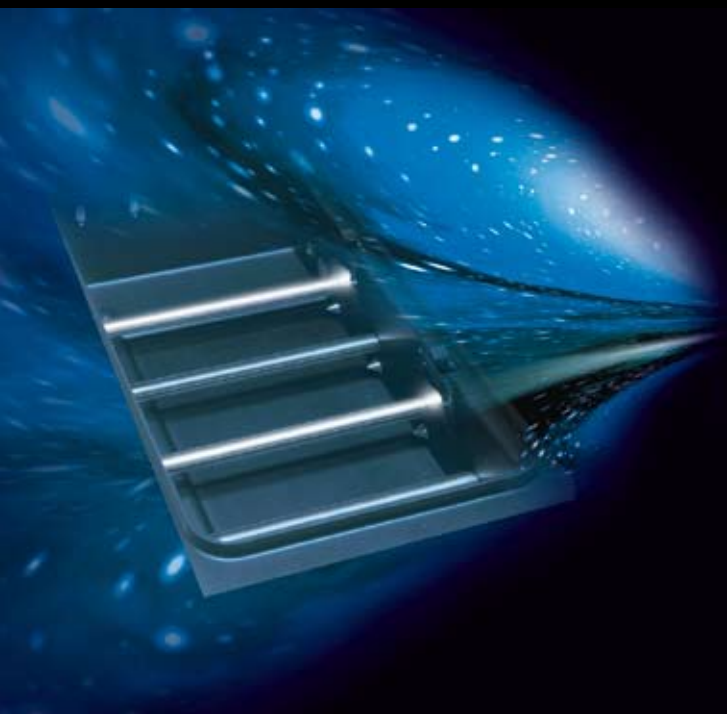


Fundamentals

Instrumentation and Techniques of UV VIS Spectroscopy



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1 Definitions and basic principles

1.1 Light absorbance and spectrum

The interaction of electromagnetic radiation with solids, liquids or gases produces various effects, such as absorbance, reflectance or scattering. UV VIS spectroscopy exclusively investigates the interaction of radiation with matter in the ultraviolet and visible range.

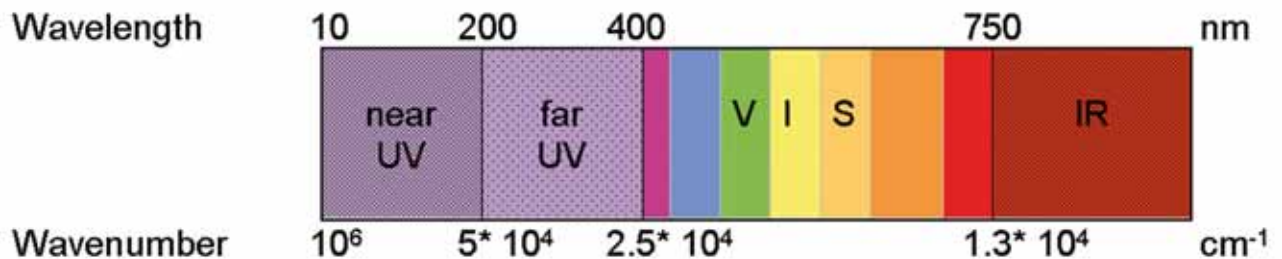


Figure 1: Electromagnetic radiation

When atoms or molecules absorb electromagnetic radiation they are transformed from a ground state into an energetically excited state. Energy of a specific wavelength is absorbed in this process. The various molecular states have a relatively broad energy range in comparison with atoms. Rotation and vibration of a molecule can be stimulated in the infrared range. The absorbance of defined packets of energy (quanta) by the valence electrons is observed in the range of visible and ultraviolet light.

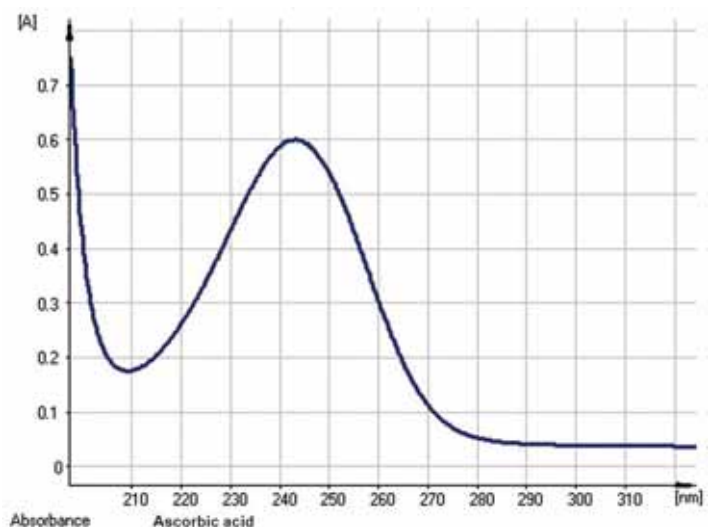
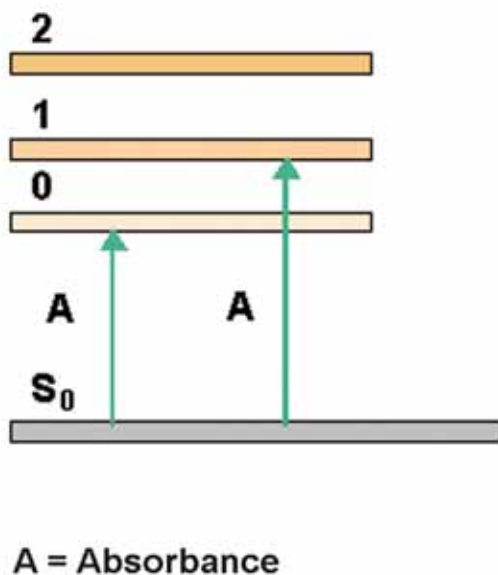


Figure 2: Absorbance and absorbance spectrum (ascorbic acid)

The energy of these quanta can be specified as the wavelength of the radiation. The shorter the wavelength, the greater the energy of the quanta. The location of the absorbance points and the relative magnitudes of absorbance can be determined with UV VIS spectrophotometers.

1.2 Transmittance and absorbance

If a light beam of intensity I_0 penetrates a medium of thickness d , the light beam is attenuated by the absorbance properties of the sample apart from reflectance and scattering losses. The exiting light beam (transmittance) now has the intensity I .

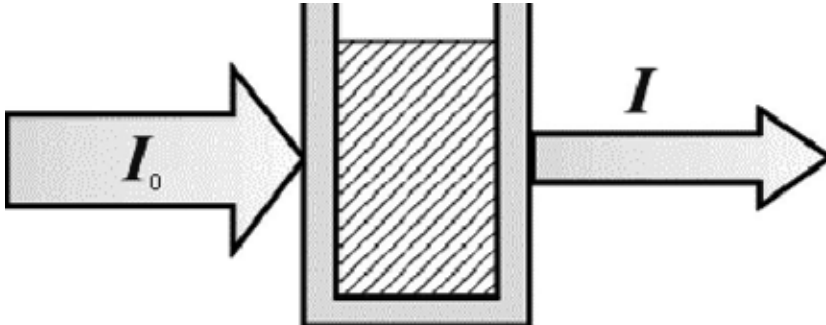


Figure 3: Transmittance

Transmittance T is defined by the following equation

$$T = \frac{I}{I_0} \quad \text{or} \quad \%T = \frac{I}{I_0} \cdot 100\% \quad (\text{Equation 1})$$

The following equation describes the absorbance A of a sample:

$$A = -\log_{10} T = \lg\left(\frac{I_0}{I}\right) \quad (\text{Equation 2})$$

In contrast to the transmittance, the absorbance of a solution therefore increases with increasing attenuation of the light beam.

1.3 Qualitative analysis



1.3.1 Spectra and structures

UV VIS spectra generally show relatively broad absorbance bands from molecules. Compared with IR spectroscopy, in which many narrow bands are produced, the qualitative information yield is relatively low. Absorbance of organic molecules in the UV VIS range is often caused by chromophoric groups (color bearing species). Table 1, as follows, provides an overview of chromophoric groups with their respective absorbance maxima.

Table 1

Chromophore	Formula	Example	Absorbance maximum
carbonyl- (ketones)	$RR'C=O$	acetone	271 nm
carbonyl- (aldehyde)	$RHC=O$	acetaldehyde	293 nm
carboxyl-	$RCOOH$	acetic acid	204 nm
amido-	$RCONH_2$	acetamide	208 nm
azo-	$-N=N-$	diazomethane	339 nm
nitro-	$-NO_2$	nitromethane	280 nm

A shift in absorbance bands towards the long wavelength range primarily occurs if chromophores are arranged conjugated to one another.

 1,5-hexadiene	 1,3,5-hexatriene
(not conjugated) Absorbance maximum 185 nm	(conjugated) Absorbance maximum 250 nm

Particularly cyclic unsaturated hydrocarbons show very characteristic absorbance bands in the UV VIS range.

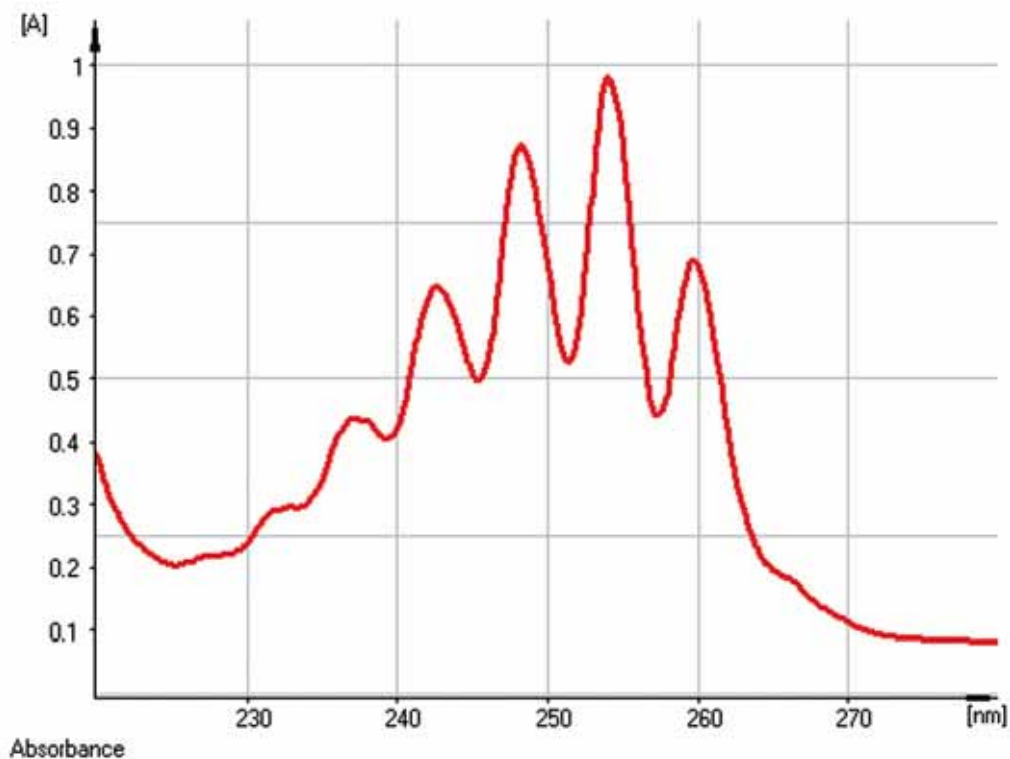


Figure 4: UV VIS spectrum of benzene vapor

1.3.2 Factors influencing the spectrum

The precise values for the wavelengths depend both on the specific substituents and the solvent. A hypsochromic (short wavelength) shift of the absorbance spectrum is generally observed with increasing solvent polarity and a bathochromic (long wavelength) shift for decreasing solvent polarity.

Table 2

Solvents (increasing in polarity)	Transmittance limit in nm
n-hexane	195
chloroform	240
diethyl ether	200
ethanol	200
water	185

Other parameters, such as pH and temperature, also affect the position and intensity of the absorbance maximum.

1.3.3 Qualitative information

UV VIS spectrophotometry can deliver the following qualitative information:

- Identification of pure substances
- Identification of substances following HPLC separation (preferably with diode array systems)
- Purity testing (for example of proteins or DNA/RNA)
- Melting point curves of proteins and nucleic acids
- Differentiation of saturated and unsaturated compounds
- Differentiation of keto and enol forms
- Identification of carbonyl bands
- Clarification of bonding relationships and substituent effects
- Enzyme activities

1.4 Quantitative analyses

1.4.1 Lambert-Beer law

The Bouguer-Lambert-Beer law describes the relationship between absorbance and concentration.

Bouguer (1696 – 1758) Absorbance is proportional to path length

Lambert (1728 – 1777)

Beer (1825 – 1863) Absorbance is proportional to molar concentration

$$A(\lambda) = \varepsilon(\lambda) \cdot c \cdot d$$

(Equation 3)

- $A(\lambda)$ - absorbance at wavelength λ
- $\varepsilon(\lambda)$ - molar logarithmic absorbance coefficient at wavelength λ ($\text{L mol}^{-1} \text{cm}^{-1}$)
- c - concentration (mol L^{-1})
- d - path length of the cell in cm

The Lambert-Beer law is a limited law and does not apply in the following cases:

- The concentration of the substance measured is too high ($> 0.01\text{M/l}$).
- There are side reactions between the substance to be measured and the solvent.
- The radiation used is not strictly monochromatic.
- Stray light (diffuse light, e.g., arising from reflectances from objects).

This means that the validity of the Lambert-Beer law has to be investigated.

This investigation is the calibration process.

1.4.2 The calibration process

Calibration means measuring samples with known concentration (standards) and the measured absorbance values are entered against the respective concentrations.

The Lambert-Beer law applies within the linear range of the calibration curve.

Non-linear calibration curves can also be used to a certain extent with the use of extended calculation models.

