DOI: 10.58895/ksp/1000044906-7 erschienen in:

OCM 2015 - 2nd International Conference on Optical Characterization of Materials, March 18th – 19th, 2015, Karlsruhe, Germany : Conference Proceedings

DOI: 10.58895/ksp/1000044906 | https://www.ksp.kit.edu/site/books/m/10.58895/ksp/1000044906/

Sensitivity and selectivity in optical spectroscopy and imaging: A molecular approach

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Abstract Intelligent manufacturing has attracted enormous interest in recent years. Optical spectroscopy will play a major role in the sensor technology as it provides simultaneously chemical (by absorption) and morphological (by scatter) information. The paper demonstrates, that the sensitivity and selectivity of each individual technology has its limitations due to the structure of the molecule and the quantum mechanical limitations by their interaction with the photons. The absorption and scatter cross sections are defined and discussed in terms of sensitivity and selectivity of the different technologies. These fundamentals cannot be overcome. Furthermore, the suitability and robustness of each technology is pre-determined by the selection of appropriate light illumination sources and the selected detectors. An overview of the different techniques is given.

1 Introduction

Intelligent manufacturing has attracted enormous interest in recent years, not least because of the Process Analytical Technology/Quality by Design platform of the US Food and Drug Administration (FDA) and now also by the German initiative Industry 4.0 [1]. The future of industrial automation will be "arbitrarily modifiable and expandable (flexible), connect arbitrary components of multiple producers (networked), enabling its components to perform tasks related to its context independently (self-organizational) and emphasizes ease of use (user-oriented)". Optical spectroscopy will play a major role in the sensor

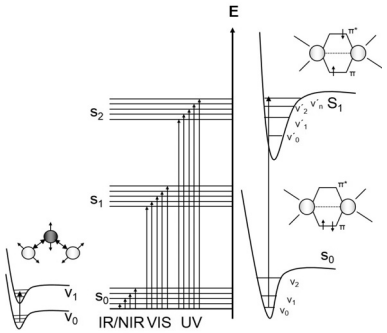


Figure 7.1: Electronic and vibrational transitions in optical molecular spectroscopy [2]

technology as it provides simultaneously chemical (by absorption) and morphological (by scatter) information [1].

Figure 7.1 shows the transitions involved using optical spectroscopy in the ultraviolet-visible range (UV-Vis: ca. 200 – 700nm, electronic transitions), near infrared (NIR: ca. 700 – 2500nm, combination vibrations, 1st, 2nd and 3rd overtones of fundamentals) and mid-infrared range (MIR: 2500nm – 25000nm, fundamental vibrations of valence bonds and fingerprint) [2].

The different ranges need suitable light sources with high intensity for illumination as well as classified detectors with as low as possible dark currents. Raman spectroscopy measures the emission (Raman scatter) from e.g. a virtually excited intermediate state into the vibrational ground state. Fluorescence spectroscopy measures commonly the emission from the first electronic excited state into the ground state (see also Figure 7.2) [2]. In most cases, both technologies use equipment also used in the UV-Vis spectroscopy.

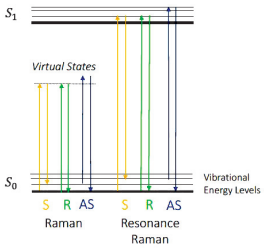
This paper will focus on the optical molecular spectroscopy because the majority of inline applications uses these wavelength ranges. The objective of this paper is to demonstrate, that the sensitivity and selectivity of each individual technology has its limitations due to the structure of the molecule and the quantum mechanical limitations by their interaction with the photons. These fundamentals cannot be overcome.

Furthermore, the suitability and robustness of each technology is predetermined by the selection of appropriate light illumination sources and the selected detectors. Furthermore, system suitability and system testing is an integral part of many analytical procedures as described in the guideline of the international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (quality: ICHQ2 (R1), Nov. 2005: validation of analytical procedures) [3]. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system which must be evaluated.

2 Sensitivity

Absorption cross section Sensitivity and selectivity are important features for the use of spectroscopy in sensor applications. "Analytical sensitivity" represents often the smallest amount of substance in a sample that can accurately be measured by a given technology. This is in general the concentration at which the mean response is statistically beyond the noise limits of the signal at zero concentration. In the pharmaceutical industry, specificity is normally used and defined: "Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.", [3]. However, the international union of pure and applied chemistry recommends strongly to use only the term sensitivity [4].

