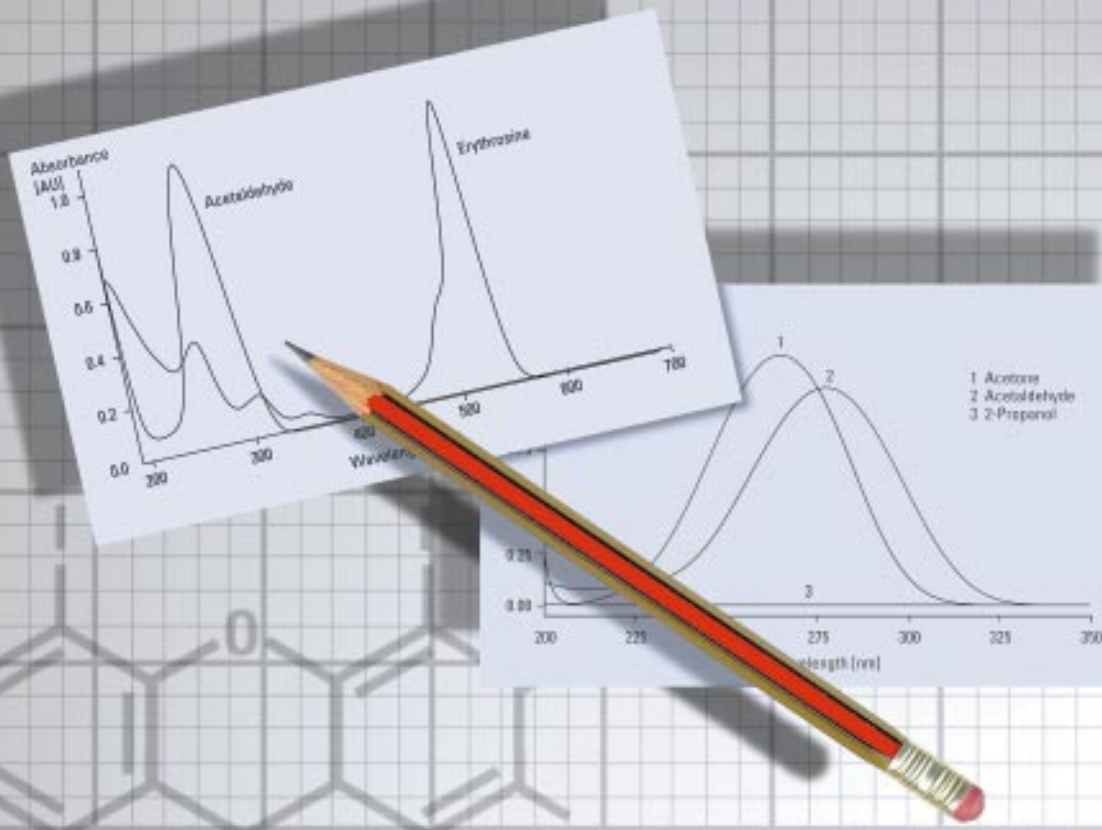
HEWLETT PACKARD

Expanding Possibilities

Fundamentals of UV-visible spectroscopy

A Workbook



Fundamentals of UV-visible spectroscopy

A Workbook

The information contained in this workbook is subject to change without notice.

Printed in Germany 08/98
Hewlett-Packard Company
Publication Number
12-5967-6357E

Preface

UV-visible spectroscopy is a well-established analytical technique with mature methods and equipment. It is commonly used in both research and science as well as in industry. Applications are found in classic analytical fields such as in the chemical industry (mainly petrochemical and dyestuff industry), the pharmaceutical industry or in environmental analyses. Other fields of application are gaining more and more importance such as biochemistry and bioscience. UV-visible spectrophotometers are available in almost all laboratories that do chemical or physical measurements. In general, they are simple to operate and modern spectrophotometers with built-in or external computer systems deliver processed results very rapidly. However, the apparent simplicity and speed of the technique can mean that spectrophotometers are used without sufficient understanding and erroneous results can be all too common.

This workbook is intended to provide information for practical training and instruction of school groups or students of engineering or natural sciences. It is also meant for self-study and independent learning of laboratory personnel and professional users. It is a companion to the primer "Fundamentals of Modern UV-visible Spectroscopy" (Hewlett-Packard publication number 12-5965-5123E) which can be used in teaching and learning the theory of UV-visible spectroscopy and instrumentation. With this workbook users can deepen the theoretical knowledge they may already have gained and complement it with practical exercises. The experiments and the results that are achieved help to better understand the theoretical background of UV-visible spectroscopy. The workbook will enable users to understand the possibilities, but also the sources of error, pitfalls and the limits when working with UV-visible spectroscopy. Soon users will develop a better understanding of their equipment and will be able to appreciate the advantages of diode array spectrophotometers compared to conventional scanning spectrophotometers.

Little prior knowledge of the equipment and of laboratory work is required before starting with the experiments. Nevertheless all users are asked to read and thoroughly follow the safety instructions for laboratory work and especially for the chemical substances they deal with. Unqualified persons have to be guided and supervised by authorized personnel. Make yourself familiar with the handbooks and users' guides of your equipment. Only if the experiments are carried out carefully according to the Good Laboratory Practice (GLP) regulations best results can be achieved.

We would be very pleased to receive feedback on this workbook, especially with suggestions for improvements or additional experiments that could be included.

The primer "Fundamentals of modern UV-visible Spectroscopy", is available from Hewlett-Packard as publication number 12-5965-5123E.

General Equipment

- ❑ UV-visible spectrophotometer.
  With a few exceptions all experiments described in this workbook were performed on an HP 8453 diode-array UV-visible spectrophotometer but, in principle, any good quality UV-visible spectrophotometer may be used. The times given (for the experiments including evaluation) are based on the use of the HP 8453 which scans full spectra in about 1.5 seconds. If a scanning spectrophotometer is used, the time for experiments that require spectral measurement will be significantly longer.
- ❑ Calculator or personal computer for evaluation of data.
- ❑ Analytical balance for accurate preparation of test samples.
- ❑ Spatula for handling powders.
- ❑ Pipette bulb (to avoid mouth pipetting).
- ❑ Weighing papers.
- ❑ Cuvettes. When using a single beam spectrophotometer only one cuvette is needed. If a double-beam spectrophotometer is used two, preferably matched, cuvettes are required.

General Aspects of Sample Handling and Preparation

To minimize possible sources of error always consider the following general aspects of good sample handling:

- Use a pipette to empty and fill the cells.
- Rinse the cell with the solvent or solution to be measured at least three times before measurement.
- Take care not to dirty the optical surfaces of the cell with fingerprints or with any other substances.
- In order to verify the quality and cleanliness of the cell used, measure a reference on air and a sample on the cell filled with distilled water before sample measurement.
- When using a conventional scanning spectrophotometer, measure the references in the same wavelength range as the sample measurements.
- Use fresh samples only. Storing the samples, especially in light and warm rooms, may create multiple sources of error due to unwanted reactions such as decomposition and sample contamination.

Caution:
Carefully read, understand and follow the safety instructions for the substances you deal with. Chemical substances must always be handled according to legal regulations for hazardous materials that are in force in your country and at your place of work. All substances used must be handled using exactly the concentrations and compositions described in this workbook.

Contents

Part 1.
Basic Principles and Applications 7

1.1. Basic Principles—What is a UV-visible Spectrum? 8
1.2. Chromophores 13
1.3. Environment of Chromophores 17
1.4. The Effect of Solvents on UV-visible Spectra 21
1.5. The Meaning of Color in Spectroscopy 25
1.6. Quantitative Analysis — Lambert-Beer's Law
Path Length Dependence of Absorbance Values 29
1.7. Concentration Dependence of Absorbance Values 37
1.8. Possible Sources of Error — Influence of Impurities 46
1.9. Influence of Temperature — Potassium Chloride 52
1.10. Influence of Temperature — Methyl Orange 57
1.11. Influence of pH — Buffered Methyl Orange Solutions 64
1.12. Influence of pH — Potassium Dichromate Solution 69
1.13. Effect of Concentration 72
1.14. Principle of Additivity 77

Part 2.
Measuring Instrument Performance 83

2.1. Wavelength Accuracy 85
2.2. Wavelength Accuracy Using the Deuterium Lines 89
2.3. Photometric Accuracy Using Potassium Dichromate 92
2.4. Photometric Accuracy Using Neutral Density Glass Filters 96
2.5. Stray Light 99
2.6. Spectral Resolution 102
2.7. Noise 107
2.8. Baseline Flatness 110
2.9. Stability 113

Part 3.
Sample Handling and Measurement 115

3.1. Sample Handling 117
3.2. Cell Types 123
3.3. Cleanliness 127
3.4. Influence of Instrumental Parameters 130
3.5. Properties of Solvents 137
3.6. Background Absorbance 141
3.7. Sample Decomposition 146

Part 4.
Applications 151

4.1. Single Component Analysis 152
4.2. Multicomponent Analysis 162
4.3. Derivative Spectroscopy 178
4.4. Kinetic Analysis — Studying Melting Temperatures of DNA 183
4.5. Isosbestic Points 189
4.6. Biochemical Spectroscopy 208

Appendix 214

Part 1

Basic Principles and Applications

1.1. Basic Principles—What is a UV-visible Spectrum?

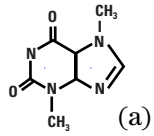
Introduction

A spectrum is a graphical representation of the amount of light absorbed or transmitted by matter as a function of the wavelength. A UV-visible spectrophotometer measures absorbance or transmittance from the UV range to which the human eye is not sensitive to the visible wavelength range to which the human eye is sensitive.

In the following experiment the spectra of two compounds, a colored one and a colorless one, are measured. The intention is to demonstrate that in many cases colorless compounds have a UV absorbance.

Reagents and Equipment

- ❑ caffeine (a)
- ❑ erythrosine
- ❑ distilled water



(a)

- ❑ two 1.0-l volumetric flasks
- ❑ 0.5-ml syringe or pipette
- ❑ disposable glass pipettes (minimum 3)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 45 min

1 Prepare the following solutions:

a) about 8 mg erythrosine in 1.0 l distilled water
b) about 5.0 mg caffeine in 1.0 l distilled water

2 Measure a reference on distilled water.

3 Measure the absorbance spectra of each solution in the range from 190 to 700 nm. If your spectrophotometer offers this functionality: overlay the spectra of the two solutions.

Evaluation

1 Enter the wavelengths of the main absorbance maxima of caffeine and erythrosine in the table below.

Evaluation Table 1.1. Measured Wavelengths and Absorbance Values of the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]
Caffeine		
Erythrosine		

2 If the color of light and wavelength are related as follows, which colors of light do caffeine and erythrosine absorb?

Color	Wavelength Range [nm]
violet	380–435
blue	435–480
green	480–560
yellow	560–595
orange	595–650
red	650–780

3 Explain the relationship between the color of light absorbed by matter and the color of light that you can observe when looking at it.

4 The following equation shows the relationship between absorbance and transmittance:

$A = 2 - \log T\ [\%]$

Calculate the transmittance values at the main absorbance maximum of each sample and enter the values in the table below.

Evaluation Table 1.2. Calculated Transmittance Values of the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]	Transmittance [%T]
Caffeine			
Erythrosine			

5 Which compound absorbs light of higher energy (per photon)? Use the following equations to calculate the energy of light in Joule that is absorbed at the maxima of both compounds.

$$E = h\nu$$

$$c = \lambda\nu$$

where:

E = energy [J]
h = Planck's constant (6.62×10^{-34} Js)
c = speed of light (3×10^{8} ms^{-1})
v = frequency [s^{-1}]
λ = wavelength [m]

Evaluation Table 1.3. Calculated Energies of Light at the Absorbance Maxima

Compound	λ_{max} [nm]	Energy [J]
Caffeine		
Erythrosine		

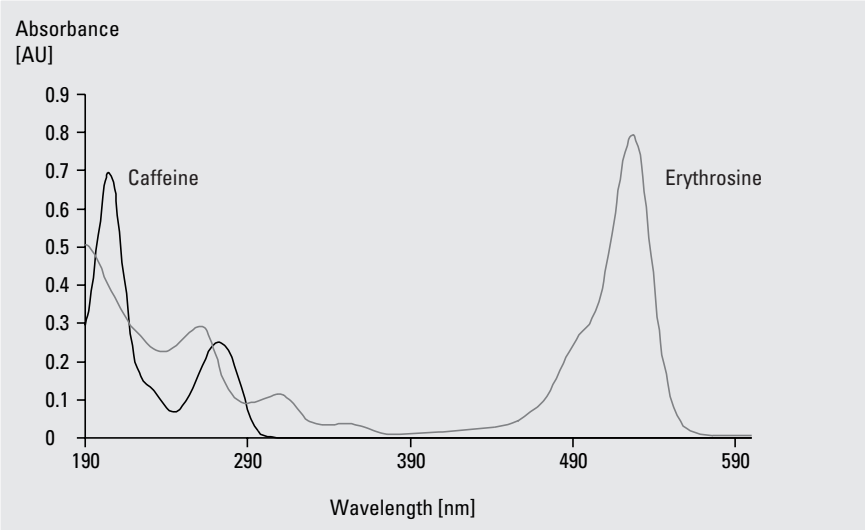
Example Results & Discussion

Samples: erythrosine in distilled water (8.0 mg/l)
caffeine in distilled water (5 mg/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 190–600 nm
absorbance range: 0.0–1.0 AU

Diagram: Measured Absorbance Spectra of the Samples



1 The measured wavelengths and the absorbance values at the absorbance maxima are listed in the table below.

Results Table 1.1. Measured Wavelengths and Absorbance Values of the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]
Caffeine	205	0.699
Erythrosine	526	0.798

2 Caffeine does not absorb light in the visible range and therefore has no color. erythrosine absorbs in the visible range so it has color. Its absorbance maximum can be found at 526 nm so the color it absorbs is green.

3 We usually see matter under sunlight or similar light which is essentially “white” light—a mixture of all wavelengths. When a sample absorbs a certain color we see the rest of the light that is not absorbed. This is called the complementary color. When green light is absorbed what we see is the red light that remains.

4 Transmittance values calculated from the absorbance values are shown in the table below.

Results Table 1.2. Calculated Transmittance Values of the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]	Transmittance [%T]
Caffeine	205	0.699	20.000
Erythrosine	526	0.798	15.922

An absorbance of 1 is measured when the sample absorbs 90 % of the incident light.

5 The calculated energies of the light at the maxima are shown in the table below.

Results Table 1.3. Calculated Energies of Light at the Absorbance Maxima

Compound	λ_{max} [nm]	Energy [J]
Caffeine	205	$9.69 \cdot 10^{-19}$
Erythrosine	526	$3.78 \cdot 10^{-19}$

Caffeine absorbs light of higher energy than erythrosine. The lower the wavelength, the higher the energy per photon.

1.2. Chromophores

Introduction

Chromophores are parts of a molecule that have electronic bands with energy differences comparable to the energies of the UV-visible light which is then absorbed by them. Chromophores, for example, dienes, nitriles, carbonyl, or carboxyl groups often contain π bonds. Other types of chromophores are transition metal complexes and their ions.

Molecules without chromophores, such as water, alkanes or alcohols, should be ideal solvents for UV-visible spectroscopy because they hardly show any absorbance themselves.

The following experiment shows that small variations in the structure of molecules can lead to significant differences in the resulting absorbance spectra.

Reagents and Equipment

- ❑ acetone
- ❑ acetaldehyde
- ❑ 2-propanol
- ❑ distilled water

- ❑ three 20-ml volumetric flasks
- ❑ 0.1-ml pipette or syringe
- ❑ disposable glass pipettes (minimum 4)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 45 min

1 Prepare the following solutions:

a) 0.1 ml acetone in 20 ml distilled water
b) 0.2 ml acetaldehyde in 20 ml distilled water
c) 0.1 ml 2-propanol in 20 ml distilled water

2 Measure a reference on distilled water.

3 Measure the spectra of the acetone, acetaldehyde and 2-propanol solutions in the range from 200 to 350 nm.

Evaluation

1 Complete the following table with the absorbance wavelengths and corresponding values of the absorbance maxima of each compound:

Evaluation Table 1.4. Wavelengths and Absorbance Values at the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]
Acetone		
Acetaldehyde		
2-Propanol		

2 Discuss the differences in the UV spectra of these compounds taking their molecular structures into consideration.

3 Which of these solvents would be best as a solvent for UV-visible analyses?

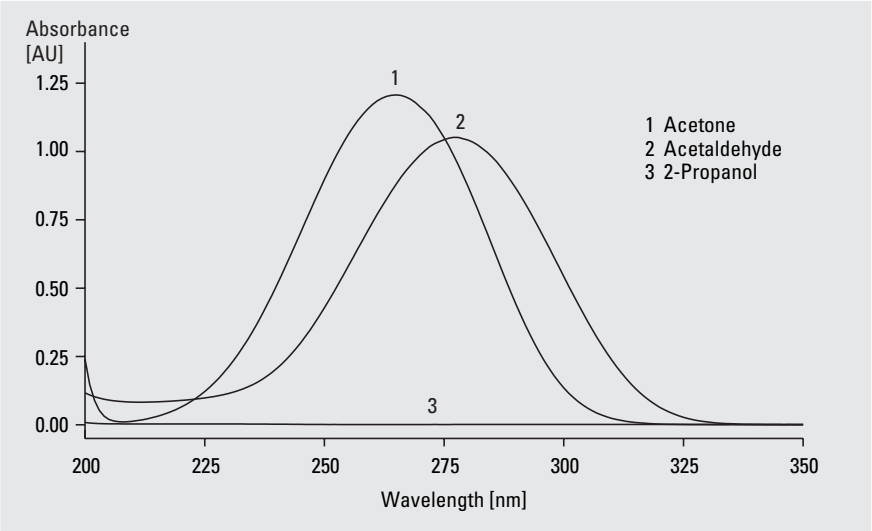
Example Results & Discussion

Samples: acetone in distilled water (5 ml/l)
acetaldehyde in distilled water (10 ml/l)
2-propanol in distilled water (5 ml/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 200–350 nm
absorbance range: 0.0–1.5 AU

Diagram: Measured Absorbance Spectra of the Different Chromophores

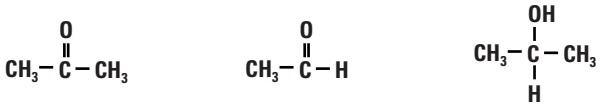


1 The wavelengths and absorbance values at the maxima for each compound are shown in the table below.

Results Table 1.4. Wavelengths and Absorbance Values at the Absorbance Maxima

Compound	λ_{max} [nm]	Absorbance [AU]
Acetone	265	1.205
Acetaldehyde	277	1.052
2-Propanol	no maximum found	not applicable

2 The molecular structures of the three compounds are:



Acetone Acetaldehyde 2-Propanol

The absorbance bands of acetaldehyde and acetone are caused by the presence of the C=O chromophore. Note that the position and intensity of the absorbance bands are similar. If either of these solvents has to be used for making sample solutions for UV-visible measurements, the data in the range from 225 to 325 nm will be affected by the absorbance of the solvent itself.

3 Propanol has no chromophore and shows no significant absorbance band in the UV-visible range. It is an almost ideal solvent for UV-visible measurements.

1.3. Environment of Chromophores

Introduction

Chromophores give rise to “characteristic” absorbance bands. Changes in their environment cause changes in their energy levels which then affect the wavelength and the intensity of absorbance.

The following experiment shows how the molecular environment of a chromophore affects its absorbance spectrum.

Reagents and Equipment

- ❑ naphthalene
- ❑ anthracene
- ❑ phenanthrene
- ❑ ethanol (CH_3-CH_2-OH)

- ❑ three 100-ml volumetric flasks
- ❑ disposable glass pipettes (minimum 4)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 60 min

1 Prepare the following solutions:

a) about 2.4 mg naphthalene in 100 ml ethanol
b) about 16 mg anthracene in 100 ml ethanol
c) about 5 mg phenanthrene in 100 ml ethanol

2 Measure a reference on ethanol.

3 Measure the spectra of the naphthalene, anthracene and phenanthrene solutions in the range from 200 to 400 nm.

Evaluation

1 Discuss the differences in the measured spectra of naphthalene, anthracene and phenanthrene solution. Take the wavelengths of absorbance maxima and the structure of bands into consideration.

2 Enter the wavelength ranges of the absorbance maxima of the compounds in the table below.

Evaluation Table 1.5. Wavelength Ranges of the Absorbance Maxima

Compound	λ_{max} [nm]
Naphthalene	
Anthracene	
Phenanthrene	

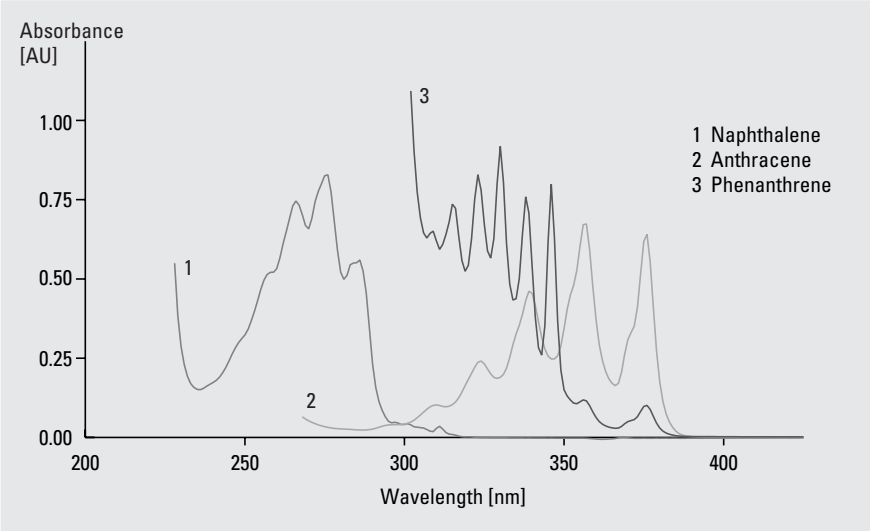
Example Results & Discussion

Samples: naphthalene in 100 ml ethanol (24 mg/l)
anthracene in 100 ml ethanol (16 mg/l)
phenanthrene in 100 ml ethanol (50 mg/l)

Cells: 2-mm path length quartz cell for naphthalene solution
10-mm path length quartz cell for the other two solutions

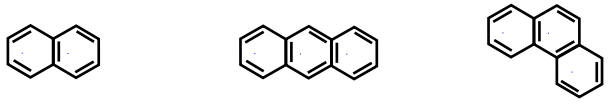
Instrument Parameters: wavelength range: 200–400 nm
absorbance range: 0.0–1.2 AU

Diagram: Measured Absorbance Spectra of the Different Chromophores



1 The spectra show that not only the kind of chromophore is important. In our example we have the benzene-rings that are chromophores with π–π^* transitions because of conjugated double bonds. The environment of the chromophores or the combination with other chromophores also have a strong influence on the position and values of absorbance bands.

The molecular structures of the three compounds are:



Naphthalene Anthracene Phenanthrene

From naphthalene to anthracene there is an increasing conjugation of the π bonds. The spectral region of absorbance changes as well as the intensity of absorbance and the form of the absorbance bands.

Anthracene and phenanthrene have the same chemical formula but their spectra are different because their structure and hence the electronic environment of the π bonds are different.

2 The wavelength ranges of the absorbance maxima are listed in the table below.

Results Table 1.5. Wavelength Ranges of the Absorbance Maxima

Compound	λ_{max} **[nm]**
Naphthalene	245–295
Anthracene	305–390
Phenanthrene	305–365

1.4. The Effect of Solvents on UV-visible Spectra

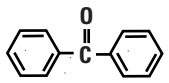
Introduction

Chromophores give rise to “characteristic” absorbance bands. Changes in their environment cause changes in their energy levels which then affect the wavelength and the intensity of absorbance.

The following experiment shows that external factors, in this case the solvent used, can also influence the chromophore. Because the substance used shows both strong and weak absorbance bands at different wavelengths, two different sample concentrations will be examined.

Reagents and Equipment

- ❏ benzophenone (a) (alternatively mesityl oxide)



(a)

Four or five of the following solution:

- ❏ ethanol (CH_3-CH_2-OH)
- ❏ cyclohexane (b)
- ❏ n-hexane ($CH_3(CH_2)_4CH_3$)
- ❏ acetonitrile (CH_3-CN)
- ❏ methylene chloride (CH_2Cl_2)


(b)

- ❏ five 25-ml volumetric flasks
- ❏ five 100-ml volumetric flasks
- ❏ 1-ml pipette
- ❏ disposable glass pipettes (minimum 15)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 90 min

1 Prepare five solutions of about 25 mg benzophenone in 25 ml of the given solvents (concentration c_{high}).

2 The following steps have to be repeated for each solution:

a) Dilute the concentration c_{high} by a factor of 100 to get the concentration c_{low}.
b) Measure a reference on the solvent used.
c) Measure the absorbance spectrum of the c_{high}-sample in the range from 300 to 400 nm.
d) Measure the absorbance spectrum of the c_{low}-sample in the range from 225 to 300 nm.

Evaluation

1 Enter the wavelengths of the absorbance maxima of benzophenone for the various solvents in the table below.

2 A measure of the polarity of a solvent is its dielectric constant. Look up the dielectric constants of the solvents used and enter them in the table below.

Evaluation Table 1.6. Wavelengths of the Measured Absorbance Maxima and Dielectric Constants

Solvent	$\lambda_{max,\ Peak\ 1}$ [nm]	$\lambda_{max,\ Peak\ 2}$ [nm]	Dielectric Constant
n-Hexane			
Cyclohexane			
Ethanol			
Acetonitrile			
Methylene Chloride			

3 Is there a relationship between a solvent's polarity and the wavelength of its absorbance maximum?

4 Can you observe any change in the form of the absorbance bands in the wavelength range from 300 to 400 nm with changes in solvent polarity?

5 Is it important to use the same solvent to achieve consistent results, for example, between the measurements made in different laboratories?

Example Results & Discussion

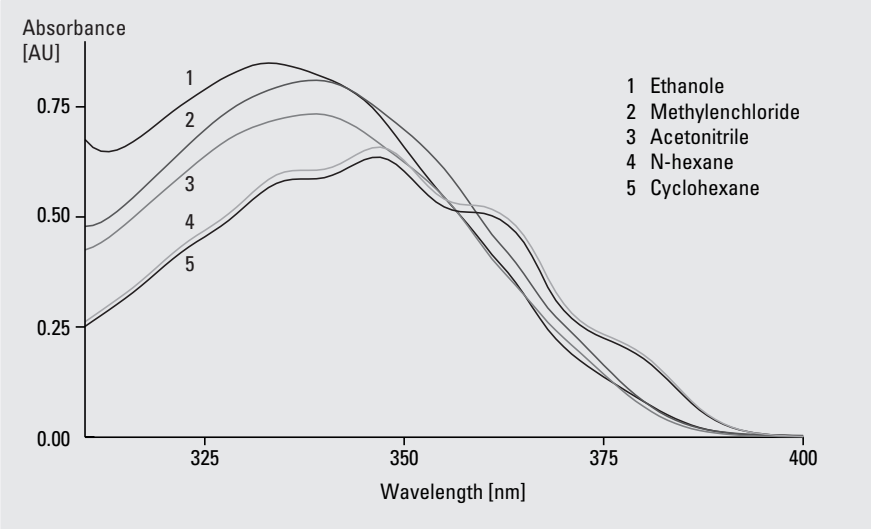
Samples: a) stock solutions of benzophenone in the following solvents:
ethanol (1 g/l)
cyclohexane (1 g/l)
n-hexane acetonitrile (1 g/l)
methylene chloride (1 g/l)

b) stock solutions diluted by 1:100

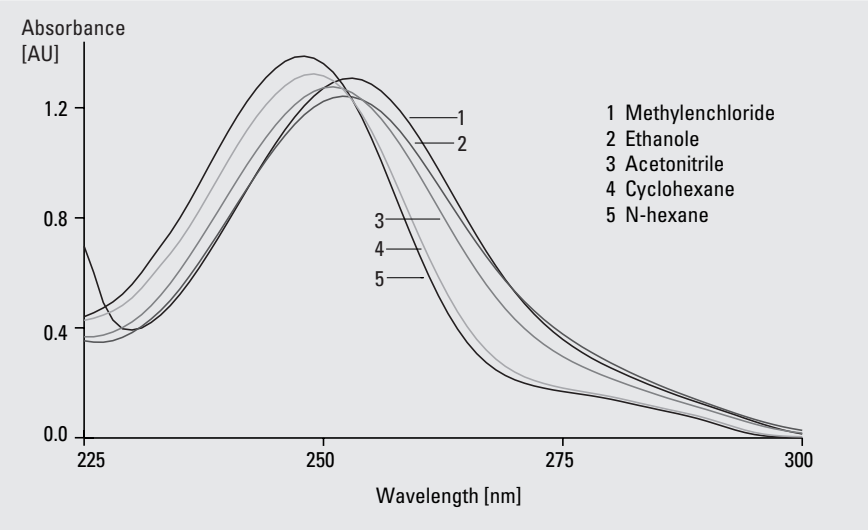
Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength ranges: a) 300–400 nm
b) 225–300 nm
absorbance range: 0.0–1.4 AU

Diagram: a) Measured Spectra Absorbance of the Sample Solutions at High Concentrations



b) Measured Spectra of the Sample Solution at Low Concentration



1 The measured wavelengths of the absorbance maxima and the dielectric constants of the solvents are listed in the table below.

