A Practical Beginner's Guide to Raman microscopy

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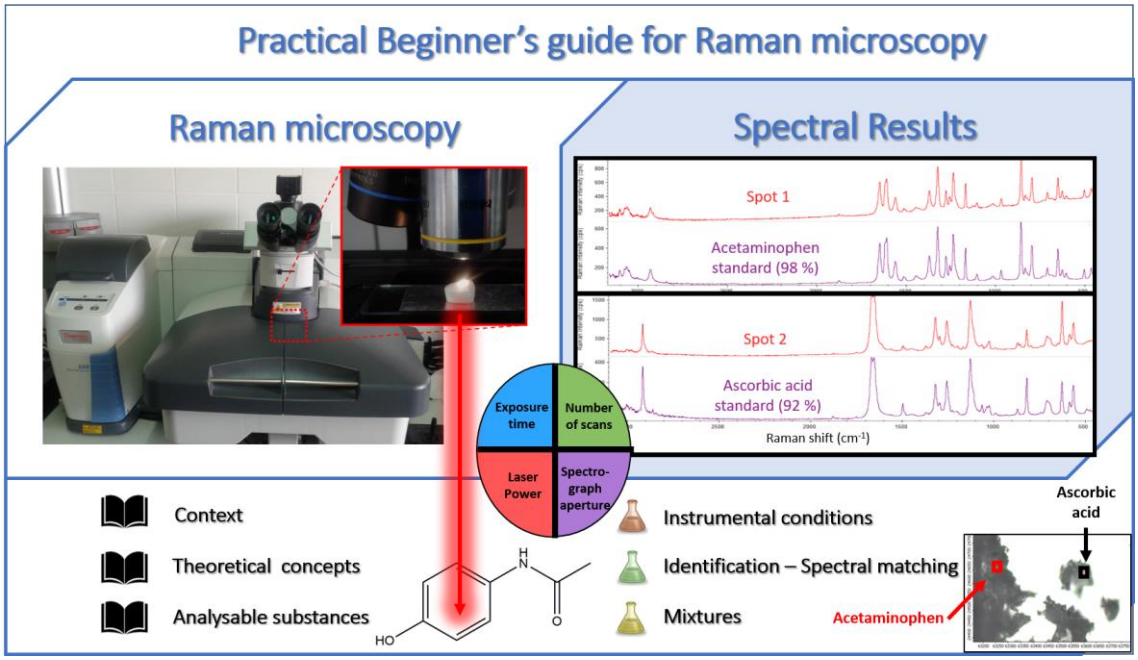


A Practical Beginner's Guide to Raman microscopy

Abstract. Raman microscopy is a highly suitable and well settled down analytical technique for qualitative determination of chemical substances. However, many university undergraduate chemical degrees do not incorporate its practical training in the experimental laboratory practises that constitute their curricula. For this reason, this work aimed at designing a practical beginner's guide to Raman microscopy useful for undergraduate students, teachers or practitioners who need to use it for the first time. After a brief explanation of the main concepts about Raman microscopy, the methodology development and results interpretation are mainly explained using paracetamol (acetaminophen) drugs as example. In addition, this guide presents an application to the identification of different components within a mixture, which shows the instrumental potential and how to use it effectively. Finally, acetaminophen, ascorbic acid, and sucrose were positively detected using Raman microscopy on a commercial drug whose major component was acetaminophen. In fact, the guide shows the detection and unequivocal identification of different components in the mixtures, even for those low concentration components (5-10 % mass ratio). This work clearly proposes different pragmatic criteria at the laboratory for identifying substances in mixtures to promote an easy implementation of the Raman microscopy technique.

Keywords: Raman microscopy; guide; chemistry; laboratory; acetaminophen; matching; identification.

Introduction to Raman spectroscopy

Historical context

Although the inelastic scattering of light was discovered in 1922 [1], it was not until 1928 when C.V. Raman understood and presented it as a new kind of radiation related to molecular vibrations [2,3]. During the following 100 years (see Figure 1), the instrumental development of components favoured the commercialization of different

Raman instruments: Raman benchtop spectrometers, Raman portable devices, Raman microscopy, and Raman imaging [4-6]. However, Raman instrumentation has not been implemented in analytical chemistry laboratories until the 21st century, because its initial low sensitivity and the often-present fluorescence from impurities/background reduced its applicability mainly to bulk highly-Raman-active substances [4-7]. In 2020, it is still very infrequent to acquire experimental experience in Raman microscopy during the pre-graduate university studies [6,7]. This happens despite the high potential of Raman microscopy for the determination of organic and inorganic substances in multiple fields [8-13] and despite its complementarity with infrared measurements.

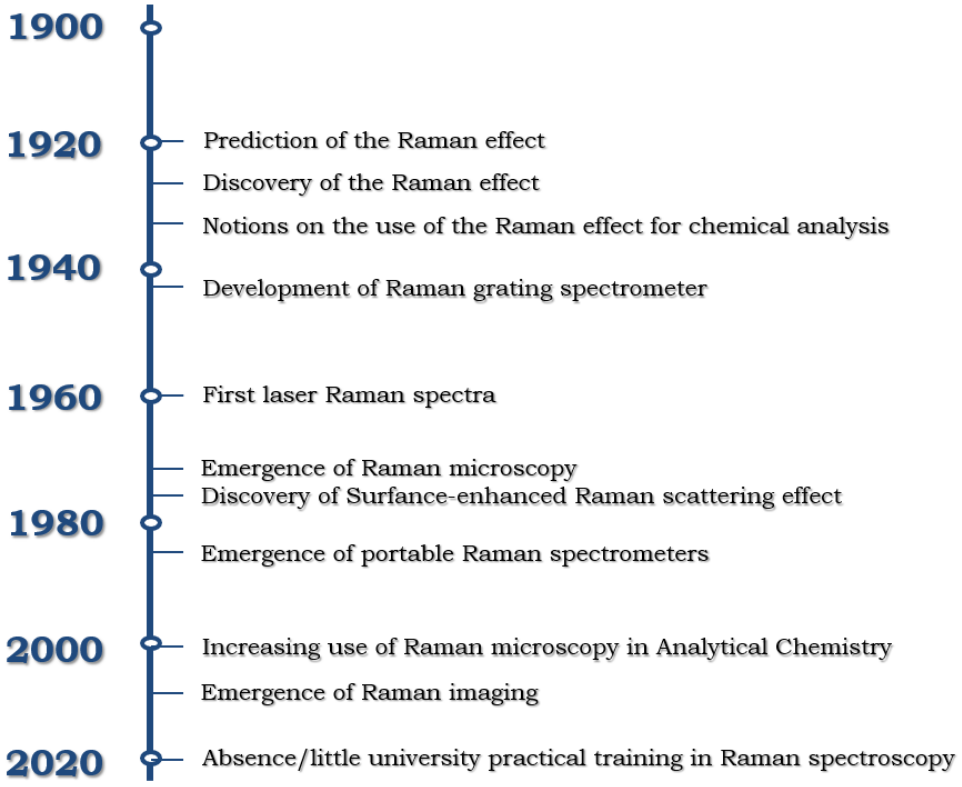


Figure 1. Schematic timeline about the Raman spectroscopy evolution (mainly based on the information extracted from the references [1-9]).

Fundamental theoretical concepts about Raman microscopy

Raman microscopy is the result of coupling Raman spectroscopy and microscopy, *i.e.*, a

Raman spectrometer in which the laser beam passes through the magnification lens of a microscope before hitting the sample.

Raman spectroscopy is based on the inelastic scattering of radiation. When substances are irradiated with electromagnetic radiation, besides the absorption phenomenon, electromagnetic scattering occurs in such a way that most of the radiation is elastically scattered without changing the wavelength of the radiation (Rayleigh scattering). However, a small part of the radiation is inelastically scattered with wavelengths that are different from those of the incident radiation (Raman scattering) [4-6]. In order to easily check the shift of the scattered wavelengths, a monochromatic and coherent radiation (laser) is used as incident radiation. To explain the Raman phenomenon, the easiest way is to consider light as quanta (remember the wave-light dual nature of light). Thus, when photons from the incident radiation hits the sample, an activated unstable "complex" is formed that "immediately" decomposes by sending out another photon. This is the scattering, that can be inelastic or elastic. The emitted photon is inelastically scattered (Rayleigh scattering) when it has not lost or gained any energy and maintain its frequency (in accordance to the Planck-Einstein equation $E = h*\nu$, where h is the Planck's constant and ν is the frequency of the radiation). The emitted photon is elastically scattered when part of its initial energy is transformed to vibratory energy. The red-shifted scattering, also called Stokes scattering, occurs when the energy is transferred from the photon to the sample, whereas the blue-shifted scattering, also called anti-Stokes scattering, occurs when the energy is transferred from the sample to the photon [4-6]. Whatever the Stokes/anti-Stokes scattering, the differences associate to the molecular vibrations of the sample, in such a way that the wavelength shift (also known as Raman shift) correlates to the infrared (IR) wavenumber of the vibration [4-7]. The same Raman information is obtained from the Stokes and anti-Stokes modes.

