UV/Vis Spectrophotometry

UV/Vis Spectrophotometry
Fundamentals and Applications

METTLER TOLEDO

1.   Introduction

Beside chemical analysis, the characterization of pure as well as mixtures of substances is achieved with physical methods. Among other techniques, such as the determination of melting point, refractive index and density, optical spectroscopy in the ultraviolet and visible light range (UV/Vis) is widely applied in almost all market segments and workplaces in research, production and quality control for the classification and study of substances. UV/Vis spectroscopy is based on the absorption of light by a sample. Depending on the amount of light and its wavelength absorbed by the sample, valuable information can be obtained, such as the purity of the sample. Moreover, the amount of absorbed light is related to the amount of sample, and thus, quantitative analysis is possible by optical spectroscopy.

For many years METTLER TOLEDO has provided instrumental solutions for sample characterization (e.g. thermal values, pH, conductivity, refractive index, density) as well as for content determination by titration. With the introduction of a new analytical technique, the application power and possibilities of METTLER TOLEDO instruments are further extended to more comprehensive multi-parameter determinations.

The new UV/Vis Excellence Spectrophotometers will additionally support the customer workflow with fast, easy-to-use and trustworthy analytical instruments. This guide provides the reader with fundamental knowledge on this technique as well as application tips and hints for accurate and precise results in daily use.

2. UV/Vis Spectroscopy

2.1 What is UV/Vis spectroscopy?

Optical spectroscopy is based on the interaction of light with matter. The following figure illustrates what is happening when light is shining onto an object:
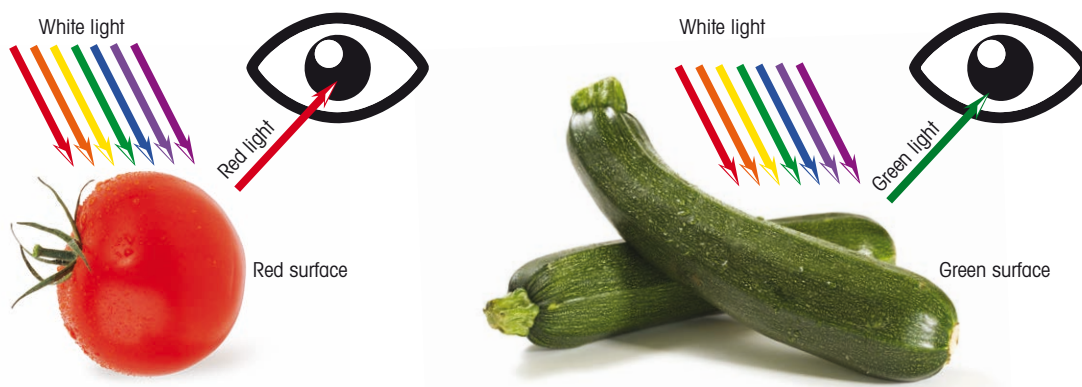

Figure 1 and 2: The light which is not absorbed by the object is reflected and can be seen by the eye

Both objects are illuminated by visible or white light, which is represented by a rainbow: the different colors represent the different components of visible light. When rays of light are shining onto an object, they might be absorbed by the object – in particular, one or more light components (i.e. its colors) are specifically absorbed.

The colors which are not absorbed by the objects are reflected. In our example, red light is reflected by the red shell of the tomato (Fig. 1), whereas green light is reflected by the green surface of the zucchini (Fig. 2). All other colors are absorbed by the two objects. The reflected light is then seen by the eyes: the tomato is seen in red while the zucchini are green.

In physical terms, light is a kind of energy propagating into space at a very high speed. More specifically, light is understood as an electromagnetic wave travelling into space – it is radiant energy. The energy of light oscillates periodically between a minimum and a maximum as a function of time – like a wave. The distance between two maxima or two minima, respectively of the electromagnetic wave is defined as the wavelength, given in nanometer (nm).
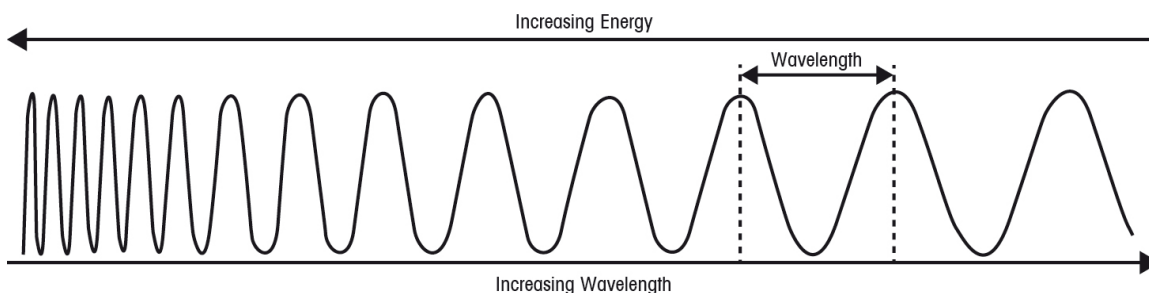

Figure 3: The energy of an electromagnetic wave increases with decreasing wavelength – and vice versa

Each color has a specific wavelength, e.g. red light has a wavelength of 660 nm, while green light has a wavelength of 520 nm. Thus, the different components of light are characterized by a specific wavelength. The sum of all components i.e. of all wavelengths, is called a **spectrum**. More specifically, a spectrum represents a distribution of radiant energy. For instance, the electromagnetic spectrum of visible light ranges from approximately 390 nm up to approximately 780 nm.
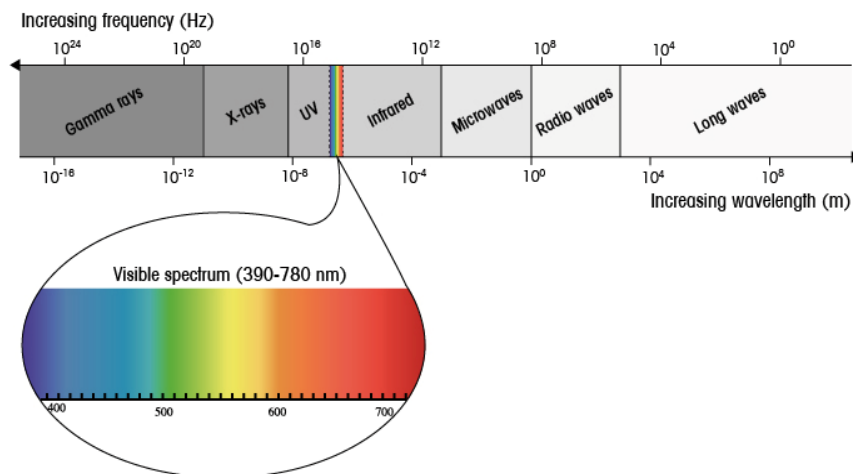

Figure 4: The visible spectrum (390 – 780 nm) represents only a small portion of the whole electromagnetic spectrum

Note that the energy of electromagnetic waves is related to their wavelengths; the shorter the wavelength, the higher the energy. For instance, violet light has a shorter wavelength than red light and therefore, a higher energy level, whereas infrared light has less energy than visible light due to its longer wavelength.

Absorption of light as analytical tool

Light absorption can be used in analytical chemistry for characterization and quantitative determination of substances. UV/Vis spectroscopy is a technique based on the absorption of light by an unknown substance or by an unknown sample. Here, the sample is illuminated with electromagnetic rays of various wavelengths in the visible (Vis, i.e. the different colors) and adjacent ranges i.e. ultraviolet (UV) and part of the lower infrared region (near IR) of the spectrum. Depending on the substance, light is partially absorbed. The remaining light, i.e. the transmitted light, is recorded as a function of wavelength by a suitable detector, providing the sample's UV/Vis spectrum.

As a result, because each substance absorbs light in a different way, a unique and specific relationship exists between the substance and its UV/Vis spectrum. The spectrum can then be used to identify or quantify a sub-stance.
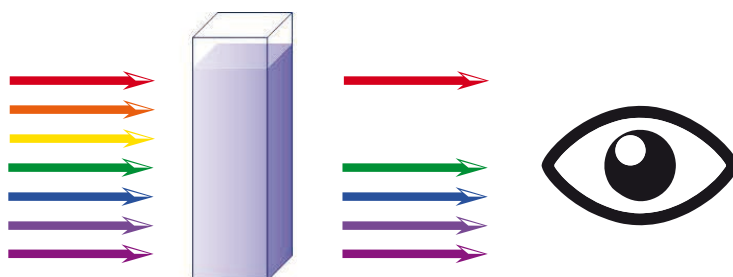

Figure 5: Light passing through a sample solution is partially absorbed by the components

UV/Vis spectroscopy is usually applied to organic molecules, inorganic ions or complexes in solutions, although solid materials such as films or glass can be analyzed as well. The obtained UV/Vis spectra are very useful for quantitative measurements of a specific compound. In fact, the concentration of an analyte in solution can be determined by measuring the absorbance at a specific wavelength. From the absorbance value of the sample, its concentration can be calculated, see the description in chapter 2.4.

UV/Vis spectroscopy is a measurement technique in which the recording of the absorption spectra of different samples using ultraviolet (UV) and visible (Vis) light is achieved by a spectrophotometer, i.e. an instrument able to measure the spectrum of a sample in the UV/Vis range.

2.2   Measurement principle

A UV/Vis spectrophotometer measures the intensity of light passing through a sample solution in a cuvette, and compares it to the intensity of the light before it passes through the sample. The main components of a UV/Vis spectrophotometer are a light source, a sample holder, a dispersive device to separate the different wavelengths of the light (e.g. a monochromator), and a suitable detector.
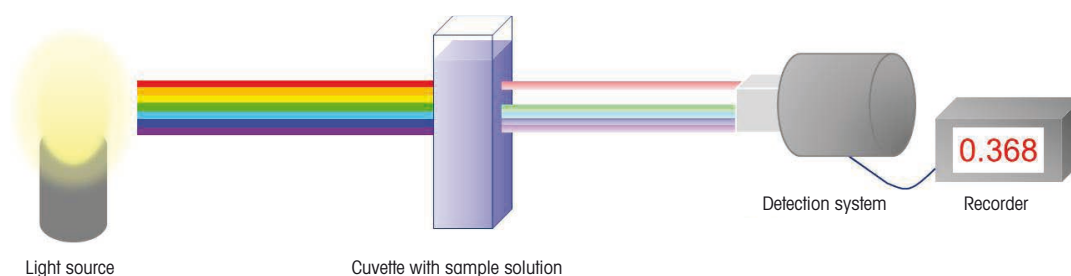

0.368

Detection system   Recorder

Light source   Cuvette with sample solution

Figure 6: Measurement principle in UV/Vis spectroscopy

The working principle of a spectrophotometer is based on the following steps:

Blank (measure of the intensity of light transmitted through the solvent):
1.   The solvent (e.g. water or alcohol) is added into a suitable, transparent and not absorbing container – a cuvette.
2.   A light beam emitted by the light source passes through the cuvette with the solvent.
3.   The intensity of the transmitted light at different wavelengths is then measured by a detector positioned after the cuvette with the solvent and recorded.

This is known as the blank, which is needed for the sample measurement.

Sample determination:
1.   A sample is dissolved in the solvent and added into the cuvette.
2.   A light beam emitted by the light source passes through the cuvette with the sample.
3.   When passing through the cuvette, the light is partially absorbed by the sample molecules in the solution.
4.   The transmitted light is then measured by the detector.
5.   The light intensity change at different wavelengths is calculated by dividing the transmitted intensity of the sample solution by the corresponding values of the blank. This ratio is finally stored by a recorder.

2.3 Transmittance and absorbance

The detector in a UV/Vis spectrophotometer measures the intensity of light after passing through the sample solution. This fraction of light collected by the detector is called the transmitted intensity, I. The intensity of the transmitted light is attenuated by the sample solution due to, for instance, absorption of light at specific wavelengths. Therefore, its value is lower than the original intensity I_0 at the light source.
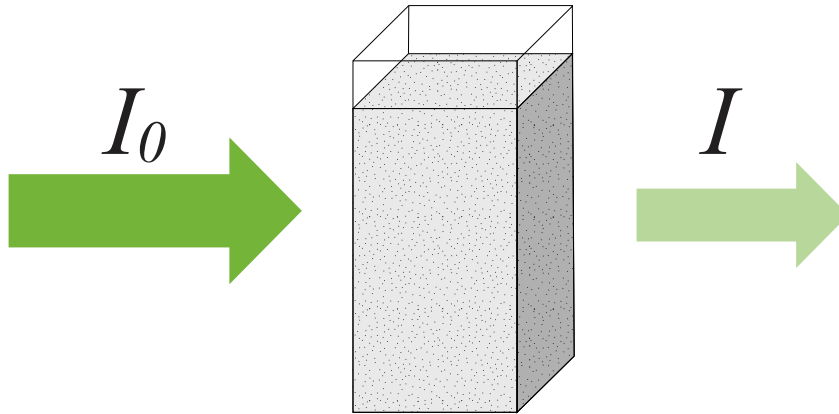
Figure 7: Light attenuation by absorption of sample molecules in solution

The ratio between the two intensities I/I_0 is defined as Transmittance T, and its unit is %.

$$T = \frac{I}{I_0}$$

Figure 8: Transmittance is the ratio of the transmitted intensity I to the original intensity I_0

The transmittance is the main value determined by UV/Vis spectroscopy, but it is not the only one. In fact, the absorbance A represents an additional result widely used when recording UV/Vis spectra. It is defined as the negative logarithm of the transmittance and it has a great advantage, which we will see in the next chapter.

$$A = -log(\mathrm{T})$$

Figure 9: Absorbance is the negative logarithm of the transmittance value

Note that the absorbance A does not have any unit of measurement. In other words, it is a dimensionless value. However, it is often represented using the letter "A" or as AU for absorbance units. For example, 0.3 A or 0.3 absorbance units respectively.

The result of a measurement using a UV/Vis instrument is shown in the following figure:
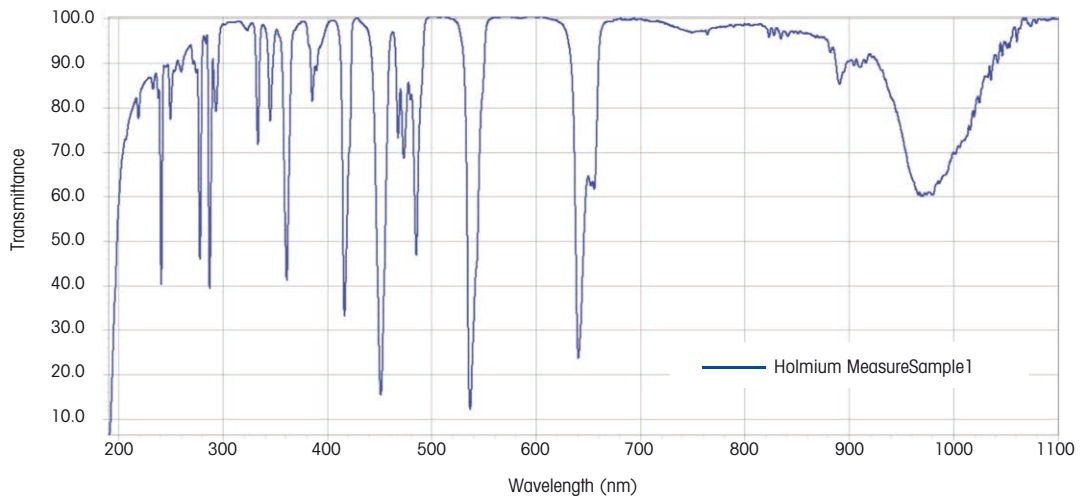
Figure 10: Transmittance spectrum of holmium solution as a function of wavelength

The transmittance spectrum of a sample is recorded as a function of the wavelength. In this particular example, the sample absorbs the light at mainly four different wavelengths, i.e. at approx. 370, 450, 480 and 540 nm. The light absorption is marked by a sharp decrease of the transmittance at these wavelengths.

In the following figure, the absorbance spectrum of the same sample is given as a function of the wavelength. Note the absorption peaks that are located at the same wavelengths. In this case, the degree of light absorption is indicated by higher absorbance values.
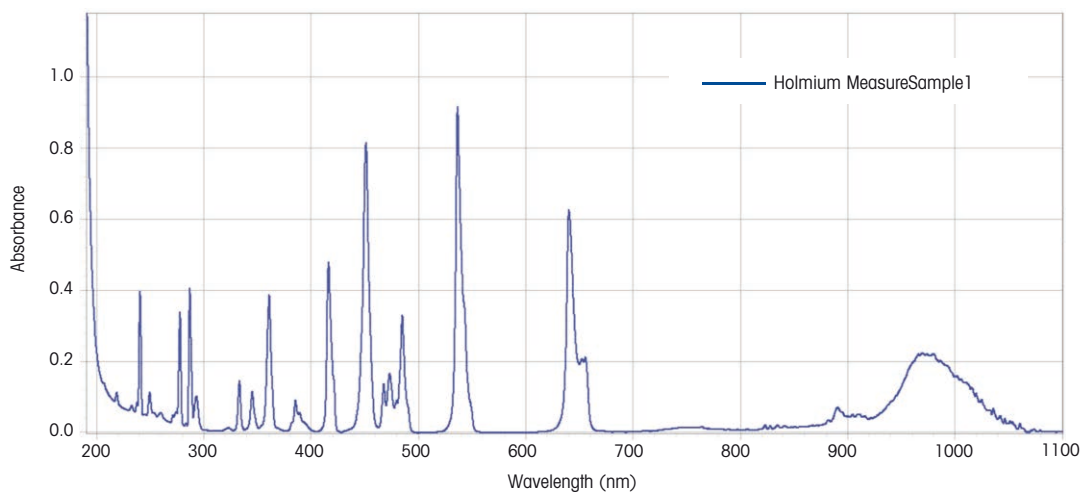

Figure 11: Absorbance spectrum of holmium solution as a function of wavelength

In general, a UV/Vis spectrum is graphically represented as absorbance as a function of wavelength.
The advantage of this representation is obvious; the height of the absorption peaks is directly proportional to the concentration of the species.