Results Table 1.6. Wavelengths of the Measured Absorbance Maxima and Dielectric Constants

Solvent	$\lambda_{max,\ Peak\ 1}$ [nm]	$\lambda_{max,\ Peak\ 2}$ [nm]	Dielectric Constant
n-Hexane	248	347	1.9
Cyclohexane	249	347	2.0
Ethanol	252	333	24.3
Acetonitrile	251	339	36.2
Methylene chloride	253	339	9.0

2 The dielectric constants are listed in the table above.

3 There is a clear relationship between the wavelengths of the absorbance maxima and the polarity of the solvent used.

4 The bands in the range from 300 to 400 nm show that nonpolar solvents such as alkanes allow considerably fine structures to be preserved in the spectra of many compounds. Polar solvents such as water and alcohols allow only broad and relatively featureless bands.

5 For comparative analyses the same solvent should be used for all measurements.

Note: Band shifts to higher wavelengths are called bathochromic shifts, shifts to lower wavelengths are called hypsochromic shifts.

1.5. The Meaning of Color in Spectroscopy

Introduction

The color of matter is a function of its ability to absorb and to emit light. Light emission can be either due to surface reflection of non-transparent samples or to transmission of source light through the samples. Matter can also be an active source of light like gases in lamps. At room temperature matter usually only reflects or transmits light. Color in its common meaning refers to daylight as illuminant.

The human eye is capable to differentiate up to 10 million different colors. It is able to detect light in a range from about 380 to 780 nm, the visible part of a UV-visible spectrum.

In the following experiment food dyes are used to correlate absorbance spectra to color impressions. Due to their wavelengths of absorbance the partly transparent sample solutions look yellow, red or blue. What a human observer sees as a yellow sample is the daylight reduced by the light absorbed by the sample. The color of the sample is therefore called complementary to the color of the light absorbed by the sample.

Reagents and Equipment

- ❑ quinoline yellow (yellow dye)
- ❑ erythrosine (red dye)
- ❑ indigotin (blue dye)

- ❑ distilled water
- ❑ three 1.0-l volumetric flasks
- ❑ disposable glass pipettes (minimum 4)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 30 min

1 Prepare the following solutions:

a) quinoline yellow, about 13 mg in 1.0 ml distilled water
b) erythrosine, about 8 mg in 1.0 l distilled water
c) indigotin, about 18 mg in 1.0 l distilled water

2 Measure a reference on distilled water.

3 Measure the spectra of the three dye solutions in the wavelength range from 350 to 750 nm.

Evaluation

1 Determine the wavelength of the absorbance maximum of each dye spectrum and enter the values in the table below.

Evaluation Table 1.7. Wavelengths of the Absorbance Maxima

Sample Name	λ_{max} [nm]
Quinoline yellow	
Erythrosine	
Indigotin	

2 Compare the visible color of the sample with the spectral color of the light absorbed at the absorbance maximum. Enter both colors in the table below.

Evaluation Table 1.8. Visible Color Compared to Color of Absorbed Light

Sample Name	Visible Color	Color of Absorbed Light
Quinoline yellow		
Erythrosine		
Indigotin		

3 What does an absorbance spectrum of a sample tell you about its color?

4 How does a sample look that has absorbance values close to zero in the entire visible range?

5 How does a sample look that has very high absorbance values in the entire visible range?

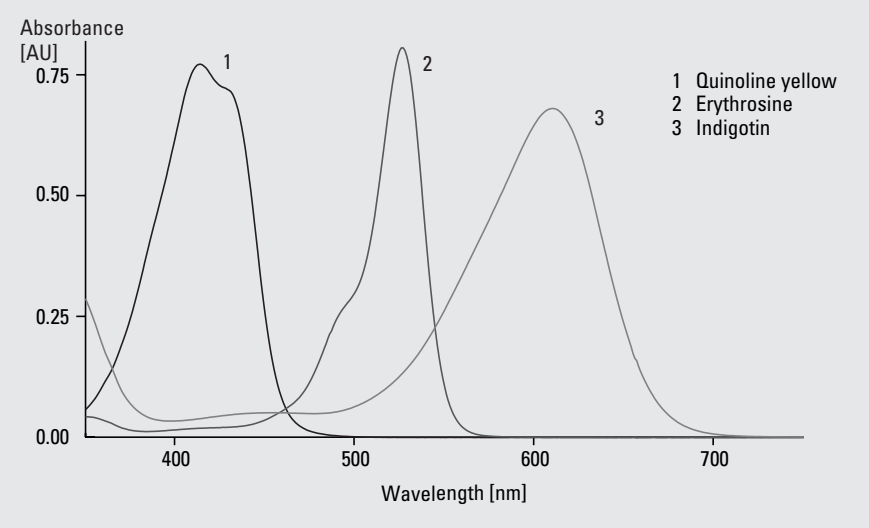
Example Results & Discussion

Samples: quinoline yellow in distilled water (13 mg/l)
erythrosine in distilled water (8 mg/l)
indigotin in distilled water (18 mg/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 350–700 nm
absorbance range: 0.0–0.8 AU

Diagram: Measured Absorbance Spectra of the Different Dyestuff Solutions



1 The wavelengths of absorbance maxima are listed in the table below.

Results Table 1.7. Wavelengths of the Absorbance Maxima

Sample Name	λ_{max} [nm]
Quinoline yellow	414
Erythrosine	526
Indigotin	610

2 The complementary colors of the sample and the spectral color of the light absorbed at the absorbance maximum are listed in the table below.

Results Table 1.8. Visible Color Compared to Color of Absorbed Light

Sample Name	Visible Color	Color of Absorbed Light
Quinoline yellow	yellow	blue
Erythrosine	red	blue-green
Indigotin	blue	yellow

3 An absorbance spectrum has all information about the color of a spectrum. For simple dye spectra with a single absorbance maximum this color is the complementary color of the spectral color of the light absorbed. Even quantitative color calculations can be done based on UV-visible spectra.

4 A sample with absorbance values close to zero in the entire visible range is colorless. It is totally transparent.

5 A sample with very high absorbance values in the entire visible range looks black and is not transparent.

References

"Measuring Colour", R.W.G. Hunt, Ellis Horwood Limited, Chichester
"Einführung in die Farbmetrik", M. Richter, Walter de Gruyter, Berlin, New York

1.6. Quantitative Analysis — Lambert-Beer's Law

Path Length Dependence of Absorbance Values

Introduction

The amount of light absorbed or transmitted by a sample depends on the path length which the light has to go through when passing the sample.

In the following experiment we will try to find out how different path lengths affect the absorbance values. We measure the same sample solution using different cells. We measure absorbance and transmittance data at three different wavelengths. The measurement data are evaluated using diagrams and linear regression calculations.

Reagents and Equipment

- ❏ quinoline yellow (yellow dye)
- ❏ distilled water

- ❏ 1.0-ml volumetric flask
- ❏ disposable glass pipettes (minimum 2)
- ❏ 1-mm path length quartz cell
- ❏ 2-mm path length quartz cell
- ❏ 5-mm path length quartz cell
- ❏ 10-mm path length quartz cell

Experiment

Time: about 30 min

1 Prepare a solution of about 28 mg quinoline yellow in 1.0- ml distilled water (or use quinoline solution from the previous experiment).

2 For each of the cells with different path lengths:

a) Measure a reference on distilled water.
b) Measure the absorbance values of the quinoline yellow solution at 224, 337 and 414 nm.
c) Measure the transmittance values of the quinoline yellow solution at 224, 337 and 414 nm.

Evaluation

1 Enter your absorbance and transmittance measurement values in the tables below.

Evaluation Table 1.9. Measured Absorbance Values at Different Wavelengths and at Different Path Lengths

Absorbance [AU] at Different Wavelengths	Path Length			
	1 mm	2 mm	5 mm	10 mm
224 nm				
337 nm				
414 nm				

Evaluation Table 1.10. Measured Transmittance Values at Different Wavelengths and at Different Path Lengths

Transmittance [%T] at Different Wavelengths	Path Length			
	1 mm	2 mm	5 mm	10 mm
224 nm				
337 nm				
414 nm				

2 Enter your measured data values in the following two diagrams.

Diagram: Absorbance as a Function of Path Length

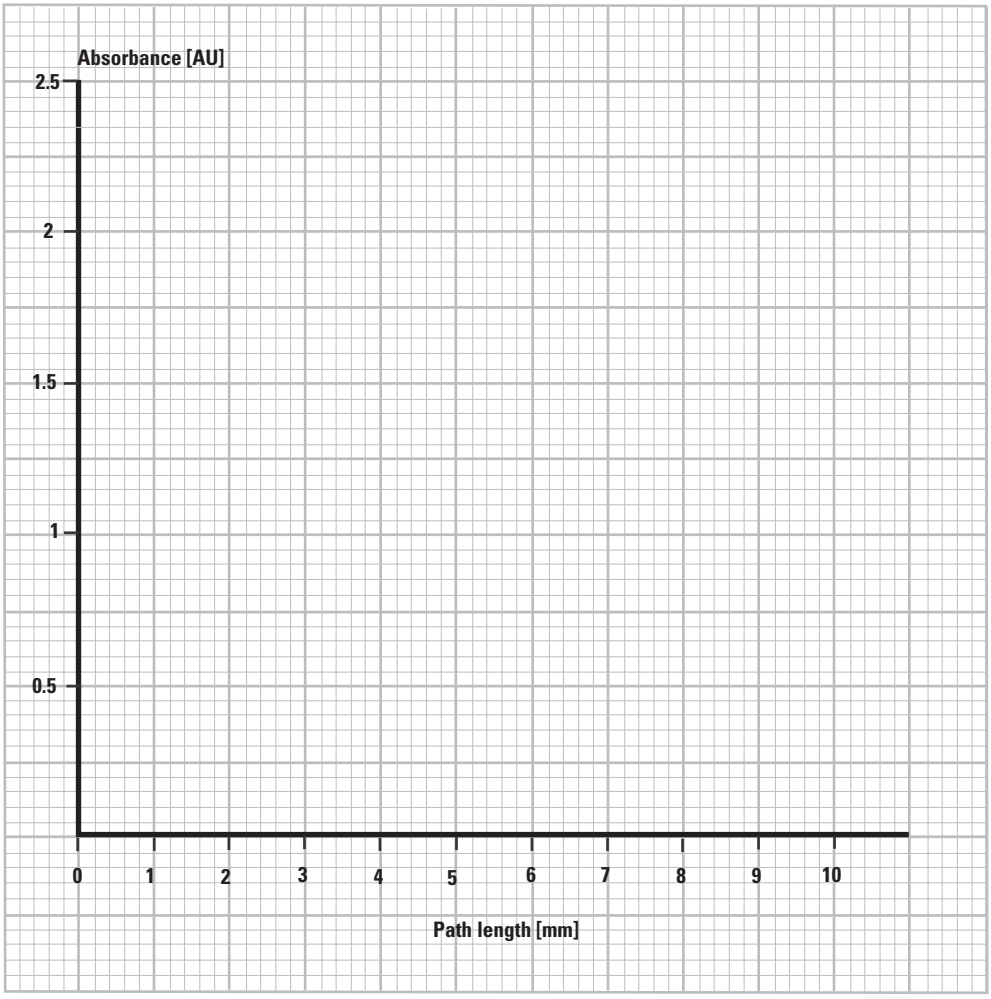
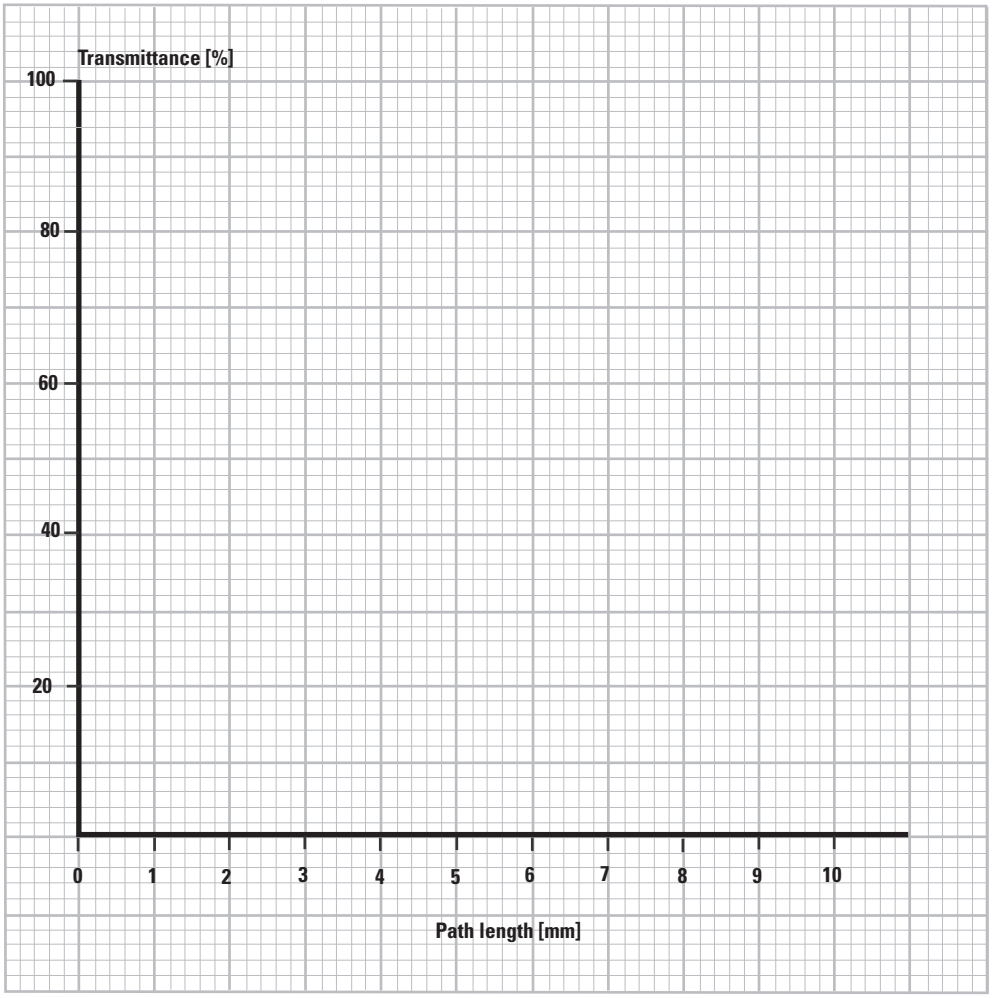


Diagram: Transmittance as a Function of Path Length



3 Calculate a linear regression with offset for the above diagrams. Use the calculation formulae given in the appendix for y = ax + b or the built-in function of a pocket calculator. Here the x,y pairs are the path length (x) and the corresponding data value (y). Enter the calculation results for all wavelengths in the following tables.

Evaluation Table 1.11. Calculation Results for the Normalized Absorbance Data

Name		Wavelength		
		224mm	337 mm	414 mm
Slope	a			
Intercept	b			
Correlation Coefficient	R			

Evaluation Table 1.12. Calculation Results for Transmittance Wavelengths

Name		Wavelength		
		224 nm	337 nm	414 nm
Slope	a			
Intercept	b			
Correlation Coefficient	R			

4 Which data values have a linear correlation with the path length?

5 Normalize your absorbance measurements to 1-mm path length by dividing your measurement data by the corresponding path length and enter the values in the table below.

Evaluation Table 1.13. Calculation Results for the Normalized Absorbance Data

Normalized Absorbance [AU/1 mm] at Different Wavelengths	Path Length			
	1 mm	2 mm	5 mm	10 mm
224 nm				
337 nm				
414 nm				

6 Are the normalized data path length specific at a given wavelength?

Example Results & Discussion

Sample: quinoline yellow in distilled water (28.3 g/l)

Cells: 1-mm path length quartz cell
2-mm path length quartz cell
5-mm path length quartz cell
10-mm path length quartz cell

Instrument Parameters:

wavelengths:	224, 337 and 414 nm
absorbance range:	0.0–1.8 AU
transmittance range:	0.0–100 %

1 The following tables show the measured absorbance and transmittance values.

Results Table 1.9. Measured Absorbance Values at Different Wavelengths and at Different Path Lengths

Absorbance [AU] at Different Wavelengths	Path Length			
	1 mm	2 mm	5 mm	10 mm
224 nm	0.178	0.348	0.874	1.692
337 nm	0.007	0.011	0.033	0.065
414 nm	0.159	0.321	0.809	1.610

Results Table 1.10. Measured Transmittance Values at Different Wavelengths and at Different Path Lengths

Transmittance [%T] at Different Wavelengths	Path Length			
	1 mm	2 mm	5 mm	10 mm
224 nm	66.37	44.92	13.37	2.03
337 nm	98.48	97.62	92.63	86.18
414 nm	69.28	47.81	15.52	2.46

2 The following diagrams show the absorbance and transmittance as a function of path length.

Diagram: Absorbance as a Function of Path Length

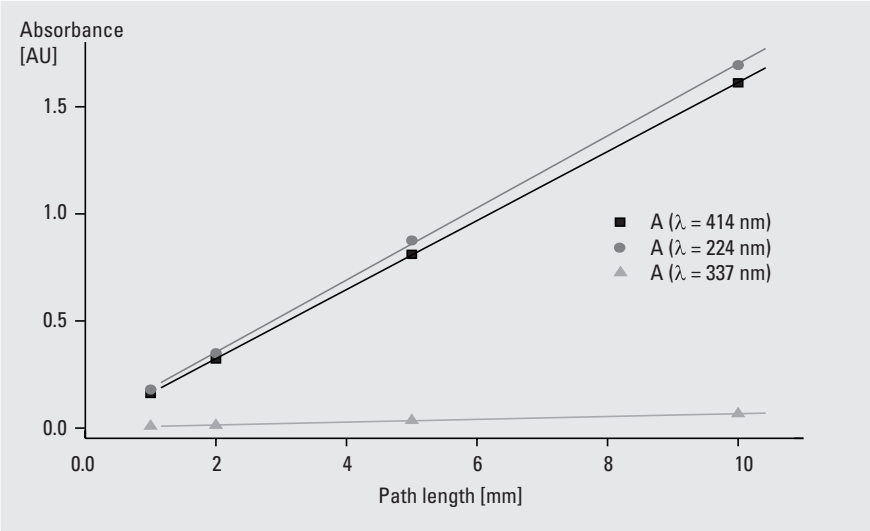
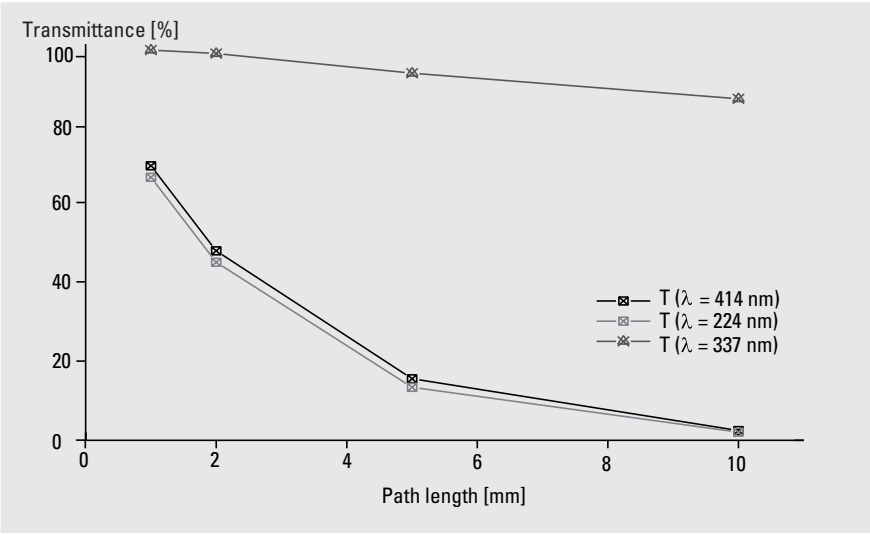


Diagram: Transmittance as a Function of Path Length



3 The following tables show the linear regression calculation results.

Results Table 1.11. Calculation Results for Absorbance Measurements

		Wavelength		
Name		**224 mm**	**337 mm**	**414 mm**
Slope	a	1.6843	0.0658	1.6124
Intercept	b	0.0150	-0.0009	0.0008
Correlation coefficient	R	0.99986	0.99863	0.99999

Results Table 1.12. Calculation Results for Transmission Measurements

		Wavelength		
Name		**224 mm**	**337 mm**	**414 mm**
Slope	a	66.68	-13.96	-69.53
Intercept	b	61.68	100.01	65.06
Correlation Coefficient	R	-0.91665	-0.99814	-0.92457

4 The absorbance values have a linear correlation with the path length.

5 The following table shows the calculation results for normalized absorbance data.

Results Table 1.13. Calculation Results for the Normalized Absorbance Data

Absorbance [AU/1 mm] at Different Wavelengths	**Path Length**			
	1 mm	**2 mm**	**5 mm**	**10 mm**
224 nm	0.178	0.174	0.175	0.169
337 nm	0.007	0.005	0.007	0.007
414 nm	0.159	0.160	0.162	0.161

6 The normalized absorbance values are not path length specific at a given wavelength. The measured absorbance values are proportional to the path length.

1.7. Concentration Dependence of Absorbance Values

Introduction

This is the basic experiment for quantitative UV-visible measurements: how do different concentrations affect absorbance and transmittance values?

In the following experiment we measure the absorbance and transmittance of five different quinoline yellow solutions to examine the effect of different concentrations. We evaluate our measurement data by using diagrams and by doing linear regression calculations.

Reagents and Equipment

- ❑ quinoline yellow (yellow dye)
- ❑ distilled water

- ❑ 1.0-l volumetric flask
- ❑ four 100-ml volumetric flasks
- ❑ disposable glass pipettes (minimum 6)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 120 min

1. Prepare a quinoline yellow stock solution of about 28 mg in 1.0 l distilled water.
2. Prepare four sample solutions in the 100-ml volumetric flasks by diluting the stock solution: sample concentrations: 14, 7, 3.5, 1.75 mg/l.
3. Measure a reference on distilled water.
4. Measure the absorbance and transmittance values at 224, 337 and 414 nm of the stock solution and of each of the other solutions.

Evaluation

1 Enter the actual concentrations of the five solutions you prepared in the list below.

c_1 = _____ mg/l

c_2 = _____ mg/l

c_3 = _____ mg/l

c_4 = _____ mg/l

c_5 = _____ mg/l

2 Enter your measured absorbance and transmittance values in the tables below.

Evaluation Table 1.14. Measured Absorbance Values at Different Wavelengths and Concentrations

Absorbance [AU] at Different Wavelengths	Sample Concentrations:				
	c_1	c_2	c_3	c_4	c_5
224 nm					
337 nm					
414 nm					

Evaluation Table 1.15. Measured Transmittance Values at Different Wavelengths and Concentrations

Transmittance [%T] at Different Wavelengths	Sample Concentrations:				
	c_1	c_2	c_3	c_4	c_5
224 nm					
337 nm					
414 nm					

3 Enter your data values measured in the following two diagrams. Use different colors for different wavelengths.

Diagram: Absorbance as a Function of Concentration

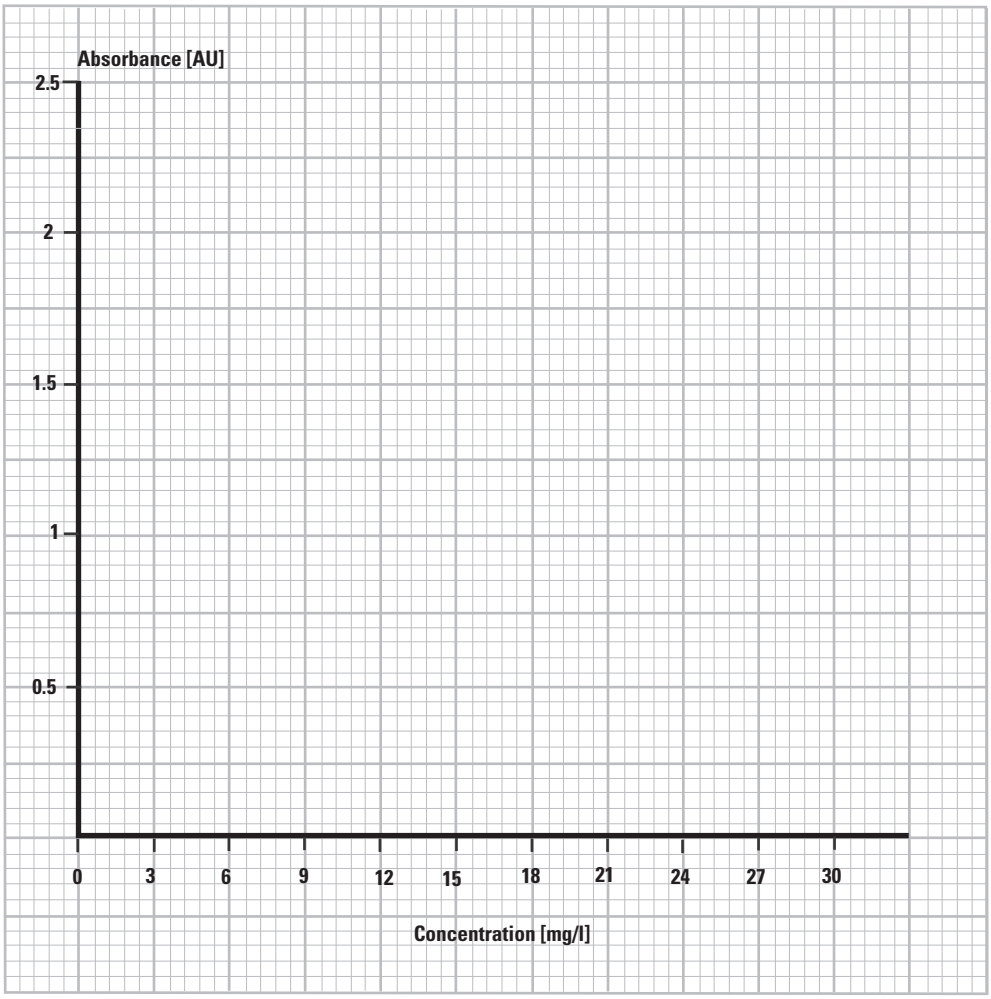
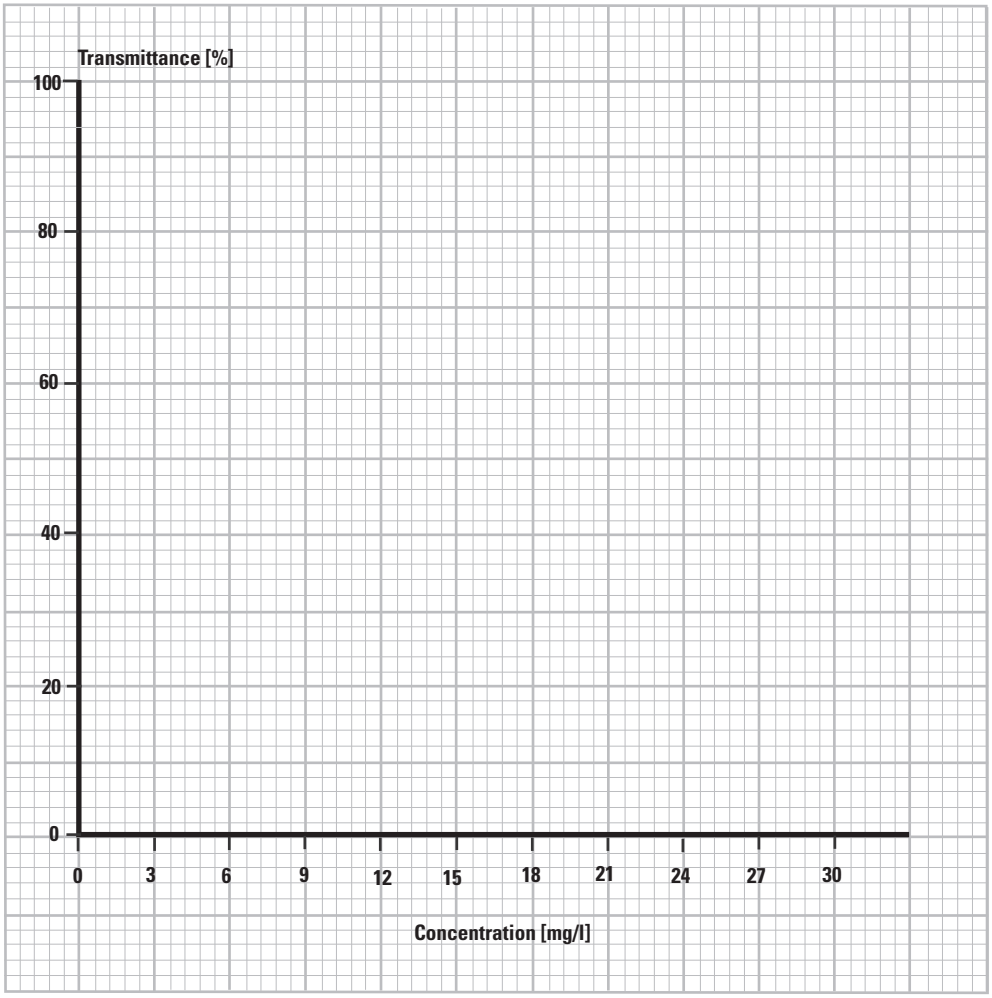


Diagram: Transmittance as a Function of Concentration



4 Which data are preferable for quantitative analysis—absorbance data or transmittance data—and why?

5 Calculate a linear regression with offset for the above graphs. Use the calculation formulae given in the appendix for y = ax + b or the built-in function of a pocket calculator. Here the x,y pairs are the concentration (x) and the corresponding data value (y). Enter the calculation results for all wavelengths in the tables below.