From the beginning, it was observed that the Raman spectra of certain molecules were different from their IR spectra. Particularly, symmetric diatomic molecules, which were non-IR-active, displayed intense Raman signals. In addition, vibrations associated to polar bonds, which were highly intense in IR spectroscopy, were less intense in Raman spectroscopy [4-6]. It was evident that different selection rules operated in IR and Raman. IR active vibrations are those that provoke a change in the molecular dipole moment whereas Raman active vibrations are those that provoke a change in the polarizability of the molecule [4-6]. Figure 2 simplifies the carbon dioxide stretching vibrations and whether they are IR or Raman active, which visually shows the difference between the IR and Raman selection rules. The symmetric stretching is not IR active (because it does not provoke any change in the molecular dipole moment), but it is Raman active (because it provokes a change in the polarizability of the molecule). On the contrary, the antisymmetric stretching is not Raman active (because it does not provoke any change in the polarizability of the molecule), but it is IR active (because it provokes a change in the molecular dipole moment). In this case, each CO_2 stretching vibration is exclusively IR or Raman active [14]. Actually, since different selection rules operate for IR and Raman, molecular vibrations might be IR-active, Raman-active or both IR-Raman active. In addition, even though certain vibrations are both IR-Raman active, they usually have different relative intensities in their IR and Raman spectra. Consequently, IR and Raman spectroscopy are complementary techniques. Since a detailed explanation about the selection rules and the precise correlation between the dipole moment/polarizability changes and the IR/Raman activity of the vibration is beyond the scope of this introductory practical guide, the interested readers are referred to advanced specialized literature on this topic [4-6,15].

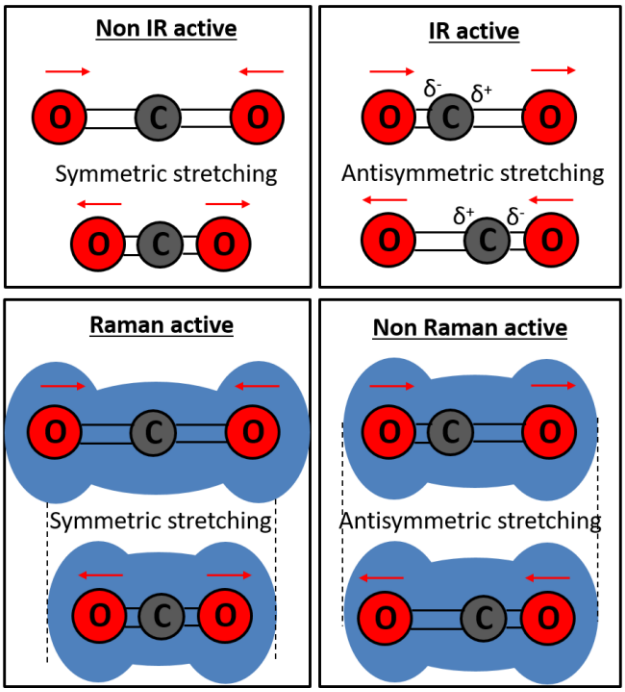


Figure 2. Simplified scheme of the carbon dioxide stretching vibrations and their IR/Raman activity, reproduced from [14] with permissions.

It should be noted that in IR spectroscopy, the incident radiation (infrared radiation) must have the same energy (wavelength) as the molecular vibrations to be absorbed. However, Raman scattering needs an incident radiation with higher energy (NIR/visible/UV radiation) than that of the molecular vibrations to be scattered. Thus, from the instrumental point of view, Raman spectroscopy usually works in the visible region, using visible monochromatic light sources. This fact is very important because it enables the easy combination of Raman spectroscopy and microscopy [4-6]. Hence, optical microscopes and microscopic lenses working in the visible region might be easily and efficiently coupled to Raman spectrometers. This way, microscopic solid traces can be selectively focused and detected using vibrational Raman spectroscopy [14]. Furthermore, whatever the incident radiation, the scattering is always the same for each molecule (*i.e.*, for each chemical bond), thus Raman can use lasers of different wavelengths.

Raman microscopy instrumentation

Figure 3 shows a scheme of a normal Raman spectrometer coupled to a microscope. It usually consists of a monochromatic light source (laser), several lenses and mirrors for controlling the beam direction, magnification objectives, binocular eyepieces, a digital camera, a sample stage, a filter, a grating as part of a spectrograph, and the detector (charge-coupled device (CCD)) [16-18].

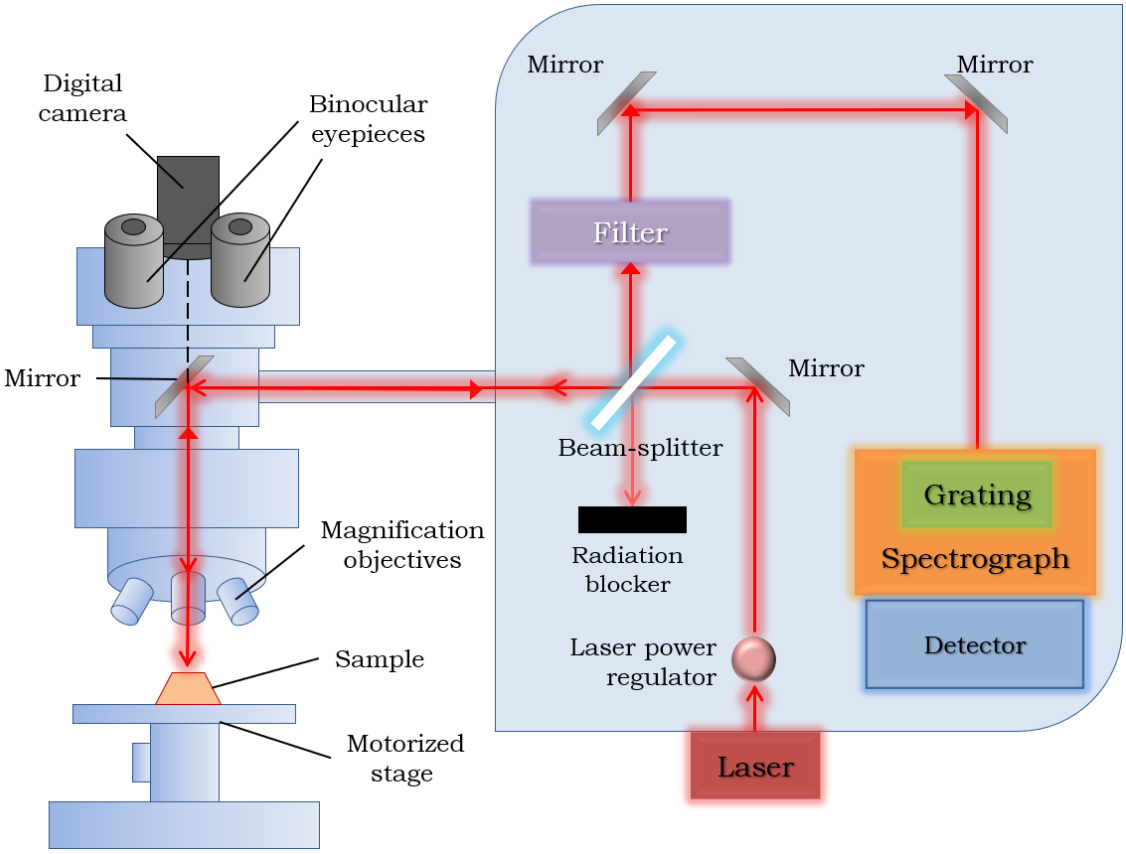


Figure 3. Schematic diagram of a Raman microscope (inspired on reference [16]).

Nowadays Raman systems normally use one or two monochromatic lasers of different wavelengths from ultra-violet (*e.g.*, 244, 364 nm) through visible (*e.g.*, 532, 785 nm) to near infrared (*e.g.*, 1064 nm). Choosing the laser wavelengths usually depends on several factors including [16-20]:

- Fluorescence: Fluorescence is the emission of light by a molecule that has absorbed electromagnetic radiation, as long as the emission occurs after a very short time since absorption (in the order of pico- to nanoseconds). The main problem of fluorescence is that it is much more intense than Raman scattering (around 7 orders of magnitude). Thus, the fluorescence, which usually comes from the background/matrix of the sample, masks weaker Raman signals. One way to reduce fluorescence is to use lasers of larger wavelengths. For more information about fluorescent suppression techniques, the reader is referred to the review by Wei et al. [19]. In practice, the short wavelengths (more energetic) increase the possibility of fluorescence.

- Sensitivity: Sensitivity is the ability of a method/technique to detect a particular analyte through its characteristic measured signal using an analytical instrument (in this case the intensity of its Raman characteristic bands). Thus, the shorter the laser wavelength, the more intense the Raman signal is.

- Spatial resolution: Spatial resolution refers to the size of the sample's surface (*i.e.* laser spot) that directly interacts with the laser and provides the Raman signal. In brief, the diameter of the focused laser spot in Raman microscopy can be estimated as $1.22\lambda/NA$ (where λ is the laser wavelength and NA is the numerical aperture of the microscope objective). The shorter the laser wavelength, the smaller the focused laser spot is.

- Application: Some applications (*e.g.*, SERS) require specific wavelengths to exploit the resonance phenomena. For example, the laser wavelength for obtaining the maximum SERS effect with certain nanoparticles depends on the nanoparticles plasmon, which varies with their composition (*e.g.*, Au/Ag), shape (*e.g.*, spherical/rod), and size.