2.4   Lambert-Beer law

When passing through a transparent cuvette filled with sample solution, the light intensity is attenuated proportionally to the sample concentration. In other words, a higher concentrated sample solution will absorb more light. In addition, the attenuation is also proportional to the length of the cuvette; a longer cuvette will lead to a higher absorption of light.
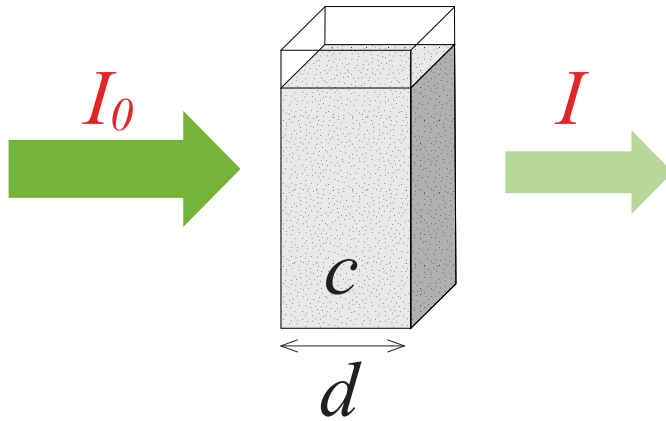

Figure 12: The attenuation of the light intensity is proportional to the concentration of the sample solution as well as the length of the cuvette

Both factors can be summarized by expressing the absorbance A as a function of the concentration and of the cuvette length. In particular, the absorbance A is equal to the product of the extinction coefficient ε, the concentration c and the path length d:

$$A = \varepsilon \cdot c \cdot d$$

This relationship is called the **Lambert-Beer** law where:
1.   The **sample concentration** c is given in mol / L or g / mL, respectively
2.   The **path length** d of the cuvette is given in cm,
3.   The **extinction coefficient** ε (epsilon) is a sample specific constant describing how much the
          sample is ab sorbing at a given wavelength (in L / (cm*mol) or mL / (cm*g), respectively).

When the path length is 1 cm and the concentration is 1% w/v, the extinction coefficient is called
specific absorbance $(E_{1cm}^{1\%})$

The Lambert-Beer law allows for the determination of the sample concentration from the measured absorbance value. If the extinction coefficient ε and the path length d are known, then concentration c can be calculated from absorbance A as given below:

$$c = \frac{A}{\varepsilon \cdot d}$$

For optimal measurement results and to comply with the Lambert-Beer Law, the absorbance shall be in the linear range of the instrument. The suitable range for optimal measurements i.e. the measurement range where the absorbance is directly proportional to the concentration is given as $0.3 < A < 2.5$. Thus, it is recommended to avoid very high absorbance values $(A > 2.5)$ as well as very low absorbance values $(A < 0.3)$ which may lead to a non-linear behavior of the calibration curve. This is shown in the following figure, where the measured values above $A = 2.5$ and below $A = 0.3$ (red dotted line) would deviate from the theoretical calibration curve (green):
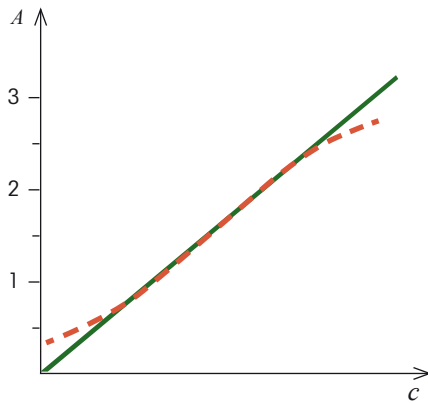
Figure 13: Non-linearity: The red measured values outside the linear range deviate from the green theoretical calibration curve

The instrument resolution, the signal to noise ratio and the stray light interference are the main contributions that may limit the linearity of the instrument. Moreover, limitations can derive from the sample itself as it follows:

- Highly concentrated sample solutions:
  - → use concentrations in the order of 0.01 M for optimum measurements
- High salt concentration in the sample:
  - → dilute sample to avoid a too high salt concentration
- Interactions between molecules in solution can lead to non-linearity, especially at high concentrations and in the presence of hydrogen bonding
- The refractive index may change in the case of large concentration changes, reducing the linearity
- Chemical equilibrium shift may take place such as dissociations and associations of molecules, or due to chemical reactions.
- Usage of right cuvette according to quality, repeadability, wavelength...

3.   UV/Vis Spectroscopy in Analytical Chemistry

3.1   Why do we measure UV/Vis spectra?

There are five main reasons to measure UV/Vis spectra:

- UV/Vis spectra allow components present in the sample solution to be identified. More precisely, the position and, to some extent, the profile of the absorption peaks allow specific compounds to be identified.
  For example, organic compounds can be identified by their spectra, or solvent purity can be easily checked by UV/Vis spectroscopy.
- Absorption peaks can be used to quantify the investigated sample. For example, the sample concentration can be calculated from the absorbance value of the peak:
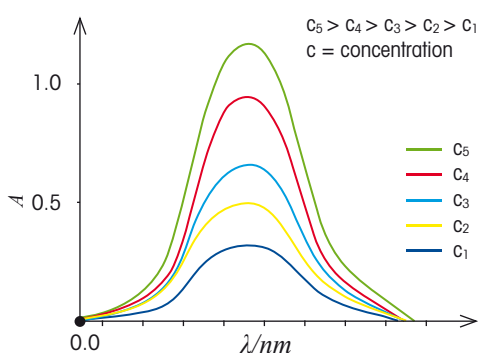

Figure 14: A higher concentration leads to higher absorbance value

- Based on the relationship between absorbance and sample concentration, UV/Vis spectroscopy is applied as a quantitative analytical technique in market segments such as e.g. Water Testing, Food and Beverages, Pharmaceutical, Chemical and Biotech Industry.
- The position of the peaks in the spectrum reveals information about the molecular structure of the sample.
  For example, specific functional groups of a molecular structure, such as carbon-oxygen, $C=O$, or carbon-carbon double bonds, $C=C$, absorb at specific characteristic wavelengths.
- The spectrum may reveal specific physical properties of the sample molecules. For instance, from the UV/Vis spectrum it is possible to:
  - calculate the extinction coefficient of the sample
  - calculate the melting point of proteins and nucleic acids by measuring the UV/Vis spectra at different temperatures
  - determine the rate of a reaction by monitoring the absorption spectra as a function of time (also known as kinetic measurements).
- Finally, position and profile of the peaks in the spectrum can give information about the microscopic environment of the sample molecules. As an example, the presence of impurities or other solvents in the sample solution has an effect on position and of the profile of the peaks. In other words, the peaks may be broader or have shifted due to impurities.

The applications of UV/Vis spectroscopy are mainly focused on qualitative and quantitative analysis, which will be addressed in more details in the next chapter.

3.2   Qualitative analysis: Identification

In qualitative analysis, UV/Vis spectroscopy can be used as a tool to identify if the analyte is pure and did not undergo decomposition. For example, this technique is used for quality control of incoming raw material, and for the purity check of biologically relevant compounds such as the nucleic acids, DNA and RNA.
Additionally, the melting point of DNA can be determined by recording its UV/Vis spectrum at different temperatures. Finally, by means of UV/Vis spectroscopy it is possible to differentiate between saturated and unsaturated fatty acids present in olive oil, and thus to monitor its quality.

Qualitative analysis is based on the specificity of UV/Vis spectroscopy. In fact, samples absorb light of one or more distinct wavelengths, with specific maximum absorbance values. For this reason, each sample has a characteristic and unique UV/Vis spectrum that can be used for its identification. In particular, this is achieved by comparing the spectrum of the sample with spectra of known, pure compounds.

As an example of UV/Vis spectrum, the spectrum of chlorophyll a is shown below. This molecule, responsible for the green color of leaves and grass, characteristically has strong absorption bands in the violet, blue and red regions of its UV/Vis spectrum.
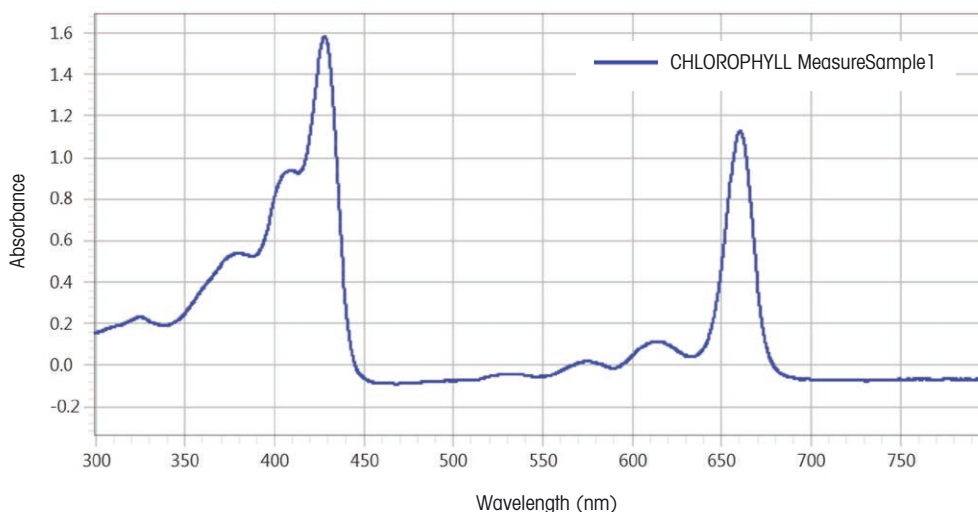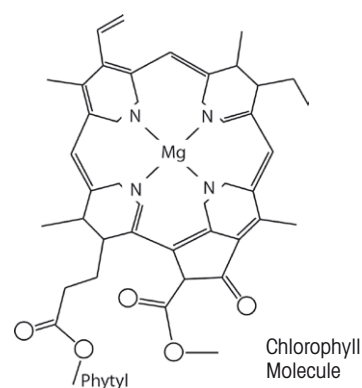

Chlorophyll
Molecule
Phytyl

Figure 15: UV/Vis spectrum of chlorophyll a

As a second example, the absorption spectrum of beta carotene is given as a function of the wavelength. Beta carotene, a strongly colored red-orange natural pigment abundant in plants and fruits such carrots, pumpkins and sweet potatoes, is characterized by a single, broad and strong absorption band in the blue-violet region of the spectrum. According to USP, the sample solution shows a shoulder at about 427 nm, an absorption maximum at about 455 nm, and a second absorption maximum at about 483 nm, whereas the absorbance ratio at 455 and 483 nm A(455) / A(483) is between 1.14 and 1.18. Note that these three absorption bands and the A(455) / A(483) ratio allow a clear identification of beta carotene as the molecule present in the solution. As a result of the absorption at these wavelengths, an intense red-orange color is seen when beta carotene is present.
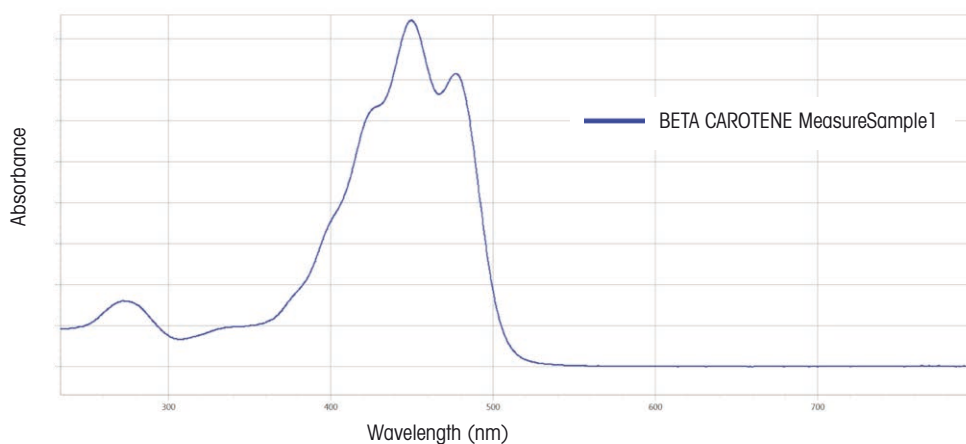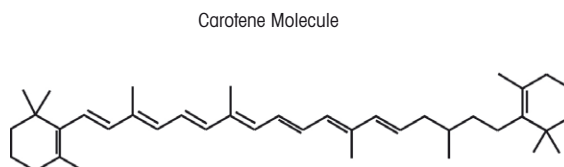
Carotene Molecule

Figure 16: UV/Vis spectrum of beta carotene – a compound present in carrots

Finally, the UV/Vis spectra of three different compounds are given in the figure below. They all have a distinctive spectrum because these molecules all have a different chemical structure. This allows for identification of the samples by UV/Vis spectroscopy.
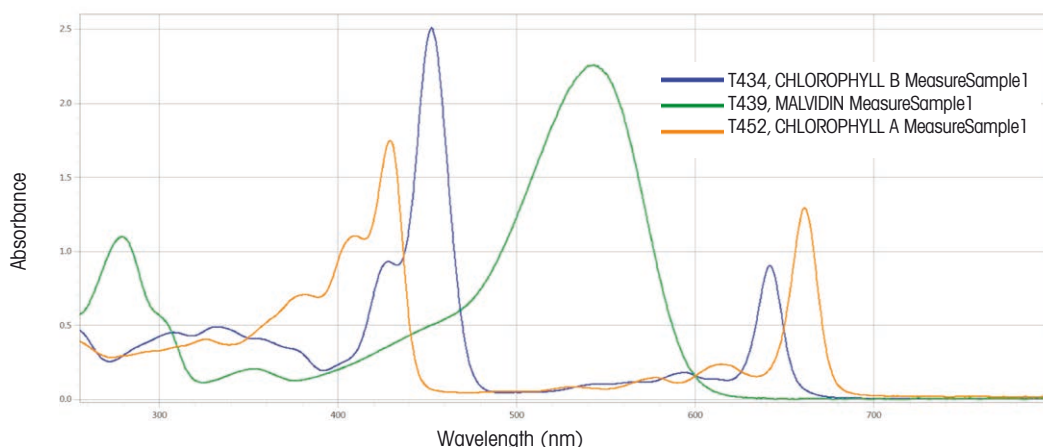
Figure 17: Each sample has a specific UV/Vis spectrum. This unique relationship allows for characterization of the sample. In this case, the compounds malvidin, chlorophyll a and chlorophyll b can be easily recognized by their individual spectra

3.3   Quantitative analysis: Concentration determination

Based on the Lambert-Beer Law, the concentration of a compound in a solution can be determined quantitatively by UV/Vis spectroscopy. To perform that, a calibration curve is first determined by measuring the absorption of several standard solutions of known concentration. In this way, the concentration of samples such as, DNA, RNA, proteins, carbohydrates or organic compounds can be determined.
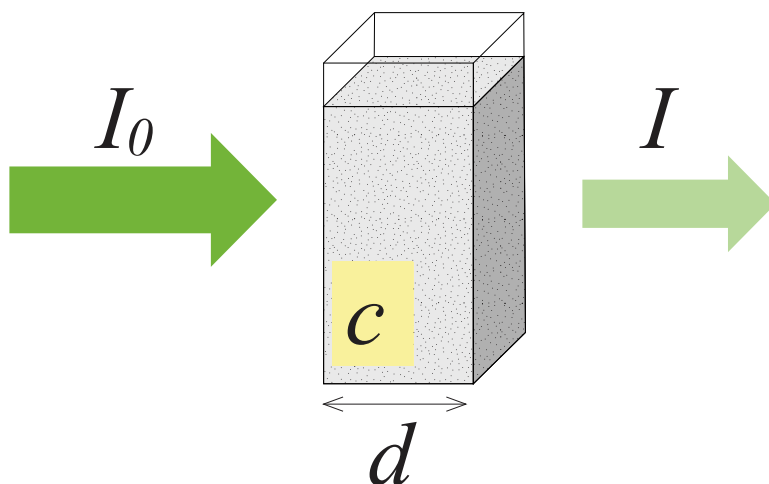
Figure 18: The attenuation of the light beam allows for the determination of sample concentration

The linear relationship between absorbance and concentration of a sample opens the door for a variety of quantitative analyses. In the following table, three main applications of the Lambert-Beer law are given:

|	Analyte / Parameter	Market segment
Concentration determination	– Metal ions e.g. iron, copper, nickel – Inorganic ions, e.g. nitrate – Chemical Oxygen Demand (COD)	– Pharma – Water – Food and Beverages – Electroplating
Monitoring analyte concentration vs. time	Enzyme kinetics: determination of rate of catalysis – Glucose oxidase catalyzes the oxidation of β-D-Glucose by oxygen (725 and 415 nm) – Oxidation and reduction of pyridine nucleotides – (NAD+/NADH, 340 nm). – Cholesterol oxidation rate by catalysis with Cholesterol Oxidase (500 nm) – GPO colorimetric kinetic test for tryglycerides (520 nm)	– Pharma (Kinetics)
Physico-chemical parameters	– Acid dissociation constant pK_a – Complex formation constant K_f – Partition/Distribution coefficient $logP$ / $logD$ – Dissolution tests	– Pharma – Research

3.3.1   Calibration curve

To determine an unknown concentration of a sample solution by UV/Vis spectroscopy, a calibration curve must first be created. This is done by measuring the light absorption of several standard solutions of different, known concentrations at a predefined, fixed wavelength. In the following example, 5 standard solutions of increasing concentrations were measured at a predefined wavelength:
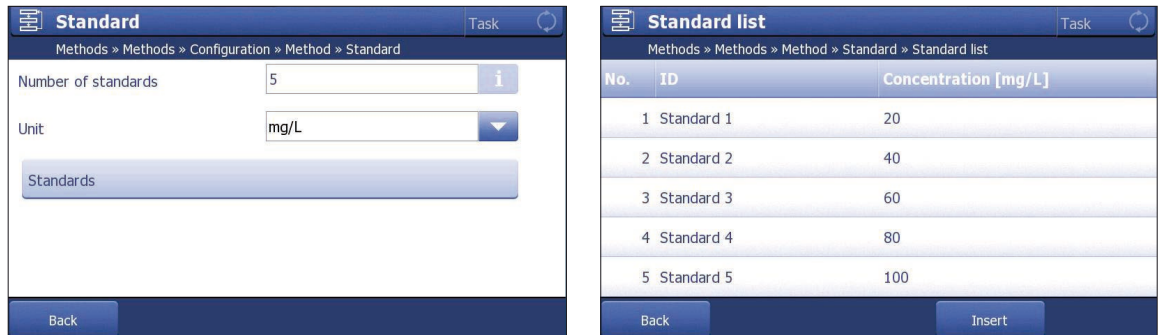
Figure 19: The calibration step and the standard list to determine the calibration curve are indicated on the instrument display