Evaluation Table 1.16. Calculation Results for Absorbance Measurements at Different Wavelengths

Name		224 nm	337 nm	414 nm
Slope	a			
Intercept	b			
Correlation coefficient	R			

Evaluation Table 1.17. Calculation Results for Transmittance Measurements at Different Wavelengths

Name		224 nm	337 nm	414 nm
Slope	a			
Intercept	b			
Correlation coefficient	R			

6 Which data values have a linear correlation with the concentration?

7 Normalize your absorbance measurements to concentration 1 mg/l by dividing your measurement data by the corresponding sample concentration and enter the values in the table below.

Evaluation Table 1.18. Calculation Results for Normalized Absorbance Data at Different Wavelengths and Concentrations

Normalized Absorbance [AU l/mg] at Different Wavelengths	Concentrations				
	c_1	c_2	c_3	c_4	c_5
224 nm					
337 nm					
414 nm					

8 Are the normalized data sample specific for a given wavelength?
How are these values also called?

Example Results & Discussion

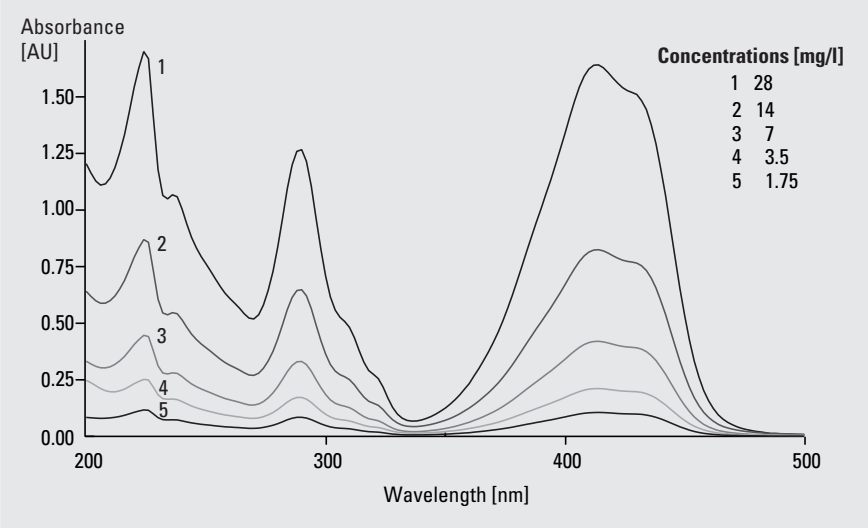
Samples: quinoline yellow in distilled water (28 mg/l)
quinoline yellow in distilled water (14 mg/l)
quinoline yellow in distilled water (7 mg/l
quinoline yellow in distilled water (3.5 mg/l)
quinoline yellow in distilled water (1.75 mg/l)

Cell: 10-mm path length quartz cell

Instrument Parameters:

wavelengths:	224, 337, 414 nm
absorbance range:	0.0–1.8 AU
transmittance range:	0.0–100 %

Spectra: Measured Absorbance Spectra for Different Sample Concentrations



1 The actual concentrations of the samples are listed in the figure above.

2 The following tables show the measured absorbance data.

Results Table 1.14. Measured Absorbance Values at Different Wavelengths and Concentrations

Absorbance [AU] at Different Wavelengths	Sample Concentrations:				
	c_1	c_2	c_3	c_4	c_5
224 nm	1.70000	0.8700	0.4472	0.2524	0.1175
337 nm	0.06846	0.0441	0.0217	0.0153	0.0072
414 nm	1.64116	0.8249	0.4206	0.2120	0.1064

Results Table 1.15. Measured Transmittance Values at Different Wavelengths and Concentrations

Transmittance [%T] at Different Wavelengths	Sample Concentrations:				
	c_1	c_2	c_3	c_4	c_5
224 nm	2.00	13.49	35.71	55.91	76.29
337 nm	85.42	90.34	95.14	96.53	98.35
414 nm	2.29	14.96	37.97	61.38	78.28

3 The following diagrams show absorbance and transmission as a function of concentration.

Diagram: Absorbance as a Function of Concentration

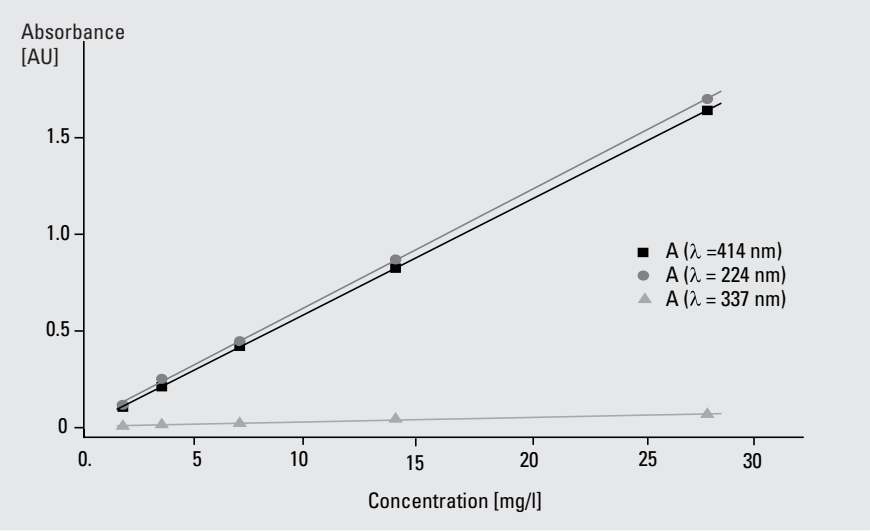
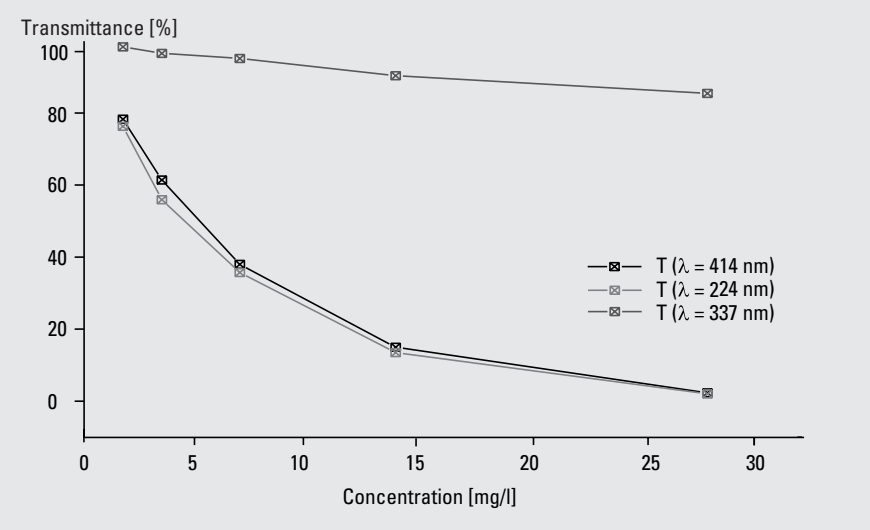


Diagram: Transmittance as a Function of Concentration



4 The following tables show the linear regression calculation results.

Results Table 1.16. Calculation Results for Absorbance Measurements at Different Wavelengths

Name		224 nm	337 nm	414 nm
Slope	a	0.060	0.002	0.058
Intercept	b	0.029	0.006	0.008
Correlation coefficient	R	0.9997	0.9809	1.0000

Results Table 1.17. Calculation Results for Transmittance Measurements at Different Wavelengths

Name		224 nm	337 nm	414 nm
Slope	a	-2.5584	-0.4855	-2.5584
Intercept	b	64.439	98.424	64.439
Correlation coefficient	R	-0.8105	-0.9759	-0.8271

5 Due to the linear relationship with concentration only absorbance values are used in quantitative analysis.

This linear relationship can be seen in the diagram and it is also indicated by the correlation coefficients close to 1. The closer this coefficient is to 1, the better is the correlation.

The diagram of absorbance versus concentration is also called a calibration curve. Such a diagram can be used to get the concentration of a sample by measuring the absorbance value at the wavelength used for calibration and reading the corresponding concentration value from the diagram. The availability of pocket calculators and data-handling computer systems also allow an easy calculation of the results. To get a quick overview of the quality of the calibration a visual check of the curve is useful.

6 The following table shows the calculation results for the normalized absorbance data.

Results Table 1.18. Calculation Results for Normalized Absorbance Data at Different Wavelengths and Concentrations

Normalized Absorbance [AU l/mg] at Different Wavelengths	Concentrations				
	c_1	c_2	c_3	c_4	c_5
224 nm	0.067	0.072	0.064	0.062	0.061
337 nm	0.004	0.004	0.003	0.003	0.002
414 nm	0.061	0.061	0.060	0.059	0.059

The normalized absorbance data are specific for a given wavelength. They are called extinction coefficients. They are usually given in units of [l/(mol · cm]. Note that extinction coefficients also depend on the path length.

1.8. Possible Sources of Error — Influence of Impurities

Introduction

Usually only data from a single wavelength is used for quantitative analyses. An impurity present in the sample solution cannot be detected by this method.

In the following experiment we see how impurities affect quantitative results and we apply a method to detect samples suspect to impurities.

Reagents and Equipment

- ❑ quinoline yellow (yellow dye)
- ❑ tartrazine (orange dye)
- ❑ distilled water

- ❑ 1.0-l volumetric flask
- ❑ two 25-ml volumetric flasks
- ❑ disposable glass pipettes (minimum 3)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 30 min

1. Prepare a stock solution of quinoline yellow, about 16 mg in 1.0 l distilled water.
2. Add a small amount of tartrazine (<0.1 mg) to one of the 250 ml volumetric flasks and fill both of them with the stock solution.
3. Measure a reference on distilled water.
4. Measure the absorbance values at 224, 270, 290, 337 and 414 nm for both solutions.

Evaluation

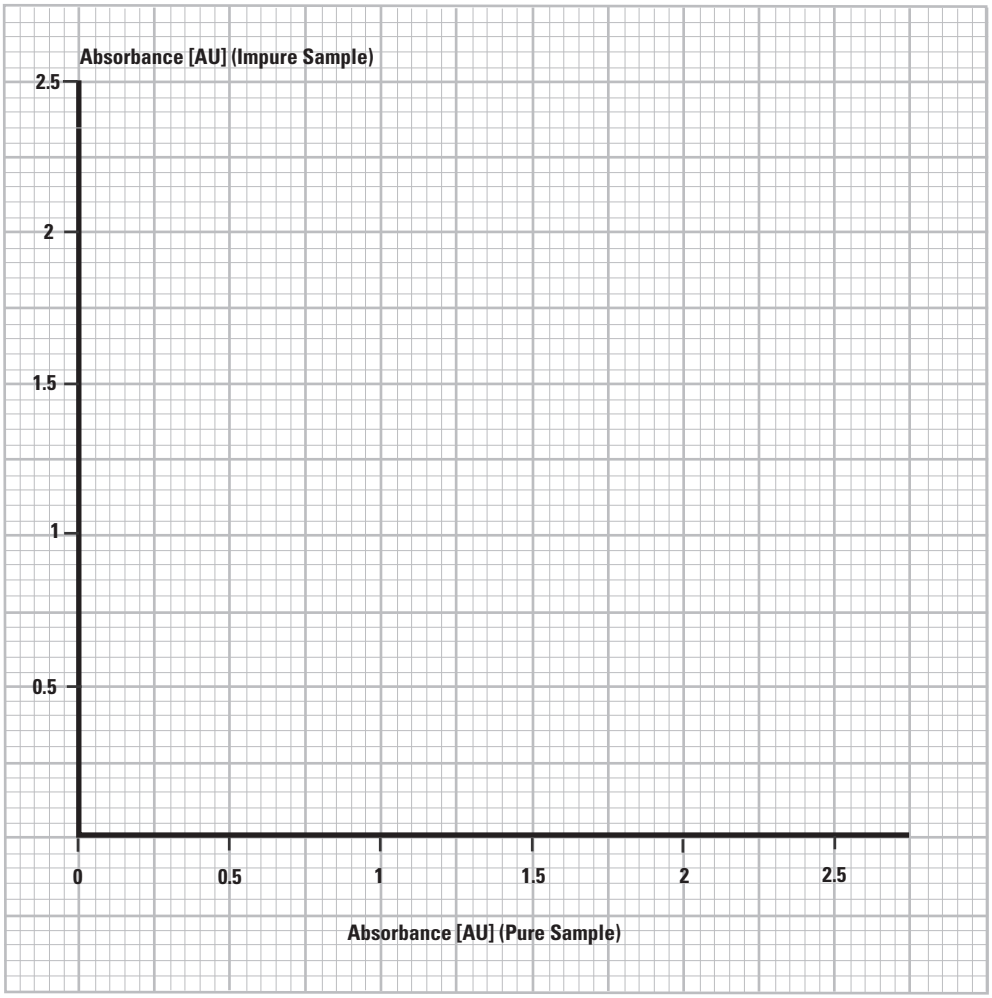
1 Enter the absorbance values for the two samples in the table below.

Evaluation Table 1.19. Absorbance Values of Pure and Impure Samples at Different Wavelengths

Absorbance [AU] at Different Wavelengths	Pure Sample	Impure Sample
224 nm		
270 nm		
290 nm		
337 nm		
414 nm		

2 Enter your measured absorbance values in the diagram below.

Diagram: Absorbance of the Impure Sample as a Function of Absorbance of the Pure Sample



3 Calculate a linear regression with offset for the diagram above. Use the calculation formulae given in the appendix for y = ax + b or the built-in function of a pocket calculator. Here the x,y pairs are the absorbance data of the pure sample(x) and the absorbance data of the impure sample(y) at the corresponding wavelength. Enter the calculation results in the table below.

Evaluation Table 1.20. Linear Regression Calculation Results

Coefficient		Value
Slope	a	
Intercept	b	
Correlation coefficient	R	

4 How can you tell that a sample is suspect to absorbing impurities?

Example Results & Discussion

Samples: pure quinoline yellow sample in distilled water (28 mg/l)
"impure" quinoline yellow sample in distilled water (28 mg/l) with a small amount of tartrazine

Cell: 10-mm path length quartz cell

Instrument Parameters:
wavelengths: 224, 270, 290, 337 and 414nm
absorbance range: 0.0–2.0 AU

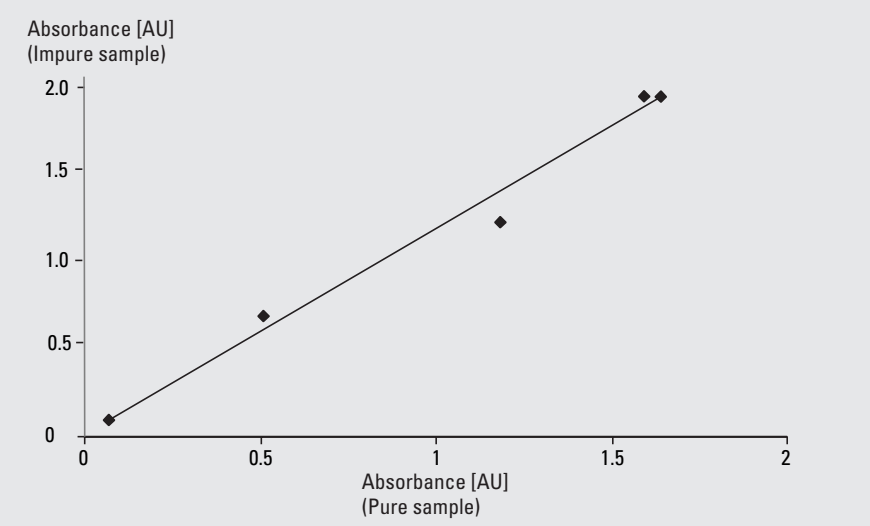
1 The following table shows the absorbance values of the pure and of the impure samples.

Results Table 1.19. Absorbance Values of Pure and Impure Sample at Different Wavelengths

Absorbance	Pure Sample	Impure Sample
224 nm	1.700	1.735
270 nm	0.519	0.603
290 nm	1.267	1.293
337 nm	0.069	0.084
414 nm	1.641	1.751

2 The following diagram shows the absorbance of the impure sample as a function of the absorbance of the pure sample.

Diagram: Absorbance of the Impure Sample as a Function of Absorbance of the Pure Sample



3 The following table shows the linear regression calculation results.

Results Table 1.20. Linear Regression Calculation Results

Name		Value
Slope	a	0.980
Intercept	b	-0.032
Correlation coefficient	R	0.9986

4 If the samples compared are absorbing at the wavelengths used in the diagram and the samples obey Beer's law, the result must always be a straight line. Any significant deviations from this straight line indicate the presence of an absorbing impurity.

For pure samples and accurate absorbance values the correlation coefficient should be as close to 1 as possible. The intercept should be close to zero. An intercept significantly different from zero indicates a constantly absorbing background.

The slope is a measure for the concentration ratio of the two samples.

1.9. Influence of Temperature — Potassium Chloride

Introduction

The temperature of a sample affects its absorbance spectrum. This is true for all samples due to volume expansion of the sample solvent with temperature. If the expansion coefficients of the solvent are known, a mathematical correction for this “dilution” effect can be applied. Chemical effects of shifting equilibria with temperature, however, can have a much higher impact on sample absorbance values than the one due to volume expansion.

The following experiment shows the effect of increasing temperatures on a potassium chloride sample.

Reagents and Equipment

- ❑ potassium chloride (KCl)
- ❑ distilled water

- ❑ 100-ml volumetric flask
- ❑ disposable glass pipettes (minimum 2)
- ❑ 10-mm path length quartz cell
- ❑ thermostattable cell holder
- ❑ temperature control unit or water bath

Experiment

Time: about 120–180 min

1. Prepare a solution of about 120 mg potassium chloride in 100 ml distilled water.
2. Measure a reference on distilled water at 20 °C.
3. Measure the absorbance spectra in the overlay mode in the range from 200 to 214 nm. Repeat the measurements in 2 degree steps in the range from 20 to 70 °C and read the absorbance values at 208 nm. Make sure that the temperature of the sample is the same as measured with the temperature sensor/thermometer and constant when measuring the absorbance spectra. Verify the temperature of the sample solution with a dipping sensor if available.

Evaluation

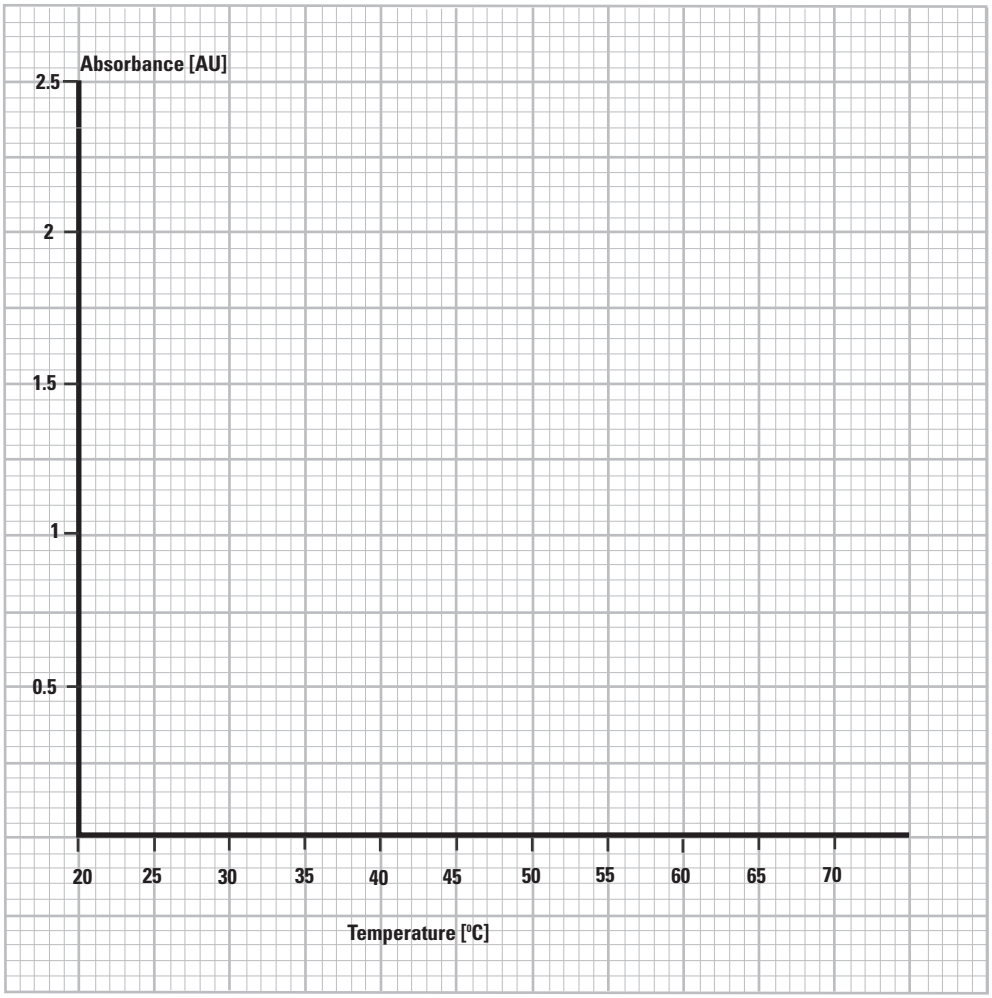
1 Enter the absorbance values at 208 nm for the corresponding temperatures in the table below.

Evaluation Table 1.21. Measured Absorbance at 208 nm for Different Temperatures

Temperature [°C]	Absorbance [AU] at 208 nm	Temperature [°C]	Absorbance [AU] at 208 nm
20		46	
22		48	
24		50	
26		52	
28		54	
30		56	
32		58	
34		60	
36		62	
38		64	
40		66	
42		68	
44		70	

2 Enter your measured absorbance values in the diagram below.

Diagram: Absorbance as a Function of Temperature



3 Calculate the difference between the absorbance value at 208 nm measured at 20 °C and 70 °C.

Example Results & Discussion

Sample: potassium chloride in distilled water (1.2 g /l)

Cell: 10-mm path length quartz cell

Instrument Parameters:

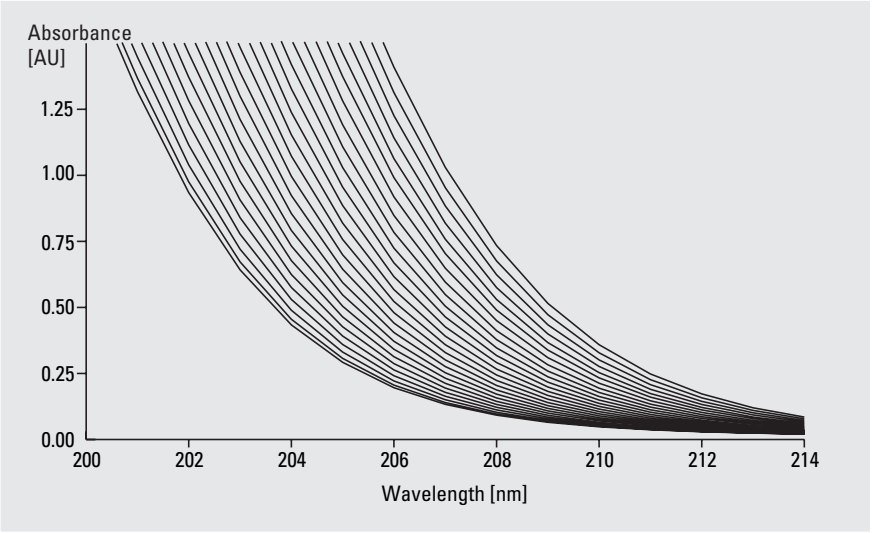
spectrophotometer:

wavelength range:	200–214 nm
absorbance range:	0.0–1.5 AU

temperature controller:

temperature range:	20–70 °C
temperature steps:	2 °C
equilibration time for temperature:	90 s

Diagram: Overlaid Absorbance Spectra for Different Temperatures



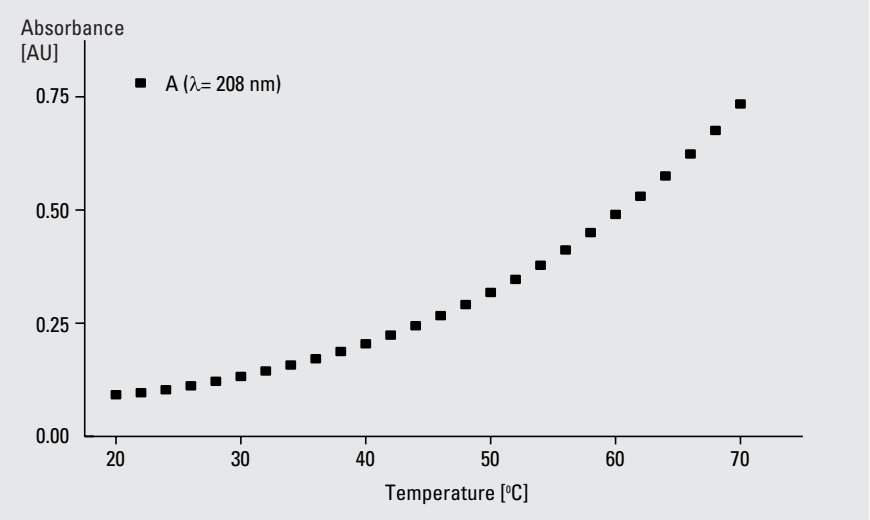
1 The following table shows the measured absorbance data at 208 nm.