A laser power regulator usually controls the laser power. The laser beam is reflected whenever necessary with the use of mirrors and lenses in order to guide the beam direction. As exemplified in Figure 3, first, the laser beam strikes the beam-splitter

in such a way that part of the light goes directly through it without changing direction. At the same time, the rest of the beam, which has changed its direction, is blocked and lost. On the contrary, the survivor beam is reflected towards the optical microscope and the microscope objective. This way, the potential of confocal microscopy adds up to that of the Raman spectroscopy. The microscopic sample can be observed (through the binocular eyepieces) and even photographed (using the digital camera) under high magnification. Different interchangeable magnification objectives are usually available including 4X, 10X, 20X, 40X, 50X, and 100X. In addition, the laser beam is focused onto a small spot of about 1-100 μm diameter. Thus, confocal Raman microscopy enables to spatially select and microscopically focus onto a certain sample spot to be analysed. Most Raman microscopes also include a motorized stage that facilitates the X-Y surface scanning (2D mapping). In addition, it is possible to do the X-Z cross-analysis (depth profiling or 3D mapping) [16]. Although the Raman light scattering occurs in all directions, the light scattered by the sample in Raman microscopes is commonly collected at *ca.* 180º. Afterwards, the beam of scattered light returns through the microscope optics and the beam-splitter before reaching the filter.

Every laser set comes with a filter and grating parts that match in wavelength. The filter blocks the undesired light (*i.e.,* laser wavelength, Rayleigh scattering, external light coming from the microscope, etc.) from reaching the spectrometer, which would hinder the detection of the relatively weak Raman signal [16-19]. Edge or notch filters are usually used to block the undesired radiation. In one hand, edge filters block all wavelengths up to a certain value. On the other hand, notch filters have a straight blocking band-range (a few nanometres wide) and transmit the wavelengths above and below that range, which seeks to block the laser wavelength. Therefore, edge filters are used only for Stokes detection, while notch filters can be used for simultaneous Stokes and Anti-

Stokes detection. Most Raman microscopes include edge filters for Raman Stokes measurements.

After being filtered, the Raman shifted radiation reaches the grating inside the spectrograph. Gratings have many parallel lines in their surface, which split the radiation into its different wavelengths. The higher the number of grating lines per unit length, the higher the spectral resolution [16]. The spectrograph sends the diffracted photons of each wavelength to a very high sensitivity CCD camera detector, whose surface is a two-dimensional array of millions of light-sensitive elements (pixels). Every photon arriving to the sensor triggers a series of proportional electric signals that accumulate and end up registered in a computer according to their corresponding wavelength. Consequently, the resulting Raman spectrum is a graph displaying the signal intensity detected for every analysed wavelength. Nonetheless, in Raman spectroscopy the wavelength axis is usually converted to Raman shift (in cm^{-1} units), in such a way that the Raman spectrum of a substance is independent of the laser wavelength used. Moreover, the Raman spectrum is also comparable to its complementary Infrared spectrum, which is usually presented in wavenumbers (cm^{-1}). The CCD detectors must be cooled (Peltier or nitrogen cooling) in order to reduce their intrinsic background noise to acceptable values [16]. The spectrograph might have different adjustable aperture (slit/pinhole) sizes in order to better control the amount of incoming radiation.

Most of the instrumental parameters (*i.e.*, laser power, Raman shift range, slit/pinhole aperture, etc.), the data acquisition and processing are controlled and set with the computer as discussed below within this guide.

Analysable compounds using Raman microscopy

Regarding the physical state of the sample, either solid, liquid or gaseous substances can be analysed by Raman spectroscopy. Nonetheless, this technique is not so suitable for

handling, focusing and analysing gaseous substances because it is difficult, and it requires specific sample containers. Hence, most commercial Raman microscopes are not prepared for the analysis of gaseous substances. Both solid and liquid samples are usually placed onto a microscope slide that is then put on the microscope stage, which is aligned with the corresponding microscope objective. The sample size to be placed onto the microscope slide consists of a tip of spatula (for solid samples) or a drop (for liquid samples). If the sample is a liquid substance, the drop is directly focused and analysed before it evaporates. If the sample comes in a liquid solution, in which the aim is to detect the analyte (instead of the solvent), the analysis might be delayed until the solvent fully evaporates. This would allow focusing better on the dried analyte, thus minimizing the Raman signals from the solvent.

Regarding the chemical composition, as previously explained, Raman scattering occurs as a consequence of the changes in the polarizability of molecules, which are mainly due to their molecular vibrations. In brief, the maximum number of molecular vibrations of a certain molecule containing N atoms is theoretically calculated as 3N-6 (for non-linear molecules) or 3N-5 (for linear molecules) [14]. This results from the combination of the three degrees of freedom that are available for every atom (3N) and the removing of those translational/rotational non-detectable modes (-6 or -5, non-linear and linear molecules, respectively).

Thus, like IR spectroscopy, Raman microscopy is very useful to identify molecular compounds, including organic and inorganic molecules [21].

Organic molecules, typically containing several and different chemical bonds (C-H, N-H, O-H, C-C, C=C, C≡C, C-O, C=O, C-N, C=N, C≡N, N-O, N=O, O-O, N-N, N=N, and C-X (X=halogen)), display characteristic Raman spectra with multiple bands. These bands are due to the large number of Raman-active fundamental molecular vibrations

associated to these bonds in these big molecules (composed of a large number of atoms). Like in IR, spectroscopic handbooks are used for Raman bands interpretation [22-25]. Most active pharmaceutical ingredients (APIs) and excipients from drugs are organic molecules, such as acetaminophen or sucrose, in whose study this work will focus later.

Inorganic molecules such as oxoacids and oxyanions (*i.e.*, carbonates, nitrates, phosphates, sulphates, silicates, chlorates, perchlorates, etc.) are normally composed of a few number of atoms, thus, they usually display characteristic Raman spectra with a small number of bands. Likewise, spectroscopic handbooks are used for inorganic Raman bands interpretation [26,27]. It should be noticed that even potassium nitrate and sodium nitrate can be selectively identified despite that their Raman bands are exclusively due to nitrate molecular vibrations. This occurs because the nitrate bands are not located within the same Raman shift for each salt (1069 vs 1051 cm^{-1}) [28]. It must be mentioned that some excipients and APIs are inorganic salts such as lithium carbonate (Li_2CO_3), calcium carbonate ($CaCO_3$) or calcium hydrogen phosphate ($CaHPO_4$).

Consequently, can Raman microscopy positively detect all solid and liquid molecular substances? No, it cannot. Unfortunately, a challenging disadvantage of Raman microscopy is known to be the fluorescence caused by some substances/backgrounds that totally overlaps the weak Raman signal [19,29]. In those cases, a thorough optimization of the Raman parameters (*e.g.*, wavelength, laser power, exposure time, optical aperture, number of scans, and sample focusing) needs to be performed for minimizing the fluorescence (although the success is not always guaranteed). In addition, the microscope glass slides are usually covered by aluminium adhesive tape when using Raman microscopy in order to avoid the fluorescence provided by some glass types. This way the sample is placed over the aluminium foil instead of the bare glass.

Objectives

This work aimed at designing a practical beginner's guide to Raman microscopy useful for undergraduate students, teachers or practitioners who need to use it for the first time. After a brief explanation of the main concepts about Raman microscopy, the methodology development and results interpretation are mainly explained through experimental examples based on various paracetamol (acetaminophen) drugs. However, they can be applied to any other chemical substance.

Experimentation using commercially available acetaminophen drugs have been accomplished because the Raman analysis of drugs is a key area of interest in several disciplines including chemistry, pharmacy, toxicology, and forensics. Besides, paracetamol (acetaminophen) drugs can be easily acquired by any teacher, researcher or practitioner in order to replicate these experiments, for preparing laboratory activities, etc. It is worth mentioning that in our laboratory we use paracetamol as reference for checking that the Raman instrument is working properly along the time.

To sum up, the main goal of this guide is to introduce Raman microscopy to beginner students, teachers, researchers or practitioners. At the same time, it will provide fundamental knowledge and know-how about Raman instrumental settings, methodology optimization, sample analysis, spectra interpretation, and its potential applications. The person using and putting this guide in practice would obtain the following specific main competences, being able:

- to identify the different components of a Raman microscope.
- to prepare and tune up a Raman instrument.
- to optimize the methodology by judiciously setting the instrumental parameters.
- to place, focus and measure solid and liquid samples.
- to interpret the Raman spectra of pure substances and mixtures.
- to make a decision on criteria for identifying substances based on their spectral-chemical features and the matching built-in function against spectral libraries.
- to detect different components within mixtures.

Experimentation with a Raman spectrometer

Materials

For the methodology optimization, and sample analysis, the following compounds were studied: Ascorbic acid (CAS 50-81-7), caffeine (CAS 58-08-2) and acetaminophen (CAS 103-90-2) standards (>99 %) (Sigma Aldrich Merck, St. Louis, Missouri, USA), and eight paracetamol-containing (*i.e.*, acetaminophen) pharmaceuticals commercially available in Spain. All pharmaceuticals had paracetamol as main active substance. All formulations including the GPE (Generic Pharmaceutical Equivalent) tablets and the oral solution were visually and microscopically homogeneous, except for the oral solution powder (composed of three main active substances), which was heterogeneous even to the naked eye. Table 1 summarizes the name, brand, pharmaceutical form, dose, and main composition of each sample.

Table 1. Name, brand, pharmaceutical form, dose, and main composition of the commercial acetaminophen-containing pharmaceuticals analysed in this experiment.