A calibration curve was obtained by plotting the absorbance values as a function of the concentration. Finally, a linear regression of the measured values gives the calibration curve:
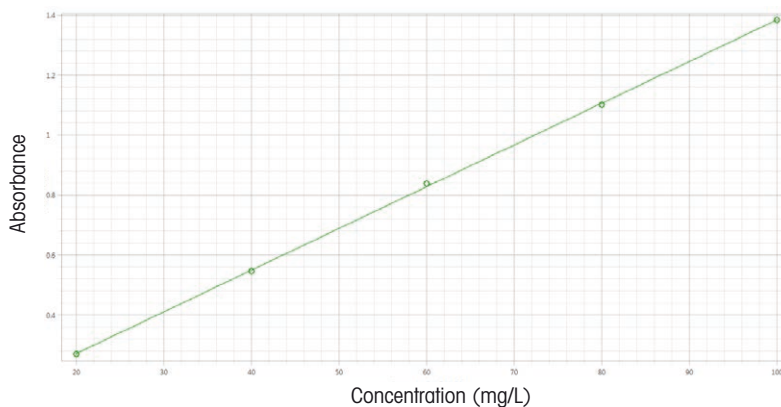
Figure 20: The absorbance values of standard solutions are determined. A linear regression of the data points gives the calibration curve

3.3.2   Sample determination

Using the calibration curve, an unknown sample can now be determined from its absorbance:
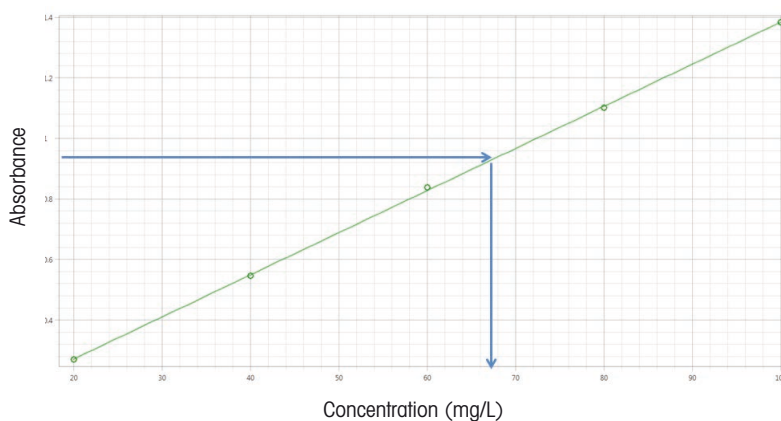
Figure 21: Concentration determination:
The absorbance of an unknown sample solution is measured and compared with the calibration curve to determine its concentration

4.   Spectrophotometer Design

A spectrophotometer generally consists of four components:

1. A suitable **light source** which covers the UV/Vis spectrum of interest. In general, a lamp containing a gas such as xenon, or a combination of two different lamps such as tungsten/deuterium is used.

An appropriate **sample holder** is needed to hold the sample.

-   **Liquid samples** are best to be placed in optical or quartz glass cuvettes. For less demanding measurements, disposable plastic cuvettes can be used as an alternative. However, glass and acrylic plastic do not transmit UV light and should only be used for measurements in the visible light range.
-   **Solid samples** can be mounted into a suitable holder to be positioned in the optical path of the spectrophotometer for measurement of the transmitted light.

2. A **dispersion element** is needed to distribute the light into separate wavelengths. It can be either a **quartz prism** or a **diffraction grating**, i.e. an optical component with a periodic structure able to diffract light.

3. Finally, the transmitted light intensity is recorded by a suitable **detector** such as photomultiplier, a multi-channel array (e.g. a photodiode array, or PDA), or a charge-coupled device (CCD), similarly to a digital camera. Both PDA and CCD detectors use a photosensitive semiconductor material to convert the light into an electronic signal which is then recorded by the instrument.

4.1   Design comparison

UV/Vis spectrophotometers can be classified according to the geometry of the components building up the optical system for the recording of spectra. The following two configurations are generally used in UV/Vis spectroscopy:

-   Scanning spectrophotometer
-   Array spectrophotometer

4.1.1   Scanning spectrophotometer

The working principle of a conventional scanning spectrophotometer is based on the measurement of the transmittance value at each single wavelength. The light is first dispersed into individual wavelengths using a reflection grating. The grating is rotated in order to individually select each wavelength that is then sent through a cuvette. The transmittance at this specific wavelength is recorded. The whole spectrum is obtained by continuously changing the wavelength of the light (i.e. scanning) incoming onto the sample solution by rotating the grating:
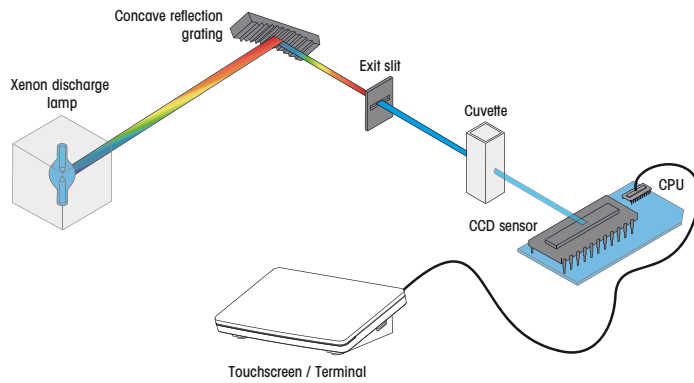
Figure 22: Scanning spectrophotometer: Light beams of specific wavelengths are directed onto the cuvette

Note that scanning spectrophotometers takes some time for a full spectrum scan because the grating has to be mechanically rotated by a motor. The scanning process may also lead to decrease in accuracy and reproducibility of the wavelength selection depending on the scanning speed of the spectrophotometer.

4.1.2   Array spectrophotometer

In this configuration, the sample is illuminated by a light beam consisting of all spectral components of the UV/Vis range – a continuum. In other words, the sample in the cuvette simultaneously absorbs different wavelengths of light. The transmitted light is then diffracted by a reflection grating located after the cuvette, as shown in the diagram below.
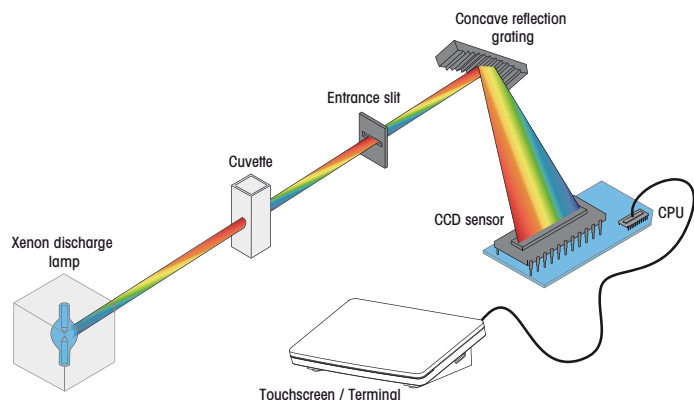
Figure 23: Array spectrophotometer: Light of the whole spectral range is directed onto the sample cuvette

This design is also known as "reverse optics"; only after passing through the sample the light is diffracted by the grating. Subsequently, the diffracted light of various wavelengths is directed onto the detector. The detector, with its long array of photosensitive, semiconductor material, allows for simultaneous measurement of all wavelengths of the transmitted light beam.

With this setup, measuring the whole UV/Vis spectrum is generally faster than using a conventional scanning spectrophotometer since the spectrum is recorded simultaneously at all wavelengths. Moreover, an array detector has an integrating function which accumulates individual measurements to enhance the signal, leading to a strongly increased signal to noise ratio, and thus to an improved signal quality of the measured spectrum. Array spectrophotometers present an innovative approach to speed up full spectrum scan based on reverse optics technology. The robust design without any moving optical parts ensures very good optical performance.

4.1.3   Optical pathways

UV/Vis spectrophotometers can have either a single beam or double beam optical pathway, i.e. the way that a light beam from the lamp passes through sample cuvette to reach the detector.

- **Single beam configuration**

  The single beam configuration is the simplest and easiest setup for UV/Vis spectroscopy. The light beam is directly guided through the sample onto the detector. A cuvette containing only the solvent has to be measured first in order to determine the blank value (see previous chap.). After measuring the blank value, the solvent cuvette is replaced by a cuvette containing the sample. The latest is measured to get the absorption spectrum of the sample.

Light source

Detector
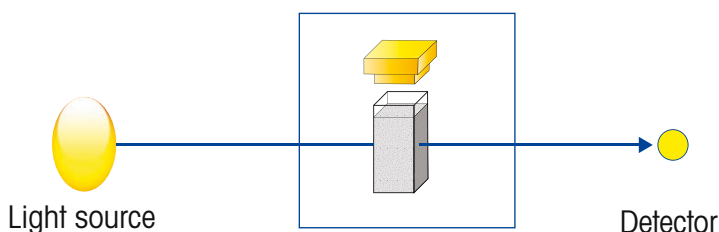
Figure 24: Single-beam optical pathway

- **Double-beam configuration**

  In a double-beam configuration, the light beam is split into a reference and a sample beam. Two different options are available for the optical pathway

  – **Simultaneous in time:**

    The light beam of the lamp is split into two beams of equal intensities. Each beam passes through a different cuvette; the reference cuvette, which is filled with solvent only, whereas the second cuvette contains the sample solution. The intensities of both beams are measured simultaneously by two detectors. For accurate results cuvettes need to be matched...

  – **Alternating in time:**

    This configuration is achieved by directing the light path with an optical chopper (OC), which is a rotating sectional mirror. The light is directed alternately through a sample and a reference cell. A unique detector measures both light beams one after the other.
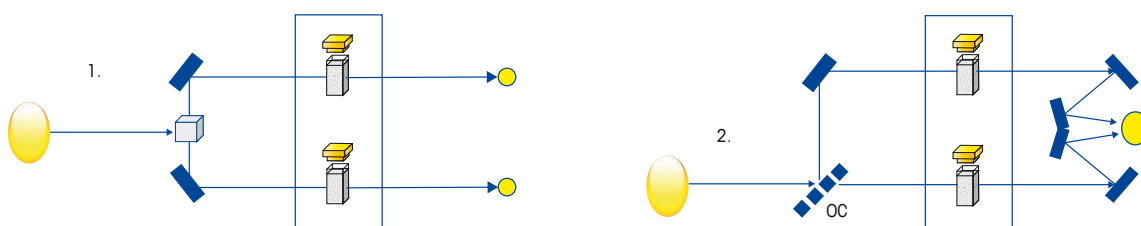
Figure 25: Double-beam optical pathway: 1. Simultaneous in time, and 2. Alternating in time with an optical chopper (OC)

4.2   Cuvette-based UV/Vis spectroscopy

METTLER TOLEDO developed a single beam array spectrophotometer that allows fast and accurate measurements over the UV/Vis range. The light source consists of a Xenon flash lamp for the ultraviolet (UV) as well as for the visible (Vis) and near-infrared wavelength regions covering a spectral range from 190 up to 1100 nm. The lamp flashes are focused on a glass fiber which drives the beam of light onto a cuvette containing the sample solution. The beam passes through the sample and specific wavelengths are absorbed by the sample components.
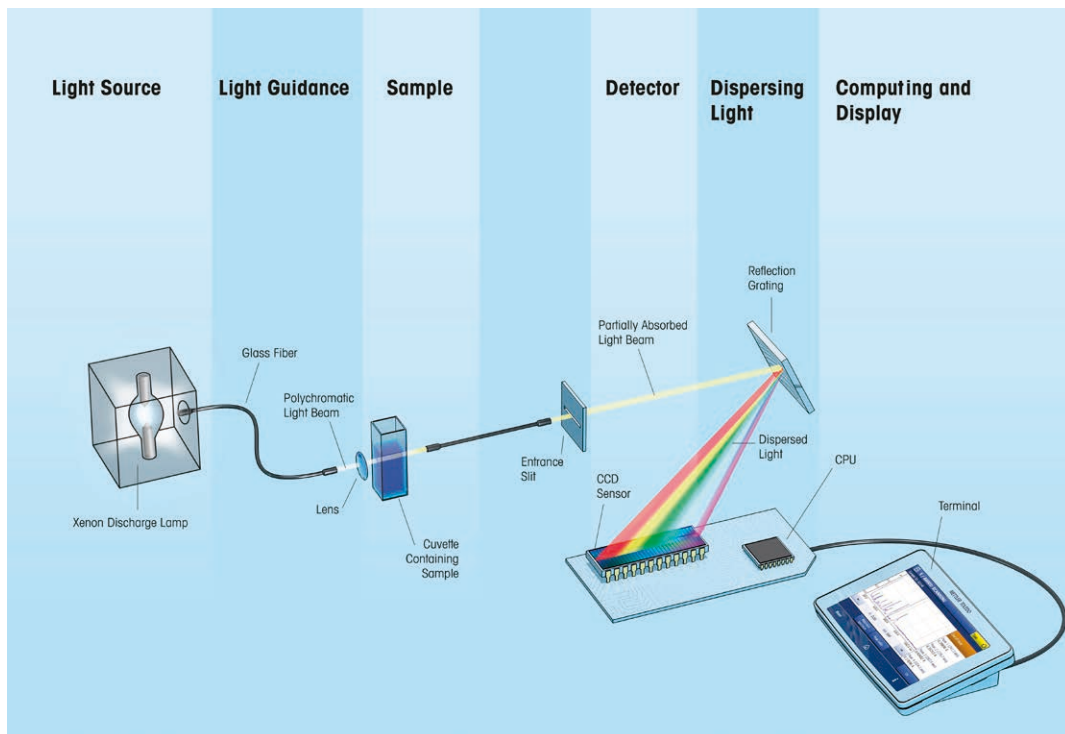
Figure 26: Cuvette-based single-beam array spectrophotometer:
The light beam from the lamp is passing through the sample, it is diffracted by the grating and directed onto the array detector

The remaining light is collected after the cuvette by a glass fiber and driven into a spectrograph. The spectrograph consists of a diffraction grating that separates the light into the different wavelengths, and a CCD sensor to record the spectra, respectively. The whole spectrum is thus simultaneously measured, allowing for fast recording.

4.3   Micro-volume UV/Vis spectroscopy

METTLER TOLEDO provides a spectrophotometer capable of performing micro-volume UV/Vis measurement. This instrument is capable of measuring very small volumes and highly concentrated samples. The method is fairly straightforward. The sample is pipetted directly onto the measuring platform, without further dilution. Therefore, manipulation errors are avoided. Moreover, the selection of a specific path length allows for the measurement over a large concentration range with as little as 1 µL of sample.

The measurements are performed at the micro-volume platform covered by a movable arm mounted on the top of the instrument. Spectrophotometer has both a micro-volume platform as well as a cuvette holder. Depending

on the selected application, the light can be directed either onto the micro-volume platform or the 1 cm cuvette. The transmitted light is then focused on the grating where diffraction occurs. The diffracted light beams of different wavelengths are then directed onto the detector.
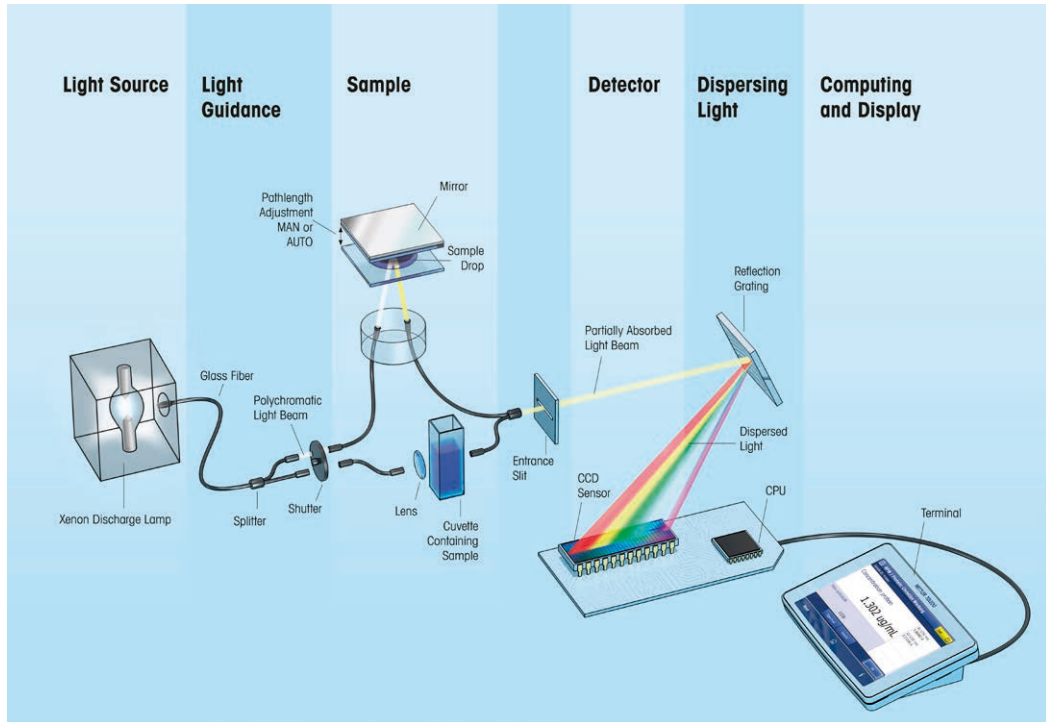
Figure 27: Micro-volume array spectrophotometer:
The light beam is directed to pass the sample on the micro-volume platform. Alternately, the beam may be directed on a 1 cm cuvette

When the arm is in open position, the micro-volume platform can be easily accessed with a pipette from either the left or the right side. The curved lid on top of the instrument allows convenient positioning of the operator's hand to securely guide the pipette tip. During measurement, the arm is securely locked to a precisely defined path length and cannot be opened until the measurement is completed.