Results Table 1.21. Measured Absorbance at 208 nm for Different Temperatures

Temperature [°C]	Absorbance [AU] at 208 nm	Temperature [°C]	Absorbance [AU] at 208 nm
20	0.092	46	0.267
22	0.096	48	0.291
24	0.103	50	0.318
26	0.112	52	0.347
28	0.122	54	0.378
30	0.133	56	0.412
32	0.145	58	0.450
34	0.158	60	0.490
36	0.172	62	0.530
38	0.187	64	0.576
40	0.205	66	0.624
42	0.224	68	0.676
44	0.244	70	0.734

2 The following diagram shows the absorbance as a function of temperature:

Diagram: Absorbance as a Function of Temperature



The effect of the temperature dependence is most likely due to changes of the hydration of the Cl-ions. The change of absorbance readings as a function of temperature is most important for quantitative analyses. For quantitative analyses all measurements must be carried out at known, constant temperatures.

3 The difference in the absorbance values at 208 nm between 20 °C and 70 °C is 0.642.

1.10. Influence of Temperature — Methyl Orange

Introduction

The environment has an influence on the absorbance spectrum of a compound, as seen in the experiment before. The following experiment demonstrates, how the temperature can cause changes in absorbance values as well as shifts of the absorbance bands.

The compound used in the following experiment is a dye which is used as a visual pH indicator of solutions during acid base titrations.

Reagents and Equipment

- ❑ methyl orange
- ❑ Na_2HPO_4 (0.2 M)
- ❑ citric acid (0.1 M)
- ❑ distilled water

- ❑ 100-ml volumetric flask
- ❑ 50-ml volumetric flask
- ❑ 50-ml pipette
- ❑ disposable glass pipettes (minimum 2)
- ❑ 10-mm path length quartz cell
- ❑ thermostattable cell holder
- ❑ temperature control unit or water bath

Experiment

Time: about 90 min

1. Prepare a solution of about 0.05 g methyl orange in 100 ml distilled water.
2. Dilute this stock solution by adding 13 ml of 0.2 M Na_2HPO_4 and 37 ml of 0.1 M citric acid to 1 ml of stock solution, in order to buffer the solution at a pH of 3.4.
3. Measure a reference spectrum on water.
4. Measure fifteen methyl orange spectra in the temperature range from 15 to 50 °C in steps of 2.5 degrees. Use a wavelength range from 250 to 620 nm. Make sure that the temperature of the sample is the same as measured with the temperature sensor/thermometer and constant when measuring the absorbance spectra. Check the temperature of the sample solution with a dipping sensor if available.

Evaluation

1 Determine the wavelength of the absorbance maximum at 15 °C.

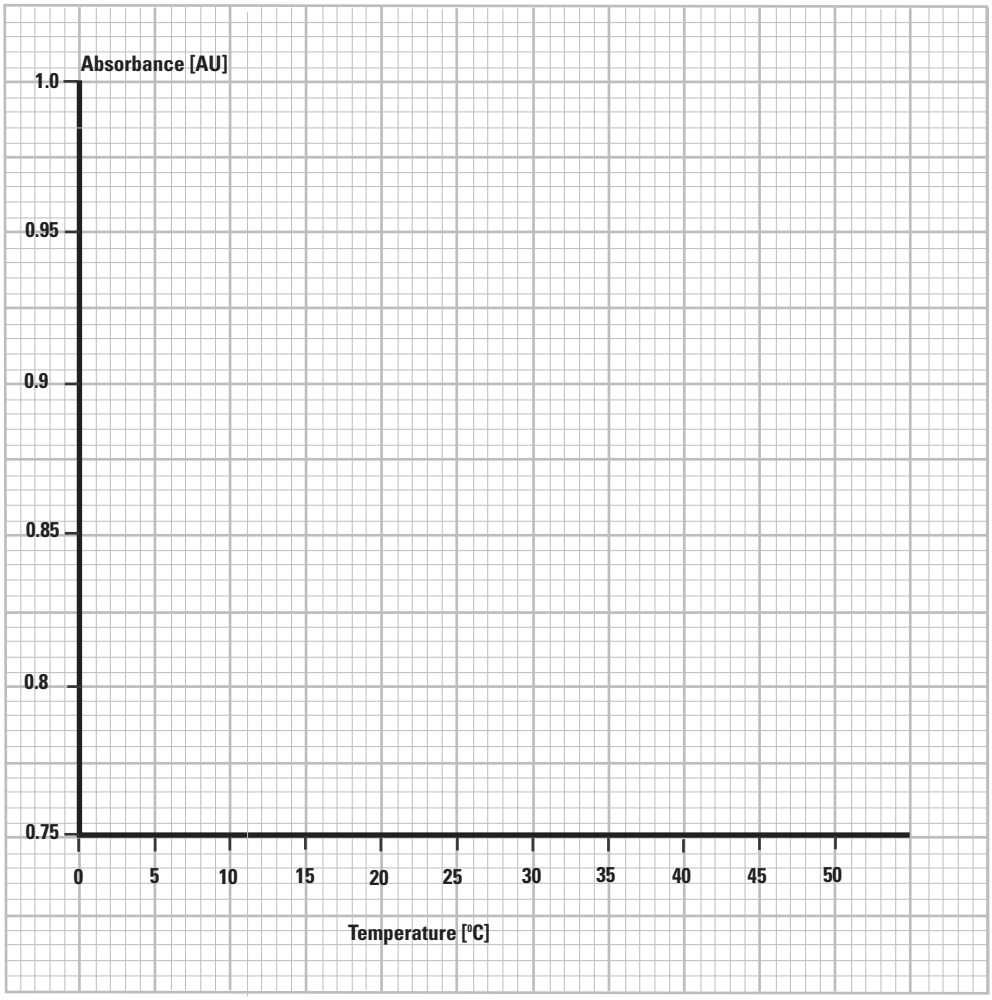
2 What is the effect of temperature changes on the absorbance values at a particular wavelength? Use the wavelength of the absorbance maximum at 15 °C for this evaluation. Enter the absorbance values in the table below.

Evaluation Table 1.22. Measured Absorbance Values at $\lambda_{max,15°C}$ at Different Temperatures

Temperature [°C]	Absorbance($\lambda_{max\ 15\ °C}$) [AU]
15.0	
17.5	
20.0	
22.5	
25.0	
27.5	
30.0	
32.5	
35.0	
37.5	
40.0	
42.5	
45.0	
47.5	
50.0	

3 Draw a graph showing the absorbance at the wavelength of the absorbance maximum at 15 °C versus temperature.

Diagram: Absorbance as a Function of Temperature



4 What is the effect of temperature on the wavelength of the absorbance maximum? Enter the wavelengths of the absorbance maxima in the table below.

Evaluation Table 1.23. Wavelengths of the Absorbance Maxima at Different Temperatures

Temperature [°C]	λ_{max} [nm]
15.0	
17.5	
20.0	
22.5	
25.0	
27.5	
30.0	
32.5	
35.0	
37.5	
40.0	
42.5	
45.0	
47.5	
50.0	

5 Determine the change in the absorbance readings, if the temperature changes by 1 °C. Quantify the error you make if the temperature of the sample changes by 1 °C.

6 What are possible reasons for the shift of the absorbance band?

Example Results & Discussion

Sample: a) stock solution: methyl orange in distilled water (0.5 g/l)

b) sample solution: 1 ml of stock solution, 13 ml of 0.2 M Na_2HPO_4 and 37 ml of 0.1 M citric acid

Cell: 10-mm path length quartz cell

Instrument Parameters:

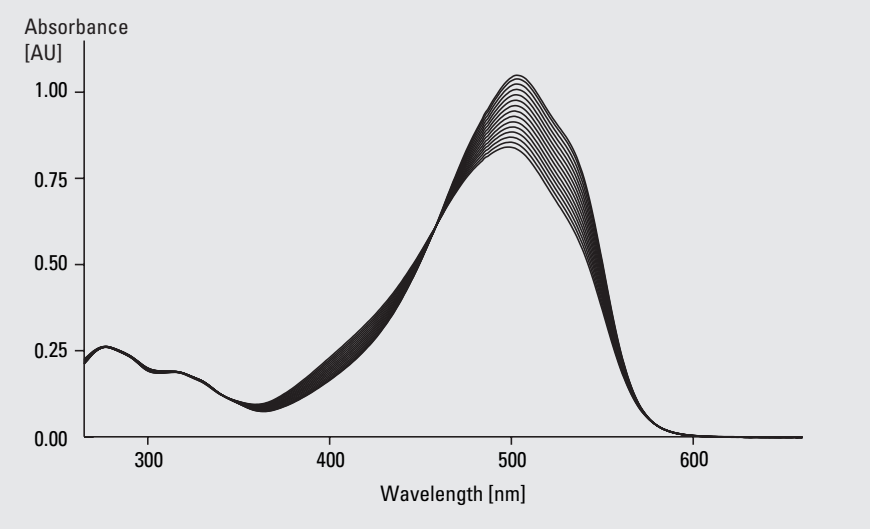
spectrophotometer:

wavelength range:	250–620 nm
absorbance range:	0.0–1.5 AU

temperature controller:

temperature range:	15–50 °C
temperature steps:	2.5 °C
equilibration time for temperature:	120 s

Diagram: Measured Absorbance Spectra for Different Temperatures



1 The wavelength of the absorbance maximum at 15 °C is 503 nm.

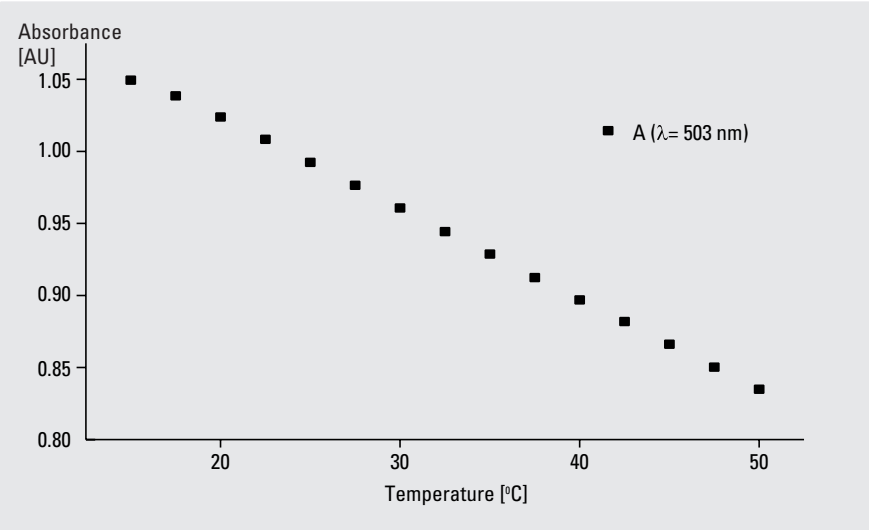
2 The following table shows the measured absorbance values at 503 nm for different temperatures.

Results Table 1.22. Measured Absorbance Values at $\lambda_{max,15°C}$ at Different Temperatures

Temperature [°C]	Absorbance [AU] (λ = 503 nm)
15.0	1.049
17.5	1.039
20.0	1.024
22.5	1.008
25.0	0.992
27.5	0.977
30.0	0.961
32.5	0.944
35.0	0.929
37.5	0.912
40.0	0.897
42.5	0.882
45.0	0.866
47.5	0.850
50.0	0.835

3 The following diagram shows the absorbance at 503 nm for different temperatures.

Diagram: Absorbance Versus Temperature at 503 nm



4 The following table shows the shift of the absorbance maximum depending on the temperatures:

Results Table 1.23. Wavelengths of the Absorbance Maximum at Different Temperatures

Temperature [°C]	λ_{max} [nm] (Interpolated)
15.0	503.4
17.5	503.0
20.0	503.0
22.5	502.7
25.0	502.5
27.5	502.4
30.0	502.2
32.5	501.8
35.0	501.4
37.5	501.2
40.0	501.1
42.5	500.4
45.0	499.3
47.5	499.3
50.0	498.7

5 The absorbance at this wavelength decreases with increasing temperature, as can be seen in the table and the graphic representation of the data. The change in absorbance is approximately -0.00625 AU/°C. Inaccurate thermostatting or too short equilibrating times cause wrong absorbance readings of about 0.6 % per 1 °C temperature deviation. Inaccurate temperature control can cause systematically wrong readings, a circumstance that must always be taken into account.

6 A possible reason for this temperature dependence of the absorbance readings is the “optical dilution” effect due to the thermal expansion of the solvent. Changing pH values of the solution cause changes in the chemical equilibrium of the compound. The dye methyl orange, used for this experiment, is a pH indicator and extremely sensitive to pH changes in a certain pH-range.

1.11. Influence of pH — Buffered Methyl Orange Solutions

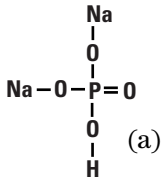
Introduction

The absorbance spectrum of a compound is related to its molecular/electronic structure. Changes of the environmental conditions can cause changes in the molecular/electronic structure. A change of the pH value, for example, has an influence on chemical equilibria and can thus change the absorbance spectrum of a solution.

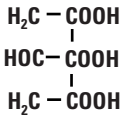
The compound used in the following experiment is a dye which is used as a visual pH indicator of solutions during acid base titrations.

Reagents and Equipment

- ❑ methyl orange
- ❑ disodium hydrogen ortho-phosphate (a)
- ❑ citric acid (b)
- ❑ distilled water
- ❑ eight 25-ml flasks
- ❑ 100-ml volumetric flask
- ❑ 200-ml volumetric flask
- ❑ 500-ml volumetric flask
- ❑ eight 50-ml beakers
- ❑ 0.5-ml pipette
- ❑ 1-ml pipette
- ❑ 5-ml pipette
- ❑ 10-ml pipette
- ❑ 25-ml pipette
- ❑ disposable glass pipettes (minimum 16)
- ❑ 10-mm path length quartz cell



(a)



(b)

Experiment

Time: about 3 h

1 Prepare a stock solution of 0.05 g methyl orange in 100 ml distilled water.

2 Prepare the following solutions:

a) a 0.2 M disodium hydrogen orthophosphate solution:
Dissolve 5.68 g Na_2HPO_4 in 200 ml distilled water. If necessary, use a magnetic stirrer for complete dissolution.
b) a 0.1 M citric acid solution:
Dissolve 9.61 g citric acid in 500 ml distilled water.
c) The eight McIlvaine's buffer solutions are prepared by mixing aliquots of the citric acid solution and the disodium hydrogen orthophosphate solution. Mix the buffer solutions shown in the table below.

Amounts of Substances for Preparing Buffer Solutions

Approximate pH	Volume [ml] of Na_2HPO_4	Volume [ml] of Citric Acid
2.2	1.0	49.0
2.6	5.5	44.5
3.0	10.0	40.0
3.4	13.0	37.0
3.8	18.0	32.0
4.2	21.0	29.0
4.6	23.0	27.0
5.2	27.0	23.0

3 Divide each mixture into two equal portions of 25 ml.

4 Add 0.5 ml of methyl orange stock solution to one of the portions of each of the buffer solutions. These are the sample solutions to be measured. The other portions of buffer have to be used for the reference measurements.

5 The following two steps have to be repeated for each corresponding pair of the buffer and the sample (same pH values):

- Measure a reference on the buffer itself.
- Measure the spectrum of the corresponding sample solution in the wavelength range from 300 to 650 nm.

Evaluation

1 Determine the absorbance maximum of all the spectra measured at different pH values of the solution. Enter the values in the table below.

Evaluation Table 1.24. Wavelengths of the Absorbance Maximum for Different pH-Values of the Samples

Approximate pH	λ_{max} [nm]
2.2	
2.6	
3.0	
3.4	
3.8	
4.2	
4.6	
5.2	

2 Check for wavelengths, at which the absorbance is independent of the pH value of the solution.

3 What are these wavelengths called?

4 What is the advantage of these wavelengths?

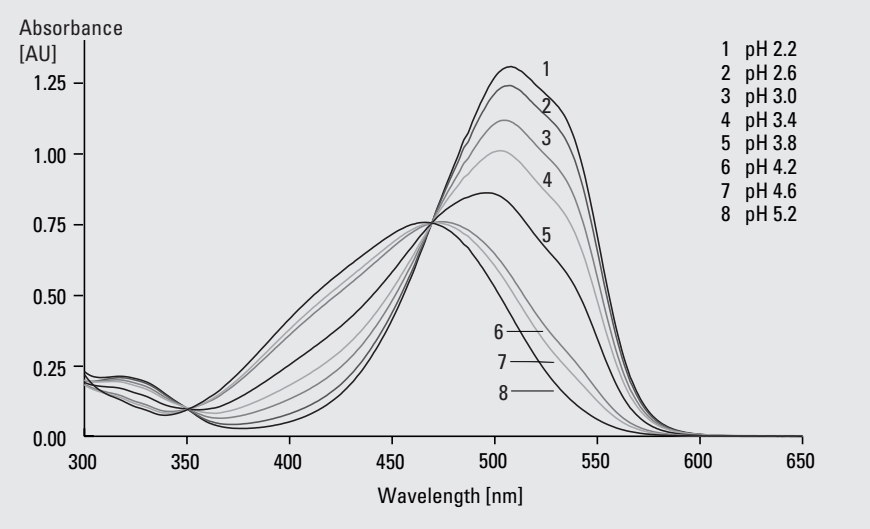
Example Results & Discussion

Sample: Stock solution: methyl orange in distilled water (0.5 g/l)
Buffered solution for pH values: 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 4.6, 5.2
preparation as described above

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 300 - 650 nm
absorbance range: 0.0 - 1.5 AU

Diagram: Measured Absorbance Spectra for Different pH Values



1 The following table shows the absorbance maximum for different pH values of the samples.

Results Table 1.24. Wavelengths of the Absorbance Maximum for Different pH-Values of the Samples

Approximate pH	λ_{max} [nm]
2.2	508
2.6	507
3.0	505
3.4	503
3.8	497
4.2	475
4.6	472
5.2	466

The wavelength of the absorbance maximum is shifted to the blue with an increasing pH value. This color change arises from a shift in the equilibrium of non-dissociated and dissociated dye molecules:

$$\text{H-Indicator} \rightleftharpoons (H^+ + \text{Indicator}^-)$$

2 The wavelengths of constant absorbance values for different pH values can be found at 350 nm and 469 nm.

3 Wavelengths at which the absorbance does not change are called isosbestic points. Isosbestic points can occur, if the absorbing species of the equilibrium have the same extinction coefficient at a certain wavelength. In this case the absorbance is independent of the position of the equilibrium and depends only on the total amount of the compounds.

4 Isosbestic points are useful for measurements in unbuffered solutions or solutions with unknown pH values because the absorbance reading is independent of the pH.

1.12. Influence of pH — Potassium Dichromate Solution

Introduction

The effect of the pH on chemical equilibria is well known and often used to influence them. The potassium chromate/dichromate equilibrium is markedly pH sensitive. The equilibrium in acid solution forms mainly dichromate ions and the one in basic solution mainly forms chromate ions.

$$Cr_2O_7^{2-} + H_2O \rightleftharpoons 2CrO_4^{2-} + 2H^+$$

Reagents and Equipment

- ❑ potassium dichromate ($K_2Cr_2O_7$)
- ❑ potassium hydroxide solution 0.1N (KOH)
- ❑ hydrochloric acid solution 0.1N (HCl)
- ❑ distilled water

- ❑ 100-ml volumetric flask
- ❑ two 10-ml volumetric flasks
- ❑ 10-ml pipette
- ❑ 1-ml pipette
- ❑ disposable glass pipettes (minimum 3)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 60 min

1 Prepare a stock solution of about 6 mg potassium dichromate in 100 ml distilled water.

2 Prepare the sample solutions:

a) Mix 9 ml of the stock solution with 1 ml potassium hydroxide solution 0.1N.
b) Mix 9 ml of the stock solution with1 ml hydrochloric acid solution 0.1N.

3 Measure a reference on distilled water.

4 Measure the spectrum of the potassium “dichromate” solution in diluted potassium hydroxide solution in the range from 230 to 500 nm.

5 Measure the spectrum of the potassium “dichromate” solution in diluted hydrochloric acid solution in the range from 230 to 500 nm.

Evaluation

1 Determine the wavelengths of the absorbance maxima of potassium dichromate dissolved in acid and basic solution (2 each) and enter the values in the following table.

Evaluation Table 1.25. Absorbance Maxima of Potassium Dichromate in Acid and Basic Solution

Potassium Dichromate Dissolved in	λ_{max1} [nm]	λ_{max2} [nm]
Basic Solution		
Acid Solution		

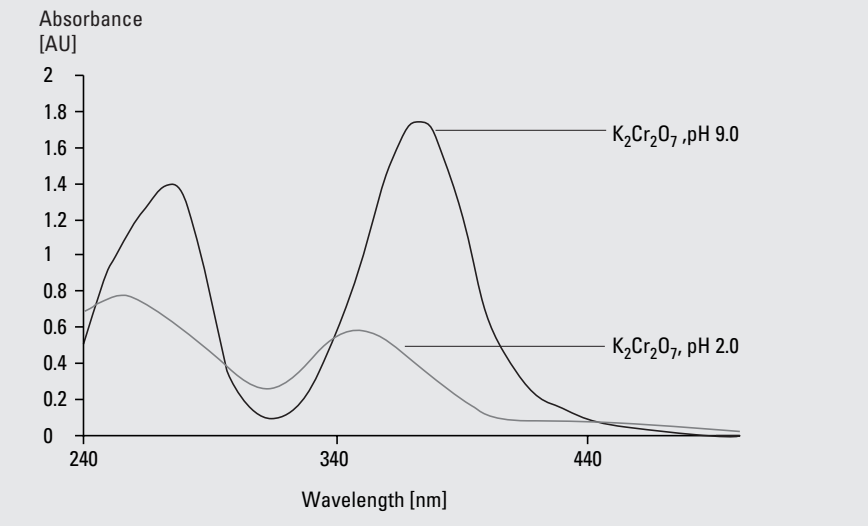
Example Results & Discussion

Sample: stock solution: potassium dichromate in distilled water (50 mg/l)
a) 9 ml stock solution mixed with 1 ml hydrochloric acid 0.1N
b) 9 ml stock solution mixed with 1 ml potassium hydroxide solution 0.1N

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 230–500 nm
absorbance range: 0.0–1.2 AU

Diagram: Measured Absorbance Spectra for Sample Solutions with Different pH Values



1 The absorbance maxima corresponding to the pH of the solution are listed in the table below.

Results Table 1.25. Absorbance Maxima of Potassium Dichromate in Acid and Basic Solution

Potassium Dichromate Dissolved in:	λ_{max1} [nm]	λ_{max2} [nm]
Acid Solution	257	352
Basic Solution	274	371

The spectra of dichromate and chromate ions are different and reflect the molecular differences of the two anions.

The pH dependence of the potassium dichromate/chromate equilibrium is of practical interest, because potassium dichromate in acid solution is used as a standard solution to check the photometric accuracy of spectrophotometers.

1.13. Effect of Concentration

Introduction

The compounds measured with UV-visible spectroscopy usually have high extinction coefficients and must be diluted before the measurements. As a consequence the concentrations of the compounds are very low and interactions between the molecules of the compounds can be neglected. Increasing the concentration of a compound can also change the spectrum by changing the environment of the compound.

The following experiment demonstrates the influence of the concentration on the spectrum by changing the concentration over three decades.

Reagents and Equipment

- ❑ methylene blue
- ❑ distilled water

- ❑ seven 100-ml beakers
- ❑ 25-ml pipette
- ❑ 10-ml pipette
- ❑ 1-ml pipette
- ❑ 0.5-ml pipette
- ❑ disposable glass pipettes (minimum 9)
- ❑ 10-mm, 5-mm, 2-mm, 1-mm and 0.1-mm path length quartz cells

Experiment

Time: about 2.5–3 h

1 Prepare an aqueous methylene blue stock solution with a concentration of

$c_0 \approx 2 \cdot 10^{-3}$ mol/l

MW (methylene blue) = 319.86 g/mol (dry)

2 Prepare 7 different dilutions of the stock solution with distilled water covering the concentration range from 1 x 10^{-3} mol/l down to 2 x 10^{-6} mol/l.

c_1 = 1:1 (distilled water: c_0)
c_2 = 5:1
c_3 = 10:1
c_4 = 25:1
c_5 = 50:1
c_6 = 100:1
c_7 = 1000:1

3 Measure a reference on water using a 10-mm path length cell.

4 Measure the spectra of all solutions using the same cell for the different solutions starting with the lowest concentrated solution. Whenever the absorbance readings exceed 2 to 2.5 absorbance units use a cell with shorter path length for the reference and the sample measurements. Use a wavelength range from 400 to 900 nm.

Evaluation

1 Determine the wavelengths of the absorbance maxima for the different solutions and enter them in the table below. Also enter the path length of the cell you used.

Evaluation Table 1.26. Absorbance Maxima Depending on Concentration

Concentration	Path Length [mm]	λ_{max} [nm]	
		Peak 1	Peak 2
c_7			
c_6			
c_5			
c_4			
c_3			
c_2			
c_1			
c_0			

2 It is useful to normalize the measured spectra at different concentrations and path lengths for convenient comparison. In this case a point between the two absorption maxima, for example, 637 nm, is suitable for normalization. Multiply each spectrum with a factor f so that the absorbance values at λ_{norm} are identical.

3 Overlay all spectra in a single plot.

4 What effect do you observe concerning the wavelength of the absorbance maxima going from high concentrations to low ones?

5 Find an explanation for the change of the absorbance band.

Example Results & Discussion

Samples: solutions of methylene blue in distilled water:

a) $c_0 = 1.876 \times 10^{-3}$ mol/l
b) $c_1 = 9.379 \times 10^{-4}$ mol/l
c) $c_2 = 3.127 \times 10^{-4}$ mol/l
d) $c_3 = 1.706 \times 10^{-4}$ mol/l
e) $c_4 = 7.216 \times 10^{-5}$ mol/l
f) $c_5 = 3.678 \times 10^{-5}$ mol/l
g) $c_6 = 1.857 \times 10^{-5}$ mol/l
h) $c_7 = 1.874 \times 10^{-6}$ mol/l

Cells 10-mm, 5-mm, 2-mm, 1-mm and 0.1-mm path length quartz cells

Instrument Parameters: wavelength range: 400–900 nm
absorbance range: 0.0–1.8 AU

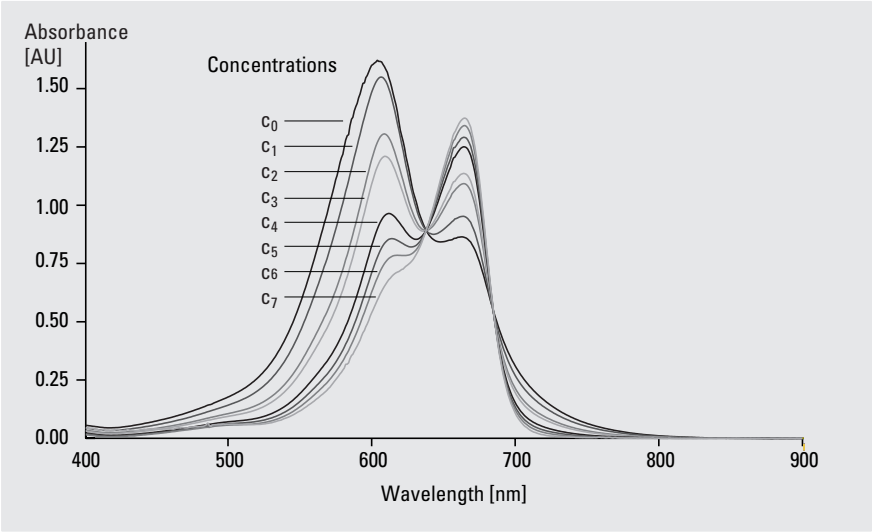
1 The table below shows the absorbance maxima depending on the concentration.