Name	Brand	Pharmaceutical form	Dose	Main composition*
Paracetamol	*Sandoz*	GPE tablets	1 g	**Acetaminophen (76 %)** Excipients (24 %)
Paracetamol	*Apotex*	GPE tablets	1 g	**Acetaminophen (76 %)** Excipients (24 %)
Dolostop	*Kern Pharma*	GPE tablets	1 g	**Acetaminophen (90 %)** Excipients (10 %)
Paracetamol	*Kern Pharma*	GPE tablets	650 mg	**Acetaminophen (91 %)** Excipients (9 %)
Paracetamol	*Teva*	GPE tablets	650 mg	**Acetaminophen (77 %)** Excipients (23 %)
Paracetamol	*Normon*	GPE tablets	500 mg	**Acetaminophen (80 %)** Excipients (20 %)
Apiretal	*ERN S.A.*	Oral solution	100 mg/mL	**Acetaminophen (10 %)** Water + excipients (90 %)
Algidol	*Almirall S.A.*	Powder for oral solution	650 mg	**Acetaminophen (30 %)** **Ascorbic acid (23 %)** **Codeine (0.5 %)** Excipients (46.5 %)

* Experimentally calculated by weighting the GPE tablet in an analytical balance (two replicates), and assuming the dose (indicated by the manufacturer) as the real amount of acetaminophen. The main composition was calculated as follows: % Acetaminophen = (dose/weighed mass)*100; % Excipients = 100 - % acetaminophen - % other APIs.

It is important to study the capability of Raman microscopy to identify minor compounds in mixtures, and particularly, the determination of the minimum ratio at which a given compound is still detectable in a mixture. Hence, eleven different mixtures (two bicomponent-mixtures at 5 different ratios each, and one tricomponent-mixture, in %, m/m) were prepared by manually mixing in a mortar the following substances:

- Mixture A: Acetaminophen + Ascorbic acid. Five mixtures were prepared including 95/5, 90/10, 50/50, 10/90, and 5/95.
- Mixture B: Acetaminophen + Caffeine. Five mixtures were prepared including 95/5, 90/10, 50/50, 10/90, and 5/95.
- Mixture C: Acetaminophen + Ascorbic acid + Caffeine. One 50/40/10 mixture was prepared.

All percentage ratios (mass percentage) were prepared by weighing in an analytical balance the exact amount of each compound to obtain the desired ratio. The total mass of each prepared mixture was around 100 mg.

Instrumentation

All masses were measured using an Ohaus (DV215CD) analytical balance (Parsippany, NJ, USA) with a precision of five decimal places (0.00001 g).

A Thermo Scientific DXR Raman microscope operated with the Omnic 8 for Dispersive Raman software (Thermo Scientific, Waltham, MA, USA) was used for the Raman microscopy analysis.

Procedure

A tiny amount (the spatula tip) of ascorbic acid, caffeine and acetaminophen standards (>99 %) were placed on microscope glass slides (previously covered by aluminium adhesive tape). Then, they were analysed by Raman microscopy (10X magnification) using the following instrumental conditions: spectral range (3200-200 cm^{-1}), laser

wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16). The resulting spectra were included into an *ad-hoc* homemade spectral library within the Omnic 8 for Dispersive Raman software. Then, similar amounts of the standard acetaminophen were again analysed by Raman microscopy using different instrumental conditions. This was done as a practical training to optimize the measurement procedure: exposure time (0.1, 0.5, 1, and 2 s); spectrograph aperture (25 µm-pinhole, 50 µm-pinhole, 25 µm-slit, and 50 µm-slit); laser power (1, 5, 10, and 14 mW); and number of scans (2, 4, 8, 16, and 32).

To obtain the Pearson coefficients, the acetaminophen spectra collected using the above-mentioned conditions were compared to the standard acetaminophen spectrum previously included in the library. The Pearson correlation option is a chemometric tool included by default in most Raman spectroscopic software that enables a matching analysis. It provides an objective statistical value for how similar/different two spectra are.

Regarding the commercial drugs, GPE tablets were directly placed onto a microscope slide previously covered with aluminium adhesive tape. The surface of the tablet was focused using a 10X magnification objective and the Raman spectrum was collected. The measurement was repeated in 10 randomly selected spots along the surface of each tablet. Those measurements were performed using the following conditions: spectral range (3200-200 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16). Likewise, two GPE tablets of each brand-dose were analysed, that is, 20 Raman spectra were collected for each sample. In the case of the liquid oral solution formula, a drop was placed onto the aluminium-covered microscope slide. The drop's interface (inside/surface) was focused using the 10X magnification, and up to 10 Raman spectra

were randomly collected using the same instrumental conditions. Exceptionally, in the case of the visually heterogeneous sample composed of three main active substances (*i.e.*, the oral solution powder), the criterion for the spots selection was not totally random. Instead, the spots were chosen to ensure the selection of at least one of each visually different particle. The Raman spectra of the different samples were compared against the acetaminophen standard spectrum previously included in the library. In each case, the analysis calculated the corresponding Pearson coefficient after matching each sample.

Regarding the homemade mixtures, three replicate spectra were collected for each mixture-ratio. The replicates were separately kept and displayed together with the image of the spot at which each Raman spectrum was collected. Whenever possible, visually different particles were focused and analysed with the Raman microscope.

Experimental guide for Raman microscopy

Preparation of the Raman instrument

Figure 4 provides a useful schematic workflow for the novel Raman experimentalists as an easy guide in preparing their Raman instrument.

In most Raman spectrometers, the laser is not turned on by default after turning on the Raman instrument, the computer and/or the spectroscopy software. In order to save the laser lifetime, normally, the laser has to be purposely turned on when needed (Figure 4). This is normally done from the software. After turning on the laser, it has to warm-up. In most Raman spectrometers, this process takes by default 5 min, during which no analysis can be performed because the Raman collection procedure is blocked.

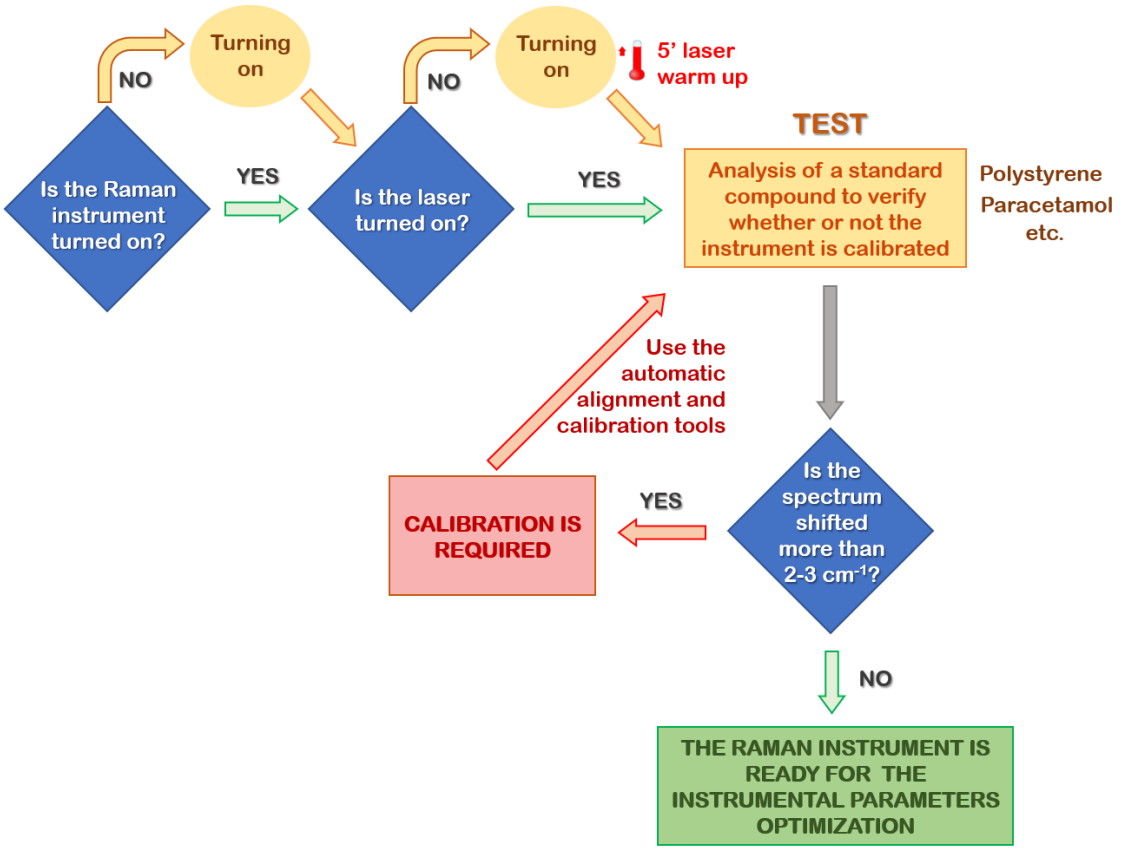


Figure 4. Preparation of the Raman instrument workflow.

The Raman instrument must be calibrated in order to work properly (Figure 4). Hence, after the warming time and before analysing any sample, it is mandatory to verify that the calibration of the Raman spectrometer is correct. The time during which the calibration remains correct mostly depends on the greater/lesser number of interchangeable components in the Raman spectrometer. Some spectrometers do not have interchangeable components, thus, they are robust equipment in which the user-calibration is not allowed, because they are designed for not to lose their calibration. On the contrary, there are Raman spectrometers (as the one used in this guide) in which the laser, filter and grating form a set working at one specific wavelength. The entire one-wavelength set (laser, filter and grating) must be changed whenever another one-wavelength set is changed. In any case, the system will trigger a warning sign if there is a non-matching component piece in the laser line. These spectrometers are not so robust, but much more versatile and flexible, although frequent user-calibration is recommended.