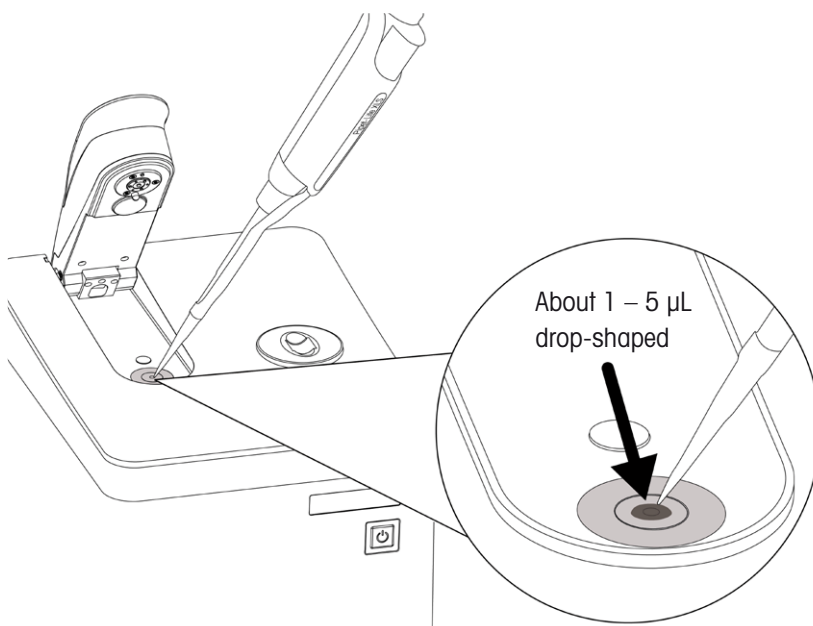Figure 28: Micro-volume array spectrophotometer: The sample is added by a pipette on the micro-volume platform.
The movable arm is then closed for measurement

5. Applications

5.1 Fixed wavelength

The Fixed Wavelength (FW) measurement is the simplest application of a spectrophotometer. It is a single or multiple wavelength measurement and, as for all other measurement types, the result can be reported in absorbance or transmittance. Further calculations can be done to obtain the final result, for example, a concentration of a substance.

5.1.1 FW application in food and beverage

In the food and beverage industry, UV/Vis spectrophotometry is used to monitor and improve product quality and consistency. Furthermore, the influence of packing material and stabilizers as well as chemical deterioration and degradation processes can also be observed with this method.

A typical application in this market segment is the check for the purity of olive oil, which enables the product to be classified as "Extra Virgin", "Virgin", or simply "Olive Oil". Standards are in place for the evaluation of olive oil based on the absorbance characteristics of certain molecules in the UV/Vis spectrum. Olive oil contains about 98% triglycerides. Unsaturated fatty acids in the oil are susceptible to breakdown and oxidation. The oxidation of free fatty acids causes the formation of peroxides. This leads to rancidity and degradation of the olive oil over time. Beside other parameters, this effect is evaluated by the conjugated di-enes and tri-enes of unsaturated fatty acids (conjugated C=C double bonds) which absorb in the range of 230 to 270 nm.

The International Olive Committee's (IOC) olive oil standards specify exactly the measurement threshold which has to be met for oils in order to be graded as extra virgin, virgin, and so on. The quality of the oil is determined by observing the absorbance behavior of a 1% solution in isopropanol between 200 and 400 nm. Elevated levels of absorbance in the spectral range from 200 to 400 nm indicate oxidized (poorer quality) oil. The absorbance at K232 nm, K270 nm and ΔK correlate with the state of oxidation by detecting specific oxidized compounds and also detect possible adulteration with refined oils. In the next figure we can see the ultraviolet spectra of good quality Extra Virgin Olive Oil showing no peaks, compared to the obvious peaks of a poor quality olive oil, presented as olive oil sample 4 and 5.
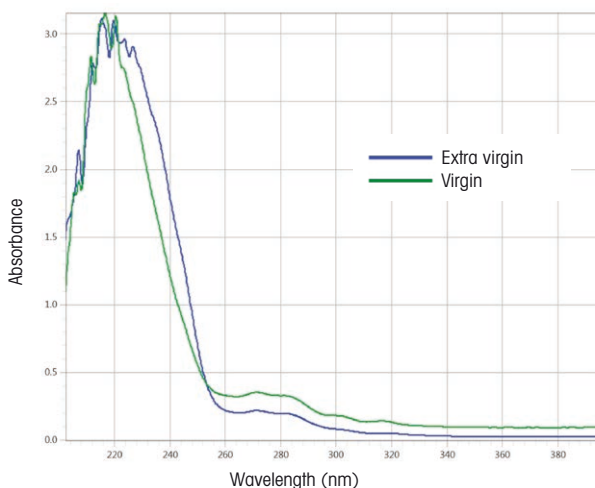

Figure 29: Absorption measured in a 1% solution, of extra virgin olive oil (blue) and virgin olive oil (green)

Figure 30: Olive oil

Low values correlate with high-quality oil, as UV absorbance detects early and later states of oxidation. This can be observed according the table below, which is regulated according to the EEC regulation (European Economic Community; The council of the European Communities):

	K232	K270	ΔK
Extra virgin	≤2.5	≤0.22	≤0.01
Virgin	≤2.6	≤0.25	≤0.01
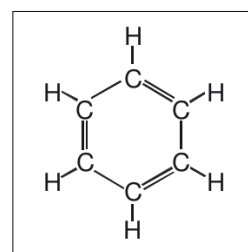
5.1.2 FW application in chemical industry

UV absorption spectroscopy is one of the best methods for the determination of the purity of organic solutions. Additional peaks appearing at specific wavelengths can be observed due to impurities in the sample.

An example in the chemical industry is the purity control in alcohol. Alcohol can be contaminated by benzene, which absorbs light at 280 nm, whereas alcohol absorbs at 210 nm. The measurement of the UV/Vis spectrum can easily tell if the sample is contaminated if an extra peak is present at 280 nm.

5.2   Concentration determination by quantification

The quantification or concentration determination of a substance by UV/Vis spectroscopy is based on the Lambert-Beer Law, described in chapter 2.4, which states that the absorbance of a solution is directly proportional to the concentration of the absorbing substance in the solution and the path length of the cuvette. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorbing substance in a solution. However, it is necessary to know how much the absorbance changes with concentration. This can be taken from references, such as tables of extinction coefficients, or more easily, determined from a calibration curve.

The first step in a quantitative analysis is to select a suitable wavelength. The wavelength is normally chosen at a peak maximum, i.e. at the peak of the absorption band, because the change in absorbance for a given concentration change is maximal, leading to greater sensitivity and accuracy in the measurements. The relative effect of other substances or impurities is thus smaller. Furthermore, the rate of change of absorbance with wavelength is smaller, and the measurement is not so severely affected by small errors in the wavelength setting.

The next step of quantification is the measurement of known standards at the chosen wavelength. The absorbance of the standards is then plotted against the concentration as shown in the figure of chapter 3.3.1. The calibration curve for a spectrophotometric analysis should approximate the sample as closely as possible and encompass a suitable range of substance concentrations. Ideally at least three different concentrations of the substance are needed, although a single one can be applied. In practice, five different concentrations will produce a more accurate calibration curve. The absorbance presents a linear relationship to the concentration and a first order regression curve can be fitted to the data points.

A sample of an unknown concentration can then be determined using the calibration curve.

5.2.1   Colorimetric determination of phosphate

Phosphorus is the eleventh most abundant element on the surface of the earth and is most commonly found as phosphate. It plays an important role in biochemical processes and is a key factor in surface water. Increased phosphate concentrations are linked with increasing rates of plant growth. Thus, the analysis of phosphorus is very important in many fields, including medical and clinical science, agriculture, metallurgy and environmental science. Moreover, in recent years large quantities of phosphate have been used in beverages, detergents, fertilizers and also in sugar industries. In this context, the colorimetric determination of phosphate in various samples of different market segments is performed using UV/Vis spectrophotometry. The basis of the colorimetric technique is the direct relationship between the intensity of the color of a solution and the concentration of the colored component (the analyte species) which is contained.

Phosphate will readily react with ammonium molybdate in the presence of suitable reducing agents to form a blue colored complex, the intensity of which is directly proportional to the concentration of phosphate in the solution. The phosphate content of an unknown sample can be obtained by first plotting the absorbances of a series of standard solutions against the corresponding concentrations, thus giving a calibration curve. The unknown concentration of phosphate in the sample can then be determined from the graph.

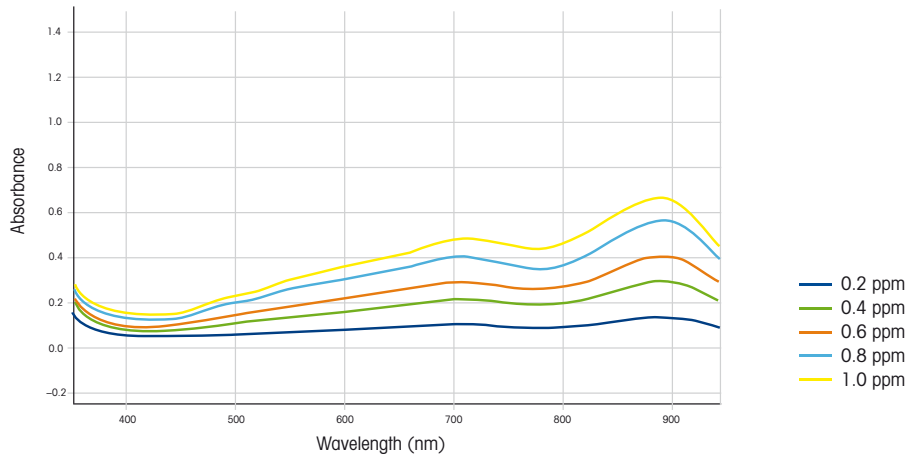Figure 31: Series of prepared phosphate standard solutions

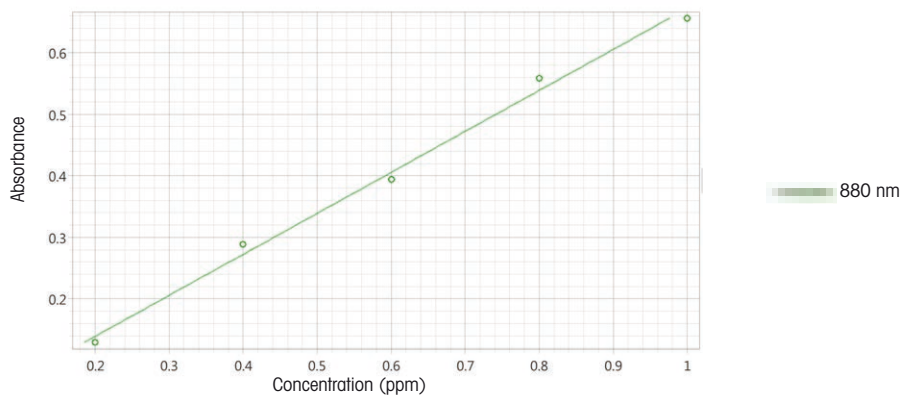Figure 32: Plotted absorbance spectra of the corresponding concentrations

Figure 33: Absorbance values at 880 nm vs. the concentration of each standard solution leading to the calibration curve. The unknown concentration of phosphate in the sample can be determined from the linear regression curve

5.3 Scanning

In contrast to the fixed wavelength measurement, the spectral scanning measurements determine the absorbance or transmittance of a sample over a specified wavelength range or over the full spectrum range, typically from 190 to 1100 nm. Following the scan measurement, the most often applied analysis is the detection of peaks and valleys in the spectrum. A peak is where the absorbance reaches a maximum and a valley is where the absorbance is smallest between two peaks. The height and location of peaks and valleys is of interest as it gives an indication of the sample composition and purity. For example, from the location of peaks and combination of peaks, it can be concluded whether the compound is saturated or unsaturated. Furthermore, the identification of a compound can be managed by comparing the spectrum to a known compound spectrum from a database. Scanning UV/Vis methods can be used for characterizing aromatic compounds and aromatic olefins.

5.3.1 Scanning application of nicotinamide in food and beverage

As an example, the spectrum of nicotinamide adenine dinucleotide (NAD^+) is shown below. NAD^+ is an important coenzyme found in all living cells. Due to the presence of several aromatic rings in the adenine base it absorbs light in the UV range. Running a scan analysis with an NAD^+ sample shows that the maximum absorbance occurs at 260 nm. The absorbance value at the maximum in the following example is 1.
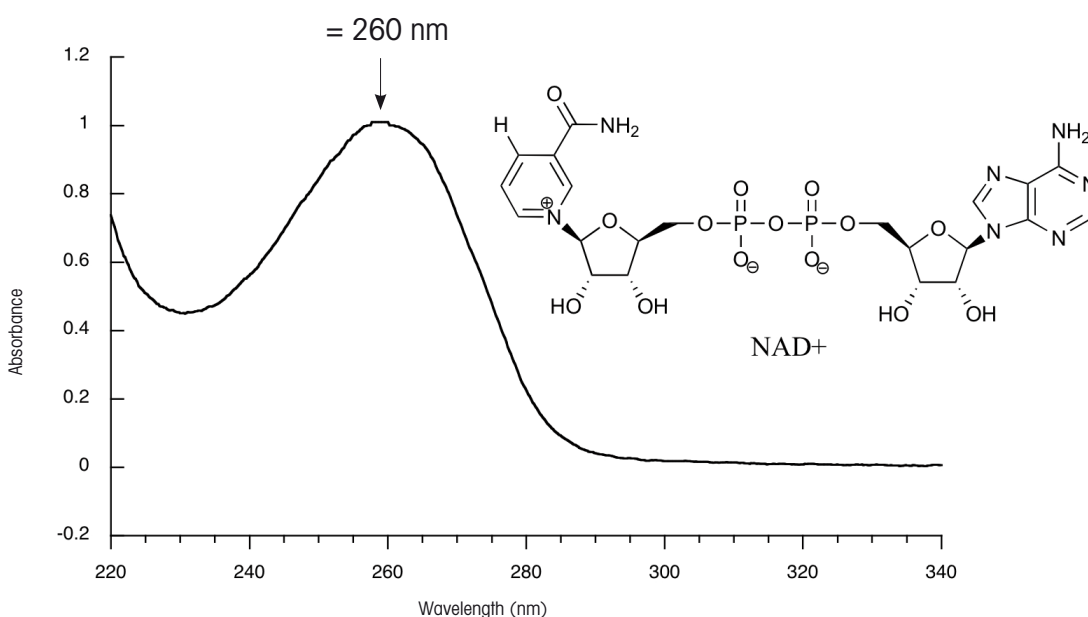
Figure 34: Spectrum of NAD^+ showing the absorbance maximum at 260 nm and the resulting absorbance of about one

Typically, two parameters are of importance and recorded from a UV/Vis spectrum:

- Lambda max: the wavelength of the analyte where the maximum absorbance is reached.
- The amount of absorbed light in absorbance units, detected at the lambda max.

5.3.2 Analysis of sunscreen in the cosmetics industry

The increasing awareness of the risks of skin cancer with sun exposure requires that sunscreen products are appropriately tested and labeled. The various sunscreen formulations available on the market demand a sophisticated analysis method.

Sunscreens either reflect or absorb the ultraviolet (UV) radiation before they reach the skin. The spectral region that must be blocked in sunscreen products is the UV-A and the UV-B region, which is between 280 – 400 nm. The more energetic UV-C light is already blocked by diatomic oxygen (from 100 – 200 nm) or by ozone (triatomic oxygen) (200 – 280 nm) in the atmosphere. The active ingredient in sunscreen that protects the skin from sunlight must be present in sufficient quantity and uniformity to ensure blocking of UV-A and UV-B light so that the skin does not get sun burned.

The traditional method for the effectiveness of sunscreen analysis is based on a quantitative analysis of a diluted sample. A series of standards based on different concentrations of the active ingredient are measured and a quantitative method based on Lambert-Beer's Law is developed. Representative spectra of two different sunscreen formulations are presented in the figure below.
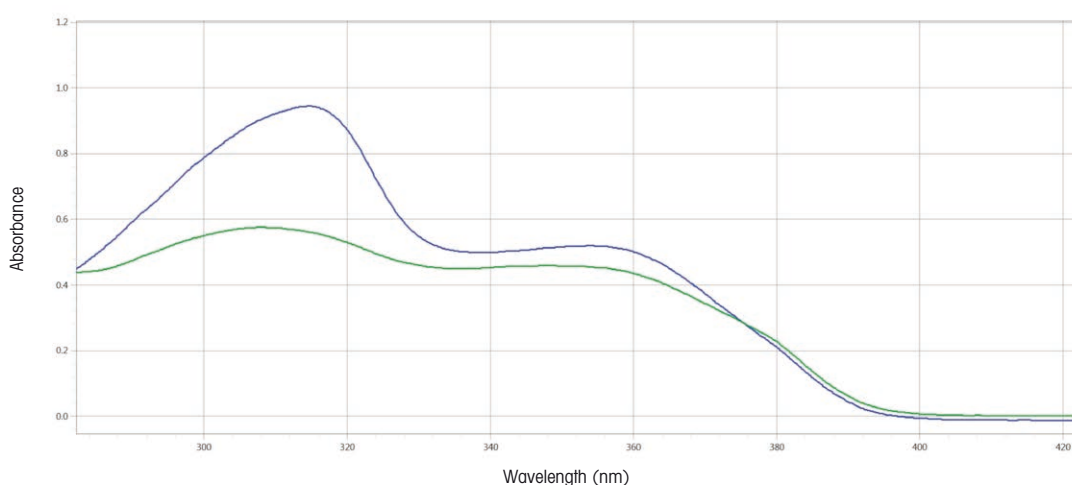
Figure 35: Sunscreen spectral scan. Formulation A (blue) with higher uv light absorbance compared to formulation B (green) in the range of 280 nm up to 350 nm

The first formulation, visualized in blue, absorbs most of the UV radiation between 280 and 350 nm, which would indicate that this sunscreen formulation would be a strong protector. In contrast, the second formulation, depicted in green in the spectrum, absorbs only around 60% of the radiation between 280 and 350 nm.