Results Table 1.26. Absorbance Maxima Depending on Concentration

Concentration	Path Length [mm]	λ_{max} [nm]	
		Peak 1	Peak 2
c_7	0.1	616	663
c_6	0.1	613	663
c_5	0.1	613	663
c_4	1	613	663
c_3	2	610	663
c_2	5	608	664
c_1	10	605	662
c_0	10	604	-

2 In this case the spectra were normalized to an absorbance value of 1.0 AU at 637 nm by dividing the spectra by their readings at this wavelength.

Diagram: Normalized Absorbance Spectra for Different Sample Concentrations



3 The wavelengths of the absorbance maxima are shifted to higher wavelengths.

4 At low concentrations, methylene blue is dissolved in the form of monomers. With increasing concentration the molecules start to interact and to form dimers.

The energies of the electronic levels are changed by forming the dimere instead of the monomers. As a result a significant shift to shorter wavelengths takes place.

1.14. Principle of Additivity

Introduction

According to Beer's law the absorbance is proportional to the number of molecules that absorb radiation at a specified wavelength. If more than one absorbing species are present the principle of additivity says, that the absorbance at any wavelength of a mixture is equal to the sum of the absorbances of each component in the mixture at that wavelength. This principle is the base for a simple approach to quantitative multi-component analysis. For calibration the absorbance values of standards of known concentration of the pure components are measured to determine their extinction coefficients (proportionality factors).

The following experiment shows an easy approach to multi-component analysis. The spectra of two food dyes are measured.

Reagents and Equipment

- ❏ quinoline yellow (yellow dye)
- ❏ indigotin (blue dye)
- ❏ distilled water

- ❏ three volumetric 1.0-ml flasks
- ❏ disposable glass pipettes (minimum 4)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 90 min

1 Prepare the following standard solutions:

a) about 12 mg quinoline yellow in 1.0 l distilled water.
b) 16 mg indigotin in 1.0 l distilled water.

2 Prepare a mixture of about 6 mg quinoline yellow and 9 mg indigotin in 1.0 l distilled water. This solution is the sample solution.

3 Measure a reference on distilled water.

4 Measure the spectra of quinoline yellow and indigotin standards in the range from 300 to 700 nm.

5 Measure the spectrum of the sample (mixture of quinoline yellow and indigotin).

Evaluation

We use the simplest method for multi-component quantification for this experiment. The method uses only the absorbance values at the maxima of the pure compounds.

1 Determine the wavelength of the absorbance maxima for quinoline yellow and indigotin and enter them in the table below.

Evaluation Table 1.27. Wavelengths of the Absorbance Maxima of the Two Pure Dyes

Compound	λ_{max} [nm]
Quinoline yellow standard	λ_1 =
Indigotin standard	λ_2 =

2 Determine the absorbance of the two standards and the sample at these wavelengths and enter them in the table below.

Evaluation Table 1.28. Measured Absorbance Values at the Absorbance Maxima

Compound	Absorbance [AU] at λ_1 = _____nm	Absorbance [AU] at λ_2 =_____nm
Quinoline yellow standard		
Indigotin standard		
Sample		

3 Calculate the concentration of the components quinoline yellow and indigotin in the mixture.

For a mixture of two components the absorbance A at the wavelengths λ_1 and λ_2, (for example, the absorbance maxima of the components x and y) is given in the following two equations:

$$A(\lambda_1)_{(x+y)} = xA(\lambda_1)_x + yA(\lambda_1)_y$$
$$A(\lambda_2)_{(x+y)} = xA(\lambda_2)_x + yA(\lambda_2)_y$$

with:

$A(\lambda_1)_{(x+y)}$, $A(\lambda_2)_{(x+y)}$	= absorbance values of the sample at λ_1, λ_2
$A(\lambda_1)_x$, $A(\lambda_2)_x$	= absorbance values of the quinoline yellow standard at λ_1, λ_2
$A(\lambda_1)_y$, $A(\lambda_2)_y$	= absorbance values of the indigotin standard at λ_1, λ_2
x	= fraction of quinoline yellow in the sample
y	= fraction of indigotin in the sample

The concentration of the components can be calculated by:

$$c_1 = xc_{01}$$
$$c_2 = yc_{02}$$

with:

c_{01}	= concentration of quinoline yellow in the standard solution
c_{02}	= concentration of indigotin in the standard solution
c_1	= concentration of quinoline yellow in the sample
c_2	= concentration of indigotin in the sample

4 Compare the calculated concentrations c_1 and c_2 of quinoline yellow and indigotin with the real ones that you have prepared.

5 Judge the certainty/uncertainty of the method described above. To get an estimation for the reliability of this method simulate noise by adding an error of x % to the absorbance reading of the sample and calculate the concentrations again.

6 How can this simple method be improved to achieve more reliable results?

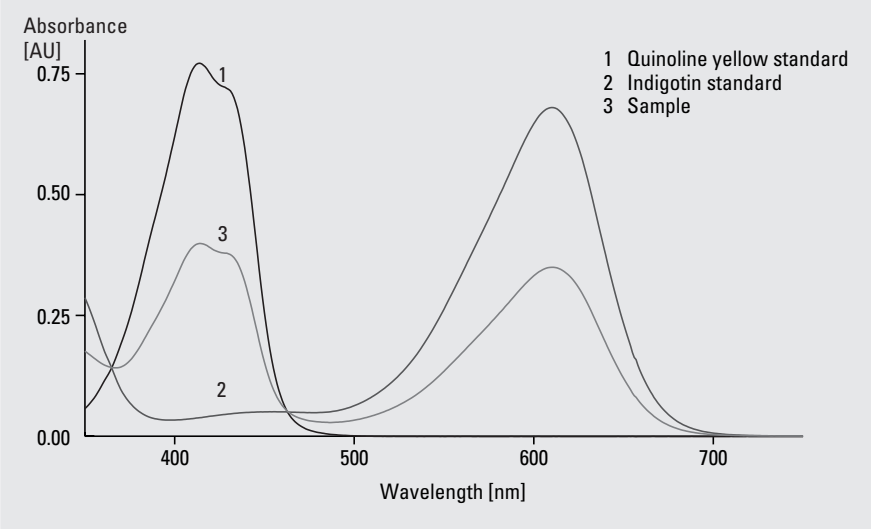
Example Results & Discussion

Samples: a) standard solution 1: quinoline yellow in distilled water (12.0 mg/l)
b) standard solution 2: indigotin in distilled water (17.5 mg/l)
c) mixture of 5.85 mg quinoline yellow and 8.9 mg indigotin in 1 l distilled water

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 300–700 nm
absorbance range: 0.0–0.8 AU

Spectra: Measured Absorbance Spectra of Pure and Mixed Sample



1 The wavelengths listed below were chosen for the calculation.

Results Table 1.27. Wavelengths of the Absorbance Maxima of the Two Pure Dyes

Compound	λ_{max} [nm]
Quinoline yellow standard	λ_1 = 414 nm
Indigotin standard	λ_2= 610 nm

2 The measured absorbance values at the absorbance maxima are listed in the table below.

Results Table 1.28. Measured Absorbance Values at the Absorbance Maxima

Compound	λ_1 = 414 nm	λ_2 = 610 nm
Quinoline yellow standard	0.772	-0.001
Indigotin standard	0.039	0.680
Sample	0.399	0.350

3 Calculation:

0.7719 x + 0.0384 y = 0.3988	-0.0007 x + 0.6801 y = 0.3496

The fractions x and y of quinoline yellow and indigotin were calculated as:

x = 0.491	y = 0.514

The concentrations of quinoline yellow and indigotin in the mixture were calculated as:

c_1 = 5.89 mg/l	c_2 = 9.00 mg/l

4 Comparing the measured concentrations with the calculated concentrations gives a good correlation with an error of about 1 %.

5 To simulate noise, we add an error of about 1 % of the absorbance measurement of the sample (0.3948 and 0.3531) results. This changes the calculated concentrations of quinoline yellow and indigotin in the mixture as follows:

c_1 = 5.83mg/l	c_2 = 9.10 mg/l

6 Any measurement error such as noise in the absorbance reading influences the calculated concentrations of the mixture directly. The effect of random noise can be reduced through the use of additional spectral information. Instead of using only two data points, a series of data points can be used for quantification. In this case we have a so-called over-determined system. A least square fit of the standard spectra to the spectrum of the measured sample yields quantitative results.

Part 2

Measuring Instrument Performance

General

Instrumental performance is one of the main factors that effect the accuracy and reproducibility of measurements. The following experiments are procedures for measuring many of the key instrumental performance parameters.

The relevance of the various procedures can best be appreciated if they are made on two instruments with different overall performance specifications. For example a research grade instrument and, for comparison, a lower priced instrument with a performance intended for less demanding routine work can be used simultaneously.

2.1. Wavelength Accuracy

Introduction

Wavelength accuracy is an important performance parameter when comparing data measured on different instruments. Wavelength accuracy is normally checked by using a calibration standard which has a series of narrow transmittance valleys. A commonly used standard is holmium perchlorate solution which is prepared by dissolving holmium oxide in perchloric acid. It has a series of narrow valleys over the wavelength range from 200 to 700 nm allowing a check of wavelength accuracy over the whole UV range and far into the visible range.

Reagents and Equipment

- ❏ holmium oxide (Ho_2O_3)
- ❏ perchloric acid ($HClO_4$)
- ❏ distilled water

- ❏ 50-ml beaker
- ❏ 100-ml volumetric flask
- ❏ 10-ml pipette
- ❏ disposable glass pipettes (minimum 2)
- ❏ 10-mm path length quartz cell
- ❏ heater with stirrer

Experiment

Time: about 120–150 min

1 Prepare a solution of about 4.0 g holmium oxide in 100 ml 10 % v/v pure perchloric acid:

a) Weigh 4 g of holmium oxide and add 10 ml of distilled water and 10 ml of pure perchloric acid.
b) Dissolve the holmium oxide by heating and stirring the mixture at about 80 °C for one hour.
c) Transfer the clear solution quantitatively to a 100-ml volumetric flask and bring to volume with distilled water.

2 Measure a reference on 10 % perchloric acid.

3 Measure the transmittance spectrum of the holmium oxide solution in the wavelength range from 210 to 700 nm. Use a slow scan speed if you are working with a conventional scanning spectrophotometer. Determine the wavelengths of the transmittance minima using the built in function, if available in your instrument. Otherwise determine the wavelengths of the transmittance minima manually.

Note: Instead of preparing the holmium oxide solution you can also use a holmium oxide solution SRM 2034 from NIST (National Institute of Standards and Technology).

Evaluation

1 Enter the measured wavelengths of the transmittance minima in the table below. The reference values for instruments with 0.5, 1 and 2 nm bandwidth are also shown (taken from NIST 2034 certificate). Calculate the deviations of the measured values from the specified values for the bandwidth closest to the instrument you used and enter them in the table below.

Evaluation Table 2.1. Measured Transmittance Minima Compared to Reference Values

0.5 nm λ_{ref} [nm]	1 nm λ_{ref} [nm]	2 nm λ_{ref} [nm]	Measured λ_{min} [nm]	Deviation $\pm\Delta\lambda$ [nm]
241.01	241.13	241.08		
249.79	249.87	249.98		
278.13	278.10	278.03		
287.01	287.18	287.47		
333.43	333.44	333.40		
345.52	345.47	345.49		
361.33	361.31	361.16		
385.50	385.66	385.86		
416.09	416.28	416.62		
---	451.30	451.30		
467.80	467.83	467.94		
485.27	485.29	485.33		
536.54	536.64	536.97		
640.49	640.52	640.84		

Note: If the optical bandwidth of the instrument you are using is not known you can estimate it using the procedure from the experiment “Spectral Resolution” on page 102.

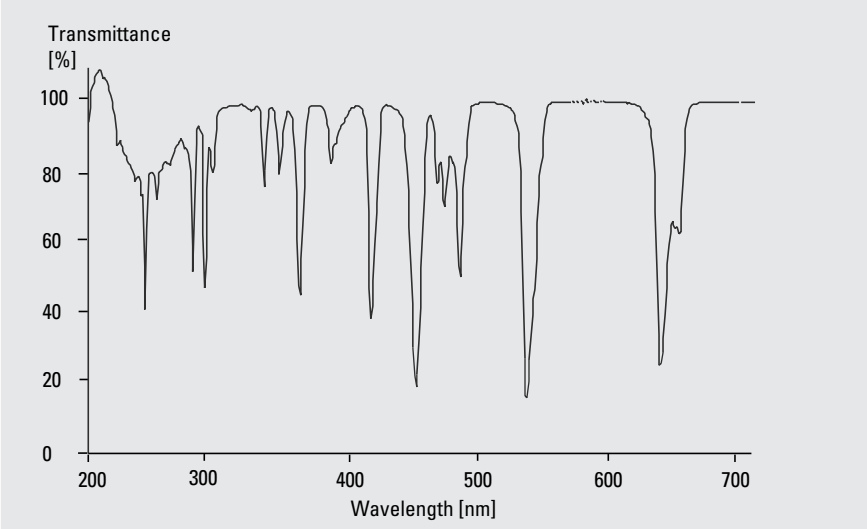
Example Results & Discussion

Sample: holmium oxide in 10 % v/v perchloric acid (40 g/l)

Cell: 10-mm path length quartz cell

Instrument Parameters:
wavelength range: 210–700 nm
transmittance range: 0.0–100 [%]

Diagram: Transmittance Spectrum of Holmium Perchloride Solution



1 The following table shows the measured wavelengths of transmittance minima compared to the reference values.

Results Table 2.1. Measured Transmittance Minima Compared to Reference Values

λ_{ref}* [nm]	Measured λ_{max} [nm]	Deviation $\pm\Delta\lambda$ [nm]
241.11	241.03	-0.08
249.93	249.90	-0.03
278.07	277.98	-0.09
287.33	287.09	-0.24
333.42	333.22	-0.20
345.48	345.16	-0.32
361.24	361.04	-0.20
385.76	385.47	-0.29
416.45	416.18	-0.27
451.30	451.43	-0.13
467.89	467.65	-0.28
485.31	485.00	-0.31
536.81	536.34	-0.47
640.68	640.27	- 0.41

* These values are interpolated between the 1- and 2-nm reference values to a nominal bandwidth of 1.5 nm which is the bandwidth of the spectrophotometer used.

References

"Holmium Oxide Solution Wavelength Standard from 240 to 640 nm - SRM 2034" Victor R. Weidner, Radu Mavrodineanu, Klaus D. Mielenz, Rance A. Velapoldi, Kenneth L. Eckerle and Bradley Adams, *National Bureau of Standards, Gaithersburg, MD 20899*

2.2. Wavelength Accuracy Using the Deuterium Lines

Introduction

Another way to check the wavelength accuracy is to use the emission lines from various sources such as mercury or deuterium arc lamps. The easiest ones to use are the deuterium emission lines of the deuterium lamp — one of the light sources in virtually all UV-visible spectrophotometers. The advantage of this method compared to the one described above is that it is quick and easy since no reagents are required. The disadvantage is that only two calibration wavelengths are available and there are no calibration wavelengths in the UV region.

Reagents and Equipment

- ❑ spectrophotometer capable of measuring intensity spectra

Experiment

Time: about 20 min

1 Measure the intensity spectrum of the deuterium lamp in the wavelength range from 200 to 700 nm. If you work with a conventional scanning spectrophotometer use the slowest scan speed and scan the wavelength ranges from 480 to 490 nm and from 650 to 660 nm. If your instrument offers this functionality determine the wavelengths of the intensity maxima using the built-in function. Otherwise the intensity maxima have to be determined manually.

Evaluation

1 Enter the measured values in the following table and calculate the wavelength errors:

Evaluation Table 2.2. Measured Wavelengths of Intensity Maxima Compared to Reference Values

Reference λ_{max} [nm]	Measured λ_{max} [nm]	Deviation $\pm\Delta\lambda$ [nm]
486.0		
656.1		

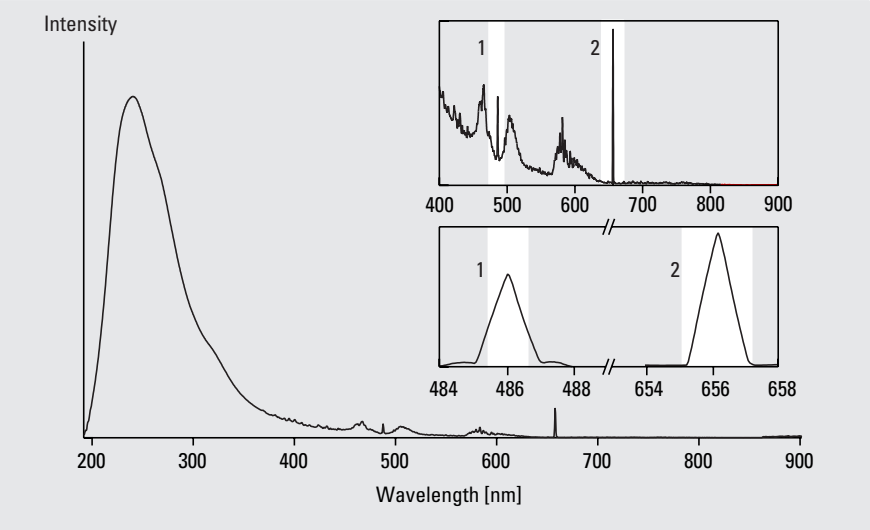
Example Results & Discussion

Sample: no sample required

Cell: no cell required

Instrument Parameters: *conventional dual beam spectrophotometer:*
scan speed: 7.5 nm / min
wavelength ranges: 480–490 nm and 650–660 nm

Diagram: Intensity Spectrum of the Deuterium Lamp



1 The following table gives a comparison of the measured and the reference wavelengths of the intensity maxima.

Results Table 2.2. Measured Wavelengths of Intensity Maxima Compared to Reference Values

Reference λ_{max} [nm]	Measured λ_{max} [nm]	Deviation $\pm\Delta\lambda$ [nm]
486.0	486.92	-0.07
656.1	656.36	0.26

2.3. Photometric Accuracy Using Potassium Dichromate

Introduction

Photometric accuracy is the most important criterion for quantitative analysis when extinction coefficients or factors are used. A potassium dichromate solution (60 mg/l) in sulphuric acid (0.01 N) is used in the following experiment.

Reagents and Equipment

- ❑ potassium dichromate, dried to constant weight at 130 °C (KCr_2O_4)
- ❑ 0.01-N sulphuric acid (H_2SO_4)
- ❑ 100-ml volumetric flask

- ❑ disposable glass pipettes (minimum 2)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 20 min

1. Prepare a solution of about 6 mg (maximum range 5.7–6.3 mg) potassium dichromate in 100 ml 0.01 N sulphuric acid.
2. Measure a reference on sulphuric acid (0.01 N).
3. Immediately afterwards do a sample measurement of the potassium dichromate solution in the wavelength range from 220 to 380 nm.

Evaluation

1 Determine the absorbance values of the peaks and valleys at the wavelengths 235, 257, 313 and 350 nm. Calculate absorbance values corrected from the actual concentration to a reference concentration of 60.06 mg/l using the following equation.

$$\text{Corrected Absorbance [AU]} = \frac{\text{actual weight [mg]}}{6.006\ \text{mg}} \cdot \text{Measured Absorbance [AU]}$$

2 Enter the measured and the corrected values in the table below. Calculate the deviation between the corrected absorbances and the reference values.

Evaluation Table 2.3. Measured Absorbance Values Compared to Reference Values

Wavelength [nm]	Measured Absorbance [AU]	Corrected Absorbance [AU]	Reference Absorbance [AU]	Deviation ΔA [AU]
235			0.742	
257			0.861	
313			0.291	
350			0.639	

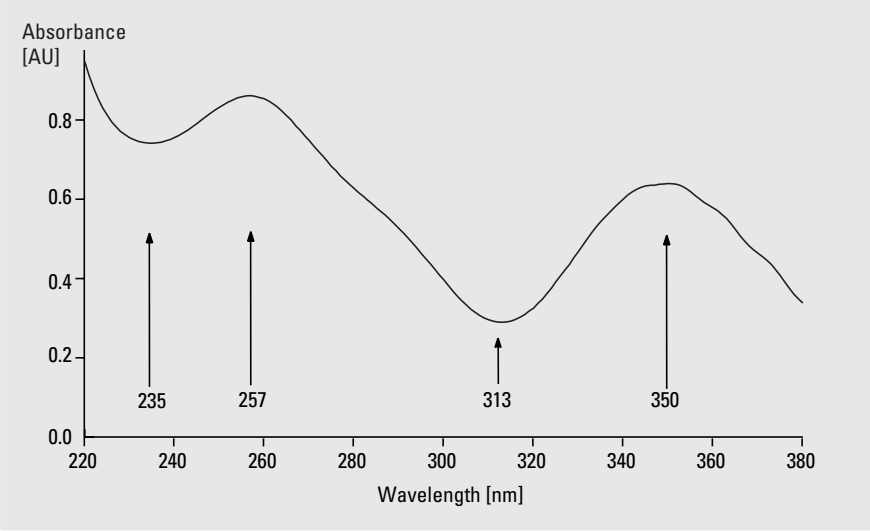
Example Results & Discussion

Sample: potassium dichromate in 0.01 N sulfuric acid (60.06 mg/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 220–380 nm
absorbance range: 0.0–1.0 AU

Diagram: Absorbance Spectrum of the Potassium Dichromate Solution



1 The following table shows a comparison of the measured and of the reference absorbance values at defined wavelengths.

Results Table 2.3. Measured Absorbance Values Compared to Reference Values

Wavelength [nm]	Measured Absorbance [AU]	Corrected Absorbance [AU]	Reference Absorbance [AU]	Deviation ΔA [AU]
235	0.741	0.741	0.742	0.009
257	0.861	0.861	0.861	0.010
313	0.290	0.290	0.291	0.004
350	0.640	0.640	0.639	0.001

2 In this example the concentration of potassium dichromate used was exactly the same as the one that was used for measurement of the reference values. Therefore no correction needs to be applied.

One problem with using potassium dichromate as a standard is that it is not only a test of the instrument accuracy but also a test, for example, of the accuracy of the balance used to weigh the dichromate. The accuracy of the volumetric flask and the experimental skill of the chemist preparing the solution also have a strong impact on the accuracy of the results.

The advantage of potassium dichromate is that it absorbs in the UV range. There are only very few suitable standards available for this part of the spectrum.

References

"European Pharmacopeia" Third Edition **1997,** *Council of Europe, Strasbourg,* ISBN 92-871-2991-6

2.4. Photometric Accuracy Using Neutral Density Glass Filters

Introduction

Another way to check the photometric accuracy is using neutral density glass filters, for example, NIST 930e or similar. Such standards are supplied with calibration values at specific wavelengths which have been measured on a highly precise reference spectrophotometer. The advantage of this method compared to using solutions is that no sample preparation is required.

Reagents and Equipment

- ❑ neutral density filter NIST 930e or similar with 10 % transmittance

Experiment

Time: about 10 min

1. Measure a reference on air (no cell in the cell holder).
2. Measure the absorbance spectrum of the neutral density glass filter in the wavelength range from 400 to 700 nm.

Evaluation

1 Determine the absorbance values at the certified wavelengths and enter them in the table below. For the NIST filters they are 440.0, 465.0, 546.0, 590.0 and 635.0 nm. Find out the specified wavelengths from the calibration certificates if another standard is being used. Enter the reference absorbance values from the calibration certificate of the standard and calculate the errors.

Evaluation Table 2.4. Measured Absorbance Values Compared to Reference Values

Wavelength [nm]	Reference Absorbance [AU]	Measured Absorbance [AU]	Deviation ∆A [AU]

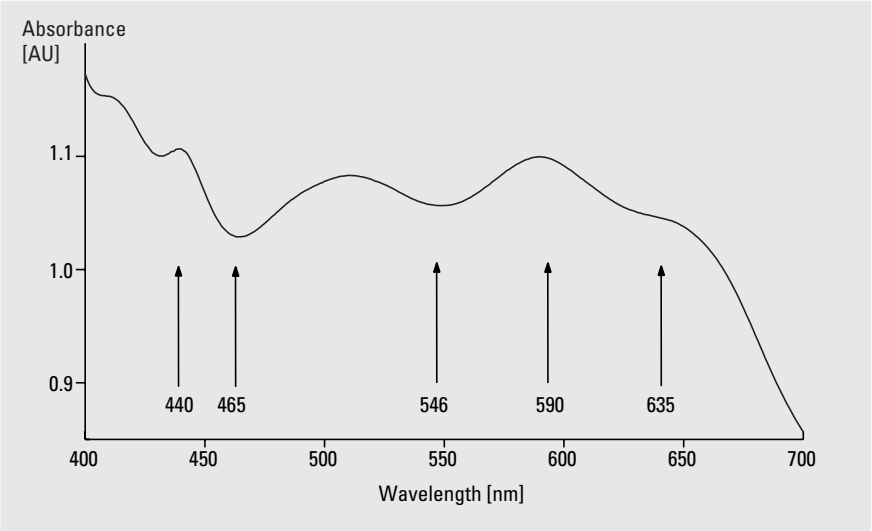
Example Results & Discussion

Sample: NIST 930e neutral density glass filter, 10 % transmittance

Cell: no cell required

Instrument Parameters: wavelength range: 400–700 nm
absorbance range: 0.8–1.2 AU

Diagram: Absorbance Spectrum of NIST 930e Neutral Density Glass Filter



1 The following table shows the absorbance values of the NIST 930e neutral density glass filter at defined wavelengths and compares the measured values to the reference values.

Results Table 2.4. Measured Absorbance Values Compared to Reference Values

Certified Wavelength [nm]	Reference Absorbance [AU]	Measured Absorbance [AU]	Deviation ΔA [AU]
440.0	1.1051	1.1067	0.0016
465.0	1.0278	1.0290	0.0012
546.0	1.0570	1.0569	0.0001
590.0	1.0996	1.0997	0.0001
635.0	1.0487	1.0484	0.0003

2.5. Stray Light

Introduction

Stray light is the factor that most strongly affects the linear relationship between absorbance and concentration at high absorbance values. 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.

To detect stray light at given wavelengths solutions of sodium nitrite (340 nm), sodium iodide (220 nm) and potassium chloride (200 nm) in water are used as cut-off filters.

Reagents and Equipment

- ❑ sodium nitrite ($NaNO_2$)
- ❑ sodium iodide (NaI)
- ❑ potassium chloride (KCl)
- ❑ distilled water

- ❑ three 100-ml volumetric flasks
- ❑ disposable glass pipettes (minimum 3)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 60–75 min

1 Prepare the following sample solutions:

a) 5 g sodium nitrite in 100 ml distilled water,
b) 1 g sodium iodide in 100 ml distilled water and
c) 1.2 g potassium chloride in 100 ml distilled water

2 Measure a reference on distilled water.

3 Measure the transmittance spectra of the stray light sample solutions in the wavelength range from 200 to 500 nm.

Evaluation

1 Determine the transmittance at the specified wavelengths for each solution and enter it in the table below.

Evaluation Table 2.5. Measured Transmittance of Different Stray Light Filters at Different Wavelengths

Stray Light Filter	Wavelength [nm]	Measured Transmittance [% T]
$NaNO_2$	340	
NaI	220	
KCl	200	

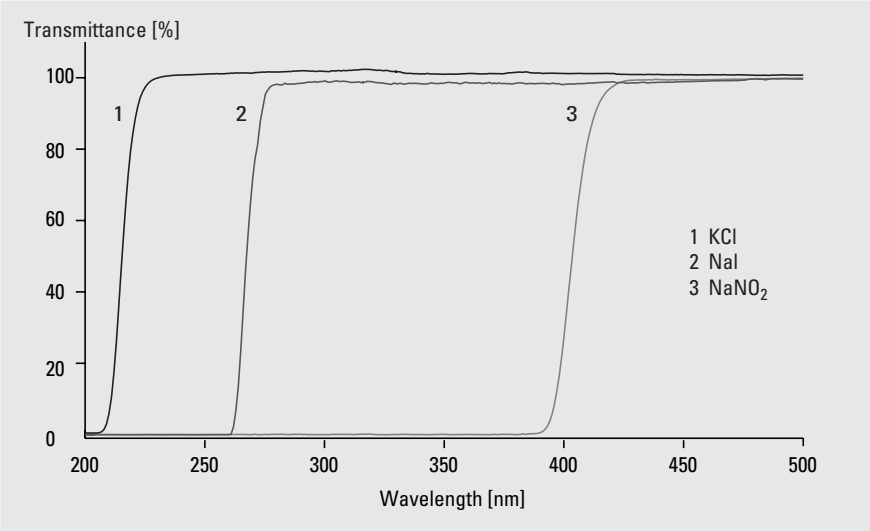
Example Results & Discussion

Samples: a) sodium nitrite in distilled water (50 g/l)
b) sodium iodide in distilled water (10 g/l)
c) potassium chloride in distilled water (12 g/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 200–500 nm
transmittance range: 0–100 %

Diagram: Transmittance Spectra of the Tested Stray Light Filters



1 The following table shows the measured transmission values compared to the instrument specifications.

Results Table 2.5. Measured Transmittance of Different Stray Light Filters at Different Wavelengths

Stray Light Filter	Wavelength [nm]	Measured Transmission [% T]
$NaNO_2$	340	0.016
NaI	220	0.010
KCl	200	0.497

2.6. Spectral Resolution

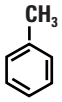
Introduction

Resolution is a critical factor in determining the shape of measured peaks. In general the instrumental resolution should be at least 10 times as high as the natural bandwidth of the peak measured. If the instrumental resolution is not sufficient, the absorbance value will be lower than the true value.