The sensitivity of a molecule can be described by the quantum mechanical cross sections which are the effective area that governs the probability of an event of e.g. elastic scattering, or absorption, or emission (e.g. in fluorescence or Raman) of a photon at a specified wavelength with a molecule. The absorption cross section σ is given usually in cm^2/molecule and depend on the individual molecular structure of the compound and the quantum mechanical selection rules. The larger the absorption cross section, the easier it is to photo-excite the molecule. The total cross section is related to the absorbance of the Lambert-Beer's law and is proportional to the concentration of the species (as a number density) and the path length. The extinction or absorbance of the ratation is then the log of the reciprocal of the transmittance [5].



Technology	–log σ_a [cm²/molecule]
Fluorescence	20 - 15
Mid-Infrared	25 - 18
Near-Infrared	28 - 22
Raman	35 - 28
Rayleigh scatter	33 - 24

Figure 7.2: Left: schematics of the Raman scattering (S=Stokes shift, AS=anti-Stokes shift, R: Rayleigh scattering), right: table of absorption cross sections of the different technologies, Remark: figures may be very different for certain molecules

Figure 7.2 depicts the transitions of the Raman Scattering and Rayleigh scattering and shows also some figures of the absorption cross sections of the different technologies [6]. The reader should be aware, that the figure provide just a rough estimation of common data, but the exact figures are molecule dependent and may vary significantly from these data. The figures are in -log σ_a [cm^2/molecule], therefore low numbers show high sensitivity. For the sake of comparison, also the figures of the Rayleigh scattering is shown which can be attributed to a molecular scattering.

Examples of NIR- and MIR absorption spectra Vibrational absorption correspond to changes in the vibrational state of the molecule and are typically found in the infrared region, which is divided into far-, mid- and near-infrared. Ab initio calculations allow to predict the theoretical vibrational states [6]. There are plentiful text books available which can be used to attribute specific peaks to the molecule entity. This is important to relate spectral features in a causal manner to the chemometric models. This feature is also important to get an impression of the selectivity of the analytical method. MIR spectroscopy is a highly sensitive and selective method whereas NIR is not. The advantage of NIR in process analytics is that, due to the low absorption cross section, no sample pre-preparation is needed during the inline measurement. Figure 7.3 shows the NIR spectra of water in transmission with different

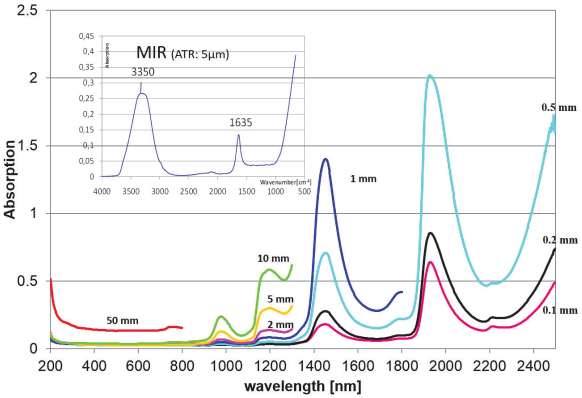


Figure 7.3: NIR spectra of water in transmission with different path lengths. Insert: ATR mid-infrared spectrum of water with a pathlength of app. 5µm [7]

path lengths. Combination bands are reasonable sensitive in the NIR region. The absorbance of the 1st, 2nd and 3rd overtone of the fundamental vibrations are successively at least one order of magnitude lower in their response. The insert shows also the ATR mid-infrared spectrum of water with a pathlength of 5µm only [7].

MIR spectroscopy is ideally suited to detect concentrations down to about the ppm concentration range whereas NIR spectroscopy may be limited to a range of 1%, at most 0.1%. Raman spectroscopy may be limited to about 1%, for specific molecules with a high Raman scattering cross section at a level of 0.1% due to its high selectivity. Fluorescence can be used for the determination of concentrations in the nanogram-range and sometimes even down to a single molecule level.

Example of UV-Vis spectra: Woodward-Fieser rules Electronic absorption corresponds to a change in the electronic state of an atom or molecule and are typically found in the visible and ultraviolet region. The energy associated with the quantum mechanical change primarily determines the frequency of the absorption, but the frequency can be shifted by several types of interactions with the molecule's environment. Absorption of a particular wavelength of light depends in UV-Vis spectroscopy mainly upon the π-electron system of the molecule.

The more the conjugation of the π-electron system within the molecule, the higher the wavelength of light it can absorb. The absorption coefficient μ_a [cm^{-1}] describes a medium containing many chromophores at a concentration described as a volume density ρ_a [cm^{-3}]. The absorption coefficient is essentially the cross-sectional area per unit volume of medium. Experimentally, the units [cm^{-1}] for μ_a are inverse length, such that the product $\mu_a L$ is dimensionless, where L [cm] is the photon's pathlength of travel through the medium.