The final sunscreen formulation can be measured directly using two UV transparent glass plates between which a thin layer of the product is spread. The squeezed glass plates are placed on a solid sample holder in the sample compartment prior to the spectrum measurement.

5.3.3   Identification of cyanocobalamin (vitamin B12) in the pharmaceutical industry

Ultraviolet and visible absorption spectroscopy is a useful technique for the identification of pharmaceutical compounds. Several examples of single-component spectroscopic assay of vitamins such as cyanocobalamin (vitamin B12), riboflavin, folic acid and vitamin A and are included in the US Pharmacopoeia (2015). Cyanocobalamin is often identified using UV/Vis Spectrophotometry by the scanning wavelength mode. Furthermore, the concentration determination by a quantification using the cyanocobalamin USP reference standard is performed at 361 nm. The content of cyanocobalamin is calculated taking into account the specific absorbance ($E_{1cm}^{1\%}$ =207), the extinction coefficient for a 1% solution measured in a 1 cm cuvette.

For the identification and characterization of cyanocobalamin the spectral range between 200 and 700 nm is selected and peaks are identified. The standard solution shows 3 absorption maxima, at 278, 361 and 550 nm. The acceptance criteria of the absorption spectrum is given as following: 278 ± 1 nm, 361 ± 1 nm, and 550 ± 2 nm. The absorbance ratio A361 / A278 is 1.70 – 1.90, and the absorbance ratio A361 / A550 is 3.15 – 3.40 in order to fulfill the acceptance criteria.
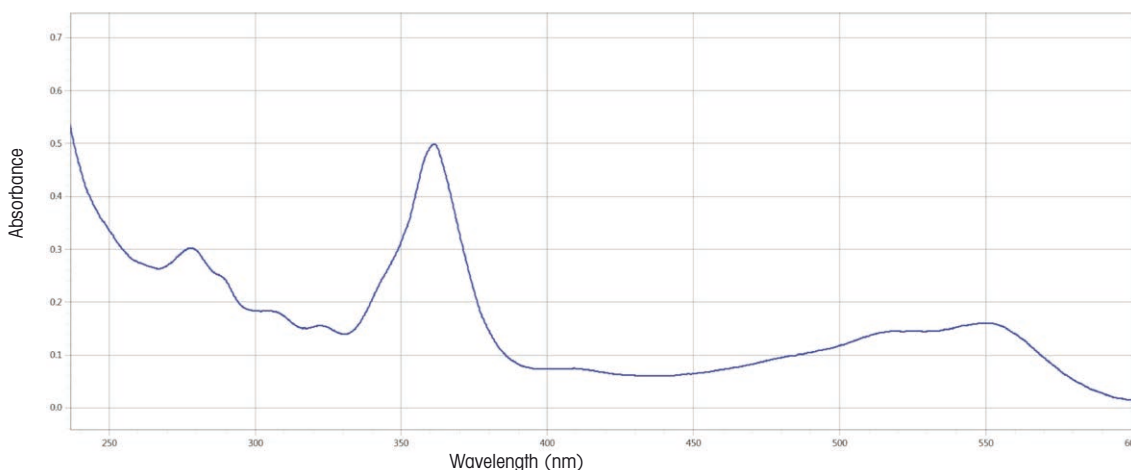
Figure 36: Spectrum of 3 mg / 100 ml cyanocobalamin is presented. Three absorption maxima at 278.5 nm, 361.5 nm and 550.9 nm can be observed.

5.4   Kinetics

UV/Vis spectrophotometry is often used in order to monitor the change of the concentration of either the reactant or the products by absorbance at a specific wavelength over time. This is a reaction as a function of time and therefore often called rate measurements. Kinetics measurements are used to investigate enzyme activity or re-action rates as well as the affinity of the enzyme-substrate interaction. This analysis type is especially prevalent in the field of biotechnology, medicine and food as well as in chemistry. Kinetic methods are particularly useful for samples in which some interfering components are present in varying concentration from sample to sample. For example, UV/Vis absorption spectroscopy is applied in colored samples such as whole blood, bottled/ canned soft drinks and juices. A specific analysis can be performed by measuring the rate of change in absor-bance of a sample without having to do complicated and time-consuming chemistry to eliminate the interfering colored background or to apply some separation method.

The most widely encountered application of spectrometric rate measurements is to study enzymes (proteins that function as catalysts). Direct enzyme measurements and concentration determination can be performed, but since this is very elaborative and chemicals are expensive, enzymes are rather analyzed for their catalytic prop-erties and the reaction they catalyze. The indirect detection and measurement of enzymes can be performed by molecules that are modified by the enzymes or substrates as well as by molecules that cooperate with enzymes, also referred to as coenzymes. Enzymes are highly specific and sensitive, permitting quantitative analysis with little or no sample preparation.

5.4.1   Enzymatic determination of glucose in food products

Typical applications in the food and beverage segment are the quantitative enzymatic determination of carbo-hydrates like sucrose, glucose, fructose, starch and total dietary fiber. Since enzymes are highly specific for a particular molecule, measurements are reproducible, rapid and are suitable for quantitative analysis without the need for any sample preparation such as purification.

Many enzymatic reactions occur concurrently with the following enzyme system: A nicotinamide adenine di-nucleotide coenzyme system in which the reduced form of nicotinamide adenine dinucleotide, abbreviated as NADH, shows the absorption maximum at 340 nm with an extinction coefficient of 6'220 mol/(L*cm). The oxidized form of the molecule, NAD^+, does not absorb at this wavelength, but both, NAD^+ and NADH, present a peak absorbance at 260 nm. NAD^+ has an extinction coefficient of 16'900 mol/(L*cm). The following figure shows the spectra of both compounds with the NADH spectrum in green and the substrate NAD^+ in blue.
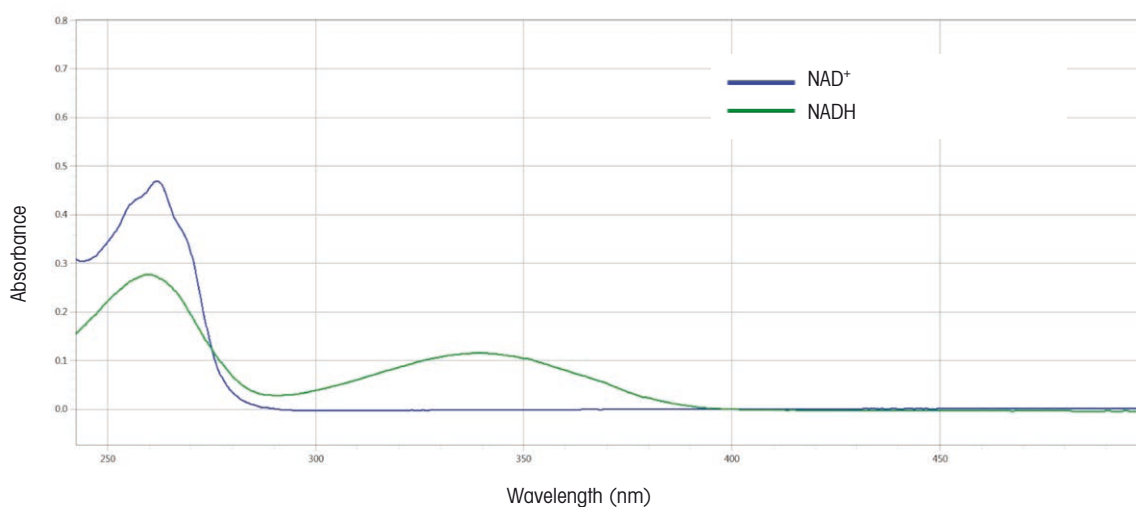
Figure 37: Absorbance Spectrum of NAD^+ and NADH

Carbohydrates can be determined by an enzymatic reaction in a kinetics approach. For the enzymatic analysis of glycose, two enzymes from the glycolysis process are typically used. Glycolysis is the degradation of glucose and an important process in all living creatures. The first step of the glycolysis is the phosphorylation of glucose by the enzyme hexokinase, where ATP is used and converted to ADP.

$$Glucose + ATP \xrightarrow{\text{Hexokinasa}} Glucose\text{-}6\text{-}phosphate + ADP$$

$$G6P + NAD^+ \xrightarrow{\text{G6PDH}} 6\text{-}Phosphogluconate + NADH$$
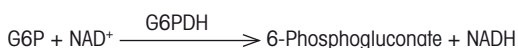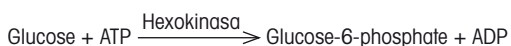
Thereafter, the glucose-6-phosphate is then oxidized to 6-phosphogluconate in the presence of oxidized NAD^+ in a reaction catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NAD^+ is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to the original glucose concentration in the sample.

This indirect enzymatic detection of glucose can equally be applied for the detection of ATP. Both substances can be determined via this coupled reaction of the hexokinase and glucose-6-phosphate dehydrogenase.

5.4.2   Alkaline phosphatase enzyme activity

Alkaline phosphatases are a group of enzymes of the Hydrolase class that split off a terminal phosphate group from an organic ester in alkaline solution. Their optimum pH is usually around pH 10, but this varies with the particular substrate and isoenzyme. These enzymes differ in amino acid sequence but catalyze the same chemical reaction and usually display different kinetic parameters or different regulatory properties. Alkaline phosphatases are available in almost every tissue in the body and serum levels of this enzyme are of interest in the diagnosis of several diseases.

Alkaline phosphatase catalyzes the hydrolysis of ρ-Nitrophenyl phosphate (pNPP) to ρ-Nitrophenol. When the enzyme alkaline phosphatase reacts with pNPP, the inorganic phosphate and ρ-Nitrophenol are produced. pNPP is colorless but ρ-Nitrophenol has a strong absorbance at 405 nm, presenting a stable yellow color in alkaline solution. At the absorption maximum, the substrate concentration $[S]$ and velocity $[v]$ can be measured. The rate of formation of ρ-Nitrophenol is measured as an increase in absorbance at 405 nm which is proportional to the enzyme activity in the sample.

The procedure is standardized under the specified conditions by the molar extinction coefficient of ρ-Nitrophenol 18.75 mol/(L*cm) at 405 nm. The results are based on the change in absorbance per unit of time. The International Unit IU/L is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

This application is mainly used in medical diagnostics. Alkaline phosphatase are found in high concentrations in the liver, in the epithelium of the biliary tract and in the bones. Normal levels are age dependent and increase during bone development. Increased levels compared to normal levels are associated mainly with liver and bone disease. Typical samples are heparinized plasma and serum, which is free from hemolysis.

5.4.3   Oxidation of iodide by hydrogen peroxide

The kinetics of a reaction, the evolution of a chemical reaction in function of time, is described by rate laws and rate constants. In order to apply the correct law and constants to a specific chemical reaction, one needs to know its order, i.e. the relation between the concentration and the rate of the reaction. When the reaction rate is directly proportional to reactant's concentration, the reaction is of the first order regarding this reactant. Similarly, when the concentration of a reactant has no influence on the reaction rate, it is of zero order regarding this reactant. Second order reactions have a reaction rate that is proportional to the square of a reactant's concentration and so on.

The goal of this study is to determine the reaction order of the oxidation of iodide by hydrogen peroxide in acid solution. The course of the reaction is monitored via the formation of tri-iodide (I_3^-):

$$H_2O_2(aq) + 3I^-(aq) + 2H_3O^+(aq) = I_3^-(aq) + 4H_2O$$

The determination of the reaction orders of the reactants is achieved by performing a series of experiments in which the concentration of one of the reactants is varied whereas the concentrations of the other reactants remain constant. The kinetics of the reaction was followed by measuring the intensity of the absorption band at 353 nm that is related to the formation of tri-iodide. The following figure shows the increase of absorbance at 353 nm as a function of time for four samples with increasing peroxide concentrations.
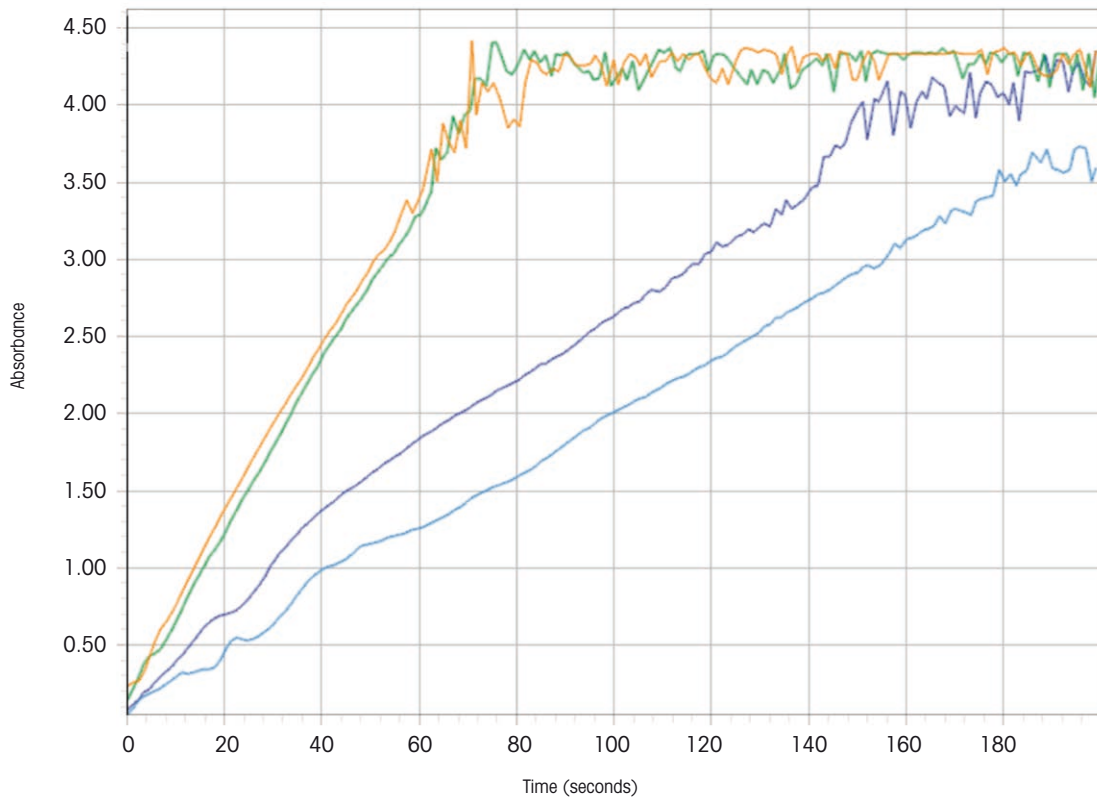
Figure 38: Absorbance values at a fixed wavelength of 353 nm as a function of time for increasing peroxide concentrations:
light blue = 0.004 M, dark blue = 0.006 M, green = 0.008 M and orange = 0.01 M of peroxide

Similar measurements have to be performed varying the amount of the two other reactants, the iodide and the acid. Reaction orders can then be determined from the measurement of the initial reaction rates. If the reaction rate is proportional to the concentration of the reactant, a plot of the initial rate (V_0) as a function of concentration will be linear.
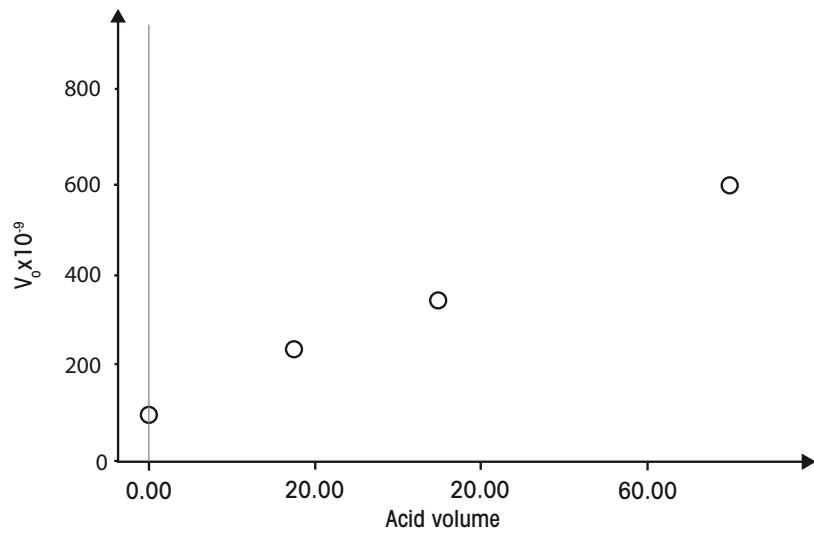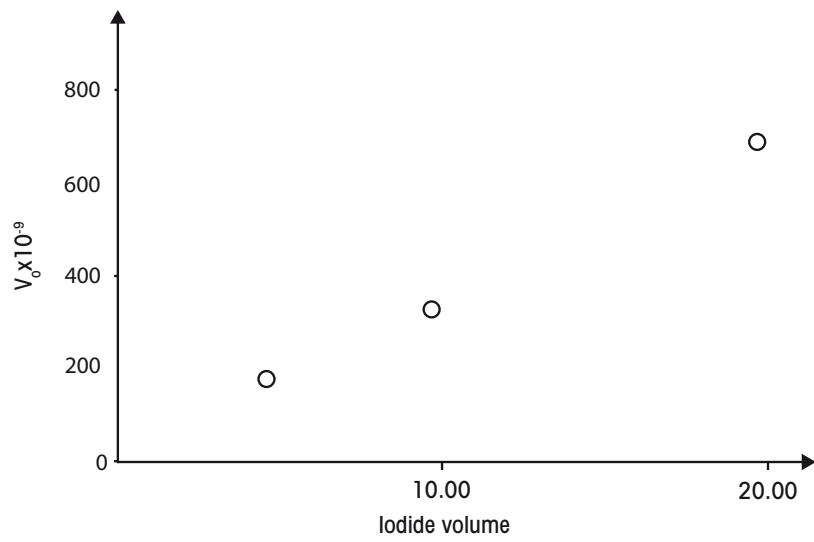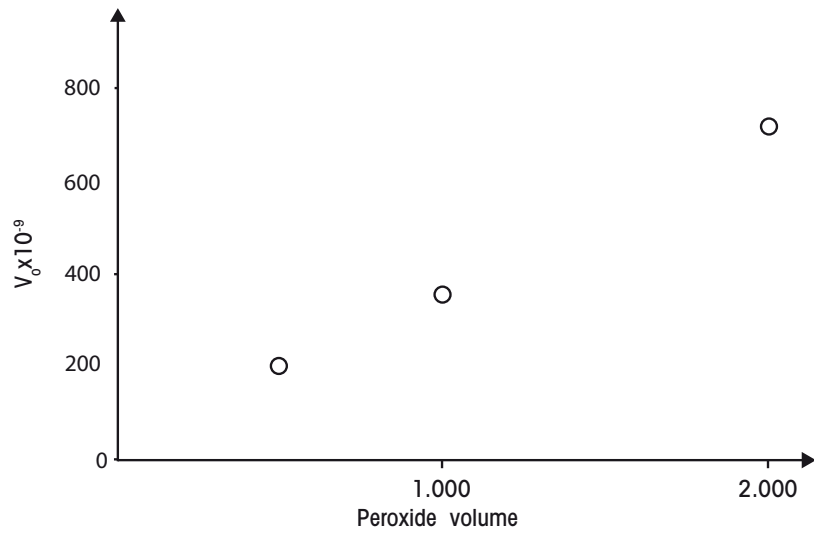
Figure 39: Initial rates as a function of the reagent volume for the three reagents; peroxide (top), iodide (middle) and hydronium ions for various acid concentrations (bottom)