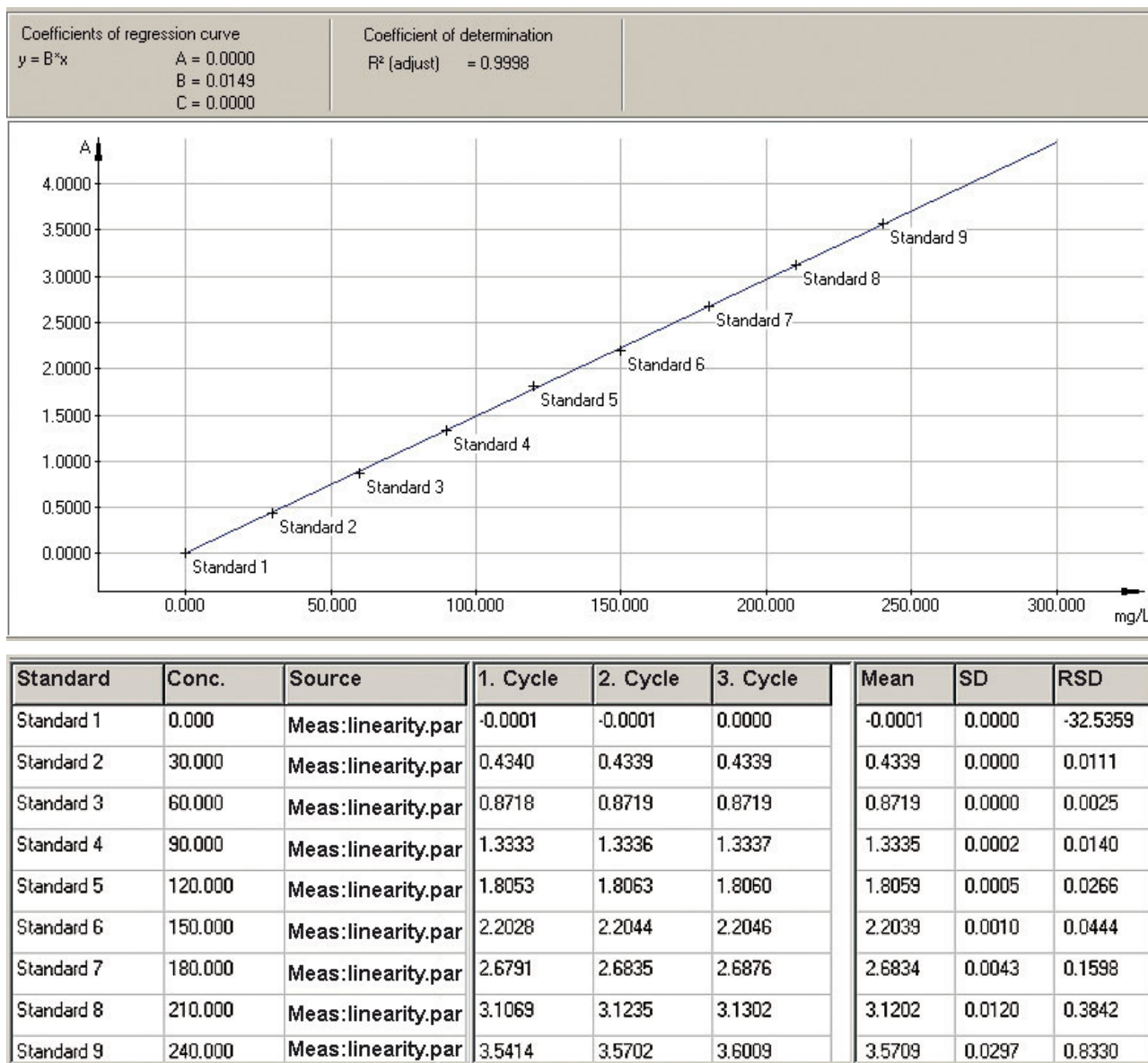


Figure 5: Calibration

1.4.3 Sample preparation for photometric measurements

In most cases, the species under investigation have to be converted to colored compounds. The reagents for this color formation process are:

- Inorganic in nature. These reagents are not very widely distributed however.
- Organic in nature. More than 7500 such organic reagents are known. The colors observed result from interligand bands and charge transfer complexes.

Table 3, as follows, shows a selection of parameters, which can be determined with UV VIS spectroscopy.

Table 3

aluminum	bromide	chloride	fluoride	manganese	nitrite	oxygen	sulfite
ammonia	BSB	chromate	gold	molybdenum	ozone	silver	surfactants (anionic)
ammonium	cadmium	CSB	iodine	sodium	phenol	nitrogen (total)	TOC
lead	chlorine	cyanide	potassium	nickel	phosphate	sulfate	peroxide
boron	chlorine dioxide	iron	copper	nitrate	residual hardness	sulfide	zinc

1.5 Kinetics

Kinetic studies serve to track time dependent changes in absorbance and therefore the speed of a chemical reaction.

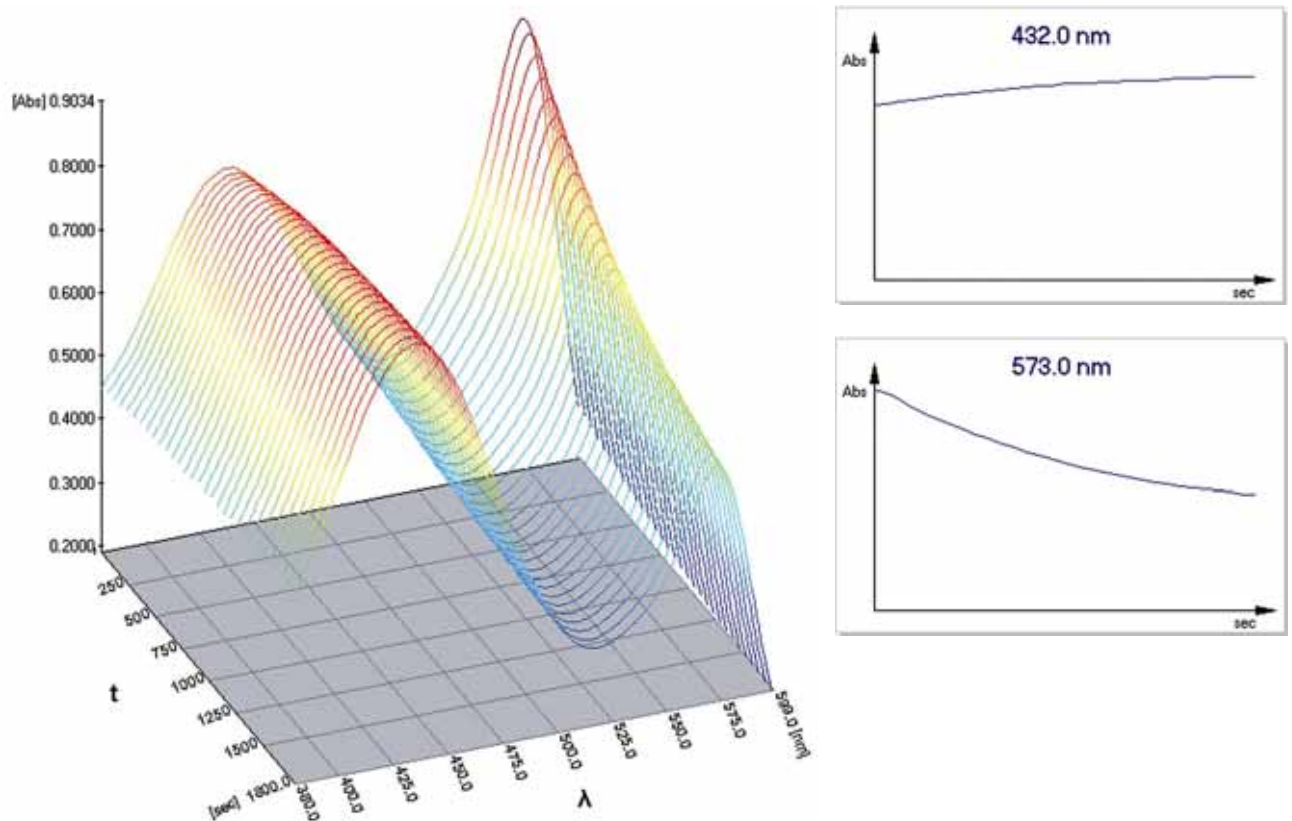


Figure 6: Three-dimensional representation of the results of a kinetic measurement with time domains of the respective absorbance maxima

These time domains can be evaluated to determine reaction speeds (e.g. determination of the slope with linear regression).

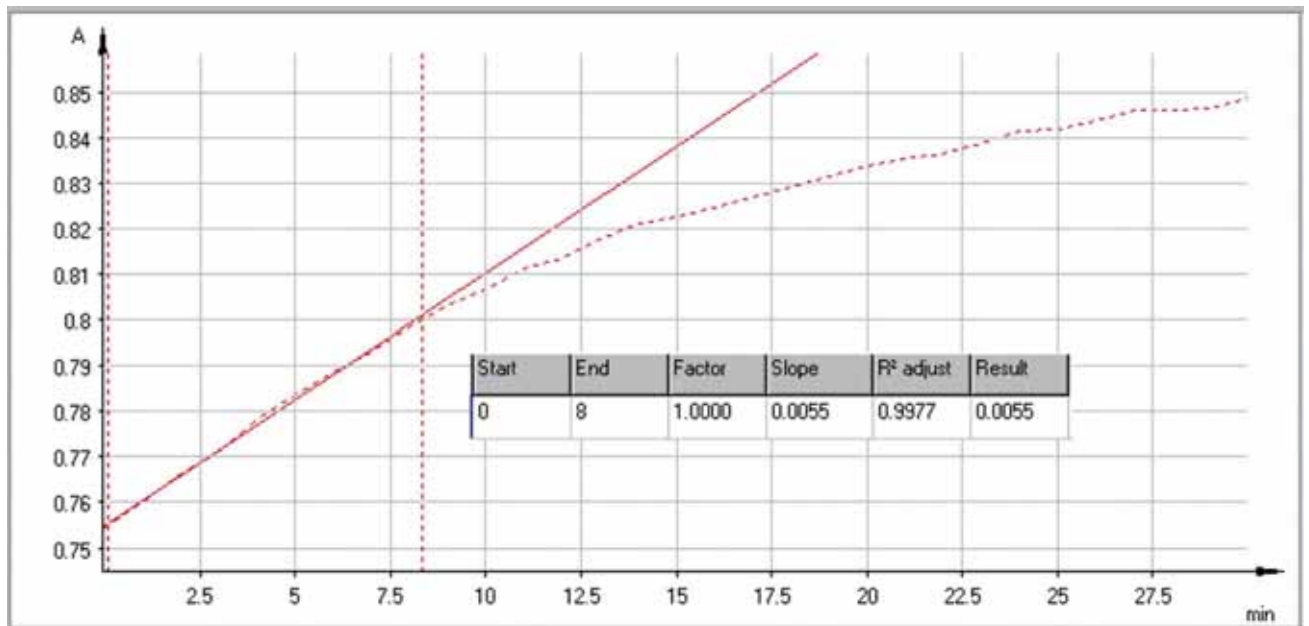


Figure 7: Evaluation option for a kinetic measurement

The concentration of enzymes can also be determined in a time-resolved manner (kinetics). This means that the concentration of an enzyme substrate changes with time. The slope of the time curve measured is proportional to the enzyme concentration.

In food chemistry, for example, enzymatic reactions are performed to determine some important parameters (see table 4).

Table 4

acetate	cholesterol	glucose	hydroxybutyric acid	oxalic acid
alcohol	citrate	glutamine	isocitric acid	sucrose
formic acid	formaldehyde	glycerol	lactate	sorbitol
ascorbic acid	fructose	urea	lactose	starch
succinic acid	galactose	hydrazine	maltose	xylitol

1.6 Further applications for UV VIS spectroscopy

1.6.1 Protein and DNA analyses

In its basic structure, DNA consists of two deoxyribose strands wound helically around an axis. The strands are connected together with hydrogen bonds between the bases purine and pyrimidine (Pu-Py), adenine and thymine (A-T), and guanine and cytosine (G-C). These bases form the UV absorbance property of DNA with a peak maximum at 260 nm. To determine content, absorbance is measured at 260 nm and the concentration is calculated using the Lambert-Beer law.

Proteins are a frequent form of contamination in DNA solutions. The purity of the nucleic acids can be estimated by determining the ratio of absorbance at 260 nm and 280 nm. If the ratio lies between 1.8 and 2 the purity is 70% - 95%.

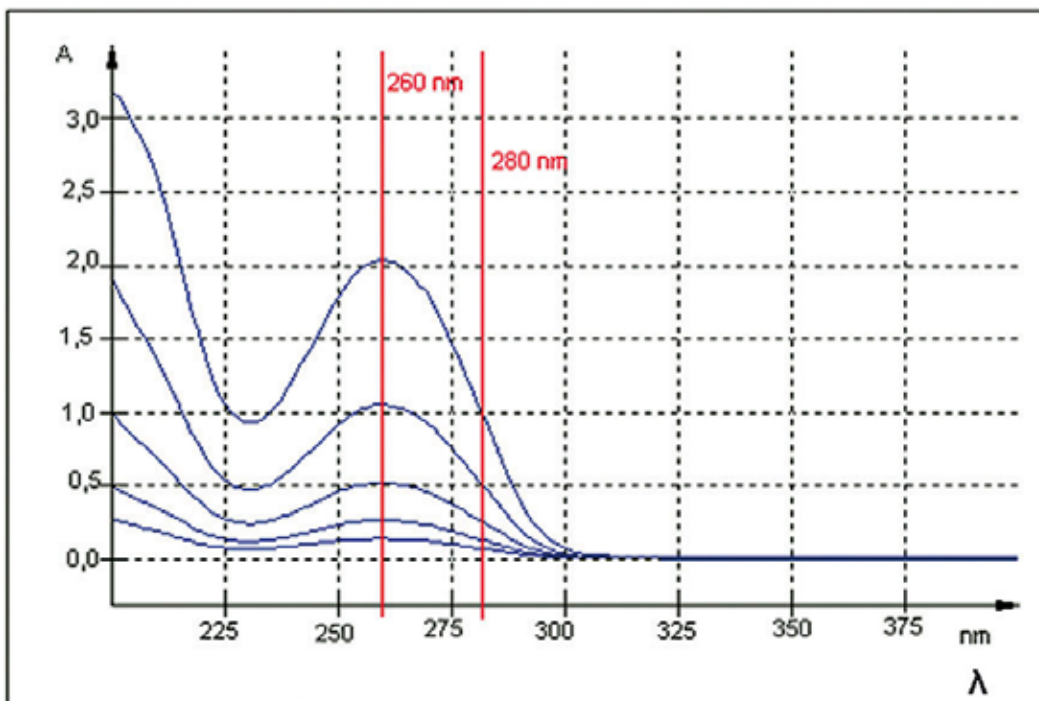


Figure 8: Absorbance properties of DNA standard solutions at 260 and 280 nm

Proteins have an absorbance maximum at 280 nm, primarily attributable to the aromatic ring system of amino acids (especially tryptophan and tyrosine). The protein concentration is therefore determined directly from the absorbance at 280 nm using the Lambert-Beer law. Determination of protein concentration is also possible by using color reagents, such as the Bradford reagent (with Coomassie Brilliant Blue G-250, absorbance maximum at 595 nm), the Biuret reagent (violet colored complex between the Cu^{2+} from the Biuret reagent and the oxygen atoms of the peptide link, absorbance maximum at 546 nm) and the Lowry reagent (coupling of Biuret, Folin and molybdenum blue reactions, absorbance maximum between 540-750 nm).

