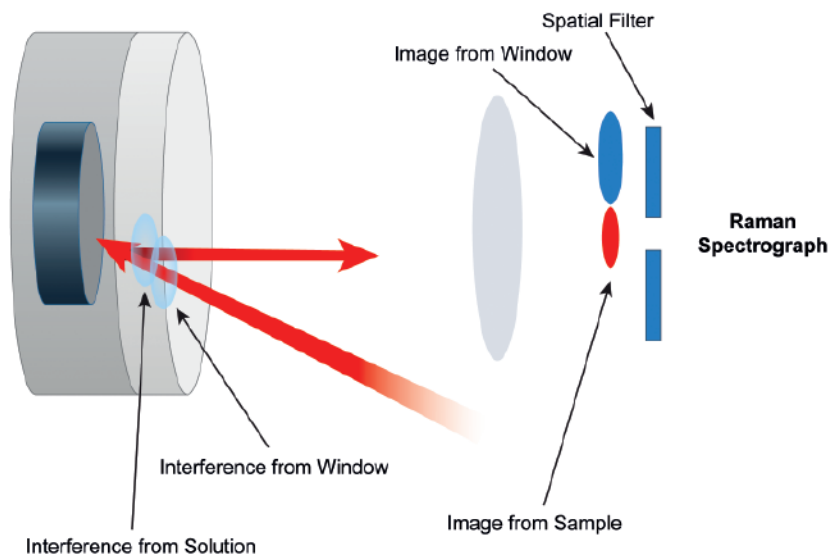
Figure 10 Illustration of angular offsets to spatial filter interferences of material before the sample.

Depth penetration

The most common collection geometry for Raman spectroscopy is 180 degrees backscattering collection. This collection geometry overcomes tedious alignment of off-axis excitation and collection. Figure 10 illustrates an older collection geometry used for spectroelectrochemistry where off-axis collection was used to eliminate scattering or fluorescence from windows and solutions in front of the electrode.

The key to understand off-axis collection is the spatial filter. Spectrographs are just imaging systems that image the entrance aperture at the focal plane. As we discussed with confocal Raman microscopy, this entrance aperture is also a spatial filter that permits, that only a small area of the spot is illuminated by the laser beam. By exciting off-axis the window and solution in front of the sample are imaged to the side of the spatial filter (entrance aperture) and they are removed from the spectroscopic signal. A Raman instrument with **O**ff-**A**xis **R**aman **S**cattering (OARS) can easily see into chemical containers to eliminate wasteful, time consuming sampling inside the container. This can be particularly useful to minimize the exposure to hazardous materials. The OARS technique permits us to completely resolve the container material from the contents; Identification/verification can be performed without opening the container.

Data processing and analysis

Chemometrics

Chemometrics is the use of multivariate statistical and mathematical analysis to simplify and improve information content of large, complex data sets. In spectroscopy, chemometrics is often used to establish correlations between sample quality parameters, or other physical properties, to the analytical data collected from the materials.

Chemometrics allows us to easily model specific patterns that exist in the data set and to predict the same quality parameters from future data that is collected. However, there are many different determination methods that can be applied in chemometrics, and each has its advantages and disadvantages for modeling data sets. While it is not that important to understand the mathematics of each method, it is important to understand which model is best suited for a particular problem to be solved.

Data analysis and chemometrics

Raman data is considered relatively simple in that it features very narrow bands that can be easily differentiated without much data processing, and the collected data stems from a single-wavelength excitation source, thus minimizing effects of overlapping wavelengths. However, there are still a number of variables that can influence the measured spectra of a sample such as influences from multiple species present in the sample, minor sample-to-sample differences (particle size, etc.), as well as external influences

from ambient light. Varying concentrations of a particular analyte will also have an effect on the resulting Raman spectrum.

Because there is a wide array of variables that can affect the measured spectrum, the resulting data can be considered multivariate. Chemometrics aims to reduce this complex data set and select only the variables that correlate to a certain property being measured. It does so by deconstructing the data into a set of vectors that retain the important information while removing unwanted information (e.g., spectral noise).

Chemometrics relies on matrix mathematics to reduce the dimensionality of the variables. These variables can be dependent or independent depending on the information that is being measured. Independent variables are those that are controlled or changed during an experiment, while dependent variables are the values that are being measured (instrument channels) or predicted based on how the independent variables change.

One example of this, as it applies to a Raman spectrum, would be to measure how Raman peak intensities change as the concentration of a molecule changes. By separating these variables into independent vector matrices we can create a chemometric model that is able to predict the concentration of an unknown material based on the intensity of a peak or a group of peaks.