The above figure shows plots of initial rates as a function of reagent volume for the three reagents: peroxide H_2O_2, iodide I^- and hydronium ion H_3O^+:

(a) the initial rate increases linearly with the concentration of peroxide

(b) the initial rate increases linearly with the concentration of iodide

(c) the initial rate increases linearly with the acid concentration but at zero concentration the initial rate is not zero

From these observations, the experimental relationship between the initial reaction rate and the concentrations of reagents (concentration is expressed with the square brackets) can be defined as it follows:

$$Rate = x[H_2O_2][I^-](y + [H^+])$$

This can also be written in a more common chemical kinetics form:

$$Rate = k[H_2O_2][I^-] + k'[H_2O_2][I^-][H^+]$$

k and k' are the rate constants or coefficients.

The rate law in this case consists of two terms: The first term is first order with respect to hydrogen peroxide and iodide concentration, whereas it is zero order with respect to acid concentration and is thus second order overall. The second term is first order with respect to the concentration of all reagents and is thus third order overall. Finally, the rate law indicates that the acid acts as a catalyst in the oxidation of iodide by hydrogen peroxide.

Knowing this rate law, the correct rate law description can be applied to fit the measurements and determine the rate constants of the reaction.

5.5   Bio applications

5.5.1   FW application in life sciences

UV/Vis absorbance spectroscopy is the preferred method to estimate nucleic acid concentration such as DNA or RNA and to analyze the purity of a preparation due to its ease-of-use. Purines and pyrimidines in nucleic acids naturally absorb light at the maximum 260 nm. In routine use, for pure samples, it is generally accepted that using a path length of 10 mm and an absorption of 1 A unit is equal to a concentration of 50 µg/ml DNA and 40 µg/ml for RNA. For oligonucleotides, the concentration is around 33 µg/ml but this may vary with the length of the chain and base sequence. Thus an oligo calculator is recommended to get a more precise result for the concentration.

An indicator of nucleic acid sample purity is the ratio of the absorbance at 260 nm over the absorbance at 280 nm. Proteins absorb at 280 nm and therefore DNA contaminated with protein will reduce the ratio value of 260 / 280. Typical sources of contamination in nucleic acid preparation are ethylene-diamine-tetra-acetic acid (abbreviated as EDTA), chaotropic salts and phenol, which produce peaks in the range of 220 to 230 nm region. For this reason, the ratio of the absorbance at 260 nm over 230 nm is often applied.

As we can see in the following graph, proteins show their peak maximum at 280 nm. Neither proteins nor nucleic acids absorb light at a wavelength of 320 nm and therefore, absorbance at 320 nm is used to apply a background correction.
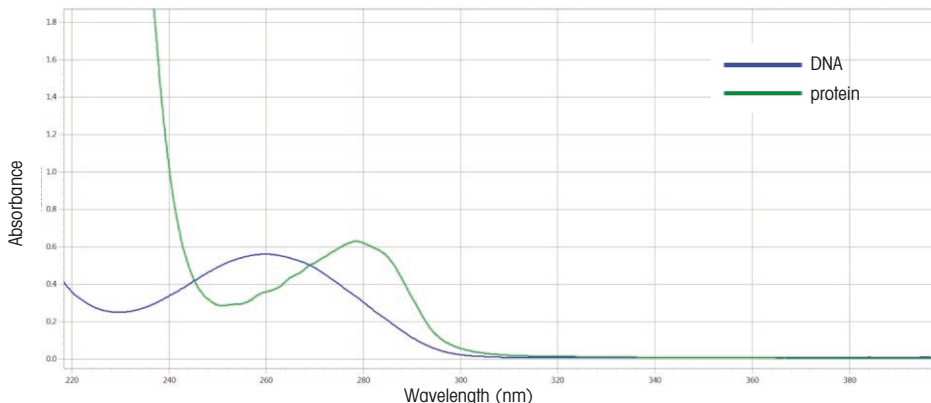
Figure 40: Absorption spectrum of DNA (blue) at 260 nm and BSA protein (green) at 280 nm

5.5.2 Nucleic acid concentration determination and purity according to Christian Warburg

The Christian Warburg method is used to determine the nucleic acid concentration in presence of protein contamination. The method is based on the fact that nucleic acids show their absorbance maximum at 260 nm while proteins at 280 nm. A nucleic acid solution contaminated by proteins will show a higher reading at 260 nm, which can be compensated by the reading at 280 nm.

Originally, Warburg and Christian developed a method to determine protein concentration using the 260 / 280 ratio for compensating the nucleic acid contamination.

Generally accepted extinction coefficients for 1 mg/mL of double strand DNA and 1 mg/mL protein are given in the following table considering 1 cm path length for both wavelengths at A260 as well as A280 nm:

1 mg/mL	A260	A280
Nucleic Acid	20	10
Protein	0.57	1.0

Thus, nucleic acids are expected to have a higher absorbance at 260 nm than at 280 nm, proteins on the other hand, exhibit the opposite behavior. Pure nucleic acid samples are expected to have a ratio of 2.0, while proteins have a ratio of 0.57 respectively. Mixtures containing protein and nucleic acids will differ from these ratios. Theoretically, the 260 / 280 ratio of a solution containing proteins and nucleic acids can be calculated in the following way:

$$\frac{A_{260}}{A_{280}} = \frac{(\varepsilon_{260}p \times (\%P) + \varepsilon_{260}n \times (\%N)}{(\varepsilon_{280}p \times (\%P) + \varepsilon_{280}n \times (\%N)}$$

%P: percentage of protein,

%N: percentage of nucleic acid

The subscripts p and n are the extinction coefficients for the corresponding molecules.

It has to be noted that the absorbance of protein at 280 nm depends only on the aromatic amino acids tryptophan, tyrosine and phenylalanine. Therefore, the reading can be inaccurate and highly depends on the amino acid composition of the protein.

The Christian Warburg formula is used for samples containing both proteins and nucleic acids. In the following example, the total amount of nucleic acid concentration is determined by subtracting protein interference concentration:

$C\ [mg/mL] = 62.9 \times A260 - 36.0 \times A280$

The constants 62.9 and 36.0 refer to specific extinction coefficients used by Warburg and Christian. For best accuracy, the factors should be determined for the particular protein affecting the analysis.

5.5.3   Direct protein concentration measurements at A280 nm

As with DNA, proteins absorb ultraviolet light at a specific wavelength, allowing direct measurement using a spectrophotometer. The aromatic amino acids tyrosine and tryptophan show a specific absorption at A280 nm, allowing direct measurement of protein concentrations. This is visualized in the figure below.
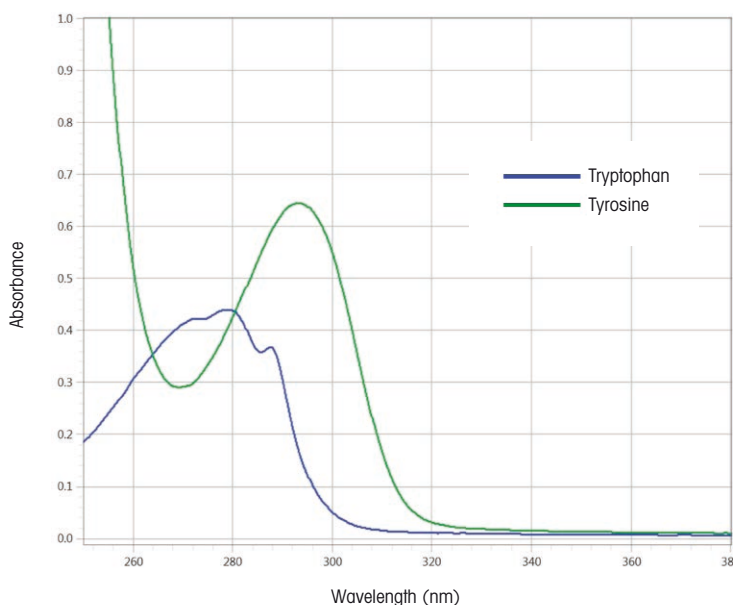
Figure 41: Absorbance spectrum of tyrosine (green) and tryptophan (blue)

The chemical composition of the protein will affect the absorption, the number as well as the type of amino acids will cause variation.

If the extinction coefficient for the protein of interest is available, the absorbance of the protein solution can be measured directly using a fixed wavelength measurement at the absorbance 280 nm. The advantages of this technique are that no special reagents are required, therefore the protein is not modified or inactivated during the process. No incubation period is required, so measurements are quick and highly reproducible, making this an incredibly simple technique.

The disadvantage is that this method relies on having an accurate extinction coefficient for the measured protein. As with nucleic acids, each protein has its own conversion factor. The common standard, protein bovine serum albumin (BSA), has a factor of 1.552. The concentration can be calculated by the following (according to the Lambert-Beer's law in chapter 2.4):

Concentration (μg/mL) = Abs280 × Factor / Path length

The A260 / A280 ratio can be used as a guide to the purity of the protein sample.

5.5.4   Nucleic acid determination using the fluorescent dye PicoGreen®

As we have seen above, the direct measurement of nucleic acids and proteins depends strongly on the extinction coefficient for the particular substance making these measurements more of a quick approximation rather than a precise result. All types of nucleic acids, dsDNA, ssDNA and RNA, show an absorbance maximum at 260 nm and can therefore hardly be distinguished by photometric methods, because the only difference between them is that single stranded nucleic acids show higher absorbances than double stranded nucleic acids (see the figure below). This effect is also known as hyperchromicity. However, it does not allow distinction between the molecules. In cases where a certain threshold is not reached, the amount of sample molecules can sometimes not be distinguished from artifacts. In the case of high, as well as low, concentrations, the specific quantification of the interested molecule is difficult if other nucleic acid molecules are present.

Other photometric methods are available that use dye molecules and result in higher accuracy.
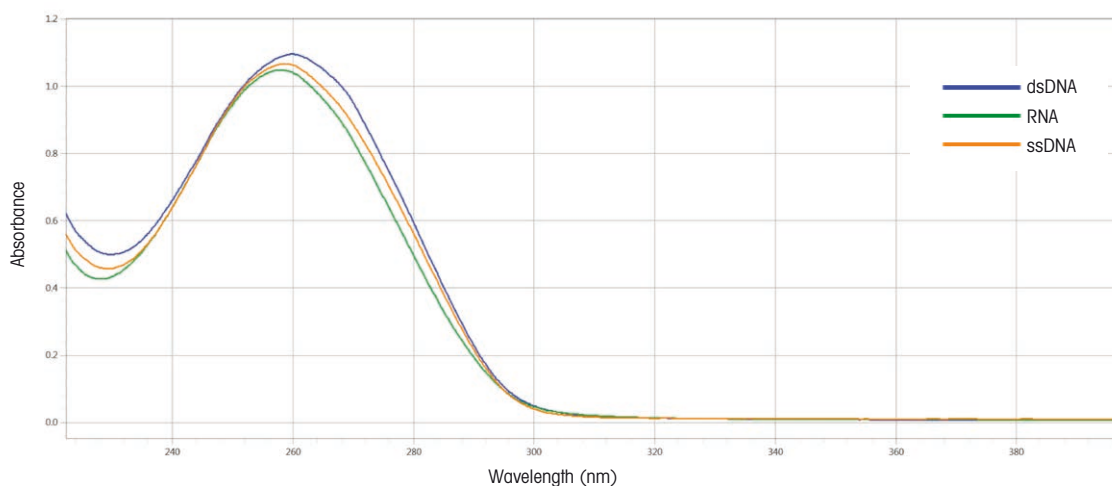
Figure 42: Hyperchromicity for different nucleic acids; dsDNA in blue, ssDNA in orange and RNA in green

Nucleic acids can be determined by dye molecules that are specifically bound to the molecule of interest before determination, e.g. binding to dsDNA but not to ssDNA. As an example, the quantification of dsDNA can be performed using the fluorescent dye PicoGreen. PicoGreen almost exclusively detects dsDNA.

The sample preparation is very simple; the PicoGreen dye solution is added to the sample and after a waiting time of 5 minutes the labelled sample can be read.

5.5.5 Quantification of proteins in biotechnology

Proteins are composed of amino acid building blocks. The protein absorbance is measured at approximately 280 nm because of their aromatic amino acids as discussed earlier. As the amount of aromatic side chains varies greatly from protein to protein, more precise techniques are often used to determine protein concentration, which are based on dyes. The dye bound to the protein forms a colored complex which can be assayed in the visible region. The three most common procedures for protein analysis are biuret, Bradford and bicinchoninic acid (BCA) assays.

Figure 43: BSA Protein Structure, Source: http://www.rcsb.org/pdb/images/3v09_bio_r_500.jpg

The Bradford test is a typical application in the biotechnology market segment. This assay is one of the most applied colorimetric methods used to determine the concentration of proteins.

The Bradford assay is based on the changing color of a Coomassie Brilliant Blue reagent when bound to a protein. The color starts off as red with an absorbance maximum at 470 nm, and ends at blue with the absorbance maximum at 595 nm. This color change occurs as protein binding takes place in the acidic medium. The reaction depends on the concentration of basic amino acids (primarily arginine, lysine and histidine). The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein.

The protein concentration of unknown samples can be quantified, using a calibration curve typically created from bovine serum albumin (BSA) samples. This quantification results in an approximation, as the ratio of positively charged amino acids is different from protein to protein.

The Bradford reagent itself, containing the dye in an acidic solution, is brown and changes to blue color when bound to a protein.
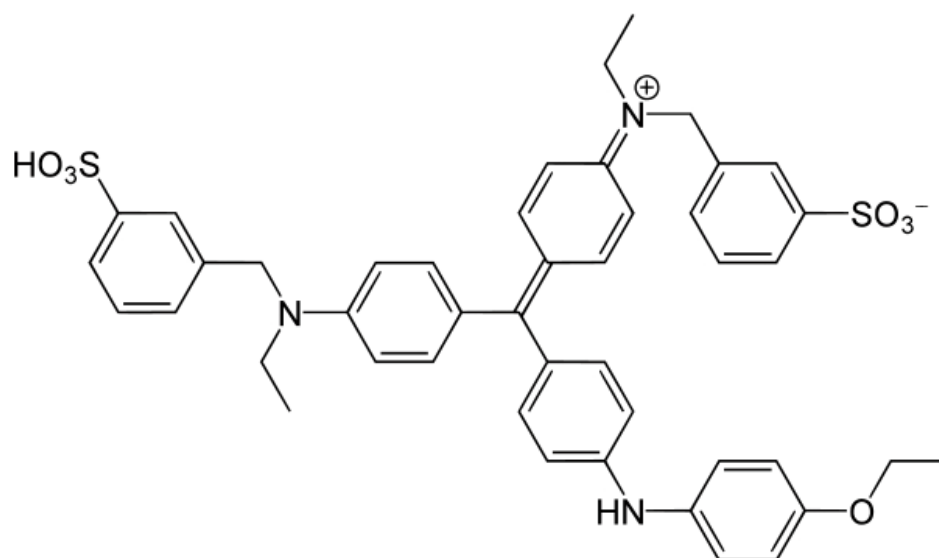
Figure 44: The molecular structure of the Coomassie Brilliant Blue Dye reagent

6.   Tips and Hints for Accurate and Precise UV/Vis Measurements

The next chapter will provide you with tips and hints from an applicative point of view in order to assure accurate and precise UV/Vis measurements.

Beside the instrument's performance which will be discussed in chapter 7, the largest sources of error in spectrophotometry are related to sample handling. The absorption spectrum of a substance can be influenced by the solvent, the pH of the solution, the temperature, high electrolyte concentrations, and the presence of interfering substances or low-quality cuvettes. Effects of these variables must be known and conditions for the analysis chosen so that the absorbance will not be affected by small, uncontrolled variations in their magnitudes. The most important effects will be described in the following paragraphs.