1.6.2 DNA melting point determination

The unwinding of the DNA double helix is termed as melting as this process takes place at an elevated temperature. Here the melting temperature T_m is the temperature at which half of the helix structure has been lost. As a consequence of the dissociation of the base pairing (hyperchromic shift), the absorbance at 260 nm rises with increasing temperature. In order to determine this temperature-dependent absorbance change as precisely as possible, it is necessary to have very accurate temperature control. Peltier temperature control with an accuracy of up to 0.1 °C is preferable to conventional water bath temperature control. A melting point curve can be evaluated at any wavelength available in the spectrum.

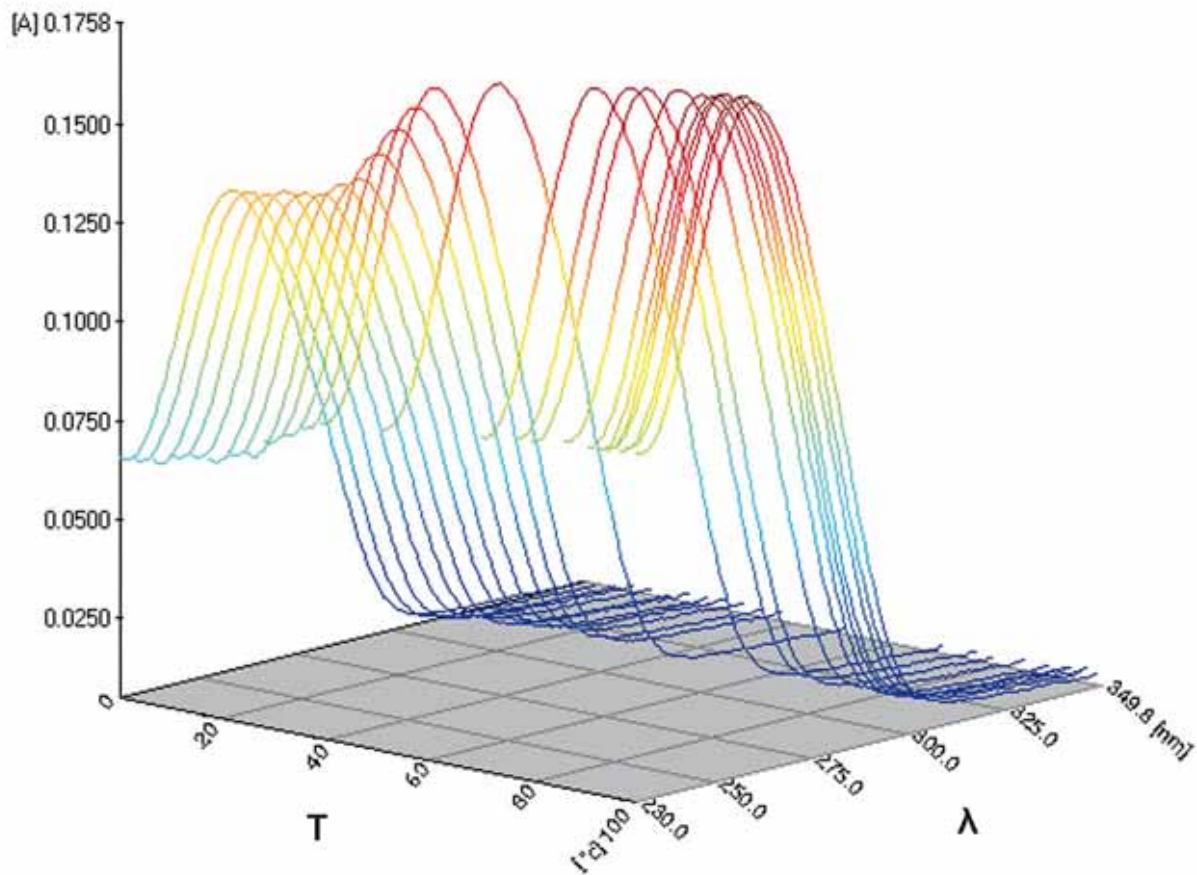


Figure 9: DNA spectrum at different temperatures

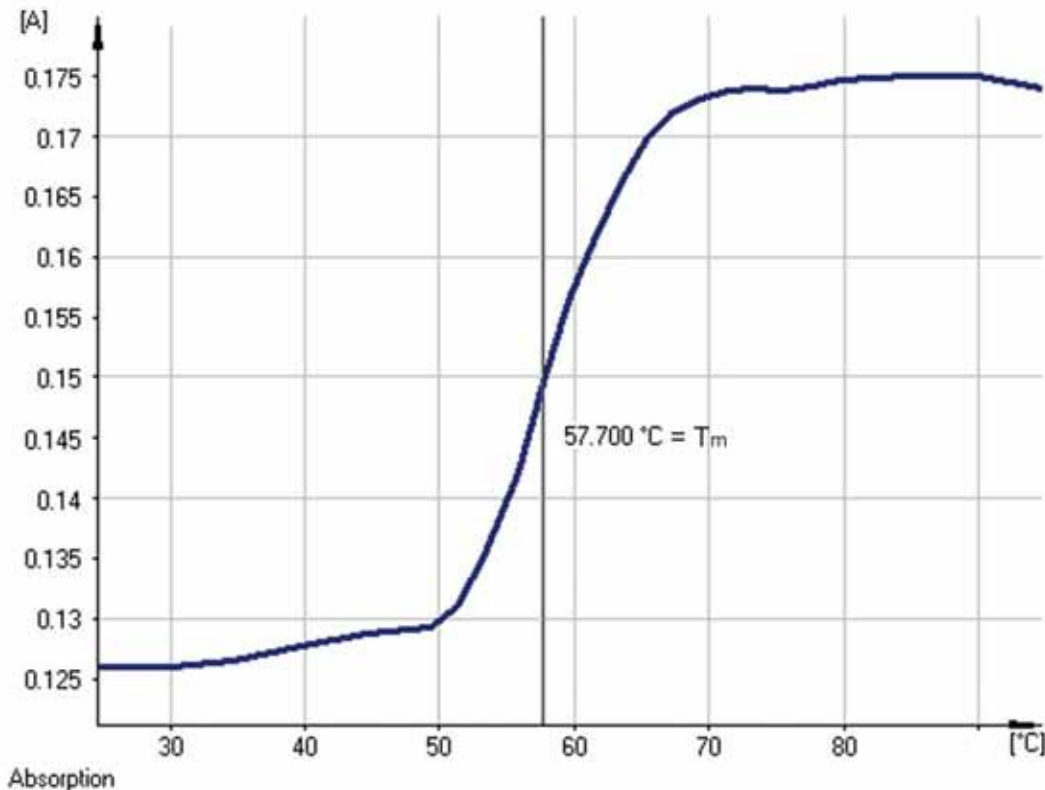


Figure 10: DNA melting point curve at 260 nm

The T_m value can, for example, be found by determining the inflection point on the melting point curve. The melting temperature is dependent on the base pairs present. DNA with a high proportion of GC pairs shows a higher T_m value than DNA with predominantly AT base pairs. A T_m value of 57.7 °C was ascertained for the calf typhus DNA used in the example.

1.6.3 Derivative spectroscopy

Derivative spectroscopy involves taking the derivative of the spectrum for both qualitative and quantitative evaluation. Many of the most important applications in derivative spectroscopy in the ultraviolet and visible range serve to qualitatively identify and analyze samples. Precise details in the fine structures of the derivative spectra allow compounds with overlapping spectra to be resolved.

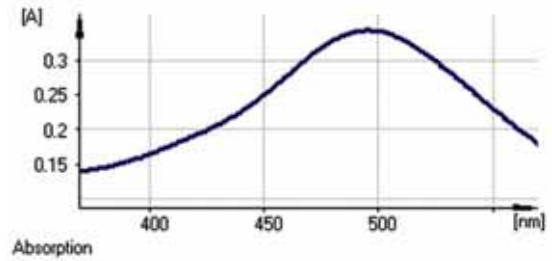
Derivative spectroscopy has also become established in investigating analytes affected by spectral interference. By taking the derivative of a spectrum, a background signal superimposed on the measurement spectrum can be suppressed and the specific absorbance more effectively emphasized.

The following derivatives of the Lambert-Beer law emerge for UV VIS spectroscopy:

Output spectrum

$$A(\lambda) = \varepsilon(\lambda) \cdot c \cdot d$$

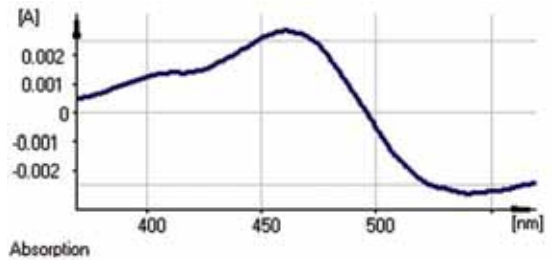
(Equation 3)



1st derivative

$$\frac{dA}{d\lambda} = c \times d \frac{d\varepsilon}{d\lambda}$$

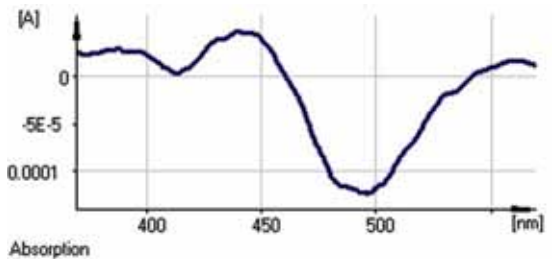
(Equation 4)



2nd derivative

$$\frac{d^2 A}{d\lambda^2} = c \times d \frac{d^2 \varepsilon}{d\lambda^2}$$

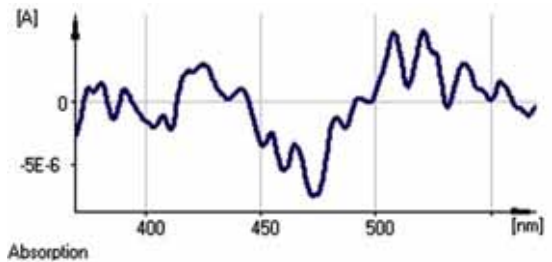
(Equation 5)



3rd derivative

$$\frac{d^3 A}{d\lambda^3} = c \times d \frac{d^3 \varepsilon}{d\lambda^3}$$

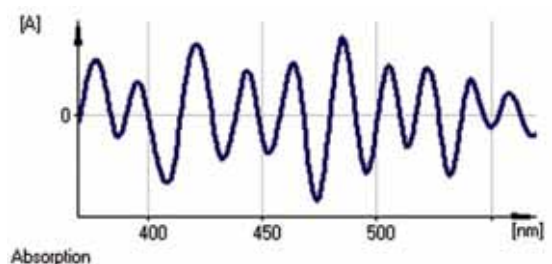
(Equation 6)



4th derivative

$$\frac{d^4 A}{d\lambda^4} = c \times d \frac{d^4 \varepsilon}{d\lambda^4}$$

(Equation 7)



The derivatives can assume both positive and negative values. As the first and second derivatives are extremely sensitive to every change in the slope of the absorbance spectrum, the method of derivative spectroscopy is very well suited for analyzing overlapping absorbance spectra. For this reason, derivative spectroscopy is often used in analytics for trace detection, as well as for quantitative determination. The tangent, peak-peak and peak-zero methods are used for evaluation.

1.6.4 Color analysis

Color is an important property of substances. Color may be determined by evaluating the reflectance spectrum of solid or opaque samples or by measuring transmittance of translucent samples, such as resins, oils or detergents.

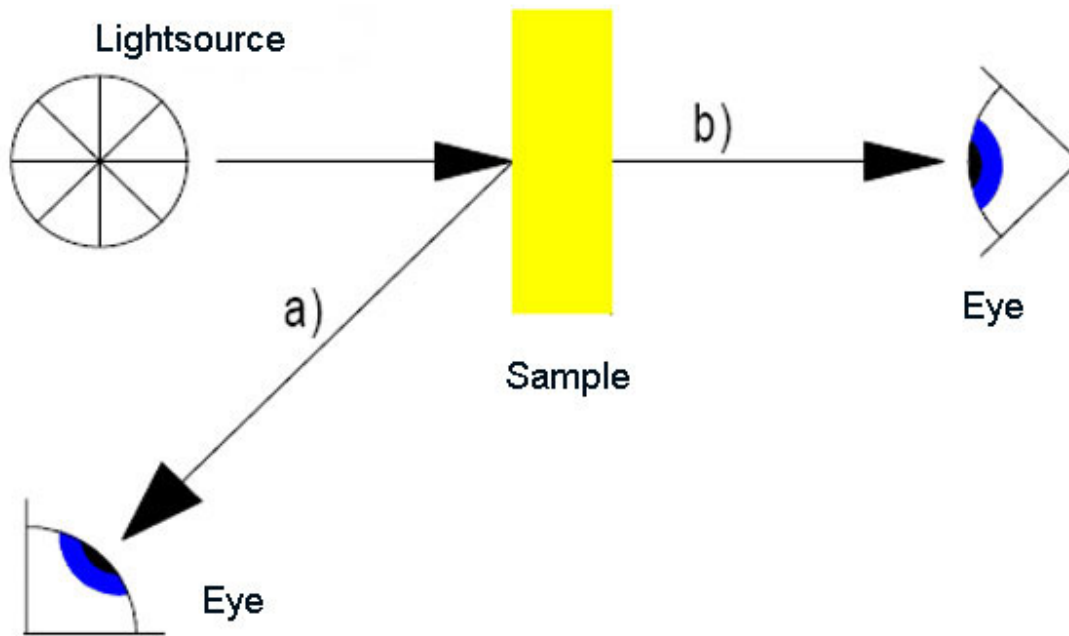
















Figure 11: Perception of color by the human eye

The human eye sees the complementary color of each color absorbed. For example, a yellow solution absorbs light in the blue range as shown in the following overview.

Absorbed color	Absorbed wavelengths [nm]	Complementary color
 violet	380 – 435	 yellow-green
 blue	435 – 480	 yellow
 green-blue	480 – 490	 orange
 green	490 – 560	 red
 yellow-green	560 – 595	 purple
 orange	595 – 650	 green-blue
 red	650 – 780	 blue-green

The visual process in the human eye is mathematically imitated with standardized datasets. The result is a set of color values that objectively and uniquely describe the color of a sample.

The evaluation takes into consideration the definitions of CIE XYZ color space* in national and international standards, such as DIN 5033 or ASTM E-308.

* CIE XYZ color space is an attempt by the *Commission internationale de l'éclairage* to represent colors based the human color perception.