Calibration and validation of Raman data

The success and robustness of any chemometric data model is very much dependent on having an appropriate data set to begin with. The starting data used to create the model must adequately represent the problem being investigated. It is of high importance that the initial data come from samples that are representative of the materials being measured. Often times it is necessary for these reference standards to be validated first using another primary technique before the samples can be used for model building. In quantitative analysis, for example, it is important to gather enough samples with varying concentrations to span the range of concentrations anticipated in the unknown material, and to first accurately measure those concentrations using a primary method. In qualitative analysis, it is equally important to input data from a sufficient number of samples needed to capture any potential sample-to-sample variation in the model.

Data pretreatment

As mentioned earlier, many physical and chemical characteristics of a sample can influence the Raman spectrum. For example, fluorescence excitation in some samples can have a major influence on the background of the Raman spectrum, as can fluctuations in the surrounding ambient light. Also, the Raman data can be influenced by random noise throughout the spectrum, and this effect is even more detrimental for samples that are weak Raman scattering molecules and, therefore, often exhibit low signal levels that can be difficult to distinguish from the random noise.

Often it can be useful to employ certain preprocessing techniques to the Raman dataset prior to creating the model to help offset some of these random effects. For example, one commonly used technique called Savitzky-Golay polynomial smoothing can be used to help remove random noise from the spectrum prior to input into the model. In this technique, polynomial functions are fit to a group of points around each point in the spectrum in order to isolate the true signal peaks versus random noise.

In the case where background fluctuations are interfering with the Raman measurement, employing certain techniques such as point difference derivatives and Savitsky-Golay derivatives can help to remove the background from the spectrum, thus minimizing its influence. This can be important depending on what metrics are being calculated since the background intensity will have an effect on the peak intensity and the determined bandwidth.

In many cases, it may also be useful to correct for spectrum intensity fluctuations by normalizing the data to a known value. This is due to the fact that there are many factors that can influence the Raman band intensity. For example, small fluctuations in laser output power and instrumental throughput can lead to dif-

ferences in the Raman scattering intensity. Also, many sample characteristics such as refractive index, opacity, and density can also cause fluctuations in intensity by affecting the interrogated volume of the sample.

For qualitative analysis where the goal is identification of a material, the peak intensity is of little importance and therefore, can be fixed or normalized to a known value so that the intensity changes do not influence the data model. With quantitative analysis, however, the peak intensity information is important in determining the corresponding concentration, and therefore peak normalization may only be utilized with an independent peak, e.g., the solvent peak.

Identification, qualification, and quantification methods

Raman spectroscopy presents a number of advantages as a quality control verification technique. The analysis is fast, it requires little or no sample preparation, and the portability of many Raman instruments allows you to implement the device at the source of the sample. In most cases Raman spectroscopy allows you to sample directly through different barriers as well (e.g., plastic bags, glass containers, etc.). In general, there are two common analysis techniques that Raman is used for: sample identification of unknown materials and sample verification for material purity/quality testing.

Basic sample identification usually involves spectral library searching techniques, in

which a spectrum of the unknown sample is compared against many different spectra in a library of known materials. In these cases, algorithms, such as Euclidean distance matching, are used to identify a list of the closest library matches and rank them based on a value of Hit Quality Index (HQI).

One drawback to spectral library search techniques is that they are only sensitive to the general shapes and patterns of the Raman spectrum, and therefore are generally not powerful enough to detect subtle variations within the sample. For example, a «good» sample that contains low level contamination or that is of a poor quality may not be correctly selected for using basic library searching. Instead the algorithm will simply report an arbitrary HQI value (0 to 1) that does not give a strong statistical measure of the true similarity of the spectra. The reason is that this type of relative measure only uses a single representative library spectrum that cannot adequately capture all potential variation of unknown samples.

In other cases where the goal is to determine if a sample is of a certain quality, then statistical discriminate techniques, such as Principal Component Analysis (PCA), may be used. PCA models are created by incorporating multiple spectra from validated reference materials that cover the allowable range of potential sample-to-sample variation. This can include the same material that is purchased from different suppliers, for example, or even the same sample that is packaged in different materials.

Principle component analysis then reduces the dimensionality of the training data set by decomposing the spectra into their most common variations in the form of a set of eigenvectors that represent the majority of the variation in the sample set, excluding those factors that represent random fluctuation in the data set such as spectral noise. Data from future unknown samples can then be deconstructed to fit the training PCA model and compared to the existing data to determine if the unknown material «passes» or «fails» the model based on the tolerances of variation that were specified. Figure 11 describes the visual projection of the PCA data into multi-dimensional space.