Resolution can be estimated by measuring the ratio of the absorbance of the maximum at ca. 269 nm to that of the minimum at ca. 266 nm of a toluene solution in hexane.

Reagents and Equipment

- ❏ toluene (a)
- ❏ hexane ($CH_3(CH_2)_4CH_3$)



(a)

- ❏ 100-ml volumetric flask
- ❏ 20-µl pipette
- ❏ disposable glass pipettes (minimum 2)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 20–60 min

1 Prepare a solution of 0.02 % v/v toluene in n-hexane in the 100-ml volumetric flask.

2 Measure a reference on hexane.

3 Measure the spectrum of the toluene in hexane solution in the wavelength range from 265 to 272 nm.

4 If your spectrophotometer allows you to vary the slit width, repeat the measurement of the toluene sample with different slit widths from 0.25 to 4 nm.

Evaluation

1 Calculate the ratio absorbance of the peak $A_p(\approx\lambda_{269})$ /absorbance of the valley $A_v(\approx\lambda_{266})$ and enter it together with the instrument slit width in the table below.

Evaluation Table 2.6. Measured Absorbances at Different Wavelengths for Different Slit Widths and Their Ratios

Nominal Instrument Slit Width [nm]	Absorbance [AU] at 269 nm	Absorbance [AU] at 266 nm	Measured Ratio

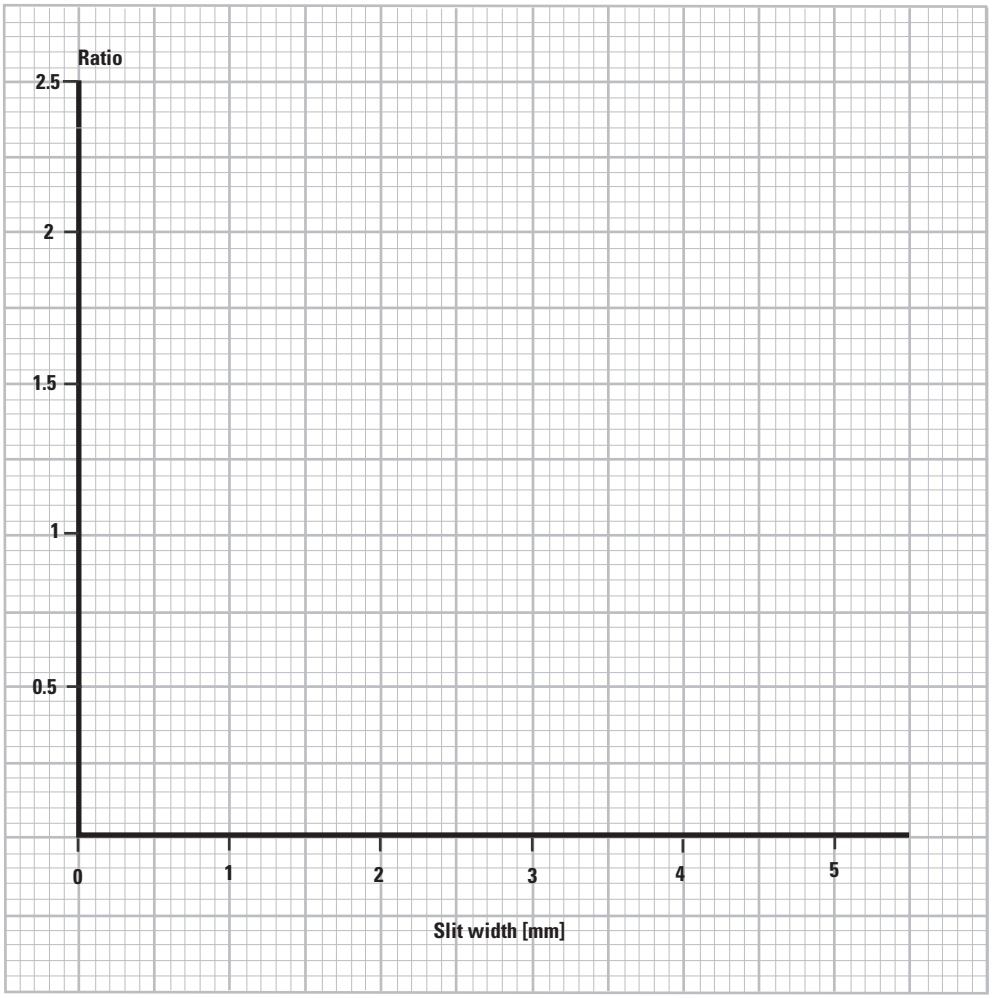
The following table shows the relationship between instrumental slit width and the 269/266 nm ratio. Use the table below to estimate the actual instrumental slit width and compare it to the nominal value.

Reference Ratio of Maxima to Minima Depending on the Slit With

Instrument Slit Width [nm]	Reference Ratio
0.25	2.3
0.50	2.2
1.00	2.0
2.00	1.4
3.00	1.1
4.00	1.0

2 Create a plot showing the calculated absorbance ratios versus slit width.

Diagram: Calculated Absorbance Ratios Versus Slit Width



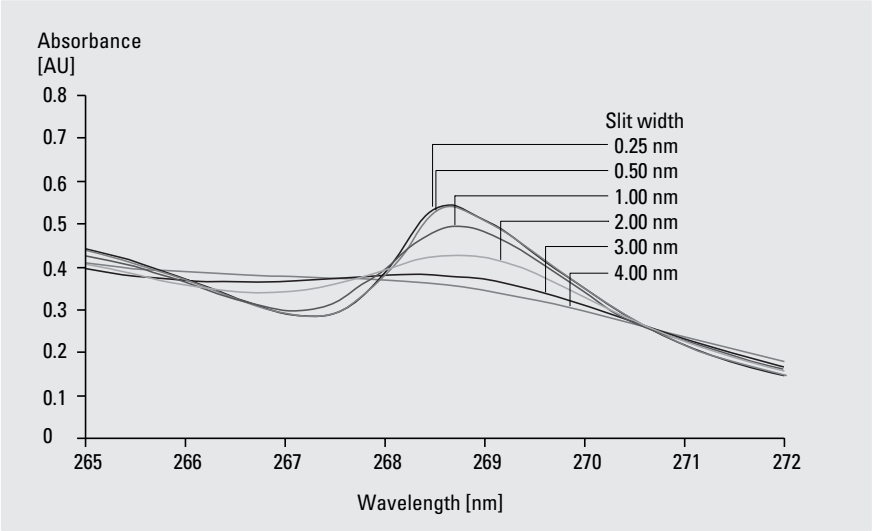
Example Results & Discussion

Sample: toluene in n-hexane (0.02 % v/v)

Cell: 10-mm path length quartz cell

Instrument Parameters: *conventional scanning spectrophotometer with variable slit width:*
slit widths: 0.25–4.0 nm
wavelength range: 265–72 nm
absorbance range: 0.0–0.7 AU

Diagram: Spectra for Different Slit Widths

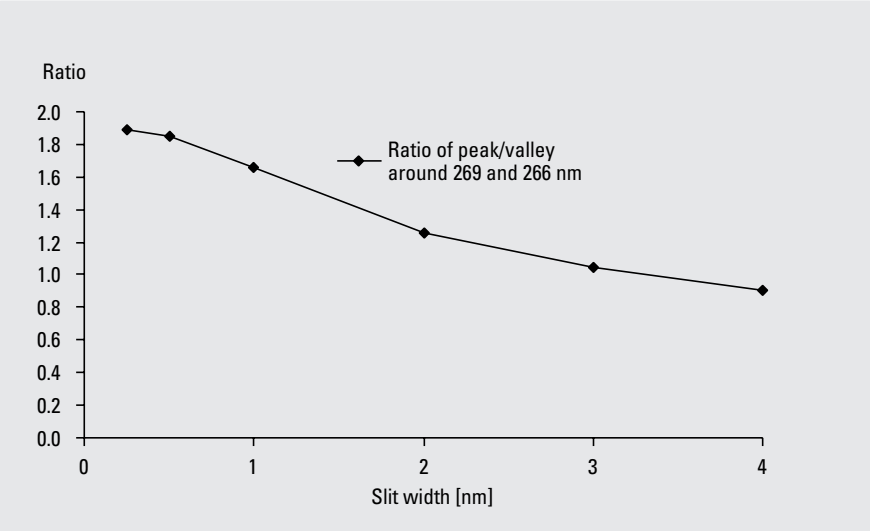


1 The following table shows a comparison between the maxima to minima ratio calculated from the measurements and the reference ratios.

Results Table 2.6. Measured Absorbances at Different Wavelengths for Different Slit Widths and Their Ratios

Instrument Slit Width [nm]	Absorbance [AU] at about 269 nm	Absorbance [AU] at about 266 nm	Measured Ratio	Reference Ratio
0.25	0.54	0.29	1.902	2.3
0.50	0.53	0.29	1.85	2.2
1.00	0.50	0.30	1.65	2.0
2.00	0.43	0.34	1.26	1.4
3.00	0.38	0.37	1.05	1.1
4.00	0.40	0.36	0.9	1.0

2 A plot of slit width versus 269/266 ratio is shown below.



Decreasing slit width improves resolution but it reduces the amount of light that reaches the detector. This results in higher noise and poorer precision.

References

"Fundamentals of Modern UV-visible Spectroscopy", Tony Owen, *Hewlett-Packard primer* **1996**, publication number 12-5965-5123E

2.7. Noise

Introduction

Noise is the major factor affecting the precision of the absorbance measurements. It is the limiting factor at low absorbances. Noise is typically measured at zero absorbance, that is, with no sample in the light path. Sometimes noise is quoted peak-to-peak but a more common method is to calculate the standard deviation value which has more statistical significance.

Reagents and Equipment

- ❑ no equipment and reagents required

Experiment

Time: about 25 min

1. Measure a reference on air (no cell in the cell holder).
2. Make sixty consecutive measurements on air (no cell in the cell holder) at 1 second intervals with 0.5 s integration time* and at 500 nm.

   * This is the standard integration time for the HP 8453 spectrophotometer. If using another kind of spectrophotometer use the standard response settings.

3. Repeat the experiment using shorter (0.1 s) and longer (5.0 s) integration times.

Evaluation

1 Calculate the noise and mean of absorbance, using the following equations:

$$\text{Noise (SD)} = \sqrt{\frac{n\Sigma x^2 - (\Sigma x)^2}{n(n-1)}} \; ; \; \text{Mean Absorbance} = \frac{\Sigma x}{n}$$

where: x = measured absorbance values,

n = number of points

2 How does the noise value change with shorter and with longer integration times?

Enter the calculated values of mean and standard deviation at different integration times in the table below.

Evaluation Table 2.7. Calculated Means and Standard Deviations

Integration Time [s]	Mean Absorbance [AU]	Noise Standard Deviation [AU]
0.1		
0.5		
5.0		

3 Would you expect the same noise level at other wavelengths?

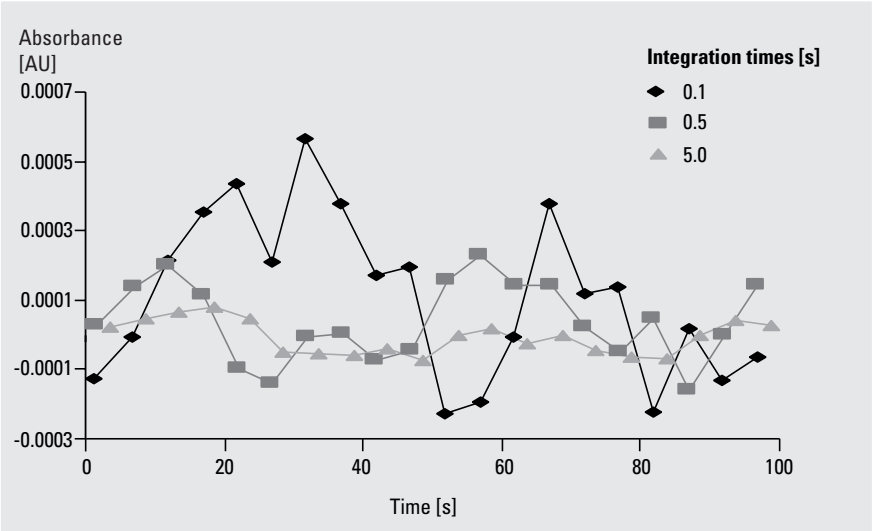
Example Results & Discussion

Sample: no reagents required

Cell: no cell required

Instrument Parameters: fixed wavelength: 500 nm
absorbance range: -0.3–0.7 mAU

Diagram: Plot of Measured Absorbance Values Versus Time



1 The following values were calculated from the measured values:

Results Table 2.7. Calculated Means and Standard Deviations

Integration Time [s]	Mean Absorbance [AU]	Noise Standard Deviation [AU]
0.1	$1.10 \cdot 10^{-4}$	$2.35 \cdot 10^{-4}$
0.5	$3.187 \cdot 10^{-5}$	$1.14 \cdot 10^{-4}$
5.0	$-7.23 \cdot 10^{-6}$	$4.79 \cdot 10^{-5}$

2 Noise increases with shorter integration times and decreases with longer integration times.

3 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.

2.8. Baseline Flatness

Introduction

In the previous experiment we measured noise at a specific wavelength. 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. Baseline flatness is typically measured at zero absorbance with no sample in the light path.

Reagents and Equipment

- ❑ no equipment and reagents required

Experiment

Time: about 30 min

1. Measure a reference on air (no cell in the cell holder).
2. Measure a spectrum on air over the full range of the spectrophotometer with 0.5 s integration time.
3. Vary the integration times to 0.1 s and to 5 s to see the effect of varying integration times.

Evaluation

1 Calculate the baseline flatness and mean absorbance using the following equation:

$$\text{Baseline (SD)} = \sqrt{\frac{n\Sigma x^2 - (\Sigma x)^2}{n(n-1)}}\ ;\ \text{Mean Absorbance} = \frac{\Sigma x}{n}$$

where: x = measured absorbance value

n = number of points

How does the noise value change with shorter and longer integration times. Enter the calculated values in the table below.

Evaluation Table 2.8. Calculated Mean Absorbance and Standard Deviation

Integration Time [s]	Mean Absorbance [AU]	Baseline Standard Deviation [AU]
0.1		
0.5		
5		

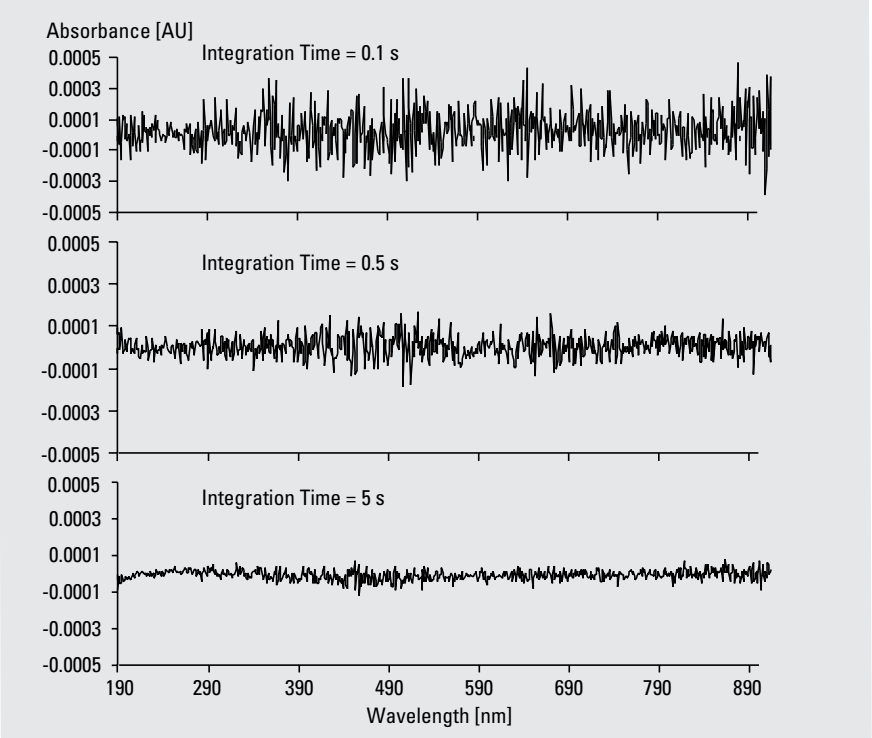
Example Results & Discussion

Sample: no reagents required

Cell: no cell required

Instrument Parameters: wavelength range: 190–900 nm
absorbance range: -0.5–0.5 mAU

Diagram: Absorbance Spectra of Baselines



1 Calculated mean absorbance and standard deviation is listed in the table below.

Results Table 2.8. Calculated Mean Absorbances and Standard Deviation

Integration Time [s]	Mean Absorbance [AU]	Baseline Standard Deviation [AU]
0.1	$3.25 \cdot 10^{-5}$	$1.65 \cdot 10^{-4}$
0.5	$7.67 \cdot 10^{-6}$	$6.47 \cdot 10^{-5}$
5	$4.76 \cdot 10^{-6}$	$3.65 \cdot 10^{-5}$

2.9. Stability

Introduction

Stability affects the accuracy of absorbance measurements as a function of time. Drift in absorbance measurements introduces systematic errors in photometric accuracy. Stability is typically measured at zero absorbance with no sample in the light path.

Reagents and Equipment

❑ no equipment and reagents required

Experiment

Time: about 75 min

1 Be sure that the instrument has warmed up and thermally equilibrated sufficiently (for example, one hour).

2 Measure a reference on air (no cell in the cell holder).

3 Measure the absorbance of air (no cell in the cell holder) at the wavelength of 340 nm. Repeat this measurement 60 times every 60 s with an integration time of 0.5 s.

Note: Be sure that the ambient temperature is constant during measuring time.

Evaluation

1 Calculate the difference between maximum and minimum values of measured absorbance.

Evaluation Table 2.9.

Maximum Absorbance [AU]	Minimum Absorbance [AU]	Stability [AU/h]
A_{max} =	A_{min} =	$\Delta A = \lvert A_{max} - A_{min} \rvert$ =

Example Results & Discussion

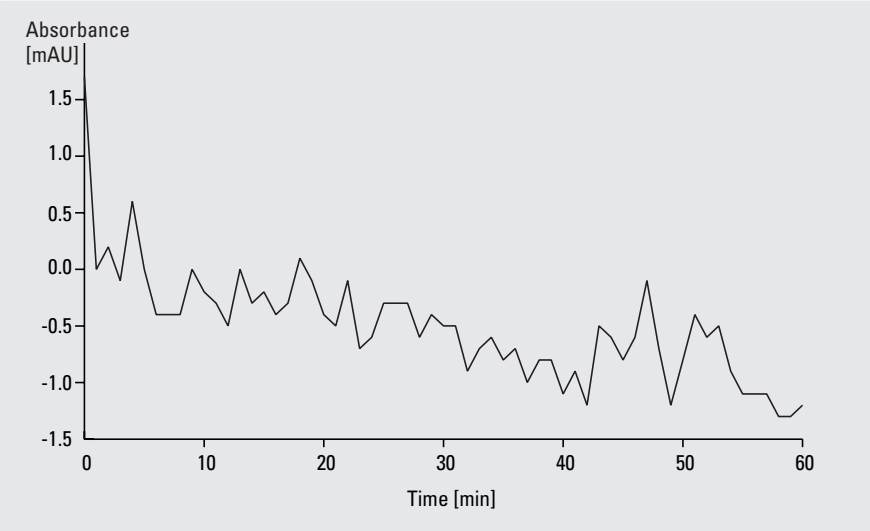
Sample: no reagents required

Cell: no cell required

Instrument Parameters:

fixed wavelength:	340 nm
absorbance range:	-1.5–2.0 mAU
integration time:	0.5 s
measuring time:	60 minutes
cycle time:	60 s

Diagram: Measured Changes of Absorbance Versus Time



1 The following table shows the measured absorbances and the figured stability.

Results Table 2.9. Measured Absorbance and Figured Stability

Maximum Absorbance [AU]	Minimum Absorbance [AU]	Stability [AU/h]
$A_{max} = 1.699\ 10^{-4}$	$A_{min} = -1.299\ 10^{-4}$	$\Delta A = \lvert A_{max} - A_{min} \rvert = 2.999\ 10^{-4}$

Part 3

Sample Handling and Measurement

General

Every chemist, laboratory staff, or user of spectrophotometric instruments must be aware, to which extent inaccurate sample or cell handling and nonreproducible measurement conditions can influence the accuracy of the results achieved.

With limitations due to instrumental performance and sample properties the greatest sources of error result from a lack of accuracy in sample and cell treatment.

This chapter shows possible sources of error and helps the user to eliminate them in order to achieve the best measurement results possible.

3.1. Sample Handling

Introduction

Assuming that the instrument works properly with given limitations due to the instrument's performance and sample properties, the largest sources of error in spectrophotometry are related to sample and cell handling.

The following experiment demonstrates the effect of sample and cell handling on the reproducibility of results.

Equipment and Reagents

- ❑ quinoline yellow (yellow dye)
- ❑ distilled water

- ❑ 1.0-l volumetric flask
- ❑ disposable glass pipettes (minimum 2)
- ❑ 10-mm path length quartz cell

Experiment

Time: about 30–60 min

1. Prepare a solution of about 12 mg quinoline yellow in 1.0 l distilled water.
2. Measure a reference on distilled water.
3. Measure the spectrum of the quinoline yellow solution in the wavelength range from 190 to 900 nm.

   If you have a slow scanning spectrophotometer, first determine the wavelength of the absorbance maximum in the visible range and set the instrument to this wavelength instead of measuring the whole spectrum.
4. Measure the first series of ten measurements without touching the cell.
5. Measure the second series of ten measurements, taking the cell out of the cell holder after each measurement and replacing it again with the same orientation.
6. Measure the third series of ten measurements rotating the cell by 180 degrees after each measurement.

Evaluation

1 Determine the wavelength of the absorbance maximum in the visible range and its absorbance value.

2 Complete the following table by entering the absorbance values at the absorbance maximum for the three series of measurements. Calculate mean $\bar{x}$, standard deviation S_x and relative standard deviation $\%RSD_x$ for each of the series of measurements and enter them in the following table below.

Evaluation Table 3.1. Measured Absorbance Values and Calculation Results at the Absorbance Maximum

	Measured Absorbance Values [AU] for Different Series of Measurements		
Number of Measurements	**First Series Cell Untouched Between Measurements**	**Second Series Cell Repositioned Between Measurements**	**Third Series Cell Rotated Between Measurements**
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
Mean			
Standard deviation			
% RSD			

The equations to calculate mean $\bar{x}$, standard deviation S and relative standard deviation $\%RSD$ are given below.

$$\bar{x} = \frac{\sum x}{n}$$

$$S = \sqrt{\frac{n\Sigma x^2 - (\Sigma x)^2}{n(n-1)}}$$

$$\%RSD = \left(\frac{S}{\bar{x}}\right) \cdot 100$$

where: n = number of values
x = values

3 Do you observe any difference in the standard deviation from one series of measurements to the next? If yes, explain why.

4 Based on these results, can you make any recommendation for cell handling to improve accuracy and precision of measurements?

Example Results & Discussion

Samples: quinoline yellow in distilled water (12mg/l)

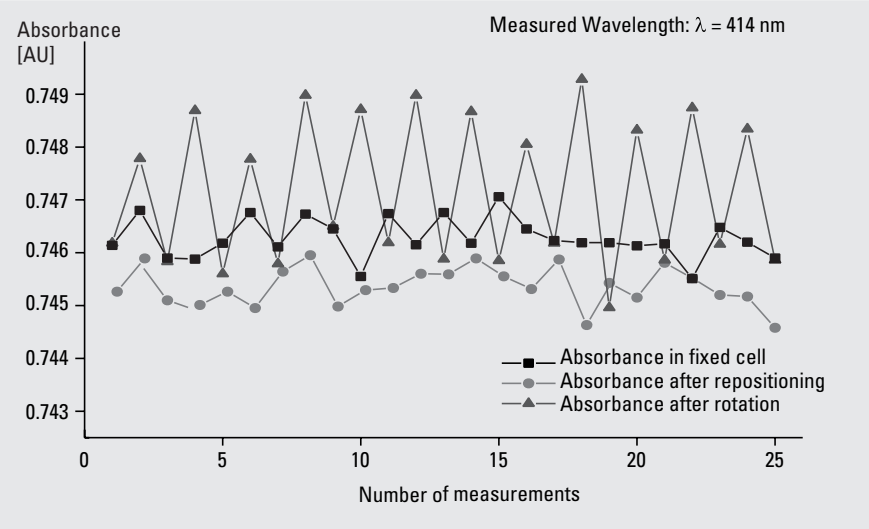
Cell: 10-mm path length quartz cell

Instrument Parameters:

wavelength range:	190–900 nm
absorbance range:	0.742–0.750 AU

1 The wavelength of the absorbance maximum in the visible range is at 414 nm. The absorbance value for the maximum is approximately 0.75 AU. In our case the y-scaling of the graphic display was set to an absorbance range from 0.742 to 0.750 AU before creating the diagram below which shows the measured absorbance values at 414 nm.

Diagram: Measured Absorbance Values at 414 nm



2 The following table shows the measured absorbance values and calculation results at the absorbance maximum in the visible range (λ = 414 nm).

Results Table 3.1. Measurements

	Measured Absorbance Values [AU] for Different Series of Measurements		
Number of Measurement	**First Series Cell Fixed Between Measurements**	**Second Series Cell Repositioned Between Measurements**	**Third Series Cell Rotated Between Measurements**
1	0.74614	0.74517	0.74618
2	0.7468	0.7458	0.74778
3	0.7459	0.7451	0.74583
4	0.74588	0.74492	0.74869
5	0.74618	0.74517	0.7456
6	0.74676	0.74486	0.74777
7	0.74611	0.74555	0.74579
8	0.74673	0.74586	0.74898
9	0.74645	0.74489	0.7465
10	0.74555	0.7452	0.74871
Mean:	0.74625	0.745252	0.747183
Standard Deviation	0.000424	0.000364	0.001345
% RSD	0.056835	0.048853	0.180016

3 The cell used to measure a sample is also part of a spectrophotometer's optical system. Therefore, the position and geometry of the cell have an influence on the accuracy and precision of absorbance measurements.

In the first series of measurements, we did not touch the cell and the precision of the measurements should be best. The standard deviation is very low (% RSD should be equal or less than 0.1 %). This demonstrates that UV-visible spectroscopy is a highly precise measurement technique.

In the second series of measurements we removed the cell after each measurement. This procedure demonstrates the influence of the position of the cell in the optical path, determined by the cell holder. Using a well-designed cell holder, that locks the cell in exactly the same position, the standard deviation of this second series of measurements should be comparable to the one of the first series. A poorly designed cell holder, or an unlocked cell will significantly worsen the standard deviation of the results.