Whatever the type of Raman spectrometer, a standard must be analysed to check whether the instrument is correctly calibrated (Figure 4). This standard can be any Raman-active substance with well-known Raman spectrum (*i.e.*, that has been previously registered in the Raman instrument). Multiple automatic tests use polystyrene to verify the correct performance of Raman instruments. Nevertheless, in the absence of polystyrene, something as common as a paracetamol (acetaminophen) tablet can be manually analysed to verify whether or not the Raman instrument works properly. Hence, if the tested Raman bands of polystyrene/acetaminophen are located at the same Raman shift values as those described in literature, or previously registered in the spectral library, the Raman spectrometer is correctly calibrated. On the contrary, if those Raman bands are shifted more than 2-3 cm^{-1} with respect to the previously registered values, a calibration is required (Figure 4). If the user-calibration is allowed, the Raman spectrometer and the software will have specific automatic alignment and calibration tools that will guide the user in that process.

Besides being properly calibrated, the Raman spectrometer must have registered the spectral background before performing any Raman analysis. This is required to automatically subtract the Raman signals of the instrumental/atmospheric background from the samples Raman spectra. In IR spectroscopy, the background needs to be frequently updated because the atmospheric carbon dioxide and water vapour concentrations might change during the analysis giving high intense overlapping on the IR signals. Conversely, in Raman spectroscopy the background is constant because the Raman active vibrations from the atmosphere molecules are far less intense. Thus, the same Raman background is usually used for hours or days. Nonetheless, in some Raman spectrometers, the background is automatically collected after a stipulated time without any user intervention.

Optimization of the instrumental conditions: developing a methodology

Once the Raman instrument working condition and the background are verified and registered, respectively, the optimum methodology for the analysis of the studied samples needs to be developed. As mentioned previously, different instrumental conditions can be optimized in Raman microscopy including the exposure time, number of scans, laser power, and spectrograph aperture. Figure 5 summarizes the influence of those instrumental parameters on the Raman spectrum.

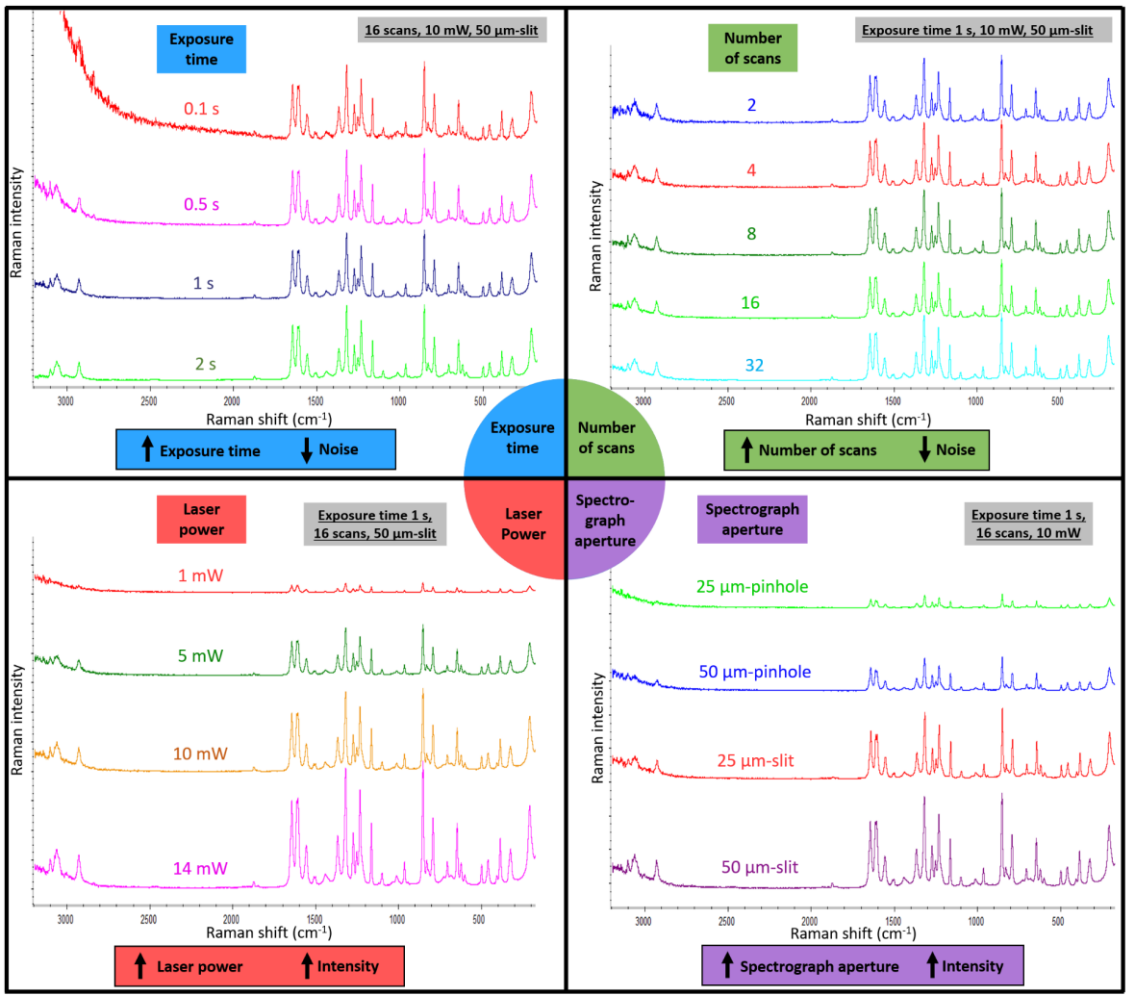


Figure 5. Raman spectra of the acetaminophen standard (>99 %) collected at different values of either exposure time (blue), number of scans (green), laser power (red), or spectrograph aperture (purple), always keeping constant the other three parameters when testing each factor.

Exposure time. Figure 5 shows the exposure times from 0.1 s to 2 s. Within this range, the higher the exposure time, the lower the noise, and thus, the higher the signal-to-noise ratio. Using this Raman spectrometer, no improvement was observed by increasing the exposure time beyond 2 s for acetaminophen. In this respect, it should be further explained that the optimum exposure time strongly depends on the substance being analysed (due to its Raman activity), and the sensitivity/speed of the CCD detector. Some substances like cellulose require long exposure times (up to 15-20 s) using this Raman spectrometer, while the optimum exposure time to detect acetaminophen using Raman spectrometers with ultimate high-speed CCD detectors might range from 100 to 300 μs. In any case, the use of longer exposure times without observing a significant improvement in the signal-to-noise ratio of the Raman spectrum is not recommended because the substance might suffer laser burning, and the laser lifetime and analysis time would be wantonly wasted.

Number of scans. Figure 5 shows the Raman spectra of acetaminophen after collecting from 2 to 32 scans. The higher the number of scans, the lower the noise, and thus, the higher the signal-to-noise ratio. A balance between number of scans and analysis time is normally established. It should be noted that in Raman two scans, at least, must be collected because of the possibility of being affected by a cosmic ray. Cosmic rays are high-energy subatomic particles coming from the sun that pass-through materials, and sometimes hit the CCD detector. This effect produces a straight vertical line in the Raman spectrum randomly located at the wavelength corresponding to the beaten CCD-pixel. By collecting a second scan and calculating the average, the signal due to the cosmic ray is eliminated because the probability of the second scan being affected by a cosmic ray in the same CCD position is infinitely small.

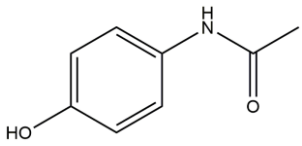
Laser power. The procedure in this guide tested the laser power from 1 mW to 14 mW (which is the maximum laser power for this Raman spectrometer) (Figure 5). The higher the laser power, the higher the Raman signal. However, the use of the higher or the maximum laser power is not recommendable because it considerably reduces the laser lifetime, and it may burn or trigger the fluorescence of some substances. Some Raman spectrometers do not indicate the absolute value of the laser power, but instead, they give a relative percentage from 1 to 100 % of the maximum laser power.

Spectrograph aperture. This procedure also tested the following spectrograph apertures: 25 µm-pinhole, 50 µm-pinhole, 25 µm-slit, and 50 µm-slit (Figure 5). The larger the aperture, the higher the Raman signal. The use of the largest spectrograph aperture is recommended as long as no fluorescence is detected. Some Raman spectrometers do not allow modifying the spectrograph aperture.

Spectral assignment of bands to fundamental molecular vibrations according to the literature

As expected for the acetaminophen standard analysis, all the Raman spectra were clearly and positively identified as acetaminophen because of the presence of all the characteristic bands of acetaminophen (Figure 5), previously reported in literature [30]. An exhaustive and formative discussion about the vibrational interpretation of the acetaminophen Raman bands is out of the aim of this guide. The reader is encouraged to revise the bibliography [30] to deepen its understanding of the acetaminophen bands interpretation (Table 2).

Table 2. Main experimentally observed Raman bands for acetaminophen and their assignment to fundamental molecular vibrations according to literature [30].