In comparison with basic library search algorithms, PCA is very effective at detecting subtle variations in data that may better reflect the actual quality of the material, not just the basic chemical structure. Additionally, PCA can also provide statistical measurement values that relate to the quality of the match, such as a p-value that yields a significance level of the sample in question «passing» or «failing» the PCA model being tested. Figure 12 demonstrates a PCA projection of various materials in 2 dimensional space. We can see how data that is similar, i.e., from the same material, clusters together based on the differing amounts of variance. Each «cluster» can be assigned a specific tolerance level, which can be seen visually in the form of an elliptical pattern. When new data is collected from unknown samples and projected onto the PCA plot, one can «accept» or «reject» the material depending on if it projects within the tolerance ellipse or outside of it.

Geometric Model for PCA (Projection)

Projection is visualizing high dimensional data into 2D or 3D. PCA is defined as the orthogonal projection of data into lower dimensional data known as principle component subspace and the variance is visually maximized.

PCA computes a new set of axes that can describe the data more efficiently. The idea is to rotate the axes so that the new axes (also called the principal components i.e., PCs for short) are such that the variance of the data on each axis goes down from axis to axis.
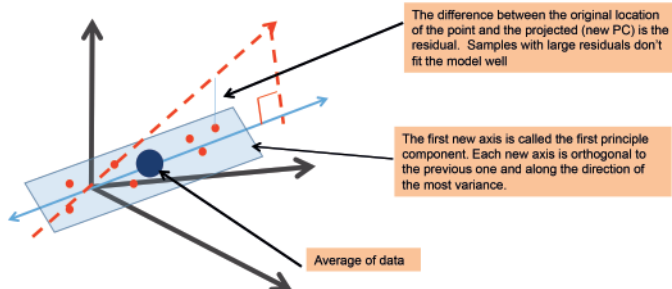
The difference between the original location of the point and the projected (new PC) is the residual. Samples with large residuals don't fit the model well

The first new axis is called the first principle component. Each new axis is orthogonal to the previous one and along the direction of the most variance.

Average of data

Figure 11 Geometric illustration of a PCA.

In addition to the identification and verification techniques mentioned above, sample quantitation by Raman spectral analysis can also be performed using a variety of chemometric techniques, such as Partial Least Squares (PLS) regression. This type of analysis relies on the presumption that the concentrations of the sample components directly correspond to the intensities or areas of the measured Raman peaks. From such a data set, PLS can deduce a linear regression line (concentration vs. peak height/area), that is able to predict the concentrations of unknown samples based on the Raman spectra that are collected.

A pertinent example of PLS with Raman is a Heck reaction between 4-bromoanisole and methyl acrylate to produce methyl 4'-methoxycinnamate. The 2010 Nobel Prize was awarded for the Heck reactions that are very useful to combine unsaturated halides with alkenes. These reactions are used in chemical industry and, in particular, with pharmaceutical preparations. This example was prepared by Drs. K. Carron and R. Corcoran at the University of Wyoming as an example of a microwave accelerated reaction. The same way a microwave oven cooks food much faster than conventional heating, they can cause reactions to take place very rapidly. Microwave assisted reaction methods are popular to screen large numbers of materials to determine the best route to a product.

Figure 13 illustrates the reaction that was performed in a small volume vial in a microwave oven. The spectra were acquired in a small autosampler vial. The solvent, DMF, is not part of the reaction and continues to be a large component of the spectra throughout the reaction. However, spectrum 4 demonstrates that it can be easily subtracted out to produce the reaction product.
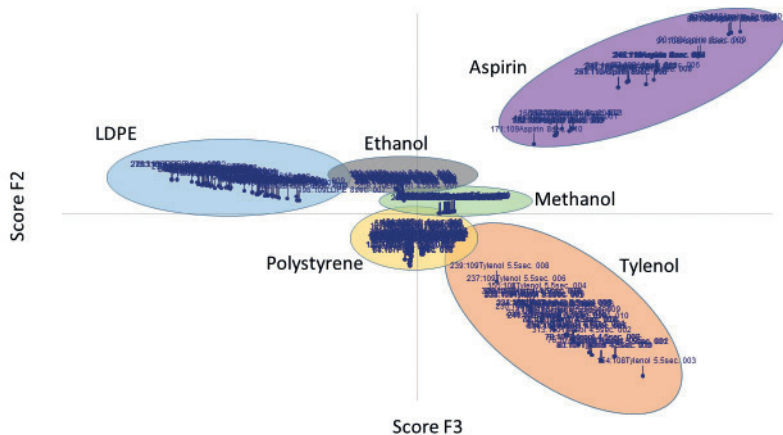
Figure 12 Illustration of a PCA score plot with 6 different materials.