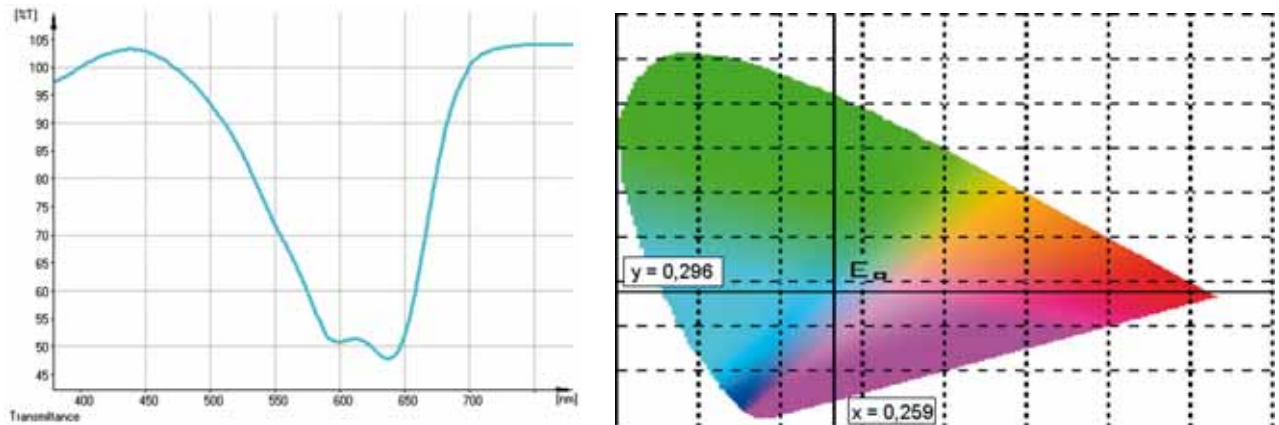


Figure 12: Color determination of a blue solution (left: transmittance spectrum, right: representation in the color chart)

1.6.5 Determination of layer thickness

The reflectance of light at layers produces interference spectra from which the thickness of the layer can be calculated. A variable angle reflectance attachment is used and a reflectance spectrum at a defined angle is taken to determine the layer thickness.

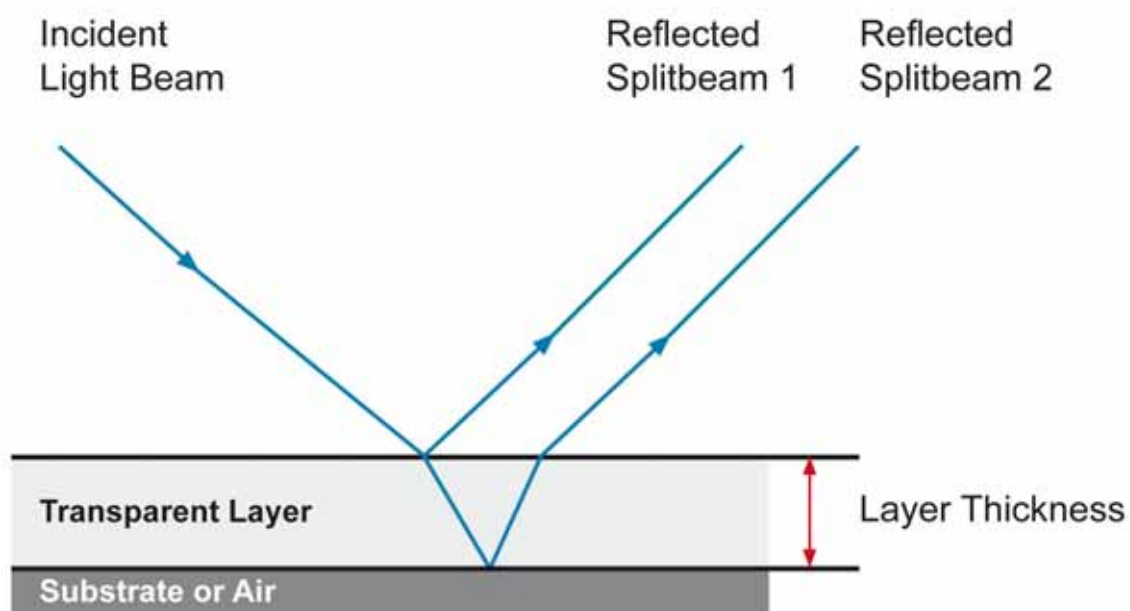


Figure 13: Interference model for determining layer thickness

The interference spectrum produced is dependent on the geometric path length and the refractive index. The optical layer thickness is calculated, which is the product of the geometric layer thickness and the refractive index.

$$d = \frac{m \cdot \frac{\lambda_2 \cdot \lambda_1}{2(\lambda_2 - \lambda_1)}}{\sqrt{n^2 - \sin^2 \Theta}}$$

(Equation 8)

Here d is the thickness of the layer, m the number of interference maxima after the zero order maximum, n the refractive index of the sample, Θ the reflectance angle used, λ_1 the wavelength of the zero order interference maximum and λ_2 the wavelength of the second interference maximum ($\lambda_2 > \lambda_1$).

The following figure shows the reflectance spectrum of a sample of polyester. The interferences occurring are easy to identify in this example.

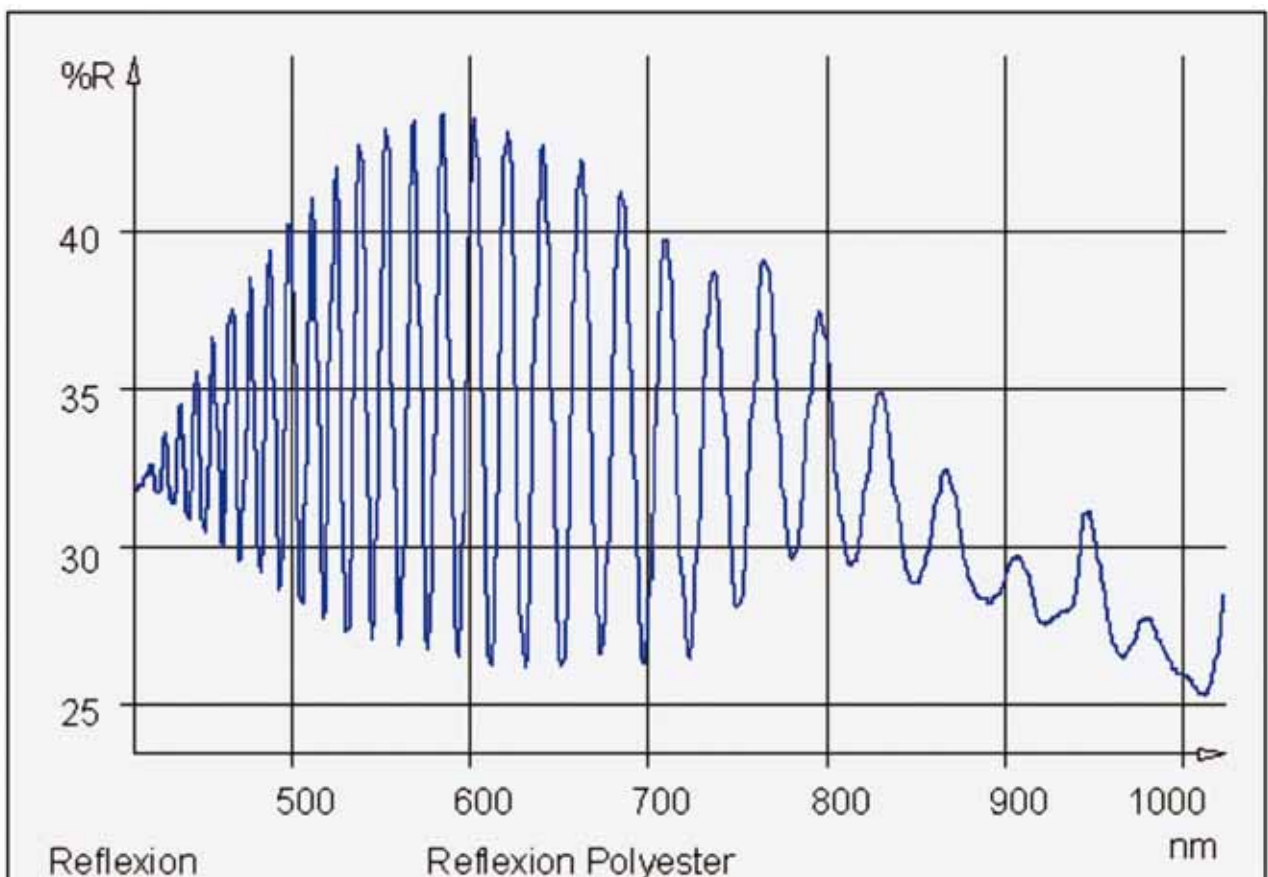


Figure 14: Example of a reflectance spectrum for determining layer thickness (an absorbance maximum of 6.45 μm was determined for a 45° angle of incidence)

2 Configuration of a UV VIS spectrophotometer

2.1 Components of the UV VIS spectrophotometer

A modern UV VIS spectrophotometer generally consists of the following components:

- Radiation source
- Light dispersing unit
- Sample space
- Detector
- Analog-digital (AD) converter
- Computer with display and other peripheral devices

2.2 Radiation sources

UV VIS spectrophotometers mainly use continuum sources, such as a combination of a deuterium lamp (UV range) and a halogen lamp (VIS range).

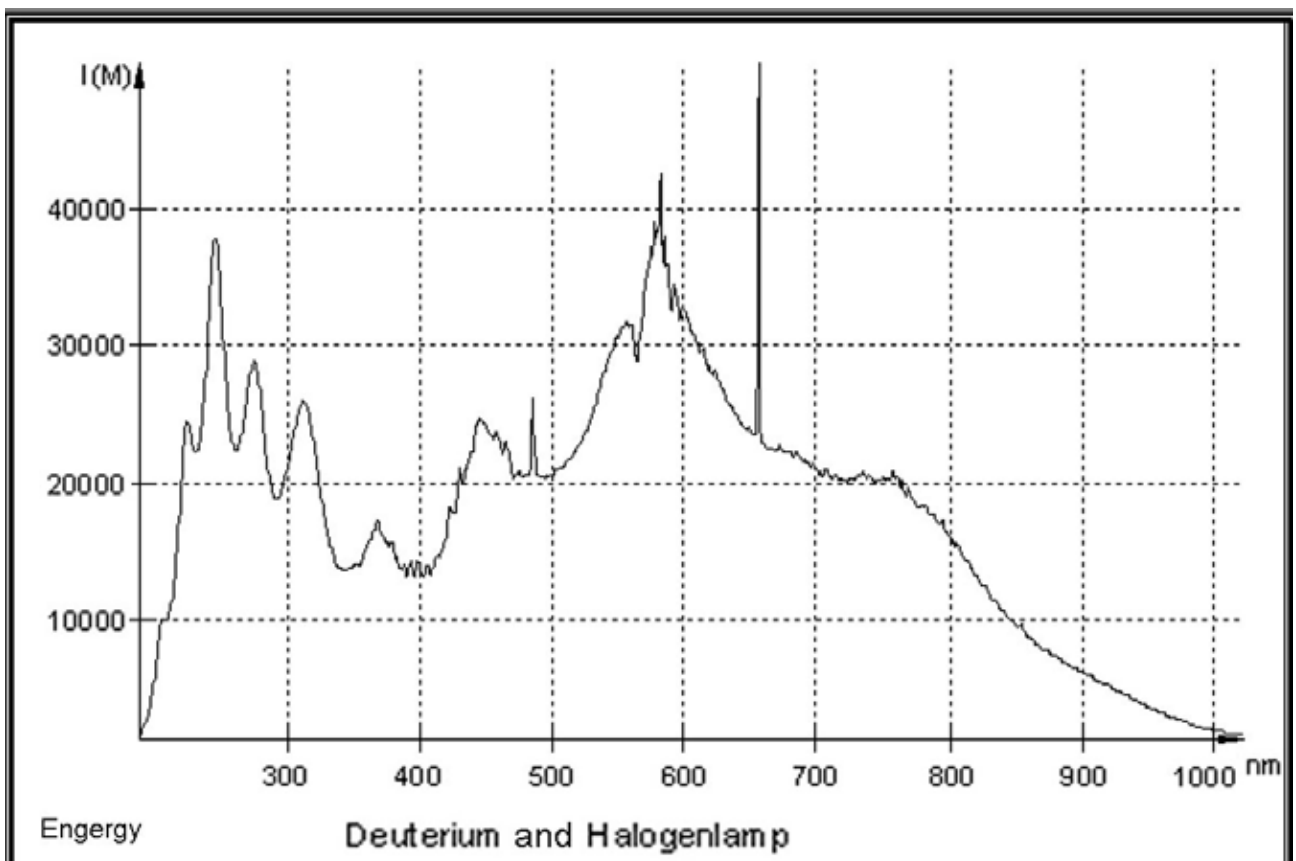


Figure 15: Energy distribution of a combined deuterium and halogen lamp

2.3 Light dispersing unit

Interference filters, prisms or holographic gratings may serve as light dispersing units in photometers. Monochromators and polychromators are also distinguished:

2.3.1 Monochromator system

There are generally two types of photometers – monochromator and polychromator systems. In the case of monochromator systems, the light is dispersing prior to the sample. Monochromatic light is therefore passed through the sample. As a result, measurements are very precise even at very low absorbance.