Substance	Chemical Formula	Raman shift (cm^{-1})	Molecular vibration [30]
Acetaminophen 	$C_8H_9NO_2$	3106	Overtone amide II
		3067	Aromatic C-H stretching
		2933	CH_3 stretching
		1649	C=O stretching
		1610	Aromatic C=C stretching
		1561	β N-H bending
		1371	CH_3 symmetric bending
		1323	Aromatic C=C stretching
		1278	C-O stretching
		1256	Aromatic β C-H bending
		1236	C-N stretching
		1168	Aromatic β C-H bending
		1104	Aromatic β C-H bending
		1016	ρ CH_3 bending
		968	Aromatic γ C-H bending
		857	Aromatic C=C stretching (ring breathing)
		834	Aromatic γ C-H bending
		797	α CCC
		710	δ CCC
		651	α CCC
		626	N-C stretching + C-C stretching
		503	β C=O
		464	β C-O
		391	β C-N + β C-O
		329	β C-N
		213	β C-N + β C-O

Substances analysis and identification by matching them against the spectral Raman libraries

In order to identify an unknown substance using Raman microscopy, the Raman spectrum of a sample is usually and automatically compared against spectral databases containing the Raman spectra of numerous standard substances. Some spectral libraries are usually included by default in the software of the spectrometer. Other spectral libraries can be purchased from spectroscopy-instrumentation companies. Finally, the experimentalists

can create homemade spectral libraries in their own spectrometers. Such libraries may increase over time by characterizing and registering new standard substances, especially those that are relevant for their specific interest, such as drugs, inks, fibres and explosives in a forensic laboratory, among others. The sample-spectrum vs. spectral database comparison performed by the software normally returns a list of potential candidate substances together with their corresponding numerical matching (given as percentage). In most spectroscopy software, this numerical matching (ranging from 0 % to 100 %) is usually calculated using the Pearson correlation coefficient, which is a chemometric tool that measures the correlation (*i.e.*, similarity) of two variables. The more similar the spectra, the higher the matching. For instance, the matching value between two spectra that are completely identical will be 100 %.

The software usually lists the matching substances exclusively according to their numerical value. However, the numerical value should not be the unique identification criterion because two potential candidates having the same matching value might have significant spectral differences. Thus, the establishment of a threshold only based on the matching percentage is not recommendable. The user must be able to make a decision on the identification criteria not only based on the numerical matching against the spectral libraries. Matching values over 90 % usually involve a positive identification. However, matching values from 40 to 90 % may sometimes involve a positive or a negative identification. It must be clear that even if a high threshold is established (>90 %), numerous samples may be erroneously detected as false negatives. On the contrary, if a low threshold is established (>40 %), numerous samples may be erroneously detected as false positives. Thus, the visual comparison of the spectra by the analyst is mandatory after obtaining the automatically selected list of potential candidates. The presence or absence of characteristic spectral bands strongly supports a positive or negative

identification, much more than a numerical matching exclusively based on the Pearson correlation coefficient. This is because the correlation of two raw spectra might be strongly affected by the instrumental conditions as experimentally tested for the Raman spectra of acetaminophen standard (Table 3).

Table 3. Raman instrumental conditions *vs* Pearson correlation coefficient matching for the acetaminophen standard (>99 %).

Exposure time (s)	Number of scans	Laser power (mW)	Spectrograph aperture (µm)	Pearson Correlation Coefficient
1	16	10	50 (slit)	100 (spectrum included in the spectral library)
0.1	16	10	50 (slit)	68.9
0.5				97.9
1				98.8
2				99.2
1	**2**	10	50 (slit)	96.0
	4			97.7
	8			98.6
	16			98.8
	32			99.3
1	16	**1**	50 (slit)	75.1
		5		98.1
		10		98.8
		14		99.1
1	16	10	**25 (pinhole)**	79.0
			50 (pinhole)	93.0
			25 (slit)	94.3
			50 (slit)	98.8

As Table 3 evidences, the influence of the instrumental parameters on the Pearson correlation coefficient (*i.e.*, matching) varies with the instrumental parameters. For instance, the number of scans does not influence the Pearson coefficient as much as the laser power, the spectrograph aperture or the exposure time. Nonetheless, all instrumental parameters have affected somehow the Pearson coefficient. In any case, the Pearson coefficient matching strongly depends on whether or not the instrumental conditions are the same for both the newly analysed sample and the standard's Raman spectrum in the library. The more similar the instrumental conditions, the higher the matching. Because of this, it is always a good practice to create homemade spectral libraries using the own

Raman instrument and its corresponding well-known Raman conditions. Matching is useful to easily and automatically list and find (within the spectral library) the potential more similar candidates aiming at further comparing the presence/absence of all the characteristic bands of the alleged standard in the inspected sample's spectrum. This is crucial for samples being a mixture of different substances, in which their Raman spectra display the signals of more than one substance, strongly reducing the matching against any of the individual components. This is a notable overcome when using Raman microscopy, as evidenced in the analysis of commercial paracetamol formulations and mixtures (discussed below).

Figure 6 shows the Raman spectra of the acetaminophen standard (>99 %) and the here tested paracetamol commercial medicines, all analysed under the same instrumental conditions and displayed using the same scale. According to these results, the samples' Raman spectra mostly composed of acetaminophen (>75 %) matched almost exactly the Raman spectrum of acetaminophen standard. Their Pearson correlation coefficient exceeded 99.5 %. However, *Algidol* (a powder composed of 30 % acetaminophen) and *Apiretal* (an aqueous solution composed of 10 % acetaminophen) gave lower Pearson correlation coefficient values when compared to the acetaminophen standard. Nevertheless, despite being only composed of a 30 % of acetaminophen, *Algidol* clearly displayed the characteristic acetaminophen bands. On the contrary, no characteristic acetaminophen bands were detected in the Raman spectrum of the *Apiretal*'s aqueous solution. Its Pearson correlation coefficient matching against the acetaminophen standard was very poor (44 %).

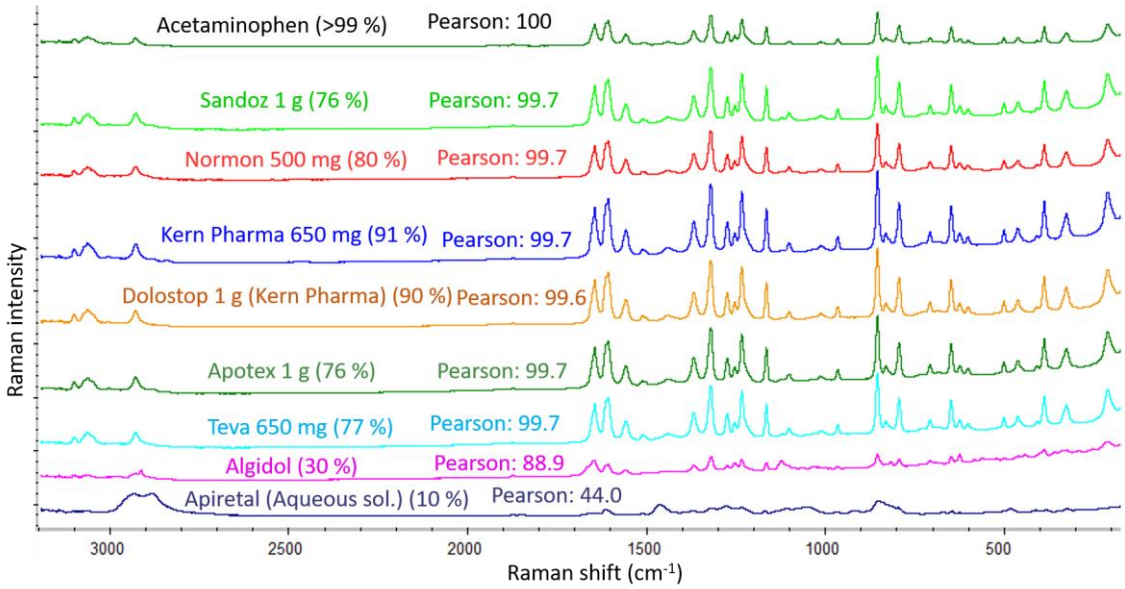


Figure 6. Raman spectra of the acetaminophen standard (>99 %) and the paracetamol commercial medicines. Each sample spectrum is the average of 20 spectra collected along the surface of two GPE tablets. The Raman conditions were as follows: Spectral range (3200-200 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16) for each of the 20 spectra.

The previous spectra are the average spectra of the 20 replicates collected from each drug. However, when using Raman microscopy different particles can be separately focused and analysed. In the case of the homogeneous drugs, this possibility became useless since the 20 spectra collected from different spots along the two tablets were already almost identical and matched the acetaminophen standard spectrum. On the contrary, up to four visually different spectra were collected from different spots when analysing the heterogeneous *Algidol* powder (Figure 7).

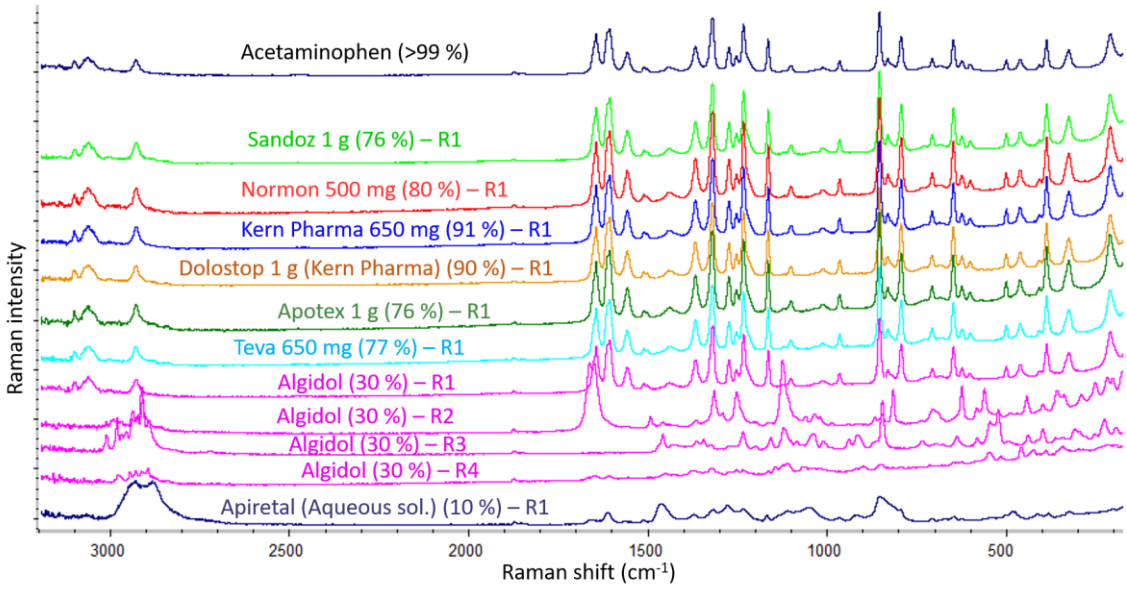


Figure 7. Raman spectra of the acetaminophen standard (>99 %) and the paracetamol commercial medicines. Each sample spectrum represents one replicate. Four different replicates are displayed for *Algidol* (composed of visually different heterogeneous particles). The Raman conditions were as follows: Spectral range (3200-200 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16).