The major challenge of microwave assisted reactions is the speed at which they are accomplished and having a spectral method that can monitor the reaction at these very high rates. Raman spectroscopy, thanks to its easy sampling, meets this challenge and can provide immediate concentrations in contrast to methods like GC-MS which require long analysis times. Figure 14 illustrates correlation spectra which is the first step to building a PLS model to provide analyte concentrations. This figure illustrates the correlation plots and the selection of masks to select regions of high correlation with the analyte of interest. The goal of this step in PLS is to select regions where each analyte is uniquely changing as their concentration is changing. The sharp bands of Raman spectroscopy makes this easy even with a large number of analytes. In this case you can see that 4 regions (masks) were found for

the 3 analytes. As the PLS model was built, only these 4 small (unique) regions were used to produce the model – a single model that can predict the concentrations of the starting materials and the product.
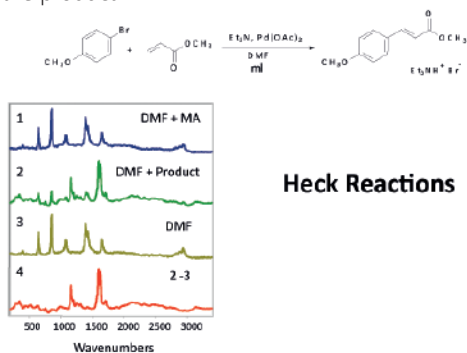
Heck Reactions

Figure 13 Spectra for the starting materials, solvent, and product of a Heck reaction between methyl acrylate and 4-bromoanisole. 1) methyl acrylate in DMF; 2) Reaction product in DMF; 3) Solvent (DMF); 4) Subtraction of spectrum 2 – spectrum 3 to show the product.

Correlation Spectra
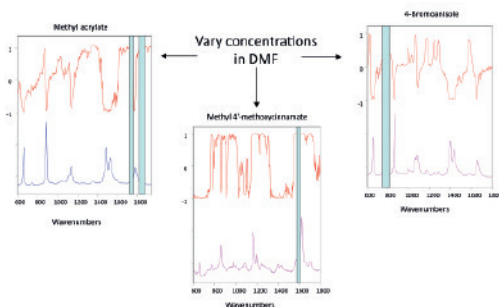
Figure 14 First step of building a PLS model is to make a training set of samples, 10 in this case, and correlate the concentration of each analyte with the spectra. The resulting correlation spectra are used to create masks that encompass spectra components that vary strongly with concentration and to exclude regions that do not contribute to information about the analyte concentration.

After correlation spectra are examined and the proper masks are placed in the spectral data the final step of creating a PLS model is to examine its correlation plots. These are plots of actual vs predicted concentrations from the training set. The correlation plots should be linear and have a large (near 1.0) correlation (R^2) value. The correlation plots in Figure 15 indicate that we have a good predictive model for the reactants and products of the Heck reaction.

This model can then be loaded with an unknown spectrum from the reaction mixture and it will provide the concentrations of the reactants and the product. Using Raman Spectroscopy and this method, one can follow the reaction progress even when microwave assistance forces the reaction to finish in as short as 10 seconds.
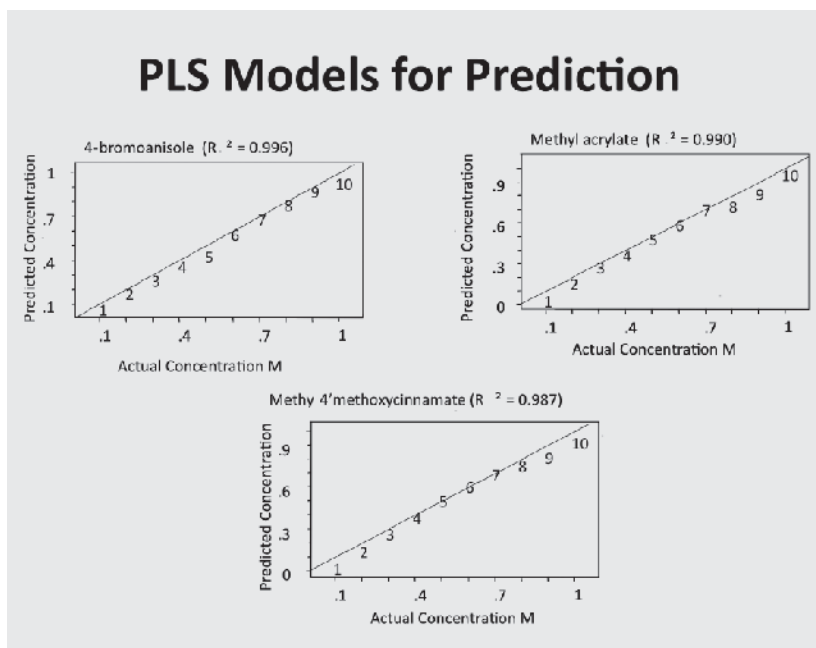
Figure 15 Correlation plots from the PLS model. These plots are used to determine if the model is accurate. In this case the large R^2 values indicate that the predicted concentrations from the PLS model match well with the actual concentrations used in the training set.