Optical configuration

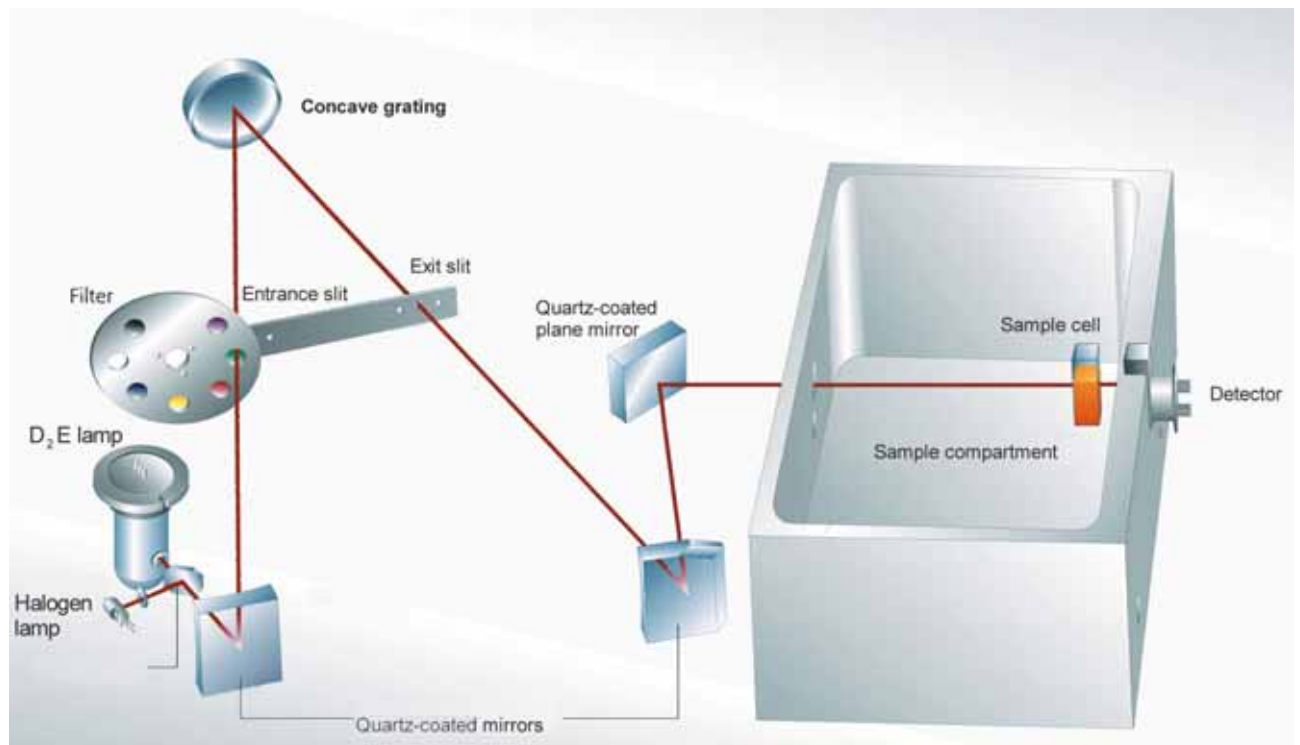
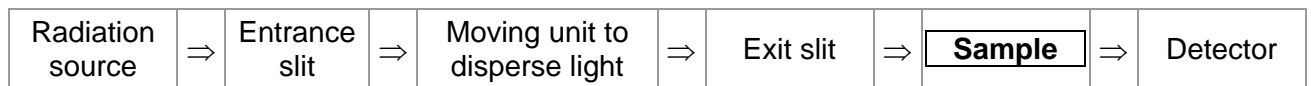


Figure 16: Optical configuration of a monochromator system (e.g., SPECORD® 40)

Properties

- Variable exit slit
- Very good linearity
- Very good S/N
- Scanning of spectra

2.3.2 Polychromator system

In the case of polychromator systems, the complete light spectrum is passed through the sample. The light is dispersing afterwards. This facilitates especially fast spectral measurements.

Optical configuration

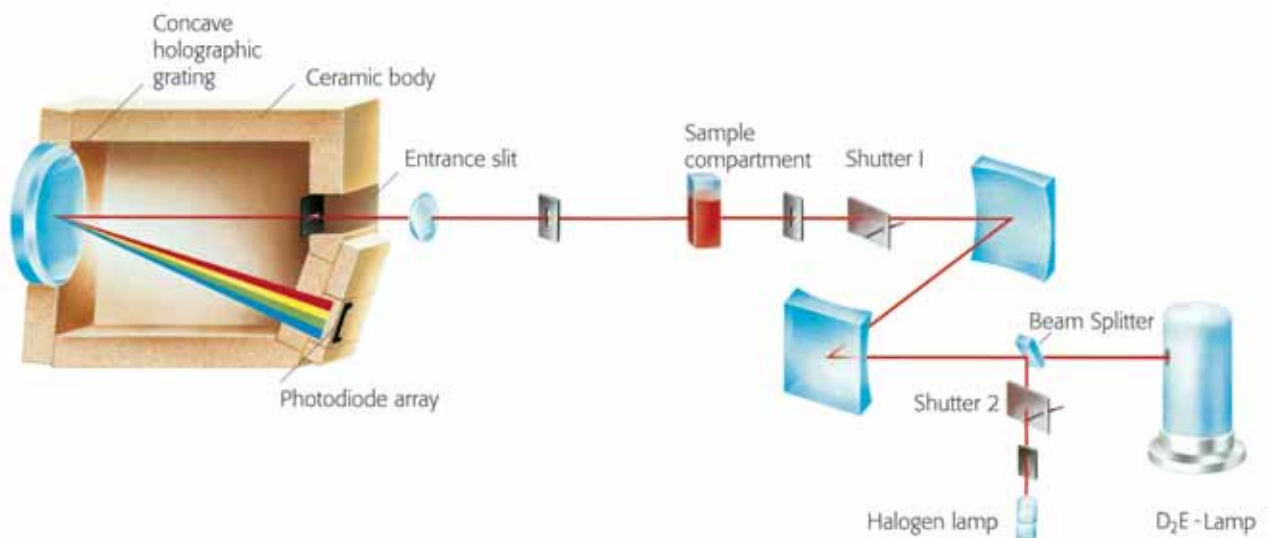


Figure 17: Optical configuration of a polychromator system (e.g. SPECORD® S 600)

Properties

- Fixed entrance slit
- Fast and simultaneous capture of spectra
- Open sample compartment
- Very good wavelength reproducibility

2.4 The effect of the exit slit on spectral resolution

A well-known example to illustrate the effect of slit width on resolution is toluene, which is characterized by a finely structured spectrum in the UV range. The ratio between minimum and maximum at 266 and 269 nm is calculated for evaluation. The larger this ratio, the better the resolution and therefore the smaller the slit.

The following table shows the spectra of a toluene standard in hexane captured with different slit widths.

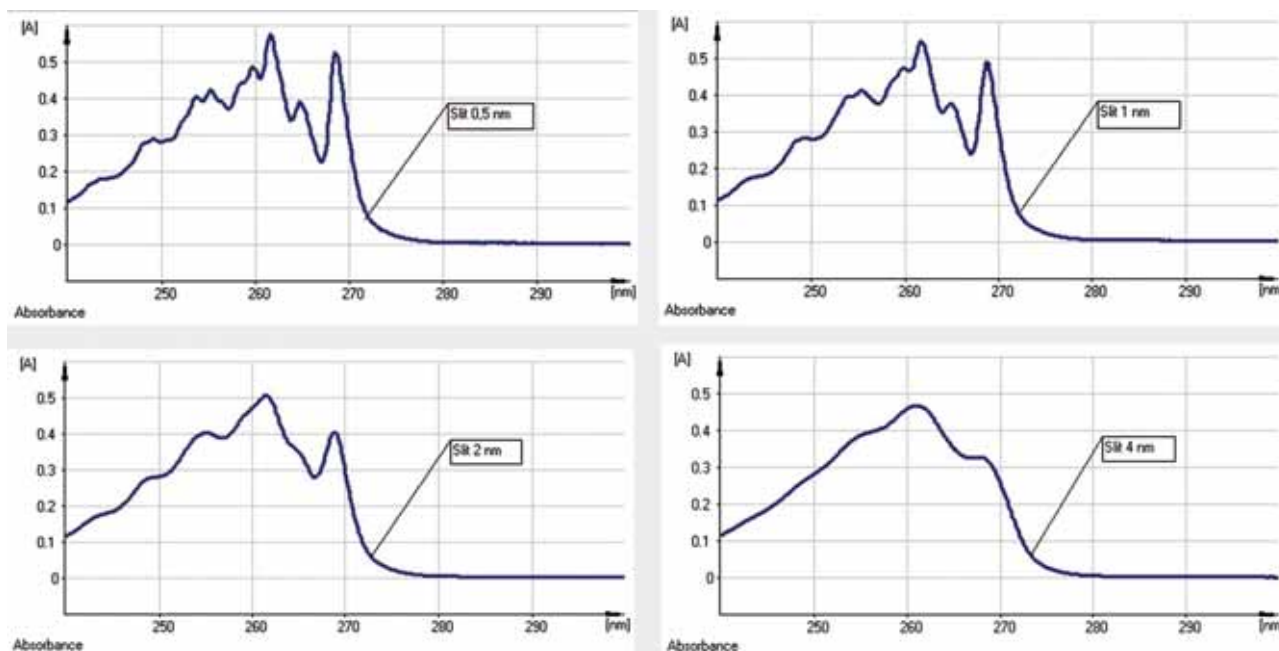


Figure 18: Effect of slit width on optical resolution

Table 5

Slit	A_{minimum} 266 nm	A_{maximum} 269 nm	Ratio $A_{\text{maximum}}/ A_{\text{minimum}}$
4 nm	0.3252	0.3265	1.0
2 nm	0.2784	0.4026	1.4
1 nm	0.2384	0.4823	2.0
0.5 nm	0.2244	0.5229	2.3

The fine structure of toluene spectrum can be clearly resolved with a 0.5 nm slit width. This is also shown by the high value for the ratio $A_{\text{maximum}}/ A_{\text{minimum}}$. However, measurements with a smaller slit show a lower signal to noise ratio than measurements with a larger slit due to the low energy falling on the detector. For measurements close to the detection limit a larger slit (e.g., 4 nm) is therefore preferable. If the exit slit is larger, more energy reaches the detector. The signal to noise ratio is therefore higher and measurements of low absorbance samples can be performed more reliably.

2.5 Sample unit

Many liquids and solutions are used as samples. These are inserted into the photometer sample space in cells.

However, gases and solids can be also photometrically measured using special accessories.

2.6 Detector

Mainly photodiodes or photomultipliers (PMT) are used as detectors in UV VIS spectrophotometers.

	Photodiodes	PMT
Advantages	<ul style="list-style-type: none"> ▪ Large spectral sensitivity range (190 - 1100 nm) ▪ Low susceptibility towards: <ul style="list-style-type: none"> - over-exposure - external mechanical disturbances - external magnetic interference ▪ Operation in the low voltage range ▪ No additional voltage or power supply 	<ul style="list-style-type: none"> ▪ High sensitivity up to 10^6 A/W ▪ Low noise ▪ Large dynamic range approx. 10^6 ▪ Large detector surface of up to 50 mm^2
Disadvantages	<ul style="list-style-type: none"> ▪ Sensitivity approx. 0.5 A/W requires subsequent amplification ▪ Dynamic range $10^4 - 10^5$ ▪ Limited receiver surface (approx. $10 \times 10 \text{ mm}^2$) 	<ul style="list-style-type: none"> ▪ Requires voltage up to 1200 V with voltage divider ▪ Sensitive to over-exposure (risk of destruction) ▪ Sensitive to external mechanical disturbance or magnetic interference ▪ Limited spectral sensitivity range (180 - 650 nm, 180 - 900 nm) ▪ Relatively expensive plus power supply

2.7 *Single and double beam systems*

Singe beam

Single beam photometers have a better signal-to-noise ratio, as the energy is not split into two partial beams.

Split-Beam Technology (SBT)

Spectrophotometers with reference beam technology combine high energy throughput of a single beam photometer with the stability of a double beam system.

Double beam

Double beam photometers show better long-term stability as the measurement result always represents the comparison of the actual energy of the two partial beams; drift phenomena are therefore largely compensated.

Double beam systems also allow direct compensation of changes in the reference sample.

3 Cells for spectrophotometry

The most favorable materials for producing cells are polymer materials, usually acrylic. The use of these cells is only possible above 300 nm as plastic absorbs strongly at shorter wavelengths.

Glass cells are slightly more expensive than plastic cells, but they have a longer service life and can be used over several years. Glass cells cannot be used for measurements below 320 nm.

Quartz cells are produced from highly pure synthetic silicon dioxide. They have the same service life as glass cells, but they have the advantage of also being usable in the UV range.

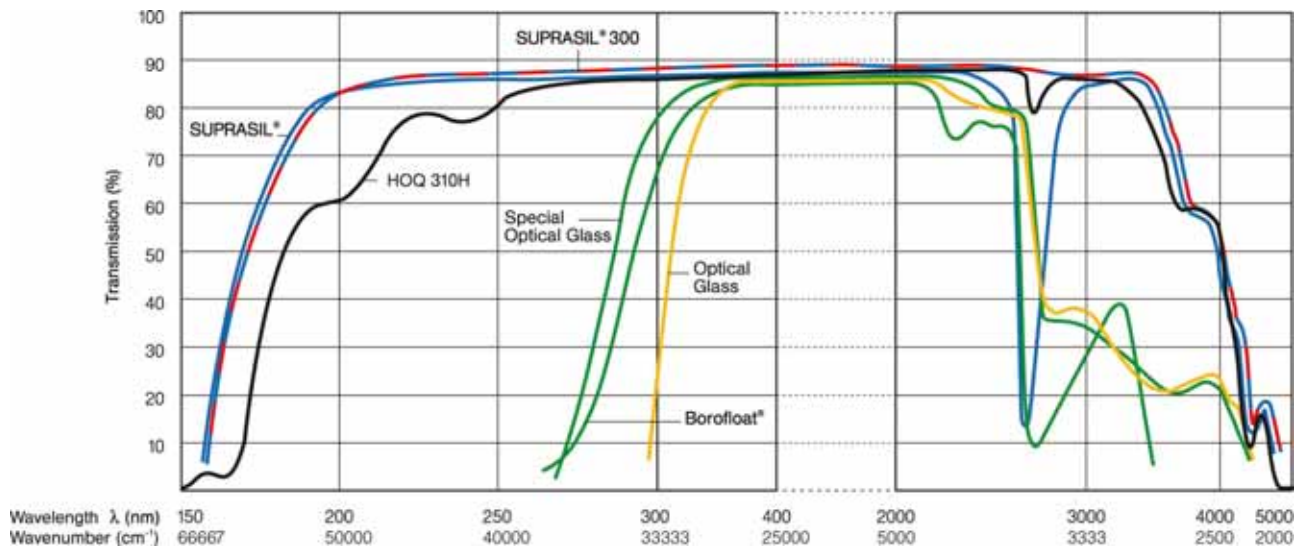


Figure 19: Optical properties of various glass materials

Various types of cells are used depending on the application.