The spectrum detected in most of the *Algidol* spots belonged to acetaminophen (R1, in Figure 8), its major active substance (30 %). This was followed by ascorbic acid (R2, as given in Figure 8), its second major active substance (23 %). The third spectrum detected in *Algidol* (R3, Figure 8) belonged to sucrose, an excipient commonly used in drug manufacturing. The fourth spectrum detected in only one of the *Algidol* spots (R4, Figure 8) was not identified, probably because of the signals' combination from several different compounds and the low intensity of the spectrum. Ascorbic acid and sucrose were identified using the spectral library automatic search (Pearson correlation coefficient matching) and later comparing the presence of all their characteristic bands (Figure 8), which were in accordance with published literature [31,32].

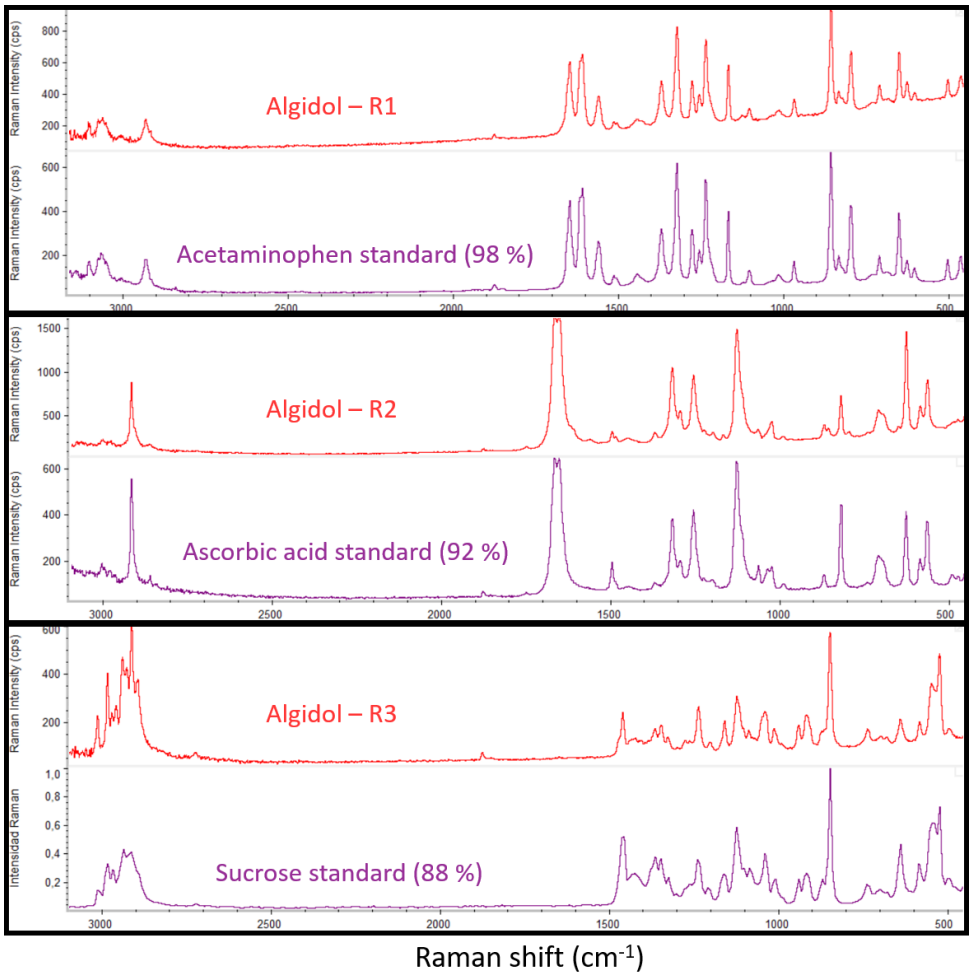


Figure 8. Three Raman spectra analysed along different spots on the *Algidol* sample and their library comparison (matching) with their corresponding standards: acetaminophen, ascorbic acid, and sucrose. Each *Algidol* spectrum represents one replicate (R1, R2, and R3). The Raman conditions were as follows: Spectral range (3200-400 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16).

The Raman microscopy potential to identify different components within a mixture

One of the specific competences that should be obtained when using a Raman microscope is being able to focus and detect different components within solid mixtures. This is a very common problem in chemical laboratories. In this particular case, after verifying that acetaminophen was perfectly detected in *Algidol* (30 % acetaminophen) but not in *Apiretal* (aqueous solution of 10 % acetaminophen), different mixtures of various acetaminophen compositions were studied in order to determine the minimum ratio at

which a particular compound is still detectable in a mixture. To this aim, several microscopically different spots on each mixture were analysed attending to small differences in colour, texture, brightness, and/or crystallinity.

Mixture A. Acetaminophen/Ascorbic acid

None of the spectra collected for the 95/5 acetaminophen/ascorbic-acid mixture displayed the ascorbic acid characteristic bands. All spots that were analysed in this mixture corresponded to acetaminophen. On the contrary, in all other mixtures (90/10, 50/50, 10/90, and 5/95), both components (acetaminophen and ascorbic acid) were detected in at least one of the replicates (Figure 9). It should also be noted that the matching of each replicate against the corresponding standard always exceeded 90 %. This is probably because the precise focusing of a particle entirely covering the laser spot maximized the capability of exclusively obtaining the Raman spectrum of that particle.

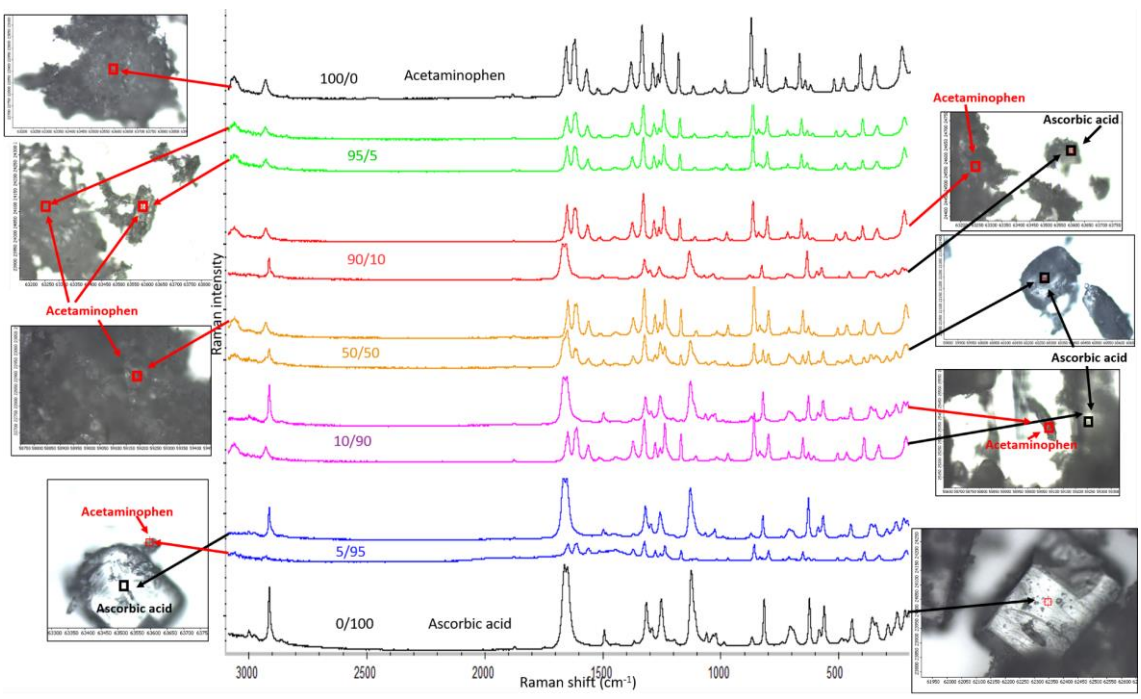


Figure 9. Raman spectra of the acetaminophen (100/0) and ascorbic acid (0/100) standards, and the five ratios of Mixture A (95/5, 90/10, 50/50, 10/90, and 5/95), together with the corresponding spot of each replicate. The figure shows two replicate spectra for each ratio, whose matching against acetaminophen/ascorbic-acid was ≥90 %. The Raman conditions were as follows:

Spectral range (3200-200 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16).