Robert Burns Woodward and Louis Fieser put down a set of rules which allows one to calculate the wavelength of maximum absorption ($\lambda_{\max}$) for a molecule empirically [8–10]. Many other authors refined these equations. The following equation (as an example: the Fieser-Kuhn rule) can be used to predict the wavelength of maximum absorption $\lambda_{\max}$ and also maximum absorptivity $\epsilon_{\max}$:

calculating $\lambda_{\max}$

$$\lambda_{\max} = 114 + 5M + n(48.0 - 1.7n) - 16.5R_{\text{endo}} - 10R_{\text{exo}}$$

calculating $\epsilon_{\max}$ $\epsilon_{\max} = (1.74 \times 10^4)n$

$\lambda_{\max}$ is the wavelength of maximum absorption in nm

$\epsilon_{\max}$ is the maximum absorptivity in [cm^{-1} mole^{-1}]

M is the number of alkyl substituents / ring residues in the conjugated system

n is the number of conjugated double bonds

R_{endo} is the number of rings with endocyclic double bonds in the conjugated system

R_{exo} is the number of rings with exocyclic double bonds in the conjugated system.

Figure 7.4 shows an example.

It can be shown, that the properties are directly related to the molecular structure of the chemical entity. Due to the high cross sections, UV-Vis spectroscopy is extraordinary sensitive. However, selectivity is limited as the absorption peaks are clearly related to the number of conjugated double bonds and not to a specific molecule. Maybe in the future the electronic transitions of σ-bonds may be available, however technology must be developed as the absorption is significantly below

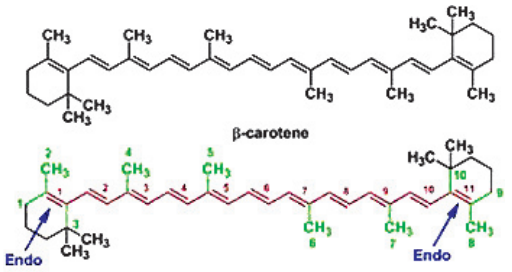


Figure 7.4: Fieser-Kuhn rules to predict the maximum absorption peak and maximum absorptivity of β-carotene (taken from [10])

200nm. Of course, the limit of detection can always be improved at higher signal to noise ratios when better detectors are used and by signal averaging.

Scattering cross section The advantage of optical spectroscopy is that simultaneously the chemical composition (by absorption) as well as the morphology can be measured in a spectrum due to scatter. Scatter perturbs the absorption spectra and is often unwanted and eliminated by chemometric tools. Scattering intensity is a function of the used wavelength and its polarisation and also depends on the angular distribution of the scattered light which in turn is dependent from the size, size distribution and shape of the particle.

As described before, Rayleigh scatter may be associated more to a molecular scatter and is wavelength dependent with $1/\lambda^4$. The scattering cross-section e.g. in the Mie or Fraunhofer regime is a hypothetical area which describes the likelihood of light being scattered by a particle, the scattering center. It is a measure of the strength of the interaction between the scattered particle and one or several scattering centres. Mie theory calculations will yield the efficiency of scattering which relates the cross sectional area of scattering, σ_s [cm^2] to the true geometrical cross-sectional area of the particle, A in [cm^2]. The scattering coefficient μ_s [cm^{-1}] is essentially then the cross-sectional area per scatterer number density ρ_s [cm^{-3}]. Depending on the geometry of the particle

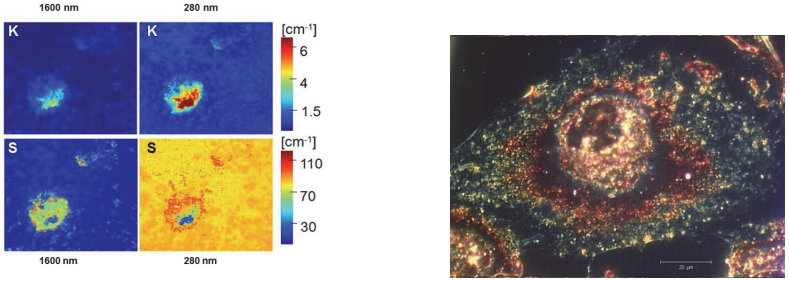


Figure 7.5: Left: Kubelka-Munk calculated scatter (S) and absorption coefficients (K) of an Aspirin particle in microcrystalline cellulose, right: white light scatter intensity of a glioblastoma cell measured in dark field arrangement

and its size, an anisotropy factor g may be integrated to account for forward and backward scattering efficiency [11, 12]. The scatter coefficient increases with shorter wavelengths app. $1/\lambda^n$, with $n = 0.2 \ldots 3$.