34

There are some disadvantages with us-
ing multivariate techniques such as PCA
and PLS. For one, a larger amount of
data must be collected initially in order
to create a robust model, and this data
must be accurate, completely represen-
tative, and verified through a primary
analytical technique. Another drawback
is that random fluctuations in the data
set (e.g. background differences, random
noise, peak height fluctuations) can
influence the outcome of the model.
Therefore, using these techniques re-
quires some basic knowledge and un-
derstanding of the data preprocessing
techniques described above and the user
must know how to properly employ
these techniques.

In the case of PCA, the user must also
have some basic working knowledge on
how to interpret the data plots so they
can select an appropriate number of

principal components (PCs) to be used in
order to avoid «overfitting» or «underfit-
ting» their model. Overfitting the model
involves selecting too many principal
components, creating a situation where
one or more of the PCs only represents
noise or other unwanted interferences.
Underfitting the model has the opposite
effect. Selecting too few principal com-
ponents can result in important informa-
tion being excluded from the model.

Industry sectors and applications

Law enforcement, defense, and security

Narcotics

Colorimetric testing is the most common testing method used for testing narcotics in the field. Chemical test kits contain ampules or plastic packages to test suspected narcotics (amphetamine, cocaine, ketamine, etc.) from color changes.

Rapid testing and the ability to non-destructively analyze the specimen through street containers is a real advantage of handheld Raman systems. Narcotics produce strong Raman spectra, and using ORS one can obtain reproducible spectra from street samples. Raman spectra for ketamine, methamphetamine, and cocaine HCl are shown in Figure 16.

Explosives

Raman spectroscopy can identify substances through sealed containers and Raman standoff systems can measure unstable samples from safe distances. Handheld allows the sample to be measured in the field rather than transporting dangerous substances to the lab for analysis. Several types of explosives can be identified with Raman spectroscopy including TATP (triacetone triperoxide), ammonium nitrate, TNT (trinitrotoluene), RDX (Research Department Formula X), and HMTD (hexamethylene triperoxide-iamine). Figure 17 illustrates the Raman spectra of RDX, PETN (pentaerythritol tetranitrate) and HMTD.
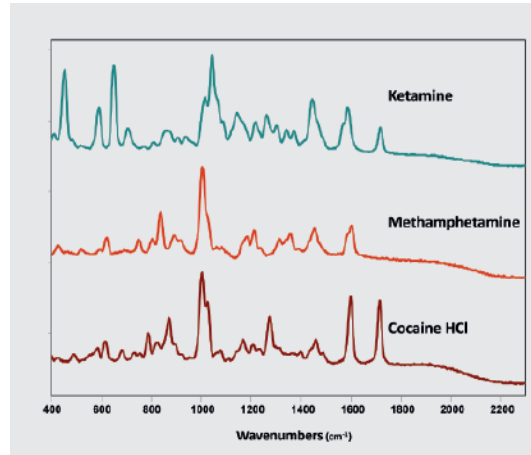
Figure 16 Spectral differences between ketamine, methamphetamine, and cocaine HCl.
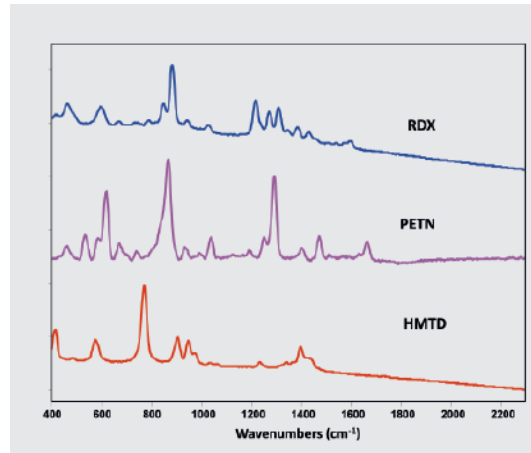
Figure 17 Spectral differences between RDS, PETN, and HMTD.