Standard cells

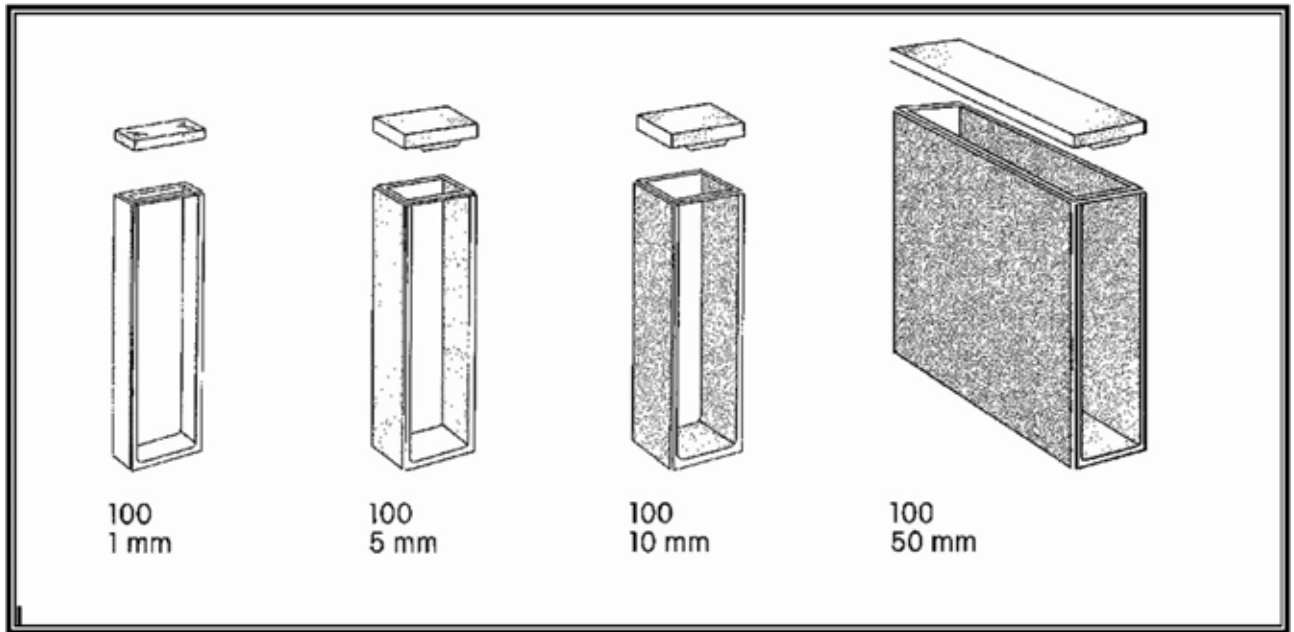


Figure 20: Macro cells with different path lengths

Cells for small sample volumes



Semi-micro cells

(10 mm, 1400 µl)

Micro cells

(10 mm, 400 µl)

Ultra-micro cells

(from 180 µl...)

Ultra-micro cells

(...down to a minimum of 5 µl)

Figure 21: Semi-micro, micro and ultra-micro cells from Hellma



TrayCell
Fiber optic ultra-micro measuring cell for sample volumes 0.7 – 5 μ l
(0.2 or 1 mm path length)

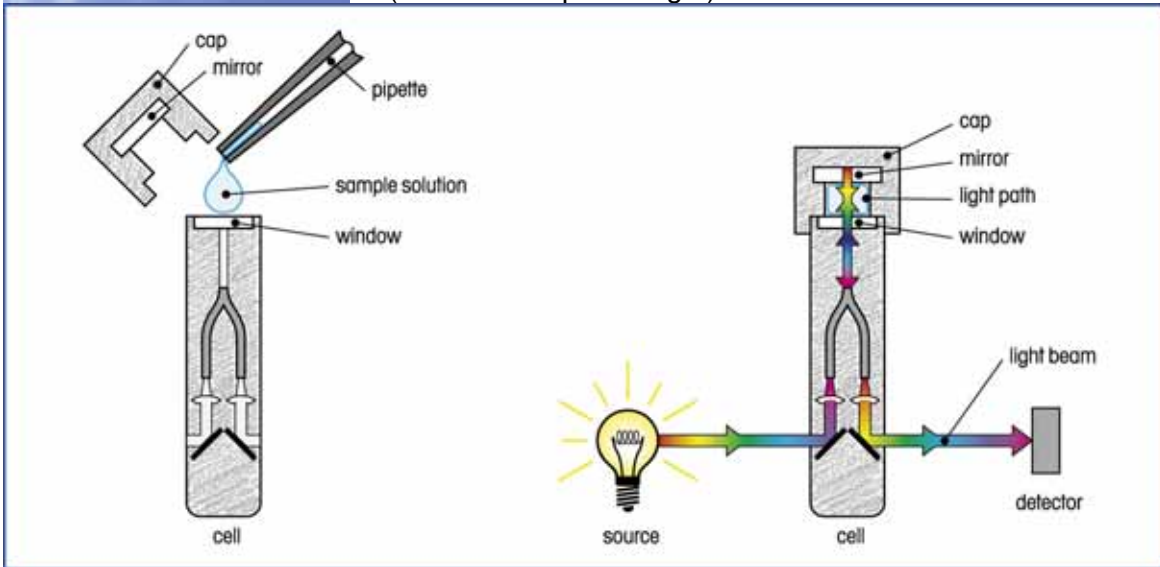


Figure 22: Functional principle of the TrayCell fiber optic ultra measuring cell

Flow-through cells



Flow-through macro cell

PL 10 mm, 750 μ l
PL 50 mm, 3750 μ l



Flow-through micro cell

PL 10 mm, 450 μ l
PL 50 mm, 2250 μ l



Flow-through ultra-micro cell

PL 10 mm, 80 μ l
PL 50 mm, 370 μ l

Figure 23: Various flow-through cells from Hellma

Suction cell



Suction cell with plastic funnel

Cell with separate windows for small path lengths



Window plates with different path lengths
(0.01 – 0.5 mm)





Holder for cells with separate windows

4 Accessories for UV VIS spectroscopy

Special accessories can be used in spectrophotometers for various applications. Whether it is a simple holder for solid samples, various thermostated cells, adjustment or automation devices for high sample throughput, the right accessory is available for almost every application. Various accessories are presented as follows with example applications.

4.1 Overview of simple cell holders

Holder for cells up to 100 mm	A large path length increases the measurement sensitivity	
Quick test cell holder	<p>Suitable for simple and quick quantitative analyses.</p> <p>The chemicals are already in the round cells or are ready for addition.</p> <p>After the reaction, the round cell can be measured directly in the photometer.</p>	
Holder for absorbance tubes	Suitable for gaseous samples.	
Thermo stated cell holder with or without stirrer	<p>Suitable for enzyme kinetics, e.g., at 37 °C.</p> <p>Temperature is controlled using an external water bath.</p>	
Adjustable cell holder for micro cells	Suitable for small sample volumes using micro and ultra-micro cells.	

Peltier thermostated cell holder with PTC 100 control unit	Suitable for very precise temperature control of enzyme kinetics or DNA melting point determinations.	
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4.1.1 Application: Determination of calcium in milk with quick test cells

Method

Photometric determination with glyoxal-bis(2-hydroxyanil) following Carrez clarification and digestion

Reagents

Spectroquant[®] calcium test, Merck, Cat. No. 1.14815.0001

Bioquant[®] Carrez, Merck, Cat. No. 1.10537.0001

Sodium hydroxide solution 1 mol/l, Merck, Cat. No. 1.09137.0001

Neutralit[®] indicator sticks pH 5-10, Merck, Cat. No. 1.09533.0001

Oxisolv[®] digestion reagent, Merck, Cat. No. 1.12936.0030

Digestion basic set, Merck, Art. 1.14613.0001

Microwave digestion device MW 500, Merck, Cat. No. 1.14672.0001

Water GR, Merck, Cat. No. 1.16754.9010

Sample preparation

5 ml milk is added under stirring to 5 ml Carrez-1 and Carrez-2 solutions respectively. The resulting thick paste is diluted with a little water for analysis and then brought to a pH of 7.5 – 8.0 with 1 mol/l sodium hydroxide (check with Neutralit[®] indicator sticks pH 5-10). The mixture is then transferred to a 250 ml volumetric flask and water added up to the mark. After thorough mixing, the undissolved constituents are filtered through a folded filter paper and 10 ml of the filtrate is mixed with 500 mg (= 5 measuring spoons) Oxisolv[®] in the digestion vessel. The closed vessel is heated for 65 s at 500 watts in a microwave oven. After cooling for 5 min the digestion vessel is shaken well and opened. The solution can now be used for calcium determination.

Analysis

The test samples are measured at 550 nm.

Calculation

Calcium content in mg/l = analysis value in mg/l x 50

4.1.2 Application: DNA analysis in micro cells and adjustable cell holder

Especially for biological samples, often only a few microliters are available for analysis as the sample material is frequently very expensive. For this reason, semi-micro or micro cells are used to perform photometric analyses. An adjustable cell holder is used for this purpose to permit reliable measurements by ensuring that the measurement beam is optimally routed through the sample cell.

To determine the DNA concentration, absorbance was measured at 260 nm against extremely pure water as a reference. To calculate the concentration for the measurement in a cell with an optical path of 1 cm, a factor of 500 was entered in the equation for double-stranded DNA (ds), 33 for single-stranded DNA (ss) and 40 for RNA.



$$\text{Concentration [ng/}\mu\text{l]} = A (260) * \text{factor } 500$$

The concentration of the ds DNA was determined using the above equation and is shown in the following Table 6:

Table 6

Absorbance	Concentration [ng/μl]	Standard deviation [ng/μl]
0.007	3.50	0.50
0.017	8.50	1.00
0.033	16.67	0.29
0.074	37.17	2.02
0.150	75.17	1.76
0.290	145.17	2.36
0.601	300.33	7.18
1.161	580.50	9.64
2.154	1076.83	13.29

4.2 Flow-through measurements

<p>Sipper system</p>	<p>Suitable for use with flow-through cells</p>	
<p>APG</p>	<p>Autosampler with sipper system</p>	

4.2.1 Application: Quantitative phosphate determination with a flow-through cell

Introduction

Phosphate determination is a method frequently applied in water and wastewater analytics. Due to the discharge of wastewater and leaching of fertilizer residues used in agriculture, surface water in particular can have a relatively high phosphate content.

In the quantitative determination of phosphate, a reaction mixture of ammonium molybdate, potassium antimony (III) oxide tartrate and ascorbic acid is added to the sample. Potassium hydrogen phosphate is used as the standard solution.

Quantitative analysis involves calibration with standard solutions and subsequent measurement of the samples. Flow-through cells are often used in routine determination to keep the workload to a minimum. A special accessory, the sipper system, is required to be able to work with these cells. This system includes an adjustable holder for 1, 2 or 5 cm flow-through cells and a pump unit to facilitate flow. The following figure schematically shows how the sipper system functions.

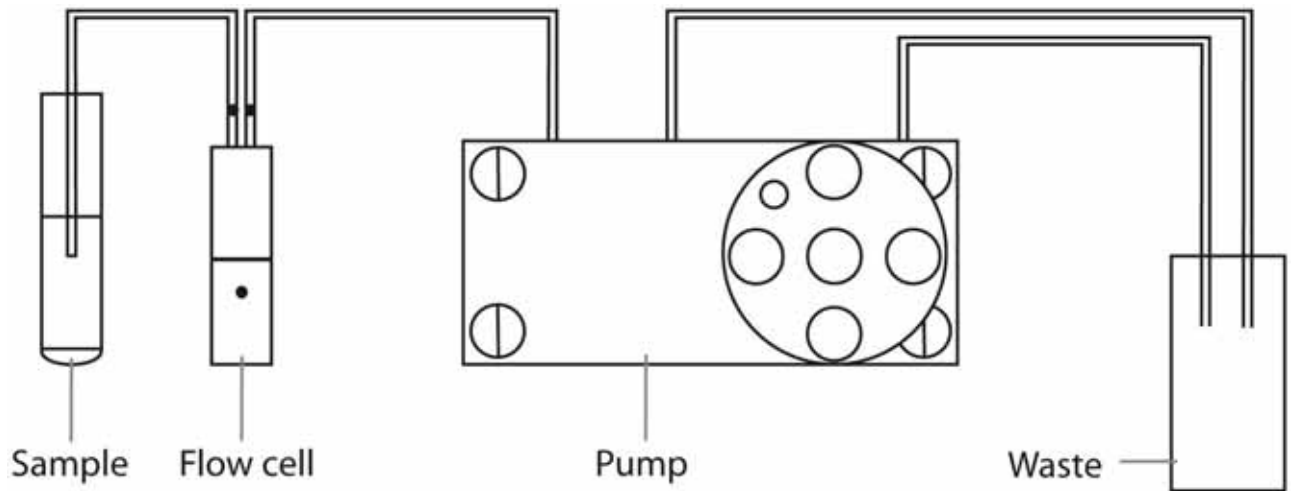


Figure 24: Functional principle of the sipper system

Sample preparation

The following solutions are prepared for the reaction mixture:

Solution 1: dissolve 40g ammonium molybdate in 1 l water (distilled)

Solution 2: dissolve 2.6 g L(+) ascorbic acid in 150 ml water (distilled)

Solution 3: dissolve 2.7 g potassium antimony (III) oxide tartrate in 1 l water (distilled)

Solution 4: 25% H₂SO₄

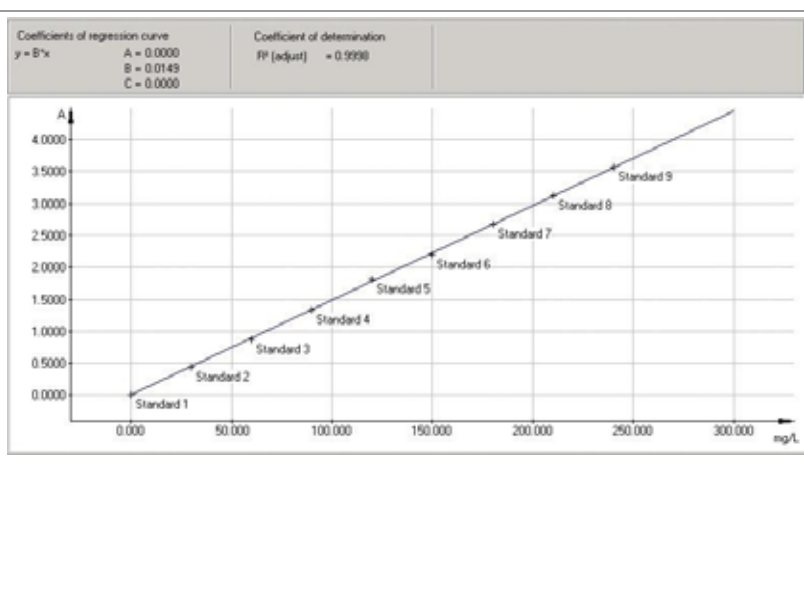
250 ml of Solution 4, 75 ml of Solution 1, 150 ml of Solution 2 and 25 ml of Solution 3 are mixed together to produce the reaction solution. The reaction solution and Solution 2 should be made fresh every day.

40 µl of the sample or standard are added to a 50 ml flask; 8 ml of the reaction mixture is added and the mixture adjusted to a final volume of 50 ml. After 10 minutes the absorbance is measured at 700 nm.