In the third series of measurements we rotated the cell by 180 degrees after each measurement. Ideally the two windows of a cell should be optically identical and absolutely parallel. In practice, the absorbance of the windows can differ slightly and the two windows can have nonflat or nonparallel surfaces. Therefore, the cell acts as an active component which changes properties with the orientation. Rotating the cell between the

measurements demonstrates this effect and the standard deviation for the third series is significantly worse compared to series one or two.

4 For best measurement practice, the cell should remain in the cell holder between the measurements, or, if removed, the cell should always face the same direction in the cell holder, for example, label to the light source. This ensures that the optical effects are identical for both reference and sample measurements.

3.2. Cell Types

Introduction

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

The following experiment demonstrates the effect of cell quality on the accuracy and reproducibility of the absorbance measurements.

Equipment and Reagents

- ❑ quinoline yellow (yellow dye)
- ❑ distilled water

- ❑ 1.0-l volumetric flask
- ❑ disposable glass pipettes (minimum 2)
- ❑ high quality matched quartz cell (cell ***a***)
- ❑ quartz cell from a different set (badly treated cell, quartz of different quality or cell with path length error, (cell ***b***)
- ❑ disposable cell made of plastic (cell ***c***)

Experiment

Time: about 30–45 min

1. Prepare a solution of about 12 mg quinoline yellow in 1.0 l distilled water.
2. Measure a reference on distilled water in cell ***a***.
3. Measure the spectra of the quinoline yellow solution in the wavelength range from 190 to 500 nm using cells ***a***, ***b*** and ***c***.

Evaluation

1 Determine the wavelengths of the three absorbance maxima in the measured wavelength range.

2 Complete the following table by entering the absorbance values at the three wavelengths of the absorbance maxima.

Evaluation Table 3.2. Absorbance Values at the Three Different Wavelengths of the Absorbance Maxima with the Different Cells

	Absorbance Values [AU] for the Different Cells		
Wavelengths of Maximum Absorbance	**Cell *a*:**	**Cell *b*:**	**Cell *c*:**
λ_1 =			
λ_2 =			
λ_3 =			

3 Explain the differences between the three cells.

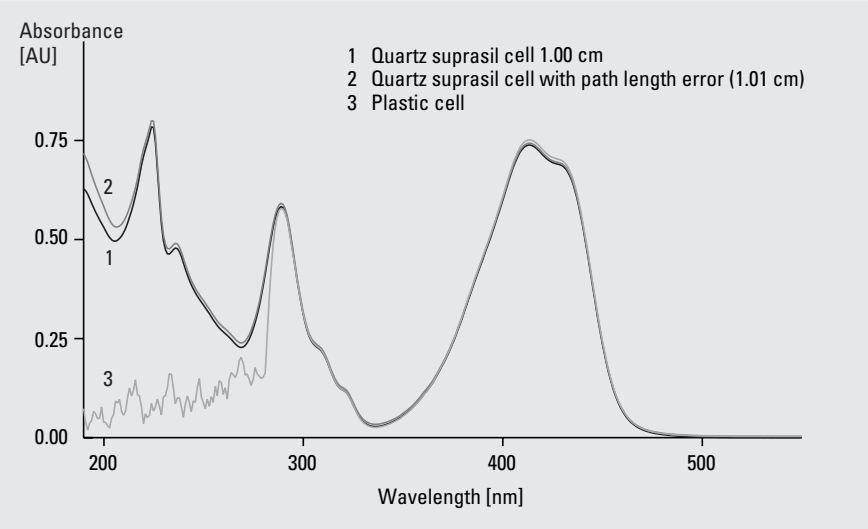
Example Results & Discussion

Sample: quinoline yellow in distilled water (12 mg/l)

Cells: high quality 10-mm path length quartz cell (cell ***a***)
10-mm path length quartz cell with a path length error (cell ***b***)
10-mm path length disposable plastic cell (cell ***c***)

Instrument Parameters: wavelength range: 190–500 nm
absorbance range: 0.0–1.0 AU

Spectra: Measured Spectra of the Sample in the Different Cells



1 The wavelengths of maximum absorbance can be found at 224, 289, and 414 nm.

2 The following table shows the absorbance values at the wavelengths of maximum absorbance for the different cells.

Results Table 3.2. Absorbance Values at the Three Different Wavelengths of the Absorbance Maxima with the Different Cells

	Absorbance Values [AU] for the Different Cells		
Wavelengths of Maximum Absorbance	**Cell *a*:**	**Cell *b*:**	**Cell *c*:**
λ_1 **= 224 nm**	0.78387	0.79980	0.08590
λ_2 **= 289 nm**	0.58291	0.59134	0.57814
λ_3 **= 414 nm**	0.73820	0.74290	0.75149

3 Cell ***a*** is used as the reference cell to which the results of the other two cells are compared to. For best results cell *a* is used for both reference and sample measurement.

Cell ***b*** is a cell of the same quality but with a slightly different path length. According to Beer's law, the absorbance reading is proportional to the path length. Any difference/error in the path length results in an error of the same magnitude of the absorbance reading.

Cell ***c*** is a disposable cell made of methylacryl. This material absorbs in the UV range and acts like a cut-off filter. No measurements can be made in the UV range.

Note that for best results reference and sample measurements must be done using the same cell.

3.3. Cleanliness

The cell, as part of the optical system of a spectrophotometer, needs the same care as all other optical components.

The following experiment demonstrates the effect of cell cleanliness on the results.

Equipment and Reagents

- ❏ quinoline yellow
- ❏ distilled water
- ❏ chalk powder

- ❏ photographic lens tissue
- ❏ 1.0-l volumetric flask
- ❏ disposable glass pipettes (minimum 2)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 30–45 min

1. Prepare a solution of about 12 mg quinoline yellow in 1.0 l distilled water.
2. Measure a reference on distilled water.
3. Measure the spectrum of the quinoline yellow solution in the wavelength range from 325 to 500 nm.
4. Remove the cell and touch the optical surfaces with your fingertips. Some fingerprints should remain in the area where the light passes the cell. Measure the spectrum of the yellow dye again using this dirty cell.
5. Remove the fingerprints by wiping the optical surface carefully with the photographic lens tissue.
6. Contaminate the outer surface of the cell with some drops of sample solution and measure the spectrum again.
7. Remove the liquid by wiping the optical surface carefully with the photographic lens tissue.
8. To examine the effect of dust or floating particles, add a small amount of chalk powder to the sample in the cell. Shake well and measure the spectrum again.

Evaluation

1 Overlay all spectra in a graph.

2 Compare the results of the various measurements and explain the reasons for the differences of the spectra.

Example Results & Discussion

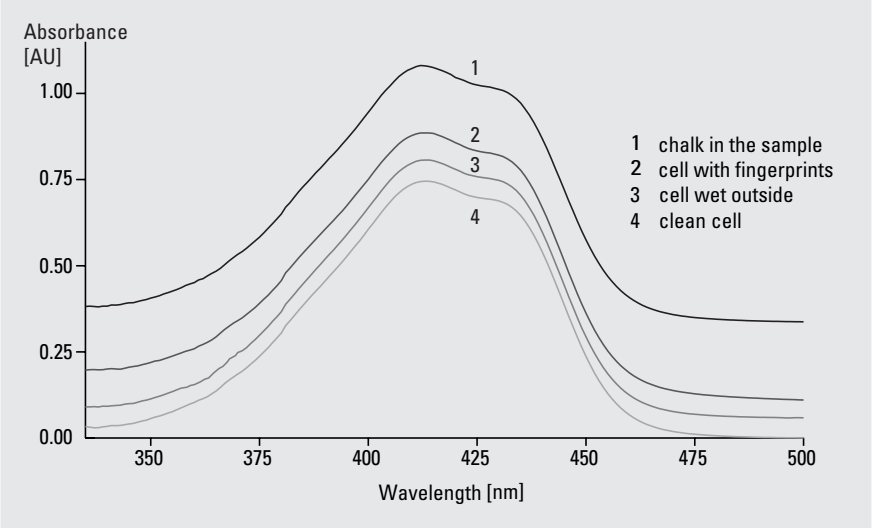
Sample: quinoline yellow in distilled water (12 mg/l)

Cell: 10-mm path length quartz cell

Instrument Parameters:
wavelength range: 325–500 nm
absorbance range: 0.0–1.0 AU

1 The following diagram shows the overlaid absorbance spectra of the different samples.

Diagram: Overlaid Spectra for the Differently Treated Samples



2 In all cases, the absorbance is higher than for the clean cell because of an additional absorbing component (see experiment “Principle of Additivity” on page 77).

The fats in fingerprints are significant absorbers in the UV region and, if left on optical surfaces, will cause erroneous results. Wipe off all fingerprints and contaminants carefully before using a sample cell. Use only high quality lens tissues to avoid scratches on the surface.

A wet outer surface of the cell can also contain absorbing components and in addition, act as a ‘lens’ and in this way influence the beam shape of the optics.

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

3.4. Influence of Instrumental Parameters

Introduction

The accuracy of results depends on the characteristics of the instrument used.

The following experiments cannot be performed with a diode array detector for two main reasons:

- Some of the parameters that must be varied are not variable with this instrument, for example, slit width and scan speed.
- Diode array detectors do not suffer from some of the effects that have to be investigated, for example, variation of peak shape and peak position depending on the scan speed.

The following experiment shows the influence of different scan speeds, different slit widths and of varying damping factors on the accuracy of the results.

Equipment and Reagents

- ❑ benzene (a)

 (a)

- ❑ conventional scanning spectrophotometer with variable slit width and variable scan speed
- ❑ disposable glass pipette
- ❑ set of two 10-mm path length quartz cells

Experiment

Time: about 120–180 min

1 Take a drop of benzene with the glass pipette and place it on the bottom of the cell. Seal the cell with a stopper. Evaporation in the cell will create enough vapor of benzene to carry out the measurements.

2 Measure a reference on air (no cell in the cell holder).

3 Measure the spectra of benzene in the wavelength range from 220 to 280 nm, varying the slit widths (0.1, 0.5, 1, 2 and 5 nm) with a constant scan speed (30 nm/min).

4 Repeat the speed measurements varying the scan speed (7.5, 30, 120 and 480 nm/min) with a constant slit width (0.1 nm).

5 Examine the effect of the damping factor (damping factors 0 and 10). Choose a small slit width (0.1 nm) and a low scan speed (15 nm/min) for your measurements.

Evaluation

1 Overlay the spectra with the identical scan speeds but different slit widths. Read the values of the five significant benzene bands in the UV range and enter them in the table below.

Evaluation Table 3.3. Absorbance Values for the Different Slit Widths

	Absorbance Values [AU] of the 5 Different Benzene Bands				
Slit Width [nm]	**at band 1: ____nm**	**at band 2: ____nm**	**at band 3: ____nm**	**at band 4: ____nm**	**at band 5: ____nm**

2 Overlay the spectra with the identical slit widths but different scan speeds. Read the values of the five significant benzene bands in the UV range and enter them in the table below.

Evaluation Table 3.4. Absorbance Values for the Different Scan Speeds

	Absorbance Values [AU] of the 5 Different Benzene Bands				
Scan Speed [nm/min]	**at band 1: ___nm**	**at band 2: ___nm**	**at band 3: ___nm**	**at band 4: ___nm**	**at band 5: ___nm**

3 Compare the absorbance values of the five significant benzene bands for the different damping factors. Enter the values in the table below.

Evaluation Table 3.5. Absorbance Values for the Different Damping Factors

Wavelength of Benzene Band [nm]	Absorbance Value [AU] for a 0.1-nm Slit Width 15 nm/min Scan Speed Damping Factor 0	Absorbance Value [AU] for a 0.1-nm Slit Width 15 nm/min Scan Speed Damping Factor 10
Band 1 _____nm		
Band 1 _____nm		
Band 3 _____nm		
Band 4 _____nm		
Band 5 _____nm		

Example Results & Discussion

Sample: benzene vapor

Cell: set of two 10-mm path length quartz cells

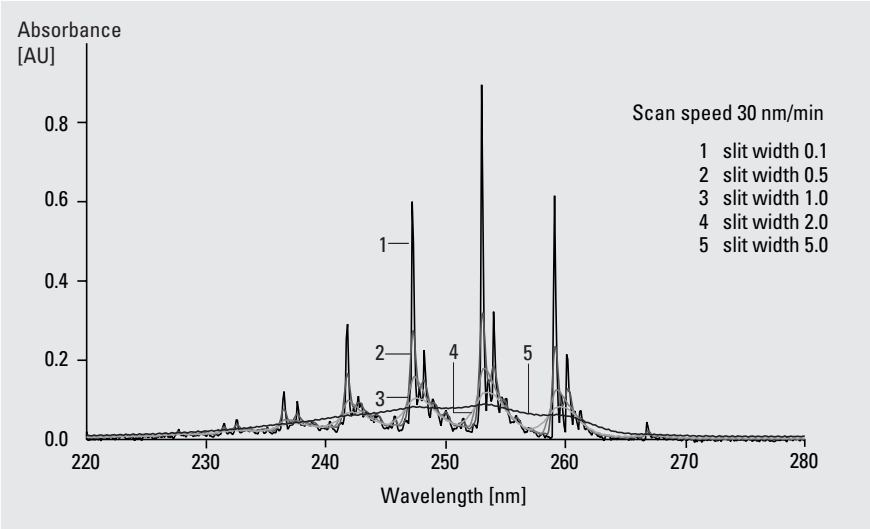
Instrument Parameters:

wavelength range:	220–280 nm
absorbance range:	0.0–1.5 AU
scan speed:	7.5 nm/min to 480 nm/min
slit width:	0.1 nm to 5 nm
damping factors:	0 or 10

Benzene vapor has several sharp absorbance bands in the ultraviolet wavelength range. The five significant bands in the wavelength range from 220 to 280 nm were used for this experiment, i.e 236.8, 241.8, 274.4, 253.0 and 259.3 nm.

1 The following diagram shows the absorbance spectra for a constant scan speed of 30 nm/min and with varying slit widths.

Diagram: Absorbance Spectra with Varying Slit Widths



The diagram above indicates that the resolution increases with the reduction of the slit width. Resolution is closely related to the instrumental spectral bandwidth (SBW). The SBW is determined by the bandwidth of wavelengths passing the slits for a certain wavelength setting. The smaller the slit width, the higher the resolution. However, decreasing the slit width also decreases the energy throughput which results in a lower signal-to noise-ratio (S/N-ratio).

Doubling the resolution requires an increase of the measurement time by a factor of 16 to achieve the same S/N ratio.

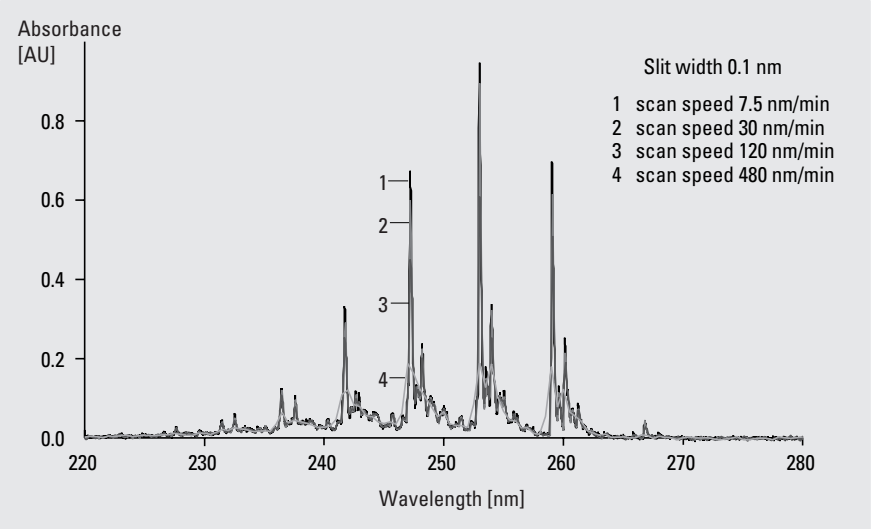
The following table shows the absorbance values detected with the constant scan speed of 30 nm/min and with varying slit width.

Results Table 3.3. Absorbance Values for Varying Slit Widths

	Absorbance Values [AU] of the 5 Different Benzene Bands				
Slit Width [nm]	**at 236.8 nm**	**at 241.8 nm**	**at 247.4 nm**	**at 253.0 nm**	**at 259.3 nm**
0.1	0.12085	0.29099	0.60012	0.89518	0.61497
0.5	0.07647	0.16698	0.26621	0.31353	0.23361
1	0.04923	0.09838	0.14884	0.16969	0.11923
2	0.03646	0.06785	0.09939	0.11305	0.0783
5	0.03926	0.06233	0.08291	0.08786	0.06154

2 The following diagram shows the absorbance spectra for a constant slit width of 0.1 nm with varying scan speeds.

Diagram: Absorbance Spectra for Varying Scan Speeds



The diagram shows the loss of information when increasing the scan speed—the absorbance values measured for the highest scan speeds are significantly lower than the values measured with the slowest scan speed. The true absorbance of the bands cannot be measured using high scan speed, because the peaks are cut off. This also creates distorted peak shapes and incorrect peak positions.

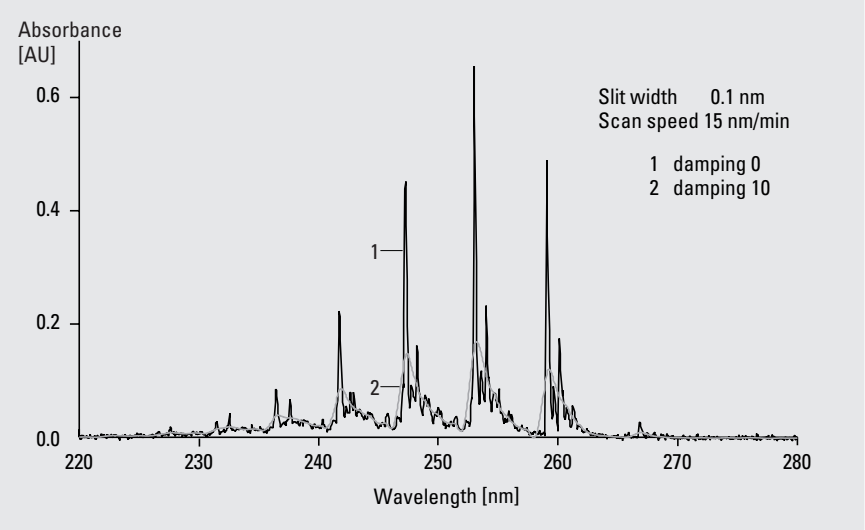
The following table shows the absorbance values detected with the constant slit width of 0.1 nm and with varying scan speed.

Results Table 3.4. Absorbance Values for Varying Scan Speeds

	Absorbance Values [AU] of the 5 Different Benzene Bands				
Scan Speed [nm/min]	**at 236.8 nm**	**at 241.8 nm**	**at 247.4 nm**	**at 253.0 nm**	**at 259.3 nm**
7.5	0.12513	0.33284	0.67429	0.9468	0.69723
30	0.11484	0.26891	0.58976	0.7549	0.45915
120	0.10664	0.24791	0.44817	0.55828	0.37854
480	0.06203	0.11479	0.1765	0.1848	0.17182

3 The following diagram shows the absorbance spectra for varying damping factors.

Diagram: Absorbance Spectra for Varying Damping Factors



The diagram above indicates that an increase of the damping factor reduces the noise, but again cuts off the peaks.

The following table shows the measured absorbance values for varying damping factors.

Results Table 3.5. Absorbance Values for the Different Damping Factors

Wavelength of Benzene Band [nm]	Absorbance Value [AU] for a 0.1 nm Slit Width 15 nm/min Scan Speed Damping Factor 0	Absorbance Value [AU] for a 0.1 nm Slit Width 15 nm/min Scan Speed Damping Factor 10
236.5	0.08286	0.03756
241.8	0.21309	0.08328
247.2	0.43881	0.13763
253.0	0.65523	0.15583
259.1	0.44824	0.11343

3.5. Properties of Solvents

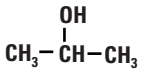
Introduction

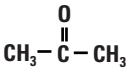
An ideal solvent dissolves a sample completely, it is easy to handle and completely transparent at the wavelengths of interest. Besides water, all common solvents absorb more or less in the UV range of the spectrum and no sample measurements can be performed there.

The following experiment demonstrates the different cut-off wavelengths in the UV range of some common solvents.

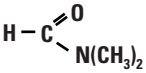
Equipment and Reagents

- ❏ ethanol (CH_3CH_2OH)
- ❏ methanol (CH_3OH)
- ❏ 2-propanol (a)
- ❏ acetone (b)
- ❏ acetonitrile (CH_3CN)
- ❏ hexane ($CH_3(CH_2)_4CH_3$)
- ❏ tetrahydrofurane (c)
- ❏ N,N'-dimethylformamide (d)
- ❏ distilled water


(a)


(b)


(c)


(d)

- ❏ disposable glass pipettes (minimum 9)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 60–120 min

1 Measure a reference on air (no cell in the cell holder).

2 Measure transmittance spectrum of distilled water in the range from 190 to 500 nm.

3 Measure transmittance spectra of the solvents listed above.

Evaluation

1 Overlay the spectra of the different solvents.

2 Determine the cut-off wavelengths (transmittance lower than 50 %) of the different solvents. Enter the data in the table below.

Evaluation Table 3.6. Cut-Off Wavelengths of the Different Solvents

Solvent	Cut-Off Wavelength [nm]
Water	
Ethanol	
Methanol	
2-Propanol	
Acetone	
Acetonitrile	
Hexane	
Tetrahydrofuran	
N,N'-Dimethylformamide	

3 What are the lowest wavelengths in the UV range that can be used with the different solvents?

4 Discuss the advantages and the disadvantages of the different solvents.

5 Does the type of solvent affect the position and intensity of the absorbance bands of molecules?

Example Results & Discussion

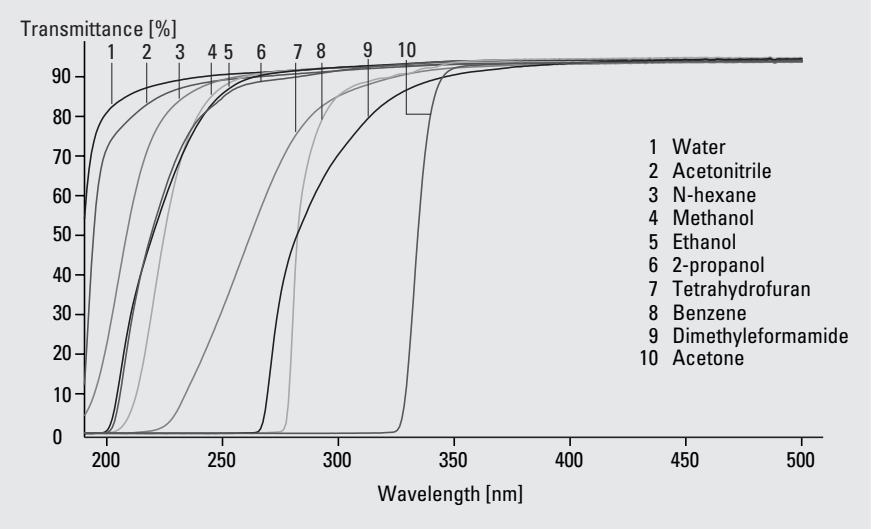
Samples: ethanol, methanol, 2-propanol, acetone, acetonitrile, hexane, tetrahydrofurane, N,N'-dimethylformamide, distilled water

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 190–500 nm
transmittance range: 0–100 %

1 The following diagram shows the overlaid transmittance spectra of the different solutions.

Diagram: Overlaid Transmittance Spectra of the Different Solutions



2 The following table shows the cut-off wavelengths of the different solvents.

Results Table 3.6. Cut-off Wavelengths of the Different Solvents

Solvent	Cut-Off Wavelength [nm]
Water	< 190
Ethanol	208
Methanol	217
2-Propanol	209
Acetone	332
Acetonitrile	191
N-Hexane	200
Tetrahydrofuran	245
N,N'-Dimethylformamide	272

3 Normally solvents can be used at wavelengths higher than their cut-off wavelength. Ideally, a solvent should absorb as little light as possible in the measured range.

4 Distilled water is transparent in the whole UV-visible wavelength range. It dissolves many polar compounds and is easy to handle. Water is not suitable for many nonpolar organic compounds. For these compounds usually organic solvents with different polarity are used. All of the organic solvents used in this experiment have a cut-off wavelength in the UV range. Below this wavelength the absorbance is too strong for sample measurements. In addition organic solvents are more difficult to handle. They can be flammable, toxic and involve health hazards.

5 The solvents can modify the electronic environment of the absorbing chromophore. This can cause a shift of the absorbance band. For more information, refer to “The Effect of Solvents on UV-visible Spectra” on page 21.

3.6. Background Absorbance

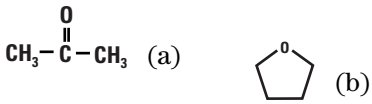
Introduction

Ideally, the measured absorbance only depends on the target compound. In practice, however, additional absorbencies which interfere with the measurement often occur for chemical or physical reasons. The presence of any other compound that absorbs in the same region as the target compound will result in an error in the absorbance measurement.

The following experiment demonstrates various methods of background correction and their influence on the results.

Equipment and Reagents

- ❏ acetone (a)
- ❏ tetrahydrofurane (b)



- ❏ 50-ml volumetric flask
- ❏ 0.5-ml pipette or syringe
- ❏ disposable glass pipettes (minimum 2)
- ❏ 10-mm path length quartz cell

Experiment

Time: about 30–45 min

1 Prepare a solution of 0.5 ml acetone in 50 ml tetrahydrofurane.

2 Measure a reference on air.

3 Measure the spectrum of the acetone solution in the wavelength range from 225 to 375 nm.

4 Measure the spectrum of the pure tetrahydrofurane in the same wavelength range.

Evaluation

1 Draw an overlay of the spectra of the acetone solution and of the pure tetrahydrofurane.

2 Determine the wavelength of the absorbance maximum of the acetone solution.

3 Use the table on the next page for the following evaluations:

Determine the absorbance value at λ_{max} of the acetone solution without any background correction.

a) Correct the absorbance value at λ_{max} of the acetone solution for the background absorbance at a single reference wavelength λ_{ref}.

The reference wavelength λ_{ref} should be selected according to the following two criteria:

- λ_{ref} should be close to the wavelength of interest (λ_{max}).
- The absorbance at λ_{ref} should only depend on the background.

b) To correct a sloped baseline, the so-called three-point drop-line or Morton-Stubbs correction can be used. This correction uses two reference wavelengths $\lambda_{ref\ 1}$ and $\lambda_{ref\ 2}$, one on each side of the wavelength λ_{max}. The absorbance due to background is estimated by linear interpolation.

- Select two reference wavelength one at a shorter wavelength, for example, 240 nm and one at a longer wavelength, for example, 325 nm as λ_{max} applying the previous rules.
- Estimate the background absorbance $A_{interpolated}$ at the wavelength λ_{max} by a linear interpolation between the absorbance values at $\lambda_{ref\ 1}$ and $\lambda_{ref\ 2}$ and subtract it from the absorbance value at λ_{max} of the sample solution (spectrum 1).

  If the spectrum of the compound which causes the background absorbance is available, it can be used for the correction.

c) Subtract the absorbance value at λ_{max} of the pure tetrahydrofurane from the absorbance value at λ_{max} of acetone.