Hazardous materials

Safe and proper disposal of hazardous materials is a growing concern. First responders, defense personnel, and remediation agencies need to rapidly analyze the contents of unspecified spilled material and illegally dumped waste. In addition, remediation facilities need to test the products they are receiving so they can be properly disposed of. Visual inspections followed by further off-site laboratory tests are commonly employed. Raman spectroscopy can reduce the time, exposure, and expenses associated with unknown material identification. On-board libraries on the device can give immediate results.

Pharmaceuticals

Infrared (MIR and NIR) spectroscopy has been the most important method for testing of incoming raw materials. Verification of final release products requires more stringent methods carried out by skilled analytical chemists, e.g., extraction procedures and titrations, liquid chromatography, dissolution testing, and mass spectrometry. As 100% validation is being encouraged by the FDA, the size, speed, and cost prohibit the latter methods from meeting this standard. Only secondary test methods like NIR, MIR, and Raman have the rapid and certified test capabilities to meet the requirements. US Pharmacopeia General Chapter <1120> recognizes Raman spectroscopy as one of the accepted techno-logies for material identification/verification, asserting that «Since the Raman spectrum is specific for a given compound, qualitative Raman measurements may be used as a compendia ID test, as well as for structural elucidation». The FDA requires a rigorous authorization of signatures on all data, and handheld Raman manufacturers are required to follow 21 CFR Part 11 which sets forth the criteria for electronic records, and signatures so that they are reliable, trustworthy, and generally equivalent to paper records and handwritten signatures.

Various types of multivariate algorithms are used for pharmaceutical analysis. While testing incoming raw material is the prevalent application, pharmaceutical manufacturers are using handheld Raman to test the authenticity of finished products, and identify counterfeit products in the field.

In the laboratory, Raman microscopes with advanced confocal and imaging systems can measure the distribution of active ingredients in tablets and capsules. Another popular application is the analysis of polymorphisms in various ingredients.

Plastics

Plastics challenge rapid identification because they can have similar appearances. The common method of analysis is «burn and sniff» and a complex chart is used as a decision tree for identifying plastics. This chart is based on several tests that leads to different branches of a decision tree. Questions are if the plastic floats on water, whether it drips or self-extinguishes when burned, the color of the flame, the odor, and the speed of burning. This method is still widely used today particularly in recycling.

Double and triple bonds tend to have much stronger signals when using Raman spectroscopy – compared to IR spectroscopy. In some cases, these bonds are totally inactive in the IR. Other vibrational frequencies work very well for Raman such as S–S, C–S, and elemental carbons. The ability to detect sulfur species can be important when studying vulcanization processes. Raman systems tend to be lower in cost and size than their NIR and MIR counterparts and show to have a good potential for the plastic recycling applications.

Besides plastic identification, there are numerous Raman applications and a quick search will provide well over 1000 references using Raman spectroscopy for polymer and plastic characterization. Raman has also been used for measuring mechanical properties, e.g., determining the modulus of pure crystalline forms of a polymer. Raman is very sensitive to the longitudinal acoustic vibrational modes along polymer chains, and
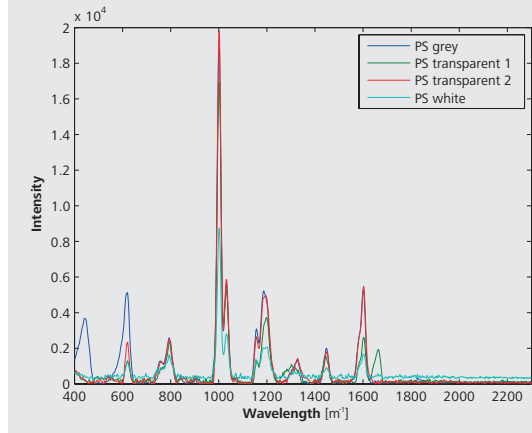
Figure 18 Overlay of polystyrene samples of various colors.

by tracking the coordinate with the experiment, the moduli of polymers like polyethylene and poly(oxymethylene) are determined. Raman band shift frequencies are also tracked when a polymer is under stress in order to gain insight into the occurences at the molecular level during stress conditions. Rotational isomers such as *cis* and *trans* conformations can give insight to the stable configuration of the polymer chain.