Parameter settings

Calibration

Name	phosphate
Cycle	none
Display	absorbance
Correction	reference
Lamp change at	320 nm
Measurement mode	wavelengths
Wavelength	700 nm
Integration time [s]	0.2
Accessories	sipper
Pump time [s]	30



4.3 Cell changer

6-position cell changer	For 1, 2 or 5 cm cells, with or without water, Peltier temperature control , with and without stirrer	
Cell carousel	With 15 positions for 1 cm cells	
8-position cell changer	With or without temperature control (water or Peltier), optionally with stirrer; can be used in double-beam photometers as 2 x 8 combination	
50-position cell changer	For very high sample throughput with positions for 50 1-cm cells	

4.3.1 Application: Enzyme kinetics with cell changer

Introduction

In kinetic measurements, the time dependent change in the concentration of a reaction component with photometrically detectable properties is determined over a longer period by measuring at one wavelength at short time intervals. The change in absorbance over a certain period of time is related to the speed of the reaction. Through the use of a cell changer several kinetic measurements can be performed simultaneously.

Cell changer operating modes

Besides the normal mode, there is also a *slow time cycle* mode for the sample changer.

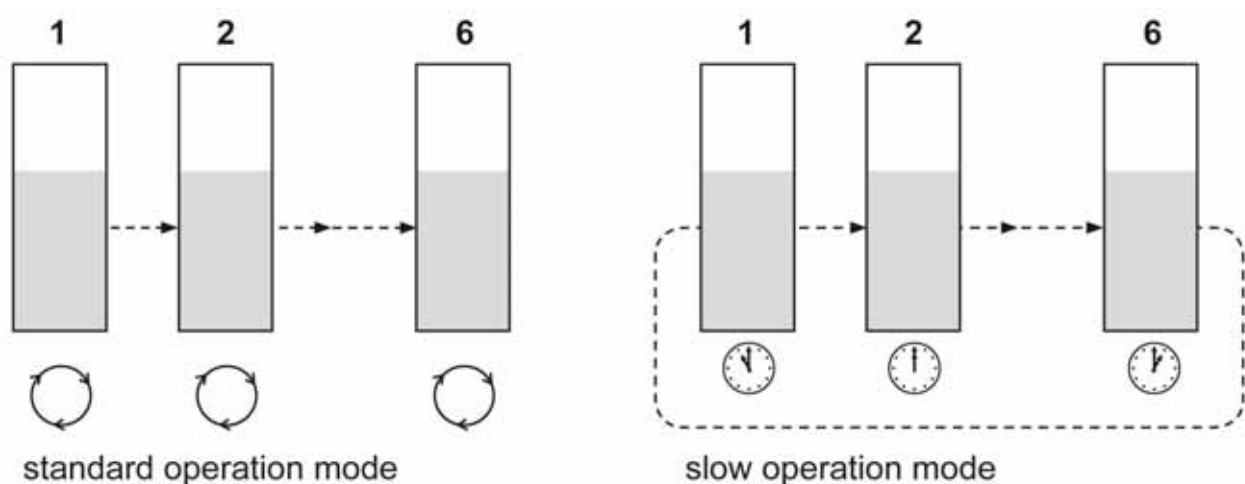


Figure 25: Cell changer operating options

In normal operation, the entire measurement of a single sample is performed before measurement of the next sample begins. For a kinetic measurement, this means that the complete reaction kinetic measurement is carried out for one sample before the next reaction kinetic is started.

For long kinetic measurements, however, the measurement can be optimized and the samples measured in cycles in a time-staggered manner. In the slow kinetic operating mode and always beginning with the same sample, all samples are measured consecutively in each cycle before the next cycle begins.

Application example

The following example presents the kinetic reaction of lactate with lactate dehydrogenase and NAD in a glycine buffer. A blank value and three different concentrations of lactate were measured. The measurement was performed at 340 nm. A measurement point was acquired every 30 seconds over a total measurement time of 10 minutes. The reference measurement was carried out in the first position of the cell changer against an empty cell. The sample table and the superimposed kinetic curves in graph format automatically appear in the window at the end of the measurement.

Sample	Start	Finish	Factor	Slope	R ² adjust	Result
Blank sample	5	30	1	0.0001	0.9994	0.0001
Conc. 1	5	30	1	0.0003	0.9994	0.0003
Conc. 2	5	30	1	0.0015	0.9988	0.0015
Conc. 3	5	30	1	0.0026	0.9984	0.0026

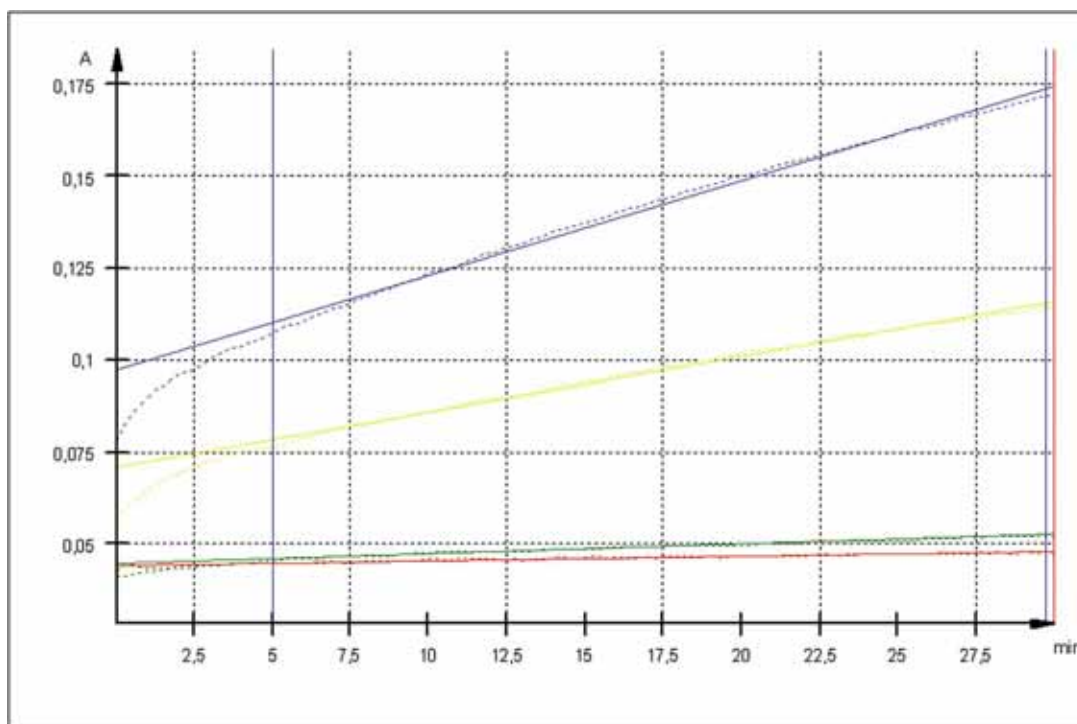

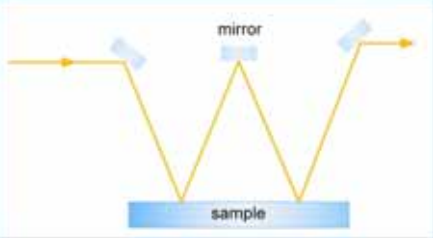

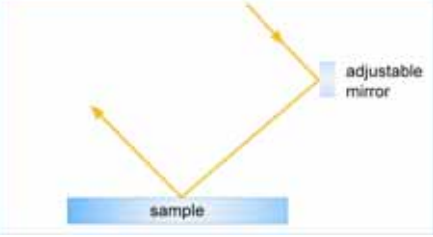

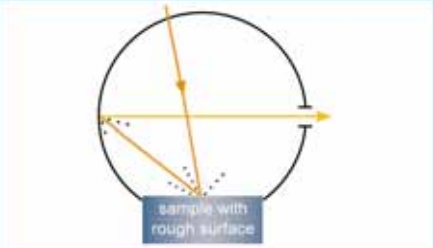



Figure 26: Presentation of the results of a kinetic measurement with cell changer

A freely selectable range can be used to calculate the slope. The slope can also be multiplied by a factor. R² adjust specifies the coefficient of determination for the straight lines applied and therefore the accuracy.

4.4 Accessories for measuring solid samples

4.4.1 Holders for solid samples and reflectance attachments

<p>Holder for solid sample</p>	<p>For transmittance measurements of solid samples (e.g. glass)</p>		
<p>Absolute reflectance</p>		<p>Determination of reflectance at smooth surfaces</p>	
<p>Variable angle reflectance attachment</p>		<p>Determination of reflectance at smooth surfaces; layer thickness measurement</p>	
<p>Reflectance measurements with the photometer integrating</p>		<p>Diffuse reflectance from powders or solids with rough surfaces, diffuse transmittance measurement</p>	

4.4.2 Application: Determination of layer thickness using transmittance measurement with the SPECORD®

The following parameter settings were used for transmittance measurement of a coated slide.

Cycle:	none	Measurement mode:	step mode
Correction:	reference	Range [nm]:	350 - 700
Display:	transmittance	Step width [nm]:	0.2
Lamp change at:	320 nm	Integration time [s]	0.2
Slit:	0.5 nm	Accessories:	none

The reference measurement was performed against air or an uncoated slide.

In both cases the transmittance spectra show an interference pattern above approximately 535 nm. The following curves show the spectra in the relevant range between 530 and 700 nm.

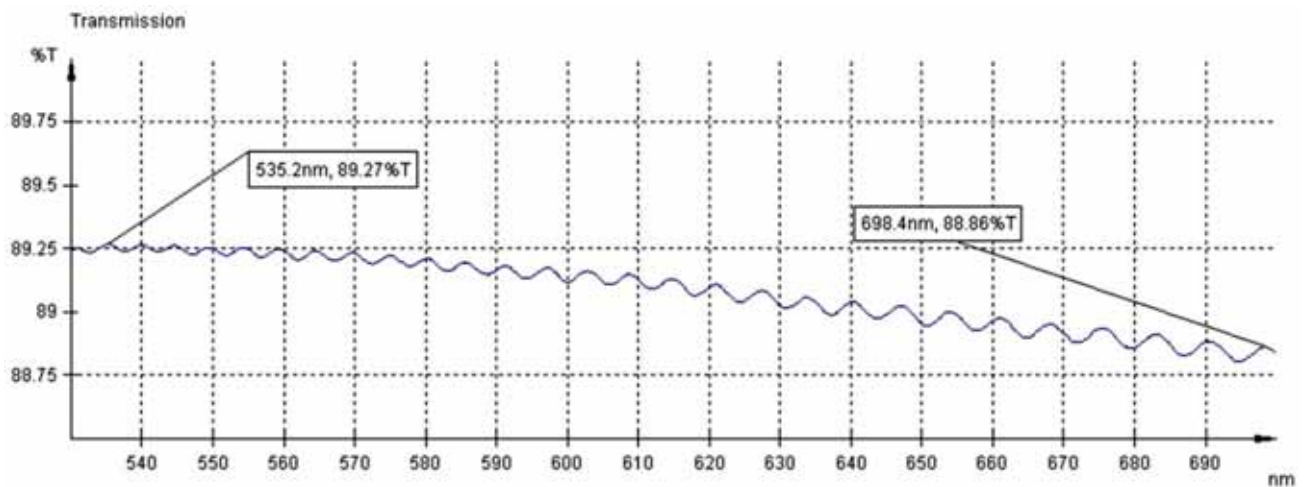


Figure 27: Transmittance spectrum of a sample-coated slide against air (smoothed with 25 sampling points)

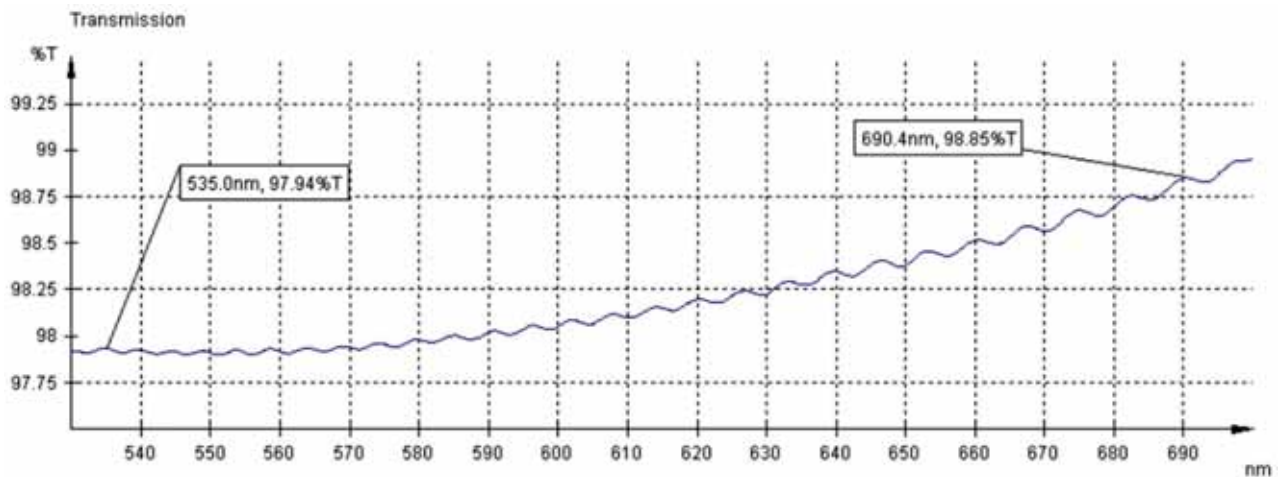


Figure 28: Transmittance spectrum of a sample-coated slide against an uncoated slide (smoothed with 25 sampling points)

Due to the decreased transmittance of the uncoated slide, other transmittance values were obtained with a reference measurement against air than with a reference measurement against an uncoated slide. Nevertheless, the interesting interference spectrum is unaffected and is attributable to only the sample.

The layer thickness can be determined from the measured spectra with a special software application based on Equation 8.

	Sample against air	Sample against reference slide
m	27	26
λ_1 [nm]	535.0	698.4
λ_2 [nm]	535.2	690.4
n	1.5	1.5
Θ	0	0
d [μ m]	20.6	20.6

4.4.3 Application: Determination of the whiteness and yellowness indices of dental surfaces with the integrating sphere

Introduction

The Ulbricht sphere (integrating sphere) is outstandingly suitable for transmittance and reflectance measurements of solid, liquid and powder samples. The following application describes a method of analyzing dental surfaces and their evaluation. The result obtained is the respective reflectance spectrum, from which the whiteness and yellowness indices can be calculated. The whiteness index is intended to compare the similarity of the shade with ideal white; the yellowness index describes the absence of brightness or shades of gray in a color (DIN 6167).