Mixture B. Acetaminophen/Caffeine

A confirmatory identification of both compounds (acetaminophen and caffeine) was possible for all the mixtures except for the 95/5 mixture, in which all analysed spots, corresponded to acetaminophen. None of the spectra collected in this mixture displayed the characteristic caffeine bands. On the contrary, as previously occurred in mixture A, in all other mixtures (90/10, 50/50, 10/90, and 5/95), both components (acetaminophen and caffeine) were detected in at least one of the replicates (Figure 10).

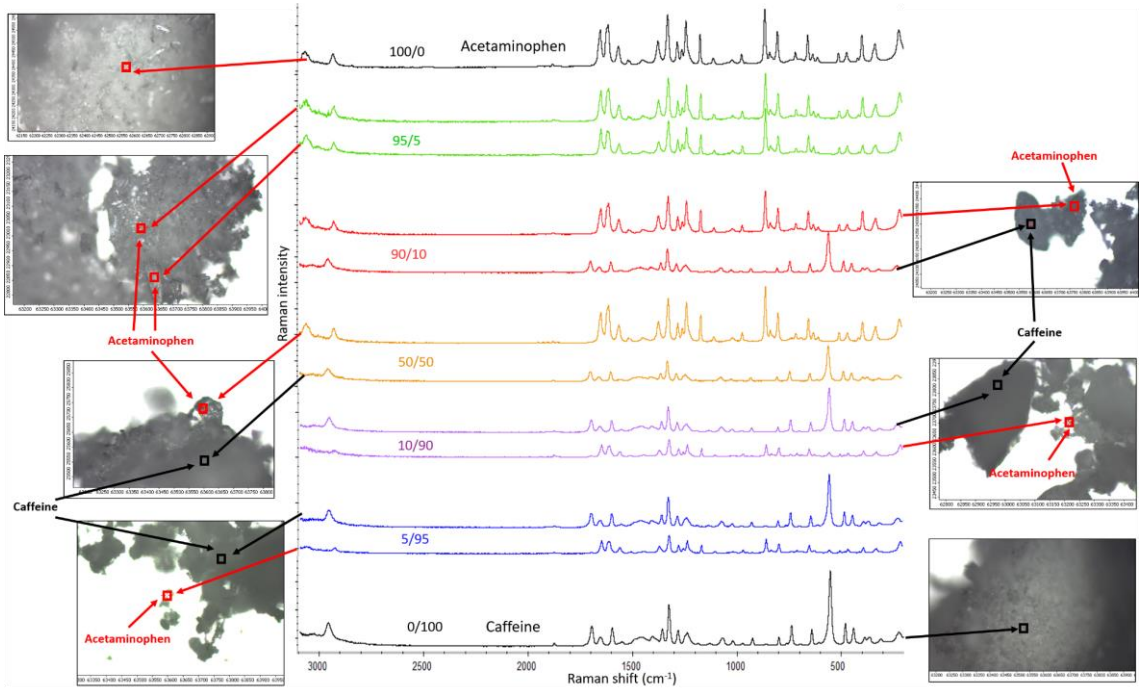


Figure 10. Raman spectra of the acetaminophen (100/0) and caffeine (0/100) standards, and the five ratios of Mixture B (95/5, 90/10, 50/50, 10/90, and 5/95) displaying the corresponding spot of each replicate. The figure shows two replicate spectra for each ratio, whose matching against acetaminophen/caffeine was ≥93 %. The Raman conditions were as follows: Spectral range (3200-200 cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16).

Mixture C. Acetaminophen/Ascorbic acid/Caffeine (50/40/10)

The great advantage of using a microscope to focus different spots along a sample was clearly demonstrated in the analysis of mixture C, because the three components (acetaminophen, ascorbic acid, and caffeine) were unequivocally detected using Raman microscopy (Figure 11). In this respect, the microscopic image clearly evidences how three different particles closely located along the microscope slide proved to be three different compounds each (ascorbic acid, acetaminophen, and caffeine). The identification was unequivocal. The visual comparison of each replicate against the acetaminophen, ascorbic acid, and caffeine standards spectra clearly confirmed a positive identification, which was supported by their statistical matchings exceeding 95 % (R1-ascorbic acid, R2-acetaminophen, and R3-caffeine).

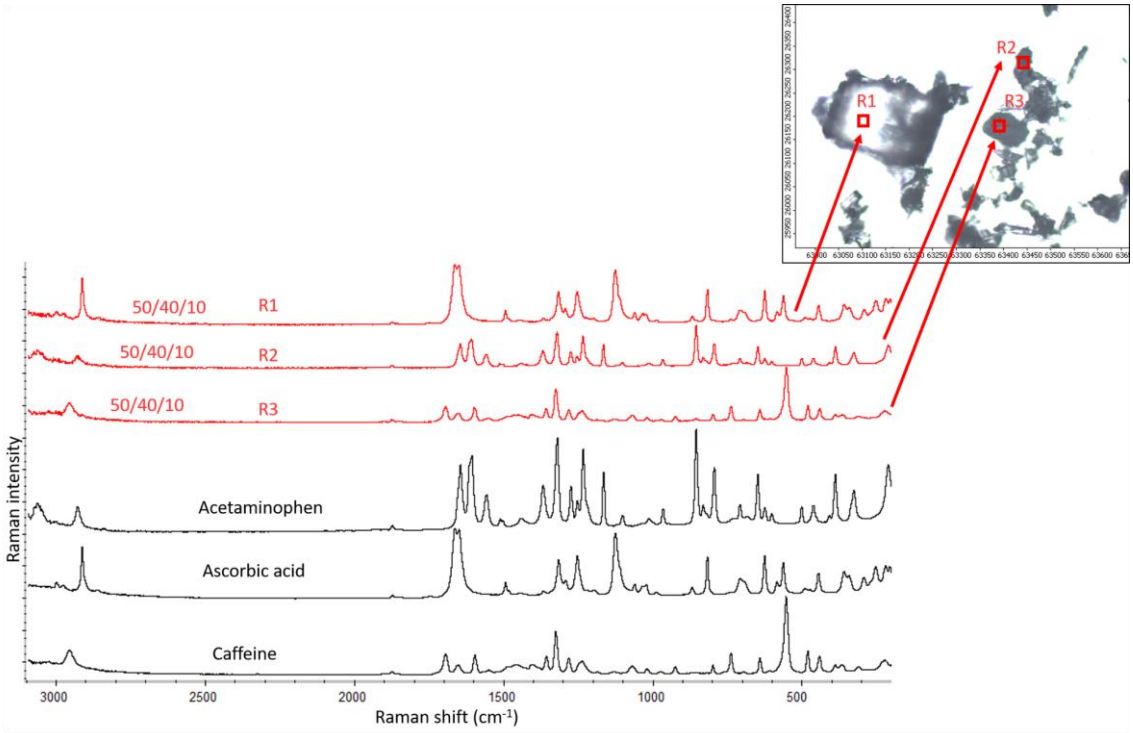


Figure 11. Raman spectra of the acetaminophen, ascorbic acid, and caffeine standards, and the replicates of the Mixture C (50/40/10) displaying the corresponding spot of each replicate, whose matching against acetaminophen/ascorbic-acid/caffeine were ≥95 %. R1 spot matches against the ascorbic acid spectrum, R2 matches against the acetaminophen spectrum, and R3 spot matches against the caffeine spectrum. The Raman conditions were as follows: Spectral range (3200-200

cm^{-1}), laser wavelength (785 nm), exposure time (1 s), spectrograph aperture (50 µm-slit), laser power (10 mW), and number of scans (16).

Conclusions

This comprehensive practical beginner's guide to Raman microscopy was written to help the teachers, technicians, students, and practical beginner's in general, to learn the basics of this technique from a practical point of view, especially because it is not usually trained during the undergraduate studies. After explaining the fundamental theoretical ideas, experimental concepts are thoroughly revised, explained and discussed. The guide specially focuses on experimental experiences that would increase the comprehension of: i) the optimization of the instrumental conditions, ii) the identification of drug samples based on spectral library matching, and iii) the potential of Raman microscopy to detect different components within mixtures.

The learning activity associated to this work, will enable users to analyse samples using the optimum instrumental conditions, as similar as possible to those conditions used for creating the standards spectral libraries. If these adequate conditions are not applied, the resulting Pearson correlation coefficients will be low (~70 %) despite that the analysed substance is the same as the substance collected in the library. The analyst should be able to make a decision on the positive/negative identification considering not only to the numerical matching, but also to the presence or absence of characteristic spectral bands.

Raman microscopy has the capability of focusing different particles using a microscope in such a way that the Raman signal of each single particle is maximized. This is particularly advantageous in the detection of several components within a mixture, especially for those components at low concentrations (<20 %, m/m). As an example, acetaminophen was detected in acetaminophen-ascorbic-acid and acetaminophen-

caffeine mixtures down to a limit of 5 % (m/m) acetaminophen. Furthermore, the three components (acetaminophen, ascorbic-acid, and caffeine) were unequivocally detected in a tri-component mixture (50/40/10) using Raman microscopy. The analyst should always keep in mind the capability of Raman microscopy to focus and detect individual particles either in homogeneous or heterogeneous solid mixtures. Thus, for the same sample, a certain number of different spots should be always analysed using Raman microscopy. The analyst should be able to focus and analyse microscopically different particles/spots along the sample paying attention to big/small differences in colour, texture, brightness, and/or crystallinity.

In conclusion, this practical beginner´s guide to Raman microscopy was born because of a clear necessity to provide technical and research personnel and teachers with pragmatic criteria to identify different substances, even in mixtures. This manual covers the pedagogical goal, and the fundamental knowledge and know-how that beginners need about the instrumentation settings.

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