Geology

Raman spectroscopy is a popular method to characterize rocks, minerals, and soils. Several hundred research applications have been carried out using Raman spectroscopy in the earth and petroleum sciences in the past 20 years. Raman spectroscopy is used to characterize inclusions embedded in mineral matrices, identify daughter minerals and other organic components in rocks, and most recently it has been implemented in several planetary rover programs. The Raman spectra of different minerals tend to have very sharp peaks and several different inorganic substances such as SO_4^{2-}, CO_3^{2-}, PO_4^{3-} and silicates, iron oxides (hematite and goethite), among others.

Standards

It is more convenient to use standards instead of emission lines from low-pressure discharge lamps (for example mercury, argon, or neon) because these light sources are not easily portable, some of the lines are difficult to distinguish, and the lamps can be difficult to properly align in the sample position. The American Society for Testing and Materials (ASTM) International has produced a Standard Guide for Raman Shift Standards for Spectrometer Calibration (ASTM E1840-96). This guide includes a list of liquid and solid standards that can be used for wavenumber calibration of Raman spectrometers (x-axis shifts). The methodology for determining the ASTM guidelines involved multiple independent laboratories measuring the Raman shifts of eight different materials using different Raman instrumentation. The average shifts and standard deviations were derived from this collaborative effort.

The standards chosen were naphthalene, 1,4-Bis(2-methylstyryl)benzene, sulfur, 50/50 (v/v) toluene/acetonitrile, 4-acetamidophenol, benzonitrile, cyclohexane, and polystyrene. These materials were chosen because they contain no known polymorphisms and are easily obtained from Sigma-Aldrich or other chemical suppliers at high purity levels. Polystyrene is one of the most common standards supplied with a Raman spectrometer because it is readily available at a low cost,

it is easy to mold, and it is easy to ship or incorporate into the Raman spectrometer. ASTM also provides a method describing how to calculate the resolution of a Raman spectrometer with $CaCO_3$.

National Institute of Standards and Technology (NIST) does not provide a frequency shift standard for Raman. NIST does provide Raman intensity correction standards which are standard reference materials (SRMs) 2241 through 2243 for 785 nm, 532 nm, and 488/514.5 nm excitation. The SRMs consist of optical glass that emits a broadband luminescence spectrum when illuminated with the Raman excitation source. The shape of the luminescence spectrum is described with a polynomial that can be applied to the Raman spectrum.

United States Pharmacopeia (USP) <1120> provides an overview of the Raman technique as well as types of instruments, instrument components, and suggestions ranging from factors affecting quantitation to sampling factors. USP <1120> provides guidelines on how Raman instrument manufactures can achieve compliance, and a statement of compliance is traditionally prepared by the manufacturer. The European Pharmacopoeia (Ph. Eur.) provides a similar document (2.248).

Annex

Quantum mechanical expression of the polarizability

The interaction of light with a molecule is described through the electric dipole moment. When light is absorbed as in UV/VIS, MIR, or NIR spectroscopy it results in a single transition from the ground state to an excited state. This can happen with an electric field oriented in a combination of x, y, or z coordinates. Scattering on the other hand involves two fields, one which induces the dipole and the second which results in the scattered radiation. This means that it always contains two vectors: xx, yy, zz, xy, xz, and yz.

The quantum term for the interaction of light with a molecule is:

$$\mu = <\psi_f \,|\, e\hat{r} \,|\, \psi_i> \cdot E_o$$
$$p = <\psi_f \,|\, \bar{\alpha} \,|\, \psi_i> \cdot E_o$$

where μ is the dipole moment for an absorption and p is the induced dipole moment for a scattering event. ψ_i and ψ_f are the initial and final vibrational wavefunctions separated from electronic and rotational wavefunctions by the Born-Oppenheimer approximation. Note that the dipole operator, $e\hat{r}$ is an *odd* function ($x \neq -x$) and the polarizability operator, $\bar{\alpha}$, is an *even* function ($x^2 = (-x)^2$). The ground state of a molecule is always an *even* function as determined by the Hermite polynomials. In order to

be nonzero, the values inside the brackets must be an *even* function. This is clear from the integral representation of the Bra-Ket formulation:

$$\langle x \rangle = \int_{-\infty}^{\infty} x\,dx = \tfrac{1}{2}\left((-\infty)^2 - (\infty)^2\right) = 0$$

The result of an odd function is a value of 0!

With respect to chemical structures this means that in absorption spectroscopies the final state must be an *odd* function; *even:odd:odd = even*. For Raman scattering the final state must be an even function: *even:even:even = even*. This notation may seem strange but it has a dramatic spectroscopic effect. Molecules that are centrosymmetric, like a benzene ring or a carbon dioxide molecule, will have totally distinct Raman and MIR spectra; even though both techniques measure vibrations of the same molecule. As molecules become less symmetric the differences between the MIR and Raman spectra decreases.