6.1   Cuvette-based UV/Vis spectroscopy

The cuvette used to measure a sample is a part of the spectrophotometer's optical system. Therefore, the position and geometry of the cuvette can have an influence on the accuracy and precision of absorbance measurements and should be carefully controlled.

For best measurement practice, the cuvette should remain in the cuvette holder between the measurements. If removed, care should be taken to always position the cuvette in the same direction in the cuvette holder, i.e. with the label towards the light source. This ensures that the optical effects are identical for both reference and sample measurements.

Furthermore, for best results, the reference and sample measurements must be done using the same type of cuvette at best, a matched pair of cuvettes or Excellence Cuvettes with a low manufacturing tolerance. Cuvettes must have windows fabricated from a material that is transparent in the spectral region of interest. For best results of measurements in the UV range, UV-transparent glass needs to be used, such as quartz glass or optical suprasil glass. Disposable cuvettes, made of poly-methyl-methacrylate, absorb in the UV range and act like a cut-off filter, making measurements in the UV range inaccurate and should only be considered for measurements within the visible range.

In optical systems, such as UV/Vis spectroscopy, the selection and cleanliness of the cuvettes has a major effect on results and repeatability. The windows of the cuvette, through which the light passes, have to be cleaned before every use with lint-free (to avoid scratches from lint on the surface) tissues. Higher absorbances are measured if residuals, such as fingerprints or grease, are deposited on the cuvette windows due to there being additional absorbing components. This can be avoided with thorough cleaning before and after use. Additional care must be taken to avoid touching the windows after cleaning is complete.

Finally, floating particles in the cell will deflect the light beam and lead to a background absorbance. This deflecting of light is also known as light scattering.

6.2   Solvent selection

A solvent for UV/Vis spectroscopy must be transparent throughout the applied region and should dissolve a sufficient quantity of the sample to give well-defined peaks. Every solvent has a UV/Vis absorbance cut-off wavelength. The solvent cut-off is the wavelength below which the solvent itself absorbs all of the light. So when choosing a solvent it is important to be aware of its absorbance cut-off as well as where the compound under investigation is expected to absorb. If they are close, a different solvent should be chosen.

Care must be taken when working below 300 nm where solvent absorption may be so high that the incremental absorption due to the sample is small compared to total absorption. The following table lists common solvents and the lower limit of their useful wavelength range.

Solvent	UV absorbance cutoff (nm)
Acetone	329
Benzene	278
Dimethylformamide	267
Ethanol	205
Toluene	285
Water	180

Generally, non-polar solvents and non-polar molecules have the least effect on one another. However, polar molecules exhibit quite drastic differences when they interact with a polar solvent such as water, alcohols esters, and ketones. Interaction between solute and solvent leads to absorption band broadening and a consequent reduction in structural resolution and maximum extinction coefficient. They tend to obliterate vibrational structures and should thus be avoided when spectral detail is desired.

The next figure illustrates the effect of iso-octane and ethanol on the spectrum of phenol. By changing from a non-polar solvent (iso-octane) to a polar one (ethanol), the fine structure due to the vibrations of phenol are attenuated. In general the spectra recorded in non-polar solvents are closer to those obtained from gas samples.
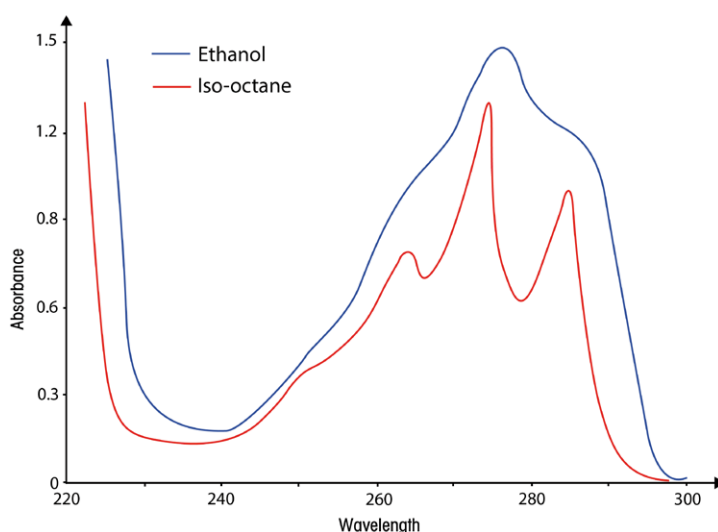

Figure 45: Spectrum of phenol in iso-octane and ethanol. Polar solvents such as ethanol show much lower spectral resolution

6.3   Sample concentration

For optimal measurement results and to comply with the Lambert-Beer Law, the absorbance shall be determined in the linear range of the instrument. Thus, it is better to avoid very high (>2.5 AU) and very low absorbance values (<0.3 AU) which may lead to non-linearity.

Nevertheless, unlike other analytical techniques, samples with low concentrations, such as 0.01 mol/L, can be measured using UV/Vis. A common example of this is seen in water analytics where very low concentrations need to be determined.

When an analyte interacts (association, dissociation) or reacts with the solvent, new chemical species are created leading to a change of absorption behavior. It is therefore recommended to use a non-interacting solvent and a low concentration of analyte to avoid any side reactions.

6.4   Wavelength selection

In order to obtain maximum sensitivity, spectrophotometric absorbance measurements are performed at a wavelength corresponding to an absorption peak. The importance of measuring absorbance precisely at the wavelength located at lambda max is demonstrated in the following figure. The wavelength setting within the narrow band indicated at the peak would have no significant effect on absorbance measured at the peak. However, a band with the same width displaced to shorter wavelength would introduce the possibility of major error, depending on the precise point within the band at which the measurement is taken.

Errors due to either wavelength setting or instrument calibration will be at a minimum when measurements are made at the wavelength of maximum absorption.
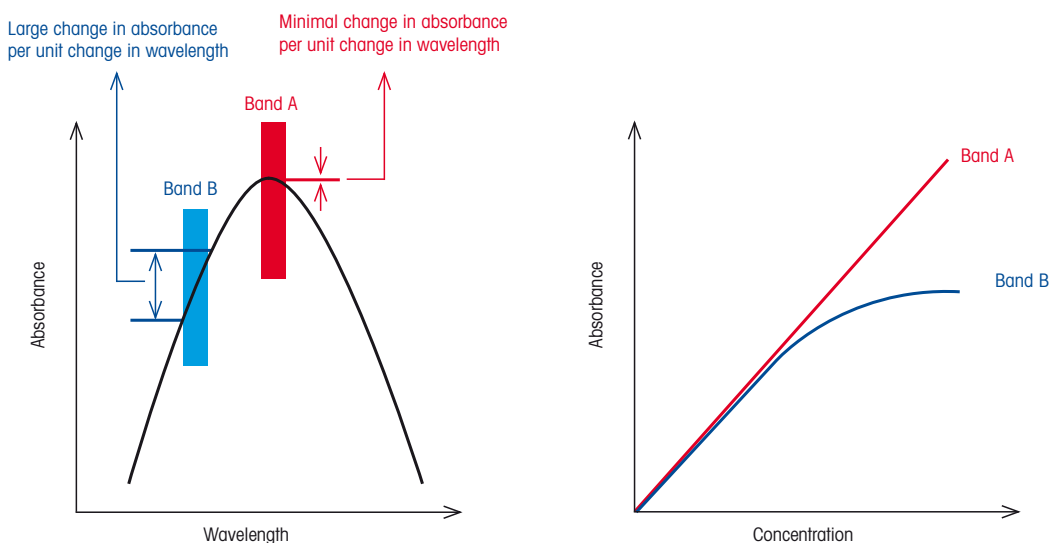

Figure 46: Left: Change in absorbance per wavelength change has significant effect in Band B, whereas in Band A the change is very small and negligible. Right: The change effects the calculated concentration of a sample in case B

6.5 Analysis of mixtures

The total absorbance of a solution at any given wavelength is equal to the sum of the absorbances of the individual components in the solution. This relationship makes it possible in principle to determine the concentrations of the individual components of a mixture even if total overlap in their spectra exists. To analyze the mixture, the molar extinction coefficients for the individual components have first to be determined at specific wavelengths, optimally wavelengths are selected so that the coefficients of the components differ significantly. For example, in a two component mixture, the extinction coefficient of component A at wavelength $\lambda 1$ is much larger than the extinction coefficient of component B at wavelength $\lambda 1$. For a second wavelength $\lambda 2$ this should be the opposite. The standard concentrations of component A and B shall encompass the absorbance range of the mixture in order to follow the Lambert-Beer law and encompass the sample mixture.

To complete the analysis, the mixture is measured at wavelength $\lambda 1$ and $\lambda 2$. With the known absorbance values, extinction coefficients and the used path length, the concentration can be calculated.

6.6 Micro-volume based UV/Vis spectroscopy

On a micro-volume platform, samples with very small volumes, such as microliters, can be measured. High concentrated samples can also be readily measured without any further dilution due to the path length availability at either 0.1 or 1 mm. This measurement technique is primarily applied for DNA and protein samples.

An initial cleaning of both measurement surfaces with dH_2O is recommended prior to performing the blank measurement in order to avoid interferences with the measurements. Do not use a spray bottle to apply water or any other liquid to the surface of the instrument.

Additional cleaning recommendations are as follows:

- Between measurements it is recommended to wipe the sample from both the upper and lower platforms with a clean, dry, lint-free lab wipe.

- A final cleaning of both measurement surfaces of the micro-volume platform with dH_2O is recommended after the last sample measurement. Depending on the sample, a 60% isopropanol solution, ethanol or ultrapure water can be used to clean the micro-volume platform. If necessary, the solvent used to dissolve the sample can be applied. When samples dried on the platform, additional cleaning can be performed using 3 µL of 0.5M HCl. Finally, after all other cleaning solutions have been applied, the platform is cleaned with a 3 µL aliquot of dH_2O.

- If detergents or 100% isopropyl alcohol are used, follow with 3 µL of dH_2O as final cleaning step.

- The polished surface allows for droplet application of aqueous solutions with high contact angle. If the droplet flattens out, it is possible that after closing the micro volume arm the measurement is performed through air, which will lead to erroneous measurement results. This is visualized in the following images:
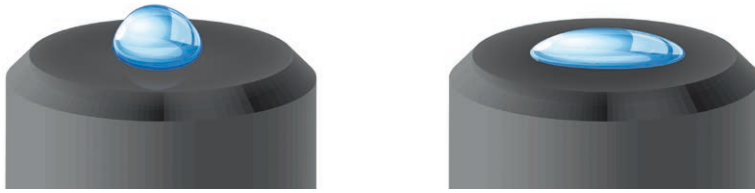

Figure 47: Droplet beads up (left). Droplet flattened out (right)

- For decontamination, a solution such as 0.5% sodium hypochlorite (commercial bleach solution 1:10 diluted) can be applied to the micro-volume platform. An additional cleansing with deionized water should follow.

7.   Performance Verification

The instrumental performance is the main factor directly affecting the accuracy and reproducibility of the measurements. For critical UV/Vis measurements, especially in clinical, pharmaceutical or industrial quality control, it is crucial that the instrument is performing according to specification. In the laboratories working according to the pharmacopeia, it is important that the instrument performance is monitored regularly and documentary evidence has to be present. Furthermore, this is mandatory for laboratories that offer an accredited measurement service (e.g. in accordance with ISO 17025). The validation is also a key requirement for laboratories working according to the Good Laboratory Practice (GLP). The following paragraphs describe the requirements of the main regulations and the procedures for measuring the key instrumental performance parameters.

7.1   Regulatory requirements

Quality requirements have become more influential in recent years. In order to obtain consistently accurate quality measurements, it is crucial that analytical instruments measure according to specifications. Some industries even require a regular monitoring and documentation of the instrument performance. In various regulatory bodies, the performance verification tests for UV/Vis spectrophotometers are described. Especially within the major Pharmacopoeias, which demand the qualification verification of the spectrophotometers used as well as tests on how to perform them. They also recommend the use of Certified Reference Materials (CRM's) from accredited suppliers. Such qualified instruments will then meet the demands for the analysis of the pharmaceutical chemicals described in the monographs of the pharmacopoeias.

The different pharmacopoeias share many of the same tests but also contain some differences by requiring additional tests and limits.

7.1.1   United States Pharmacopeia (USP)

Chapter 857 of the United States Pharmacopeia (USP) describes the performance verification in the context of Installation Qualification (IQ), Operational Qualification (OQ) and Performance Qualification (PQ). In the chapter "Operational Qualification" it describes performance verification as preparing an instrument to be "fit for purpose", or in other words, to be ready to perform reliable measurements. The pharmacopeia lists and describes the tests to be performed and gives limits which have to be fulfilled.

The 2nd supplement to USP 42 – NF 37 has taken effect at the beginning of 2020. With the release, the USP introduced major changes to chapter 857. The most relevant ones are the introduction of mandatory photometric linearity test and the introduction of new reference materials.

7.1.2   European Pharmacopeia (Ph. Eur.)

The requirements governing UV/Vis spectrophotometers used for pharmaceutical analyses in Europe are contained in the European Pharmacopoeia (Ph. Eur.).

Comparably to chapter 857 of the USP, chapter 2.2.25 of the Ph. Eur. gives some definitions which are important

for UV/Vis measurements and lists tests and limits that must be fulfilled. Many of these tests are identical to those described in the USP. Like the USP counterpart, chapter 2.2.25 was updated with the inclusion of mandatory photometric linearity tests and new reference substances for photometric tests at the beginning of 2020 in Ed. 10 of the Ph. Eur.

7.1.3 Other regulations

Many other countries have specific national regulations, which are often based and partially identical to either the USP or Ph.Eur. Some of them are:

- UK: British Pharmacopoeia (BP)
- Germany: Deutsches Arzneimittelbuch (DAB)
- Switzerland: Pharmacopoeia Helvetica (Ph. Helv.)
- Australia: Therapeutic Goods Administration (TGA)
- China: Pharmacopoeia of the People's Republic of China (PPRC)

Also the World Health Organization (WHO) issues a pharmacopoeia which is the International Pharmacopoeia (Ph. Int.).

7.1.4 American standard testing method

American Standard Testing Methods (ASTM) International, is one of the world's largest international standards developing organizations. They enhance performance and help everyone have confidence in the things they buy and use.

7.1.5 National Institute of Standards and Technology (NIST)

The US National institute of standards and technology (former National Bureau of Standards, NBS) is a measurement standards laboratory. The preparation of many CRM's employed for performance tests are described by NIST. They also produce CRM's base materials as a basis for the production of CRM cuvettes for UV/Vis.

7.2 Performance verification tests

Performance verification tests assess the quality of the measurements. For UV/Vis measurements specifically, this is the spectrum. The tests have to guarantee:

- that the wavelength positions (x axis) are correct (Wavelength accuracy) and stable (Wavelength repeatability)
- that the intensities, absorbances or transmittances (y axis) are correctly measured (Photometric accuracy) and stable (Photometric repeatability)
- that the measured shape of the spectrum is correct and not distorted (Resolution Toluene, Stray light)

7.2.1 Wavelength accuracy

Wavelength accuracy is an important performance parameter especially when comparing measure-ments on different instruments. Wavelength accuracy is normally checked by using a certified reference standard which has a series of narrow transmittance lines throughout the ultraviolet and visible range. A common used standard is holmium perchlorate which is dissolved in perchloric acid. Over the wavelength range from 200 to 700 nm a series

of narrow lines allow a check of the wavelength accuracy in the UV as well as in the visible spectral range. For diode array instruments, only one wavelength accuracy measurement is required, and no precision determination needs to be performed, since no optically moving parts are available. Diode array instruments are extremely reproducible and stable and with no moving parts, there are no mechanical components to adjust or recalibrate.

The difference between the certified and measured value of the CRM must not exceed ±1 nm in the UV region (200 – 400 nm), and in the visible region (400 – 700 nm) must not exceed ±2 nm.

The wavelength accuracy is defined as the deviation of the wavelength reading from an absorption band as compared to the known wavelength of the band. A deviation of the wavelength can cause errors in the qualitative and quantitative results of the UV/Vis measurement. It is quite obvious that if the spectrophotometer is not able to maintain an accurate wavelength scale, the spectrum of the sample measured by the instrument will be inaccurate and the true λ_{max} of the analyte cannot be characterized accurately.

7.2.2 Photometric accuracy

Photometric accuracy is the most important criterion for quantitative analysis when extinction coefficients or factors are used, as well as for spectral identification and purity control. Photometric accuracy is determined by the difference between the measured absorbance and the established standard value. A potassium dichromate solution (60 mg/L) in sulphuric acid (0.01 N) is most commonly used for checking absorbance accuracy. The method tests the absorbance at four wavelengths; 235, 257, 313 and 350 nm.

Additionally, neutral-density (ND) filters are often used to determine the photometric accuracy in the visible range between (five wavelengths between 440 nm and 635 nm).

USP 42 and Ph. Eur. Ed 10 have introduced nicotinic acid as a possible alternative to PDC. A solution of 12 mg/L of nicotinic acid in 0.1 M hydrochloric acid can be used to test the photometric accuracy at 213 and 261 nm.

7.2.3 Photometric linearity

As discussed in chapter 3.3, the quantitative determination of the concentration of a substance in a sample is based on the Lambert-Beer Law, which describes a linear relationship between the concentration and absorbance. The linearity of the response of the instrument is therefore of crucial importance if quantitative assays are routinely performed.

Traditionally, the linearity of the instruments was tested indirectly by determining the stray light of the instrument (see next chapter). USP 42 and Ph. Eur. Ed. 10 introduced mandatory photometric linearity tests to directly determine the linear behavior of the instrument.. The photometric linearity of the spectrophotometers is tested using the same substances used to test the photometric accuracy. However, instead of using a single concentration/absorbance level, at least three filters with increasing concentration are measured.

While the measuring procedure and the reference substances are the same, USP and Ph. Eur. apply different ac-

ceptance criteria: the Ph. Eur requires the calculation of the correlation coefficient (R-squared), whereas the USP requires all measured concentration to fulfill the photometric accuracy criteria.