Procedure

The integrating sphere can be used together with SPECORD®. The following parameters were selected for analyzing different dental samples:

Spectrum	380 -780 nm
Mode	scan mode
Display	reflectance
Integration time	0.10 s
Step width	1 nm
Speed	10 nm/s
Accessories	integrating sphere
Cycle	none
Slit width	4 nm

Results

The following spectra were acquired from the dental samples A, B and C:

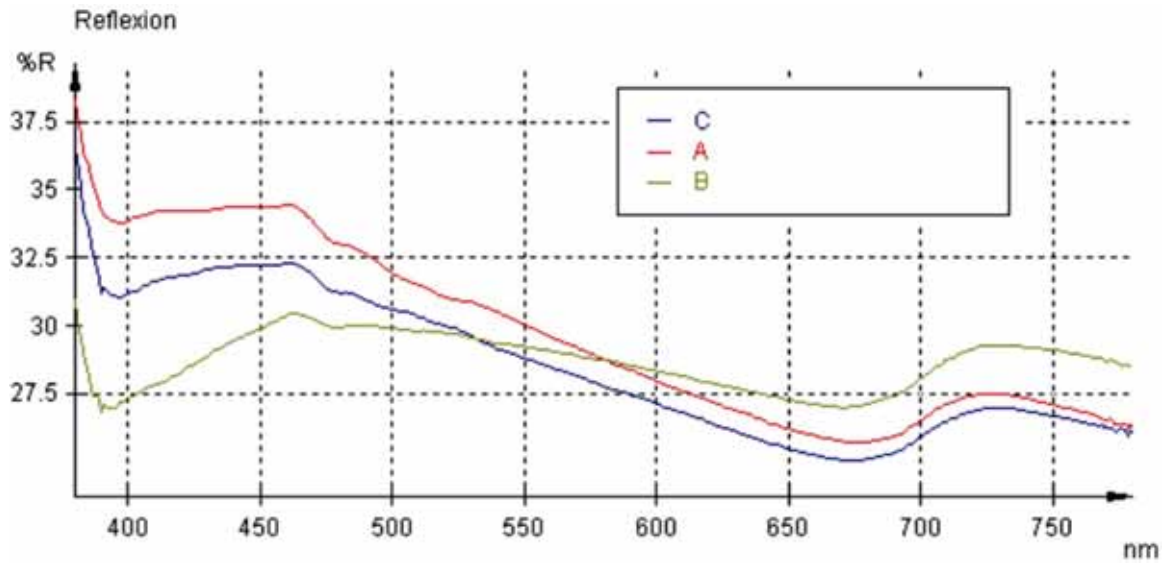


Figure 29: Reflectance spectra of dental samples measured with the photometer sphere

Evaluation

The evaluation was performed using the Analytik Jena AG color software. This software application offers the option of calculating the whiteness and yellowness indices. The following calculation formula was used with an angle of observation of 10° and the D65 light source (corresponding to natural light).

*Whiteness and yellowness indices (ASTM calculation: E 313-67): white=4*B-3*Y, yellow=100*(1-B/Y) with B=0.8475*Z*

Y and Z are the respective CIE standard color value components in the color systems.

Table 7

Sample	Whiteness index	Yellowness index
A	46.53	-14.13
B	31.66	-2.25
C	41.62	-11.36

It was possible to differentiate the various polished dental samples on the basis of the whiteness and yellowness indices shown in the Table and from the above spectra. The methods can be very successfully applied for analyzing dental surfaces.

Similar applications include the determination of the whiteness index for textiles, paper or other materials.

4.4.4 Application: Characterization of sun cream

Characterization of UVA protection

Until recently there was no recognized method in Europe for determining UVA radiation. For this reason, many manufacturers of sunscreen products used the Australian Standard 2604:98. The Australian standard is considered to be fulfilled if the sunscreen product reduces the transmittance of UVA radiation in the 320 to 360 nm range by at least 90%. If this transmittance is exceeded at any point in this range, the standard is not fulfilled. However, since 2004 the DIN standard 67502 for characterizing UVA protection has been in effect. Here UVA protection is calculated as UVA balance and so provides better differentiation of the UVA protective effect.

Equipment and materials

Six different sunscreen products available on the European market were investigated. The sun protection factor (SPF) and the UV filter systems used in the sunscreens as specified by the manufacturers are presented in the following Table 8. These are all oil-in-emulsion compositions.

Table 8

Sample number	Type	Specified SPF	UV filter system
1	sun lotion	6	octyl methoxycinnamate phenylbenzimidazole sulphonic acid
2	sun lotion	12	ethylhexyl methoxycinnamate octocrylene buthyl methoxydibenzoylmethane
3	sun lotion	15	ethylhexyl methoxycinnamate octocrylene buthyl methoxydibenzoylmethane
4	sun spray	15	ethylhexyl methoxycinnamate octocrylene buthyl methoxydibenzoylmethane
5	sun cream	20	ethylhexyl methoxycinnamate octocrylene buthyl methoxydibenzoylmethane
6	sun cream	30	octocrylene zinc oxide ethylhexyl methoxycinnamate phenylbenzimidazole sulphonic acid

Almost all the sunscreen products investigated have at least a UVB and a UVA filter system. Sample 1, however, only contains a UVB filter and is therefore not a representative sample for the European market.

SPECORD[®] was used to investigate the sunscreen products with an integrating sphere (Ulbricht sphere) as an accessory. The sphere with a diameter of 75 mm consists of two Spectralon[®] hemispheres with openings for entry and exit of the beam, beam deflection optics and holders for the samples and cells for transmittance and reflectance measurements. Spectralon[®] is a teflon-type plastic with extremely high reflectivity over a broad spectral range. The integrating sphere evenly illuminates the detector surface in the spectrometer, independent of any effects (scattering, deviation) of the beam caused by the sample. This serves to increase the accuracy of the measurement. Measurement of solid, transparent samples of a high thickness with the integrating sphere is the only way of avoiding the systematic measurement errors caused by effects on the beam.

Procedure

A quartz plate was selected as the sample carrier. The uncoated sample substrate was first weighed. The products were then applied to the quartz plate at 0.75 mg/cm² in several evenly distributed points. The samples were then evenly spread over the quartz plate with a powder-free latex glove which had previously been saturated with the sample for approximately 1 min. The plate was weighed again as a control. The samples were first measured after a 15-minute equilibration period. This process was carried out twice for each sample and the individual measurements were repeated twice at different positions. The transmittance measurement was performed in SPECORD[®] in the range 290 to 400 nm. An uncoated quartz plate was used for the reference measurement. Glycerol can also be spread over the plate and used for reference measurement.

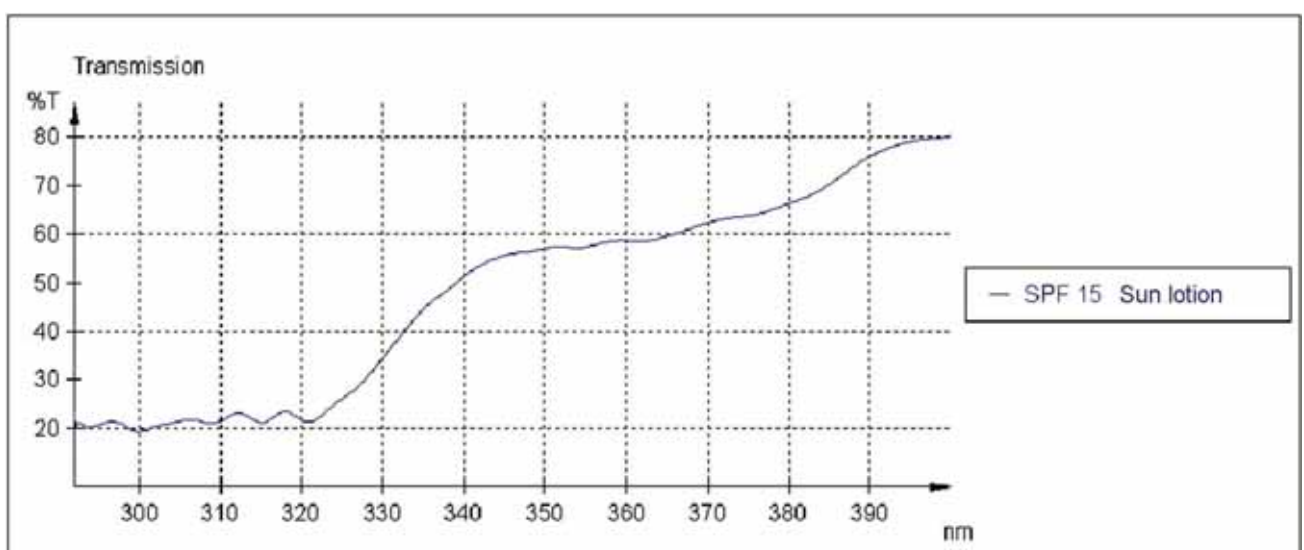


Figure 30: Transmittance spectrum of sun lotion 3 with SPF 15

Result and discussion

The measurement method was used for all products without difficulties. The transmittance spectrum of Sample 3 is presented as an example. It was found that all samples reduce the radiation in the transmittance spectrum between 320 and 360 nm by 60%. Only the transmittance of Sample 1 with SPF 6 was higher. There was no indication on the packaging of Sample 1 that the product had been tested according to the internationally recognized Australian Standard 2604 for UVA protection. After evaluating the spectra it could not be concluded that higher protection factors also had higher UVA protection. There is no correlation between UVA protection and SPF. For some samples, the UVA protection remained the same with increasing SPF without violating the standard. It was also observed that sunscreens with the same SPF (Samples 3 and 4) provide different UVA protection. Applying DIN 67502 may improve differentiation between sunscreen products.

4.5 *Measuring probes*

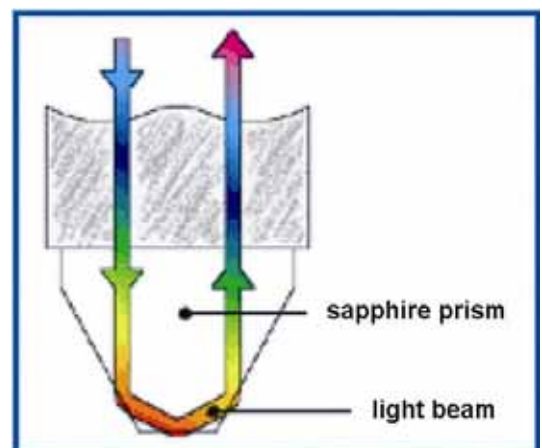
- Fiber optic cable with optional measuring probe
- For online measurements outside of the photometer



4.5.1 **Application: Measurement of dyes with the ATR probe**

Introduction

ATR probes can be used to analyze the absorbance properties of optically dense media. The principle of the ATR (Attenuated Total Reflectance) probes is based on the different refractive indices of the ATR crystal (high refractive index) and the sample (low refractive index). The light beam is totally reflected at the interface between the ATR crystal and the sample. A portion of the light beam penetrates the sample to a small depth and can be absorbed. Hence, the amount of reflected light is attenuated. An absorbance or transmittance spectrum of the sample is obtained as a result.



Procedure

The ATR probe can be used together with SPECORD[®]. The light is passed via a fiber optic adapter into the fiber optic cable and reaches the probe. The attenuated light finds its way back to the photometer via a second fiber optic cable and is detected.

Absorbance spectra between 370 and 800 nm were taken with the SPECORD[®] to analyze various pigments.



Results

The following spectra were acquired:

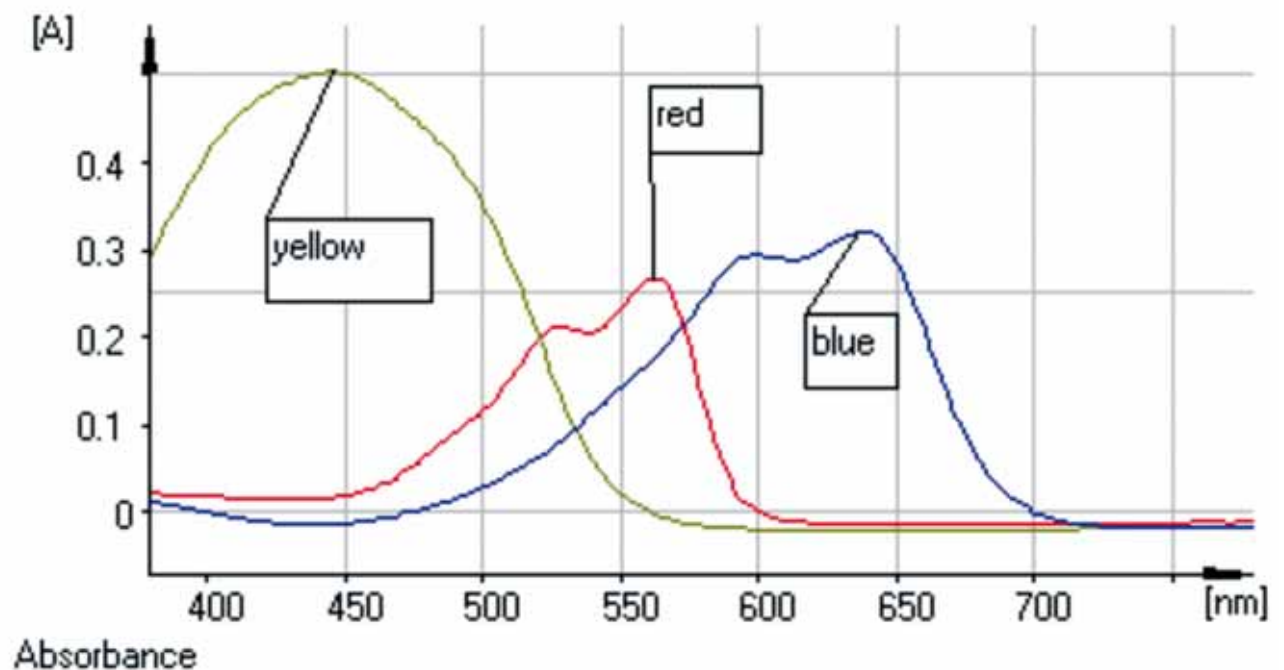


Figure 31: Absorbance spectrum of various printing inks

Absorbance maxima were found for the red pigment at 528 nm and 562 nm, for the yellow pigment at 446 nm and for the blue pigment at 600 nm and 638 nm.

Evaluation

The evaluation was performed using the Analytik Jena AG color software. This software provides the possibility of calculating different color numbers taking into account the different angles of incidence and light sources.

The color systems used were the standard color values as well as the CIELAB system. Here an observation angle of 10° and the D65 light source (corresponding to natural light) were selected.

The standard color coordinates x, y and z characterize the color in a three-dimensional Cartesian space. In the CIELAB system, the brightness of the color shade is characterized by the L value. Here the 'a' value stands for the red-green component and the 'b' values for the yellow-blue component.

Color	Standard color coordinates			CIELAB		
	X	Y	Z	L	a	b
red	81.36	74.23	99.59	89.03	22.42	-13.98
yellow	83.35	83.35	36.36	93.17	8.44	48.80
blue	63.32	72.27	108.81	88.10	-11.65	-21.43