Glossary

Anharmonicity: Refers to the vibration of atoms and describes the deviation from harmonic oscillation.

Beer's law: A= εcl, where A is the absorbance, ε is the molar attenuation coefficient, c is the molar concentration, and l is the pathlength.

Boltzmann's equation: A relationship between the energy of the system and the temperature.

Born-Oppenheimer approximation: The assumption that the motion of atomic nuclei and electrons in a molecule can be separated.

Charge-Coupled Device (CCD): Electronic light sensor used commonly in visible Raman spectrometers. The CCD stores charge created from electron hole pairs that is equivalent to photon intensity.

Chemometrics: The use of mathematical and statistical methods to extract chemical information by analyzing chemical data.

Czerny-Turner spectrograph: A spectrograph design that uses two off-axis mirrors to collimate onto a diffraction grating and to focus onto an aperture. It is characterized by its astigmatic focal image.

Dipole: The charge distribution created by oscillating positive and negative charges.

Etendue: A characteristic of dispersive spectroscopy between light collection and resolution. The more light collected, the lower the resolution.

Fellgett's advantage: Also known as the multiplex advantage. It is an advantage of FT techniques whereby all wavelengths are collected at once on the detector. It becomes a disadvantage when the detector is shot noise limited.

Fluorescence: The emission of light by a substance that has absorbed light or other electromagnetic radiation.

Hit Quality Index (HQI): A value used in library searching techniques that provides a rank of the closest library matches.

InGaAs (Indium gallium arsenide): A detector material for >1000 nm Raman detection.

IP67: Specifies the environmental protection of enclosures around electronic equipment. IP67 is used for enclosures designated to be totally protected against dust and protected against the effect of immersion between 15 cm and 1 m.

LASER: Light **A**mplification by **S**timulation of **E**mission **R**adiation. Lasers are used as the source in Raman spectrometers.

Mid-infrared spectroscopy (MIR): An absorption spectroscopy in the mid-infrared wavelengths of light, approximately 2.5-25 μm wavelength (400-4000 cm^{-1}), mainly used to study the fundamental vibrations and associated rotational-vibrational structure.

Near-infrared spectroscopy (NIR): Another form of absorption spectroscopy that uses energy in the near-IR, approximately 0.8–2.5 μm wavelength (12500–4000 cm^{-1}) used to study overtones or harmonic vibrations.

OARS (Off-Axis Raman Scattering): An off-axis collection method to eliminate scattering or fluorescence from windows and containers.

ORS (Orbital-Raster-Scan): A method using a rastering mechanism to spatial cover a large area of the sample.

PCA (Principal Component Analysis): a statistical procedure used to convert potentially correlated data in to linearly uncorrelated variables called principal components.

PLS (Partial Least Squares): A statistical method in the same family as principal component analysis where PLS finds a linear regression model by projecting the proposed variables and the discernible variables to a new space.

Polarizability: Polarizability is related to the ability of the electronic cloud surrounding the molecule to interact with an electric field.

Quantitation: The statistical regression techniques used to determine information about a component in a matrix.

Raman spectroscopy: Raman scattering occurs when incident photons in the near infrared, visible, or ultraviolet range, interact with the electron cloud of a molecule resulting in the inelastic scattering of photon at a different energy.

Rayleigh scattering: The elastic scattering of light during interaction of light with molecules.

Resonance Raman: Occurs when the wavelength of the incident light is at or near the frequency of an electronic absorption of a molecule, the efficiency of Raman scattering of these wavelengths can be increased by as much as 10^3.

Selectivity: The ability of a spectroscopic technique to correctly separate and identify a component or components in a matrix.

Sensitivity: The ability of a spectroscopic technique to detect low levels of analyte.

Stokes scattering: The inelastic scattering of Raman light where the material absorbs energy and the emitted photon has a lower energy than the incident photon.

Anti-Stokes scattering: The inelastic scattering of Raman light where the material loses energy and the emitted photon has a higher energy than the incident photon.

SERS (Surface-Enhanced Raman Scattering): An enhancement of Raman scattering from nanoparticles or nanostructures of silver and gold.

UV/VIS: An absorption spectroscopy in the ultraviolet or visible region of the electromagnetic spectrum, electronic transitions are observed in the region.

Volume Bragg Grating (VBG): A grating separates light in to individual components. The VBG is comprised of periodically alternating refractive index components so that a large reflectivity may be reached in some wavelength that meets the Bragg condition.

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Layout Ecknauer+Schoch ASW, printed in Switzerland by Metrohm AG, CH-9100 Herisau
8.108.5038EN – 2015-04