7.2.4   Stray light

Stray light is radiation emerging from imperfections in the dispersing element or in other optical surfaces, from diffraction effects, other optical aberrations or from damaged or worn components. Stray light will cause deviations from Lambert-Beer's law. At high absorbance values the linear relationship between absorbance and concentration is strongly affected by the factor of the stray light. It introduces a systematic bias to lower absorbances at increasing concentrations. Stray light is also the primary influence on the upper limit of the linear dynamic range for an analysis. See the figures below with some visualized stray light effects.
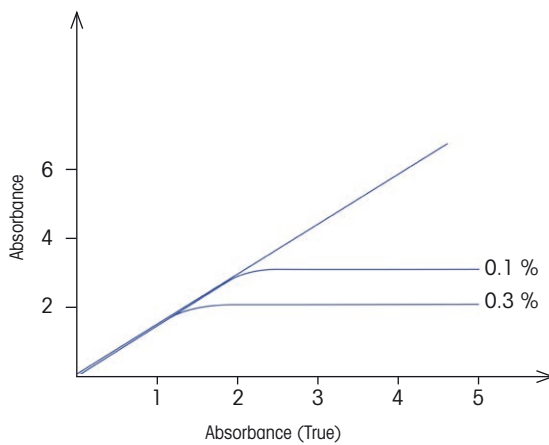
Figure 48A: Limiting factor Stray light   B: Stray light effect visualized at the peak maximum
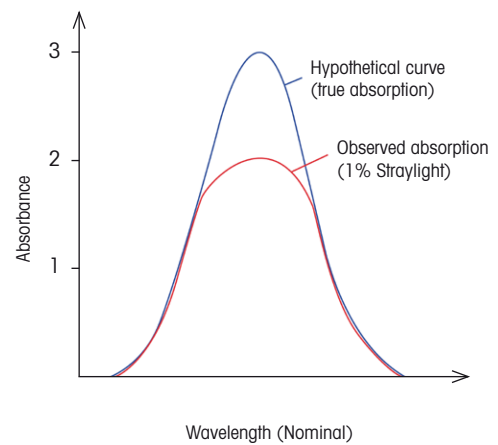
To detect stray light at specific wavelengths, solutions of sodium nitrite (340 nm), sodium iodide (220 nm) and potassium chloride (200 nm) in water are applied. These solutions have a very sharp cut-off in the UV region. This means that if light is detected at a wavelength where the sample is supposed to be completely absorbing, it must be due to stray light reaching the detector from another source without passing through the sample. These filters are applied mainly in the ultraviolet range due to the fact that stray light is more intensive in this range.

7.2.5   Resolution

The performance of a spectrophotometer is mainly dependent on the resolving power of the instrument, in other words, on its resolution. Resolution is a critical factor in determining two closely located peaks. Sufficient instrument resolution is required to be able to distinguish between different absorbance peaks in the UV/Vis spectrum of a sample. If the spectral resolution is not sufficient, then the absorption bands become 'unresolved' and they cannot be clearly distinguished from each other. Thus, the identification of absorbance peaks becomes very challenging and the absorbance value will be lower than the true value. The effect of high and low resolution is illustrated in the following two graphs:
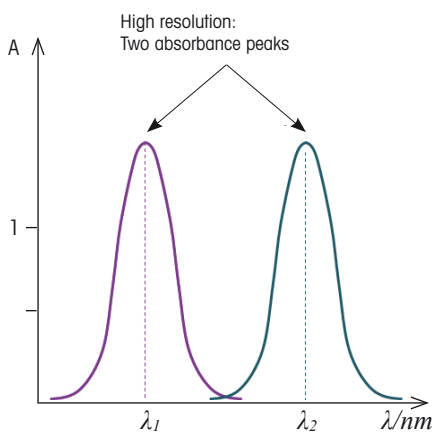

Figure 49A: High resolution instrument with clearly resolved peaks   B: Low resolution where peaks cannot be resolved
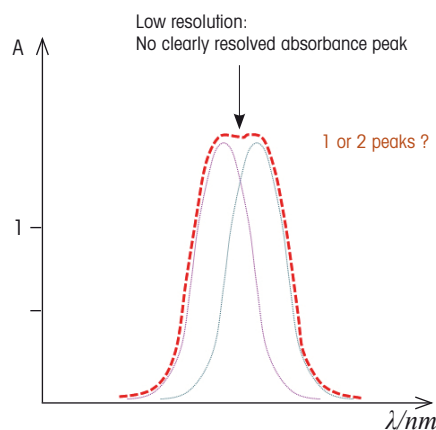
In the left graph, an example of an instrument with high resolution is presented in which two absorbance peaks are clearly visible along the spectrum. The instrument's resolution is high enough to easily separate the two different absorption bands. On the right, the instrument has a too low resolution and the two absorbance peaks cannot be resolved. The instrument cannot differentiate the two different absorption bands.

The ability to differentiate between peaks is indicated by the spectral resolution. The spectral resolution of an instrument is the measure of an instrument's ability to differentiate between two adjacent wavelengths in the recorded spectrum.

To achieve spectra where absorption bands are clearly defined, a high resolution parameter setting must be selected.

In the following example, the spectrum of benzene in the gas phase is shown at three different resolution settings on the spectrophotometer. This parameter is called the spectral bandwidth.

In the high resolution spectrum on the left, with the lowest bandwidth, several absorption peaks are clearly defined. In the medium and low resolution spectra to the right, with increasing bandwidth, the detail is progressively lost. In the right-hand graph, with the lowest resolution, only a single, broad absorption band is displayed.
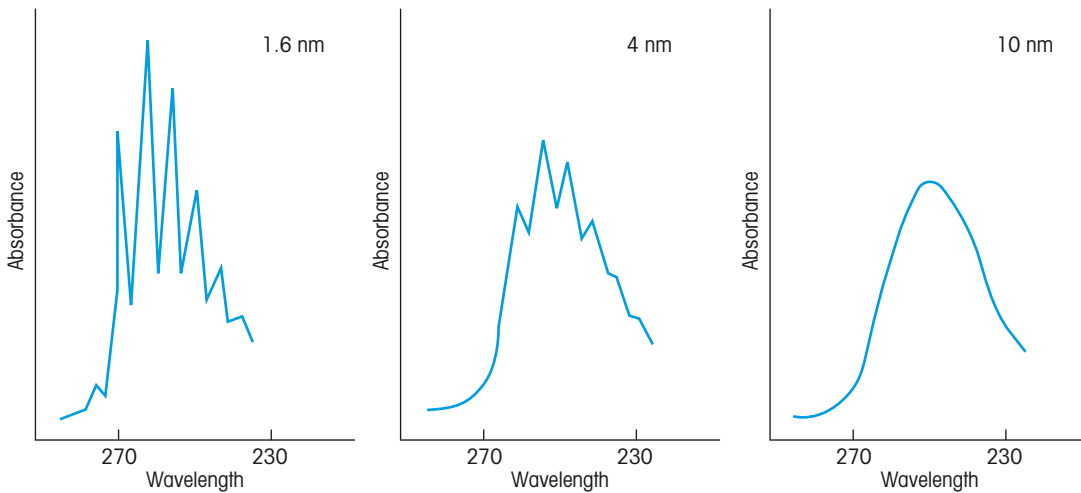

Figure 50: The same spectrum presented using different bandwidth. Left: peaks are clearly defined with a low bandwidth. With increasing bandwidth the resolution gets lost and less spectral details are visible. Right: A large bandwidth of 10 nm presents one single absorption band out of the 5 peaks that were visible using 1.6 nm bandwidth

This example clearly illustrates the importance of selecting the correct instrument resolution for accurate measurement of a UV/Vis spectrum.

Resolution is therefore a critical factor in determining the shape of measured peaks. If the instrumental resolution is not sufficient, the absorbance value will be lower than the true value.

The US and EU pharmacopoeia resolution tests consist in the measurement of 0.02% w/v solution of toluene in Hexane. The ratio of the absorbance maximum near 269 nm to that of the minimum near 266 nm is calculated. The larger the ratio the better is the resolution. The following table gives the relation for a temperature of 25 °C:

Ratio	2.3 – 2.4	1.9 – 2.0	1.6 – 1.7	1.3 – 1.4	1.0 – 1.1
Spectral bandwidth	0.5 nm	1.0 nm	1.5 nm	2.0 nm	3.0 nm

7.2.6 Photometric noise

Photometric noise is measured through the CCD light detector and derives from short-term fluctuations caused by the light source and electronic components. Noise is the major factor affecting the precision of the absorbance measurements. The amount of photometric noise will define the precision and minimum detection limit of the spectrophotometer.

Photon noise from the light source is especially apparent at low absorbance levels, whereas electronic noise from electronic components affects the accuracy of the measurements at high absorbance.
Noise is typically measured at zero absorbance, that is, with no sample in the light path.

A common method to calculate the noise is taking the Root Mean Square (RMS) of multiple measurements at a

defined wavelength.

Noise increases with shorter measurement times and decreases with longer measurement times. Furthermore, noise varies from wavelength to wavelength. This is due to the fact that the intensity of the light sources and detector characteristics vary with wavelength.

7.2.7 Photometric drift

It is expected that the measured signal is stable on a long time scale. This is measured with the photometric drift test. The instrument drift is commonly measured at zero absorbance with no sample in the light path. A measurement is taken each minute during one hour at one defined wavelength. Ideally this gives a horizontal line. Deviations are characterized by a linear fit of the curve which gives a slope as an indication of the drift direction.

7.2.8 Baseline flatness

Ideally, for a proper characterization of an instrument, its noise characteristics should be measured at all wavelengths. However, this would be extremely time-consuming. One way to get an overview of the relative noise level at all wavelengths is to measure the baseline flatness. It also reveals wavelengths with instrumental problems resulting from switching filters or light source exchanges.

The instrument is auto-zeroed with nothing in the sample compartment across its working range and air is then simply measured as the sample for the baseline flatness measurement. The Root Mean Square of the baseline spectrum in the defined range gives an indication of the flatness of the baseline.

7.3 Instrumental self-tests

The complete performance verification required for pharmacopeia compliance of a spectrophotometer is normally performed at given intervals. These intervals are determined according to the stability of the instrument. To ensure that any deviations from the performance happening in between these verifications are detected, the instrument should provide self-tests. Self-tests are for the routine analysis of the instrument performance that can be run on a daily basis. These tests should include an electronic and optical operation check of the spectrophotometer as well as wavelength accuracy checks with the xenon line in order to verify the installed lamp performance. Therefore, the drift for stability, the wavelength accuracy and repeatability using the xenon lamp are implemented additionally to the photometric noise and the baseline flatness tests, which were already described. Please find more details about the instrumental self-test in the service documents.

7.4 Certified reference materials for spectrophotometry

Certified reference materials or calibration standards comply with the requirements of the major pharmacopoeias (Ph.Eur., USP). These allow performing the instrument qualification according to the regulatory requirements and ensure that the instrument fulfills the quality demands. These materials are certified for the absorbance value at a number of wavelengths in the UV and visible spectral regions

The certified reference materials are classified into liquid and solid (glass) filters.

7.4.1 Glass filters

ND filters are typically certified using air as the blank. This is, however, not ideal, because the optical medium of the blank measurement (air) is different from the optical medium of the filter measurement (glass).

When light passes from one medium to another (e.g. from air to glass or vice versa) the light beam is slightly distorted due to the optical phenomena of refraction and reflection. Different media will exhibit different refraction and reflection behaviours, which can negatively affect the accuracy of the results.

This is usually compensated by measuring an appropriate blank sample: It is common practice to use the same cuvettes and solvents for both blank and sample measurements to ensure that the same optical properties of both measurements are the same. The measurement of an appropriate blank not only compensates for the absorbance of the cuvettes and solvents, it also ensures that the distortion of the light beam due to refraction and reflection affects blank and sample measurements equally.

While the effect on the measured values is negligible on some instruments, it has a noticeable impact on other instruments. Therefore, it is recommended to use a certified clear glass filter as blank, to ensure that the optical medium is the same during blank and sample measurement.

Glass filters are certified reference materials made of specific glasses and manufactured specifically for their calibration. These filters are robust and solid which makes handling easy and straightforward. Furthermore, they are relatively insensitive to temperature and have a good stability over time. However, for solid standards, the homogeneity cannot be ensured from batch to batch. Therefore each standard must be calibrated on a reference spectrophotometer. Due to this time-consuming step, solid standards tend to be expensive.

Glass filters can simply be mounted into a single cell holder. Such solid filters allow qualifying the spectrophotometer for the photometric accuracy (absorbance) and also for the wavelength accuracy.

Figure 51: Neutral density glass filters from Starna Scientific Ltd

Available filters are Neutral density glass filter to check the photometric accuracy, as the above example shows. The certificate from Starna, one of the accredited manufacturers of reference materials, is delivered on the USB memory stick.

Furthermore, Didymium glass filters are available for the wavelength accuracy test. There are Holmium glass filters to assess the wavelength scale accuracy in the UV and visible region of spectrophotometers. These produce characteristic peaks just like its liquid counterpart.

These filters are traceable to the primary standards of the National Institute of Standards and Technology (NIST).

7.4.2   Liquid filters

Liquid filters are certified reference materials, which are strictly prepared using solid NIST primary materials and manufactured at the solution concentrations according to NIST publications. The filling of these references is performed under controlled conditions with all of the cells hermetically and permanently sealed by heat fusion and the values certified.

The liquid filters have the advantage of being equivalent to real measurement applications.
With a certified liquid filter, the following parameters of the spectrophotometer can be qualified according to the requirements of the pharmacopoeia:

• Photometric accuracy
• Wavelength accuracy
• Resolution
• Stray light

Figure 52 Liquid filters from Starna Scientific Ltd

The liquid filters are inserted into the single cell holder of the spectrophotometer where the absorbance is then measured at the wavelength indicated on the calibration certificate. The resulting measurement values are then

compared with either the reference values published within the Pharmacopoeia or given in the corresponding filter certificate. The test is then either deemed as passed or failed according to the acceptance criteria. Liquid filters are less stable than solid standards; therefore they have to be recalibrated more often, which increases the costs of such standard material.

7.5   Performance verification tests with automated modules

The CertiRef™ and LinSet™ automated performance verification modules are Pharmacopeia (USP and Ph. Eur.) accessories, which allow an automatic calibration and performance tests of the UV7 Excellence spectrophotometer. The UV5 and UV5Bio models can only be tested with the CertiRef module.

All CRMs in the CertiRef contained in the CertiRef and LinSets modules are manufactured and certified by accredited European CRM suppliers. The data of the certificates needed for the tests are electronically stored in the individual modules. The CRMs in the modules can be re-certified. The recommended re-certification period depends on the CRM type and varies between one and two years. Although the references are covered by a lifetime guarantees this is subject to regular recertification.
The data on the chip contains information about the type of module, the CRM set serial number to trace it to the certificate, all required certified values, the certification date and expiry date and information about each of the CRM tests.
When the CertiRef materials need to be recertified or inspected for any reason, please contact your local METTLER TOLEDO service representative.

CertiRef
The CertiRef consists of an 8-place cuvette changer containing liquid certified reference materials (CRM) in sealed cuvettes. The CertiRef module is compatible with the METTLER TOLEDO UV7, UV5 and UV5Bio instruments and is mounted at the front of the instrument.
Two different configurations of the CertiRef are offered for users working according to either the European Pharmacopeia (called CertiRef EUP) and United States Pharmacopeia (called CertiRef USP). They differ in the reference materials used for photometric accuracy and repeatability test, as well as in the procedure used to test the stray light of the instrument.

LinSet
The LinSet consists of an 8-place cuvette changer containing liquid and solid certified reference materials (CRM) in sealed cuvettes. The LinSet module is compatible with the METTLER TOLEDO UV7 instruments and is mounted at the back of the instrument. It can be mounted at the same time as the CertiRef to allow the complete testing procedure to be completed in a single run.
Two different configurations are offered, which differ in the CRMs used to test the photometric linearity in the UV range. The LinSet PDC contains three potassium dichromate in perchloric acid, while the LinSet Niacin uses three nicotinic acid in hydrochloric acid solutions.

The following tables shows the tests covered by each module and the corresponding CRMs:

CertiRef™ Test	Certified Reference Material	EUP v2	USP
Photometric noise Photometric drift Wavelength accuracy with internal Xenon lamp Wavelength repeatability with internal Xenon lamp Baseline flatness	Air	•	•
Resolution Hexane blank	Toluene blank	•	•
Wavelength accuracy Wavelength repeatability	Holmium oxide	•	•
Photometric accuracy UV Photometric repeatability UV	Perchloric acid blank		•
	Potassium dichromate		•
	Hydrochloric acid blank	•	
	Nicotinic acid	•	
Stray light Ph.Eur.	Water blank	•	
	Potassium chloride	•	
Stray light USP	Potassium chloride 0.5 cm		•
	Potassium chloride 1.0 cm		•

LinSet™ Test	Certified Reference Material	PDC	Niacin
Photometric linearity UV	Perchloric acid blank Potassium dichromate 40 mg/L Potassium dichromate 80 mg/L Potassium dichromate 140 mg/L	•	
	Hydrochloric acid blank Nicotinic acid 6 mg/L Nicotinic acid 18 mg/L Nicotinic acid 36 mg/L		•
Photometric linearity VIS	Clear glass blank Neutral density filter 0.5 A Neutral density filter 1.0 A Neutral density filter 2.0 A	•	•
Photometric accuracy VIS Photometric repeatability VIS	Clear glass blank Neutral density filter 0.5 A	•	•

GUVP™ Lifecycle Services
5 Steps to Minimize Risks

GUVP – Good UV/VIS Practice improves measurement quality by minimizing risks through a 5-step lifecycle program including specific services. It provides professional evaluation and selection tools, comprehensive installation and qualification services and tailored training and maintenance programs to ensure correct operation. As each step is thoroughly documented, traceability and up-to-date regulatory compliance are guaranteed.

GUVP will help you to:
- Minimize risks
- Improve quality
- Protect your investment
- Ensure regulatory compliance
- Educate your lab

Learn more about the Good UV/VIS Practice program
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