Scattering has a diagnostic value e.g. in biomedical sciences. Scattering depends on the ultrastructure of a tissue, e.g. the density of lipid membranes in the cells, the size of nuclei, the presence of collagen fibers, the status of hydration in the tissue, etc. Cells and nuclei are in the range of micrometres, mitochondria and lysosomes are in the range of 100nm to a few microns, membranes or collagen fibrils may be in the range 10nm up to 100nm. Scattering of light by structures on the same size scale as the photon wavelength is described by Mie theory. Scattering of light by structures much smaller than the photon wavelength is called the Rayleigh limit of Mie scattering, or simply Rayleigh scattering.

Using lager particles there is no universal theory to describe scatter. The radiative transfer equation, photon diffusion theory or a more empirical theory like Kubelka-Munk theory is used [1,2]. In any case, more than one measurement is needed to separate the two unknown absorption and scatter. Figure 7.5 shows an example of the scatter of a glioblastoma cell as well as the calculated scatter and absorption coefficients of an Aspirin particle in microcrystalline cellulose [1, 2, 12].

It is important to emphasize, that the scatter coefficient in this example is by a factor of 10 – 100 more sensitive to changes than the absorption coefficient. This means, any change in particle size or particle size distribution will have a significant influence on the spectral signature.

3 Selectivity

As described before although specificity is still used in the pharmaceutical industry, IUPAC recommends strongly to use selectivity to describe its capability to deliver signals that are free from interferences and give "true results" [3,4]. This implies, that if the signal of interferent and analyte can be separated, the sensitivity increases. This is the main reason why Raman spectroscopy can be as sensitive as NIR spectroscopy or even UV-Vis spectroscopy as the peaks of the components can directly be attributed and deconvoluted to the molecular entity, although the Raman cross section may be inferior to NIR and UV-Vis.

One clear definition is the following: "Selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components in the mixture" [4]. Raman- and MIR-Spectroscopy are very selective spectroscopic methods and in most cases, the spectral features can easily be attributed to the individual molecules and entities. A good example is the selectivity of Raman spectroscopic investigations in aqueous solutions. The Raman signal of water is very weak, thus organic components can be detected without the interference of the water peak. This is not the case with MIR spectroscopy unless the signature lies in the fingerprint region between roughly 1000 – 1500 cm^{-1} (see insert in Figure 7.3). With an increasing photon flux, e.g. using lasers, and increasing path lengths even difficult analytical questions can be solved. UV-Vis and NIR spectroscopy and also fluorescence spectroscopy show broad peaks in solutions or particle mixtures and thus selectivity is significantly reduced. The advantage of UV-Vis and fluorescence spectroscopy is that water is not an interferent.

Using derivative spectroscopy greatly enhances the separation of even small changes in a spectrum with overlapping peaks [13]. Higher order derivative spectroscopy need spectra with a high signal to noise ratio together with a high reproducibility of the spectral signature of the overall system (see robustness below). Due to the technological developments in the silicon semiconductor industry (wavelength range from 170nm up to 1050nm) as well as the high absorption cross sections allow to quantitatively determine the concentration of many-components mixtures with a high precision. Especially in biotechnology, where aqueous systems are common with low concentrations of the compo-

nents, UV-Vis spectroscopy may be favourable upon NIR spectroscopy [7].

Furthermore, chemometric tools like principal component analysis (PCA) or partial least square regression (PLS) together with a pre-treatment of spectra can increase selectivity and sensitivity simultaneously. The responses are then based on interactions usually evaluated in a mathematical domain (chemometrics), giving what has been called "computational selectivity". In fact, selectivity is improved by a higher number of different measurements especially by use of a whole spectrum over a wavelength range instead of single wavelengths and processing the spectral data by chemometric methods with different spectral pre-treatments. The handling of near-infrared spectra in this way is a very good example of this approach.

The combination of several methods may enhance selectivity: multi-modal-sensor technology or multi-modal-spectroscopy [1]. A good example is 2-dimensional fluorescence spectroscopy (Excitation-Emission-Plot) where a mixture of components can spectroscopically be separated due to variations in the emission spectra excited at different wavelengths.

4 Robustness in inline spectroscopy and imaging

According to the ICHQ2 guideline, "robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [3]. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure [3]".

As a consequence a series of system suitability parameters must be established to ensure that the validity of the analytical procedure is maintained whenever used. This means that e.g. wavelength drifts, drifts of the intensity and colour temperature of the illuminations source, polarisation changes of the light must be controlled, preferable inline, as well

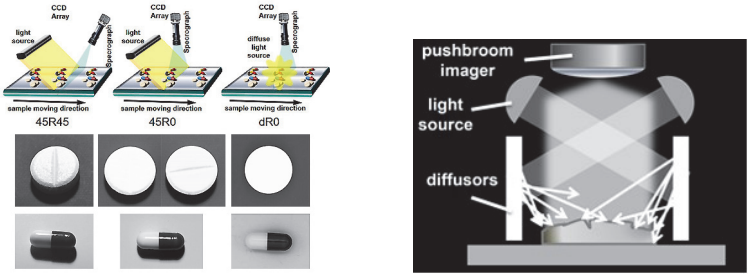


Figure 7.6: Left: example of the different optical set ups realized with an inline pushbroom imaging system and depicts the outline of the results which happen when the different illumination concepts are applied to tablets with a scratch or round shaped tablets with a strong specular reflection. Right: possible inline illumination with an even illumination from all directions

as a robust optical set up including flutter tolerance must be ensured [2]. Figure 7.6 shows an example, how a proper illumination and detection set up can influence the measured information [12].

In diffuse reflection, only scattered photons should be detected and thus all specular reflected photons from the surface of the material should be rejected. The standard setup which is commonly used in practical applications is an illumination at e.g. 45° and detection at 0° (denoted as 45R0). This works fine as long as the macroscopic sample plane is identical to the optical axis of the reflectors. However, specular reflected light can be superposed to diffuse reflected light when micro mirrors are present at the front phase of the sample (e.g. flat microscopic plates inside a lacquer), or a macroscopic curvature of the sample like a round shaped tablet directs the light into the detector. A planar sample like the presented tablet can show at e.g. 45° illumination and detection (45R45) some spots of specular reflection together with a shade. At 45R0 the specular reflections are minimized, but the sample still shows a shadow on the background and some minor shade at the boundaries of the surface.

Please note that if the scratch on the surface is in the direction of illumination (Figure 7.6, 45R0), no or little shade is produced, but in case of a mismatch of the orientation between illumination and the scratch, shades and even specular reflection may be observed. This optical arti-

fact can be eliminated by a proper data pretreatment and analysis, but very often at the expense of robustness of the chemometric model.

With diffuse illumination, no shadow on the surface and on the background material is present: the sample is almost ideally and homogeneously illuminated. If the sample is not planar like the capsule, a specular reflection spot is still present even at diffuse illumination but at a detection at 0° (dR0). The solution in this case may be to illuminate and detect the sample with an integrating sphere. Ideally the sample is therefore illuminated by a perfect Lambertian source and the detector is also integrated into an integrating sphere. A good approximation to a Lambertian illumination can be realized as shown in Figure 7.6, right, with the advantage to use it also for a continuous inline control.

5 Summary

Selectivity and sensitivity are key elements for an analytical chemist. Besides these key elements, many other aspects must also be taken into account to ensure robustness of the inline measurement [1, 2]. If the existing data are objectively classified, for example, from spectroscopic measurements, a direct and causal correlation of the spectral information to the functionality of the material is possible. The spectrum (the "spectral fingerprint" or "process trajectory") represents the entire morphology (due to scattering) and chemistry (due to absorption) of a substrate and can therefore be directly linked to the particular functionality of a product. Table 7.1 tries to summarize some features of the different techniques.

It is important to emphasize, that inline quality control by spectroscopic techniques is a holistic approach. Process chemists, process engineers, chemometricians, and many other technologists must work together where multimodality will be a bedrock supporting the production of smart materials in smart factories [1].

	UV/VIS/ s-NIR	NIR	MIR	Fluorescence	Raman
Selectivity	+	++	+++	++	+++
Sensitivity	+++	+(+)	+++	+++(+)	++(+)
Sampling	+++	+++	+	++	+++
Working in aqueous media	+++	+	+	++	+++
Applicability	+++	++	+	+	+
Process analytical tool	+++	+++	+	+	+++
Light guide glass	+++	+++	(+)	+++	+++
Signal	Absorption	Absorption	Absorption	Emission	Scattering
Samling online/inline	s, l, g	s, l	s, l, g	s, l (g)	s, l, (g)
Techniques	Transmission Reflectance ATR	Transmission Reflectance ATR	ATR (Transmission)	Reflectance Transmission	Reflectance
Relative costs	1	3 - 5	6 - 10	4 - 6	8 - 12

Table 7.1: Summary of properties of optical spectroscopic techniques for inline control (modified from [1] and [2])

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