Evaluation Table 3.7. Correcting Results with Different Correction Methods for Background Absorbance

Applied Background Correction	Resulting Absorbance Values [AU]	
No correction	Absorbance at λ_{max} :	
Constant background single reference wavelength) λ_{ref} = _____nm	Absorbance at λ_{max}: Absorbance at λ_{ref}: Corrected absorbance at λ_{max}:	 - =
Three-point drop-line or Morton-Stubbs correction $\lambda_{ref\ 1}$ = _____nm $\lambda_{ref\ 2}$ = _____nm	Absorbance at λ_{max} : Interpolated background Absorbance at λ_{max}: Corrected absorbance at λ_{max}:	 - =
Subtraction of the underlying background spectrum	Absorbance at λ_{max}: Background absorbance at λ_{max} : Corrected absorbance at λ_{max}:	 - =

4 Which kind of baseline correction is the most accurate one?

Example Results & Discussion

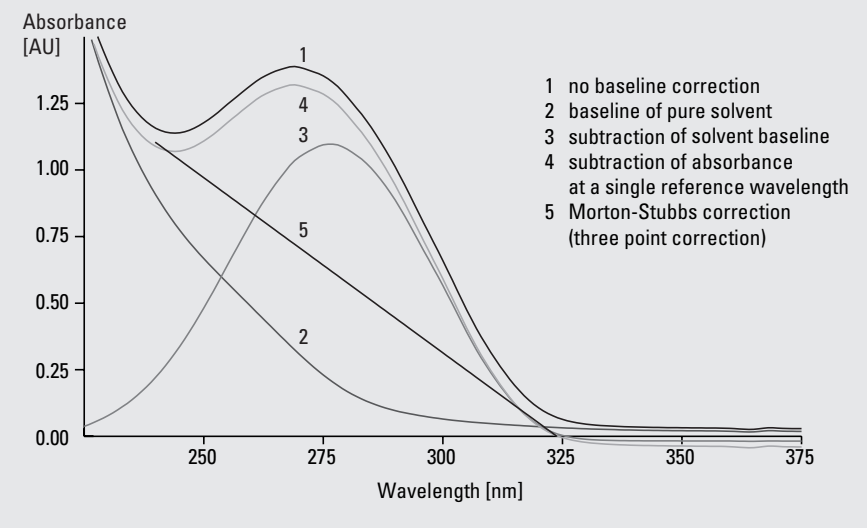
Sample: acetone in tetrahydrofurane (10 ml/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 225–375 nm
absorbance range: 0.0–1.5 AU

1 The following diagram shows the overlaid spectra for the different methods of background correction.

Diagram: Overlaid Spectra for the Different Methods of Background Correction



2 The wavelength of maximum absorbance is 269 nm.

3 The following table shows the results of the background correction calculations.

Results Table 3.7. Correcting Results with Different Correction Methods for Background Absorbance

Applied Background Correction	Resulting Absorbance Values [AU]	
No correction	Absorbance at λ_{max} :	1.388
Constant background (single reference wavelength)	Absorbance at λ_{max}:	1.388
λ_{ref} = 335 nm	Absorbance at λ_{ref}:	- 0.039
	Corrected absorbance at λ_{max}:	= 1.349
Three-point drop-line or Morton-Stubbs correction	Absorbance at λ_{max} :	1.388
$\lambda_{ref\ 1}$ = 240 nm	Interpolated background absorbance at λ_{max}:	- 0.783
$\lambda_{ref\ 2}$ = 325 nm	Corrected absorbance at λ_{max}:	= 0.605
Subtraction of the underlying background spectrum	Absorbance at λ_{max}:	1.388
	Background absorbance at λ_{max} :	- 0.335
	Corrected absorbance at λ_{max}:	= 1.053

Background absorbance is an additional absorbance which is not caused by the compound of interest, but by the matrix of the sample, an interfering impurity, or scattering. Different techniques can be used to minimize the influence of the background absorbance on the analytical result depending on its nature.

- Correcting a constant background absorbance.
  A constant background absorbance over a large wavelength range can be eliminated using a single reference wavelength.
- Correcting a background absorbance with a constant slope
  A background absorbance with a constant (linear) slope over a large wavelength range can be eliminated by a three-point drop line.
- Correcting a background absorbance by subtracting a spectrum.
  Best results can be achieved by this correction, if the background absorbance can be measured separately.

4 In this experiment, the subtraction of the background absorbance spectrum gives the most accurate result. A non-constant background cannot be eliminated by a linear correction.

Note: Internal reference can also be used for a dual wavelength measurement to correct the additional absorbance of a second interfering compound. In this case, the absorbance of the second compound at the reference and the analytical wavelength have to be identical.

3.7. Sample Decomposition

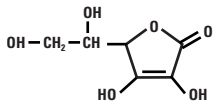
Introduction

In some cases samples are very sensitive to oxygen, especially in the presence of impurities acting as catalysts. Ascorbic acid is oxidized fast in the presence of even low concentrations of Cu^{2+}– ions. These concentrations of Cu^{2+}– ions can already be found in tap water.

The following experiment shows the ongoing oxidation of ascorbic acid in the presence of oxygen with Cu^{2+}–ions present in tap water as catalysts.

Equipment and Reagents

- ❑ ascorbic acid (a)
- ❑ tap water



(a)

- ❑ 100-ml volumetric flask
- ❑ disposable glass pipettes (minimum 2)
- ❑ thermostattable cell holder with stirring capabilities
- ❑ stop watch or time drive program included in the spectrophotometer software
- ❑ 10-mm path length quartz cell

Experiment

Time: about 90–120 min

1 Prepare a solution of about 8 mg ascorbic acid in 100 ml tap water.

2 Seal the flask immediately to avoid contact with air (oxygen).

3 Measure a reference spectrum on tap water.

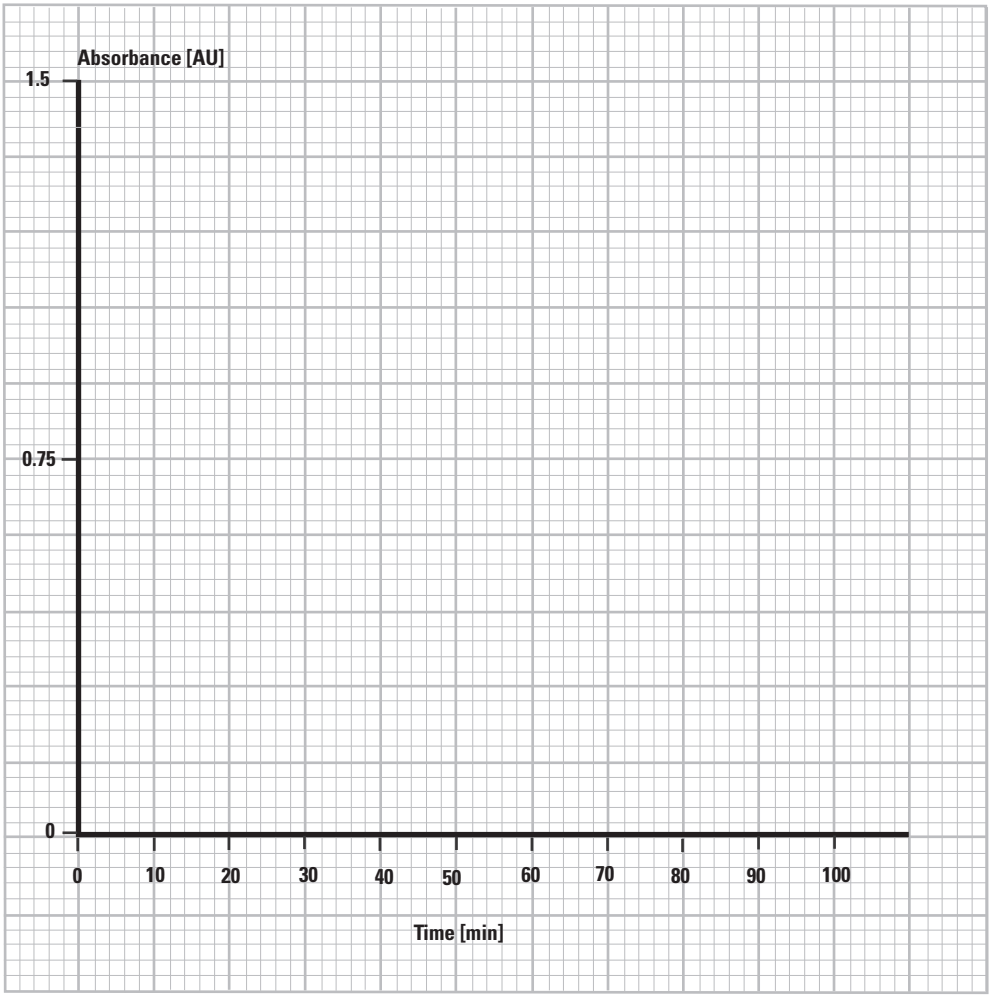
4 Measure a spectrum of the ascorbic acid solution in the wavelength range from 190 to 400 nm using a cell with a stopper. If you use a conventional scanning spectrophotometer, determine the wavelength of maximum absorbance of the solution and continue your decomposition measurements at this single wavelength.

5 Open the cell and measure the absorbance at the wavelength of the maximum absorbance every two minutes for 80 minutes. The cell has to be kept open during this time to allow contact with air. Stir the solution in the cell to accelerate the oxidation process. Set the cell temperature to 30 °C.

Evaluation.

1 Draw a graph showing the change of the absorbance value at λ_{max} versus time.

Diagram: Absorbance as a Function of Concentration



2 Guess the order of the reaction.

Example Results & Discussion

Samples: ascorbic acid in tap water (82 mg/l)

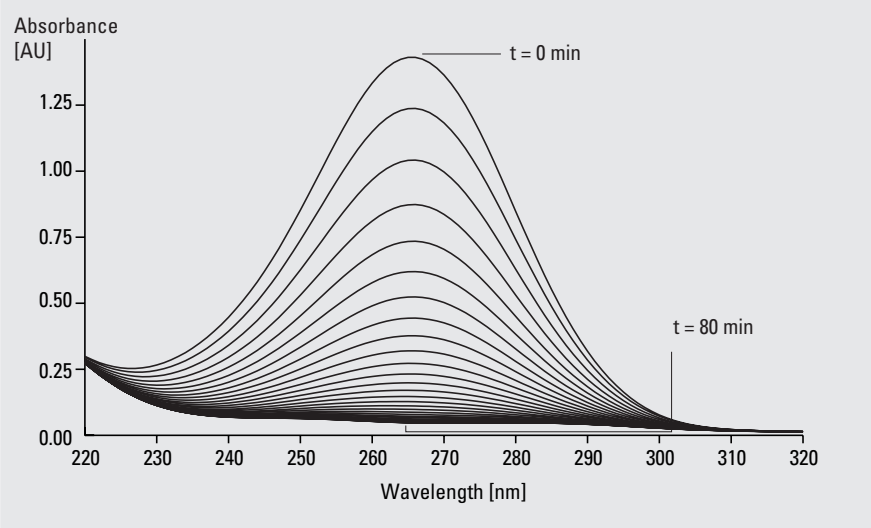
Cell: 10-mm pass length quartz cell

Instrument Parameters:

wavelength range:	220–320 nm
absorbance range:	0.0–2.0 AU
acquisition time:	every 2 minutes for 75 minutes
Sample temperature:	30 °C
stirrer speed:	300 RPM

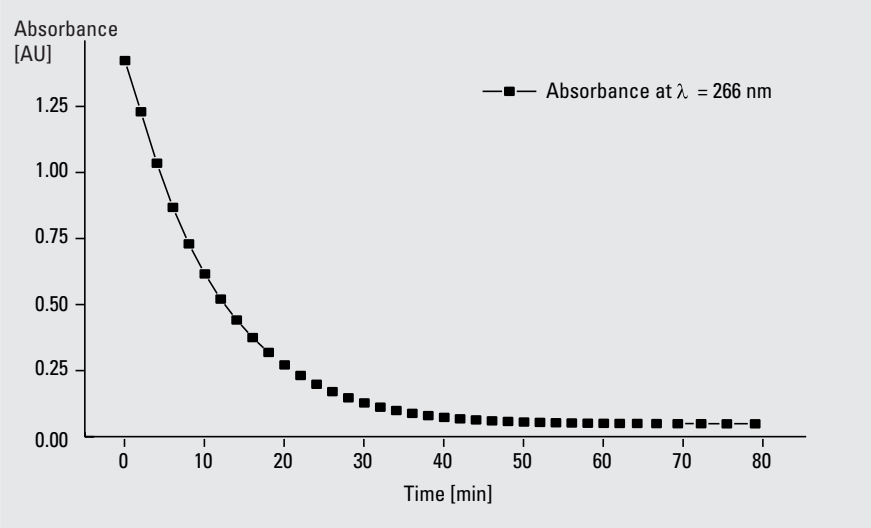
The wavelength of maximum absorbance is 266 nm.

Diagram: Overlaid Spectra Indicating Sample Decomposition with Advancing Time



1 The following diagram shows the change of the absorbance value at λ_{max} versus time.

Diagram: Change of the Absorbance Value at λ_{max} Versus Time



2 The absorbance shows a exponential decay over time. From this point of view the reaction can be described by first order kinetics.

Part 4

Applications

4.1. Single Component Analysis

Introduction

The most common quantitative method in UV-visible spectroscopy is single component analysis. Here we analyze an analgetics tablet containing acetyl salicylic acid as the only active ingredient.

Reagents and Equipment

- ❑ acetyl salicylic acid
- ❑ an analgetics tablet containing acetyl salicylic acid as the only active ingredient (for example, aspirin)™
- ❑ distilled water

- ❑ 100-ml volumetric flask
- ❑ 500-ml volumetric flask
- ❑ disposable filter (size of pores < 1 µm)
- ❑ disposable glass pipettes (minimum 3)
- ❑ 10-mm path length quartz cell
- ❑ magnetic stirrer

Experiment

Time: about 60–90 min

1 Prepare a standard solution:

- acetyl salicylic acid (about 13 mg in 100 ml distilled water).
  Make sure that the acetyl salicyclic acid is completely dissolved. Use a magnetic stirrer if necessary.

2 Determine the tablet weight.

3 Break the tablet into small pieces and prepare a sample solution:

- tablet material (about 50 mg in 500 ml distilled water)

4 Stir this solution for at least 30 min.

5 Use distilled water for the reference measurement in the wavelength range from 200 to 400 nm.

6 Measure the acetyl salicylic acid standard absorbance spectrum in a wavelength range from 200 to 400 nm.

7 Use the filtered sample solution to measure the sample spectrum in the range from 200 to 400 nm.

Evaluation

1 Determine the wavelength of the absorbance maximum of your standard solution.

λ_{max} =	nm

2 Get the absorbance value $A^{std}_{\lambda max}$ of your standard solution at the absorbance maximum.

$A^{std}\lambda_{max}$ =	AU

3 Calculate the molar concentration c^{std} of your acetyl salicylic acid standard solution. The molecular weight of acetyl salicylic acid MW is 180.15 g / mol.

c^{std} =	mol/l

4 Determine the sample concentration using Beer's law:

$$A_\lambda = \varepsilon_\lambda \, c \, d$$

where:

λ = wavelength [nm]

A_λ = absorbance value [AU] at λ

ε_λ = molar extinction coefficient [l/(mol ·cm)] at λ

c = concentration [mol/l]

d = path length [cm]

According to Beer's law we determine the molar extinction coefficient ε_λ using the standard absorbance value $A^{std}_{\lambda max}$ first.

$$\varepsilon_{\lambda max} = A^{std}_{\lambda max} / (c^{std} d)$$

$\varepsilon_{\lambda max}$ =	l/(mol·cm)

Get the absorbance value $A^{smp}_{\lambda max}$ of your sample solution at the absorbance maximum.

$A^{smp}_{\lambda max}$ =	AU

Based on the molar extinction coefficient $\varepsilon_{\lambda max}$ and the measured sample absorbance $A^{smp}_{\lambda max}$ we can calculate the sample concentration.

$$c^{smp} = A^{smp}_{\lambda max} / (\varepsilon_{\lambda max} d)$$

c^{smp} =	mol/l

5 Determine the total amount of acetyl salicylic acid contained in your tablet.

Using the sample concentration result c^{smp} and the total volume V^{smp} of our sample solution we get the number of molecules.

$$n^{smp} = c^{smp} V^{smp}$$

n^{smp} =	mol

With the molecular weight of acetyl salicylic acid MW we can calculate the weight w^{smp}.

$$w^{smp} = n^{smp} \; MW$$

w^{smp} =	mg

Based on the fraction of the tablet m^{smp} we used to prepare our sample solution and the total tablet weight m^{tab} we can calculate the total amount of acetyl salicylic acid / tablet m^{ass}.

$$m^{ass} = w^{smp} m^{tab} / m^{smp}$$

m^{ass} =	mg

6 Compare the determined value m^{ass} with the specified content on the packing note of your tablets.

Evaluation Table 4.1. Comparison of Labeled Content and Determined Content

Labeled Content	Determined Content
m^{ass} = mg	m^{ass} = mg

7 What can be improved in the single component analysis procedure?

Advanced Experiment

Time: about 60–90 min

1 Prepare five standard solutions:

- acetyl salicylic acid about 6 mg in 100 ml distilled water
- acetyl salicylic acid about 8 mg in 100 ml distilled water
- acetyl salicylic acid about 9 mg in 100 ml distilled water
- acetyl salicylic acid about 11 mg in 100 ml distilled water
- acetyl salicylic acid about 13 mg in 100 ml distilled water

2 Use distilled water for the reference measurement.

3 Measure all acetyl salicylic acid standards at 274 nm in absorbance.

Advanced Evaluation

1 Calculate the concentrations of your five standard solutions in mol/l based on your actual acetyl salicylic acid weights.

c_1 = _____ mol/l

c_2 = _____ mol/l

c_3 = _____ mol/l

c_4 = _____ mol/l

c_5 = _____ mol/l

2 Enter your absorbance measurement data in the table below.

Evaluation Table 4.2. Measured Absorbance Values at 274 nm

Name	Absorbance[AU] at 274 nm
Standard (c_1)	
Standard (c_2)	
Standard (c_3)	
Standard (c_4)	
Standard (c_5)	

3 Calculate a linear regression y = ax. The x,y pairs are the measured absorbance data (y) and the corresponding concentration values (x). Enter the calculation results in the table below.

Evaluation Table 4.3. Correlation Coefficient and Slope of Measured Curve

Name		Value
Slope	a	
Correlation coefficient	R	

4 What does the slope value tell you?

5 How is the slope value related to the molar extinction coefficient ε_λ ?

6 What can the correlation coefficient be used for?

7 Calculate the total amount of acetyl salicyclic acid of your tablet using the statistically improved molar extinction coefficient and evaluation data from the basic experiment.

m^{ass} = mg

Example Results and Discussion

Standard: acetyl salicylic acid (131 mg /l)

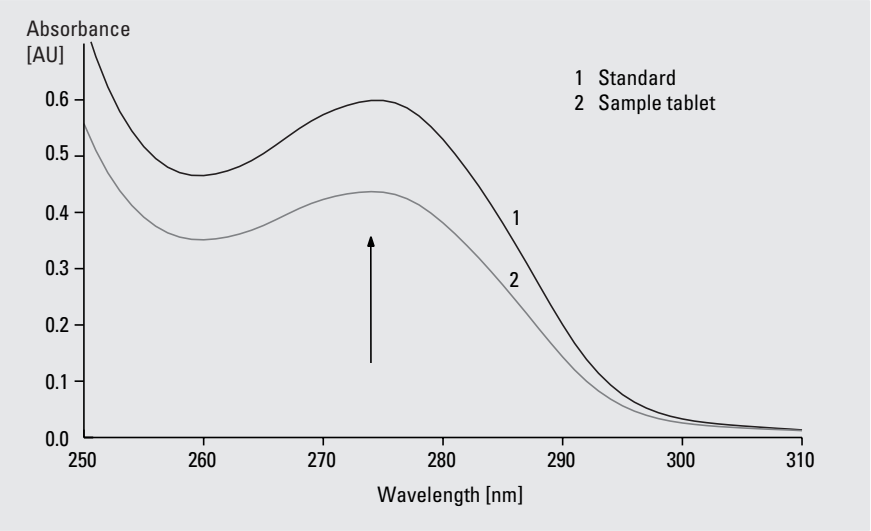
Sample: tablet material (116 mg/l)

Tablet: tablet weight (604.85 mg)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 250–310 nm
absorbance range: 0.0–0.8 AU

Diagram: Measured Absorbance Spectra of Acetyl Salicyclic Acid Standard and of Sample Tablet



1 The wavelength of the absorbance maximum of the standard solution is:

$\lambda_{max} = 274$ nm

2 The absorbance value of the standard solution is:

$A^{std}{}_{\lambda max} = 0.5988$ AU

3 Based on the concentration of 131 mg/l the molar concentration can be calculated as:

$$c^{std} = 7.272 \cdot 10^{-4} \text{ mol/l}$$

4 The molar extinction coefficient of 274 nm can be calculated as:

$$\varepsilon_{\lambda max} = 8.2343 \cdot 10^{2} \text{ l/(mol} \cdot \text{ cm)}$$

The measured absorbance of the sample solution at 274 nm is:

$$A^{smp}{}_{\lambda max} = 0.4368 \text{ AU}$$

Using the molar extinction coefficient at 274 nm, the molar concentration of the sample solution is:

$$c^{smp} = 5.305 \cdot 10^{-4} \text{ mol/l}$$

5 Using the volume of the sample solution (100 ml) the number of acetyl salicyclic acid molecules can be found:

$$n^{smp} = 2.653 \cdot 10^{-4} \text{ mol}$$

Using the molecular weight of acetyl salicyclic acid the amount of the substance can be determined as:

$$w^{smp} = 47.79 \text{ mg}$$

The total amount of acetyl salicylic acid per tablet can be calculated as:

$$m^{ass} = 498.3 \text{ mg}$$

6 The table below shows a comparison of the labeled content with the analysis result:

Results Table 4.1. Comparison of Labeled and Determined Content

Labeled Content	Determined Content
m^{ass} = 500 mg	m^{ass} = 498.3 mg

7 The analysis result depends on the quality of the determination of the molar extinction coefficient. Usually a series of standards is prepared to minimize the probability of errors. These errors are due to the limited precision that can be achieved in standard preparation. Weighing errors and volume errors affect the standard concentration accuracy. If these errors are not systematic, a series of standards minimizes those errors and improves the quality of the molar extinction coefficient.

Advanced Example Results and Discussion

Standards: acetyl salicylic acid (65.5 mg /l)
acetyl salicylic acid (81.75 mg /l)
acetyl salicylic acid (90.69 mg /l)
acetyl salicylic acid (109.17 mg /l)
acetyl salicylic acid (131.05 mg /l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength: 274 nm
absorbance range: 0.0–0.8 AU

1 Standard concentrations:

c_1 =	$3.636 \cdot 10^{-4}$ mol/l
c_2 =	$4.538 \cdot 10^{-4}$ mol/l
c_3 =	$5.034 \cdot 10^{-4}$ mol/l
c_4 =	$6.060 \cdot 10^{-4}$ mol/l
c_5 =	$7.272 \cdot 10^{-4}$ mol/l

2 The following table shows the measured absorbance values.

Results Table 4.2. Measured Absorbance Values at 274 nm

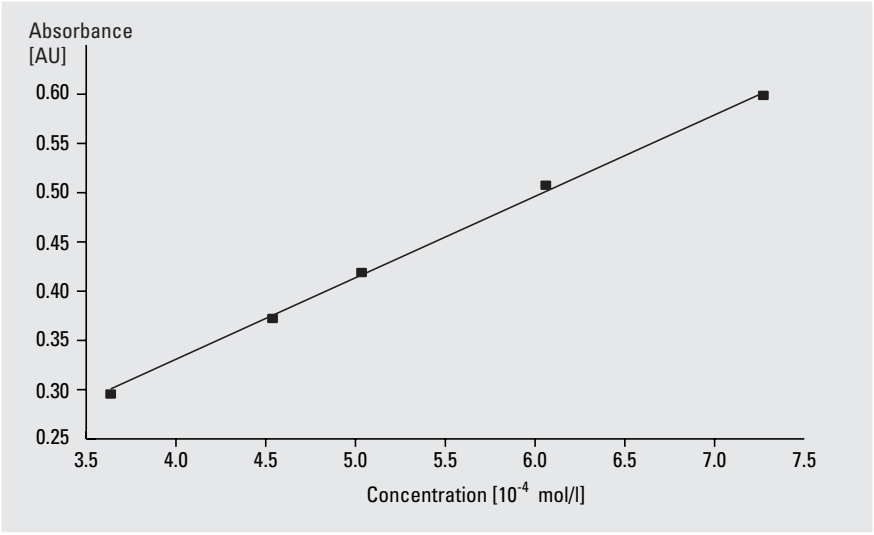
Name	Absorbance [AU] at 274 nm
Standard (c_1)	0.2955
Standard (c_2)	0.3723
Standard (c_3)	0.4189
Standard (c_4)	0.5075
Standard (c_5)	0.5988

3 The following table shows the linear regression calculation results.

Results Table 4.3. Correlation Coefficient and Slope of Measured Curve

Name		Value
Slope	a	$8.27 \cdot 10^{-2}$
Correlation coefficient	R	0.9984

4 The slope is the linear dependence of the measurement data as a function of the concentration values of the standards. The graphical representation of this relationship is called calibration curve and shown below.



5 Due to the regression formula used in the calculation, the slope is the reciprocal of the molar extinction coefficient ε_λ. This is true because we used a 10-mm path length cell. The statistically improved molar extinction coefficient ε_λ is $8.271 \cdot 10^2$ l/(mol ·cm). The unit of the molar extinction is [l/(mol · cm)] because we used a 10-mm path length quartz cell.

6 The correlation coefficient is a measure of the quality of a calibration and tells you how good a straight line fits all of your calibration points.

7 Using the statistically improved molar extinction coefficient the total amount of acetyl salicyclic acid can be calculated as:

$$m^{ass} = 495.7 \text{ mg}$$

4.2. Multicomponent Analysis

Introduction

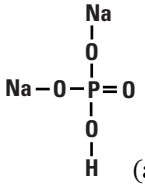
Mixtures can be analyzed using UV-visible spectroscopic data. The method is based on the assumption that Beer's law is obeyed by all components of the mixture and all possible components forming the mixture are known. This method is called multicomponent analysis (MCA).

As an example methyl orange solutions at different values of pH are evaluated. Due to the spectra shown in the experiment "Influence of pH — Buffered Methyl Orange Solutions" on page 64 where the isosbestic points are in the pH dependent spectra, the assumption is made that an equilibrium of two different molecular forms exists. Therefore the two solutions with the highest pH (pH = 5.2) and lowest pH (pH = 2.2) are used as standard solutions of two hypothetical pure forms A and B.

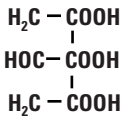
The solutions with pH 3.0, 3.8, and 4.6 are considered to be mixtures of these two hypothetical components A and B.

Reagents and Equipment

- ❑ methyl orange
- ❑ disodium hydrogen ortho-phosphate (a)
- ❑ citric acid (b)
- ❑ distilled water



(a)



(b)

- ❑ five 25-ml volumetric flasks
- ❑ 100-ml volumetric flask
- ❑ 200-ml volumetric flask
- ❑ 500-ml volumetric flask
- ❑ five 50-ml beakers
- ❑ 0.5-ml pipette
- ❑ 1-ml pipette
- ❑ 5-ml pipette
- ❑ 10-ml pipette
- ❑ 25-ml pipette
- ❑ disposable glass pipettes (minimum 5)
- ❑ 10-mm path length quartz cell
- ❑ magnetic stirrer
- ❑ calculator with matrix operation or spreadsheet program with matrix operation capabilities

Experiment

Time: about 4 h

1 Prepare a stock solution of 0.05 g methyl orange in 100 ml distilled water.

2 Prepare the following solutions:

a) a 0.2 M disodium hydrogen orthophosphate solution:
Dissolve 5.68 g Na_2HPO_4 in 200 ml distilled water. If necessary, use a magnetic stirrer for complete dissolution.
b) a 0.1 M citric acid solution:
Dissolve 9.61 g citric acid in 500 ml distilled water.
c) The five McIlvaine's buffer solutions are prepared by mixing aliquots of the citric acid solution and the disodium hydrogen orthophosphate solution. Mix the buffer solutions shown in the table below.

Approximate pH	Volume [ml] of Na_2HPO_4	Volume [ml] of Citric Acid
2.2	1.0	49.0
3.0	10.0	40.0
3.8	18.0	32.0
4.6	23.0	27.0
5.2	27.0	23.0

3 Divide each mixture into two equal portions of 25 ml.

4 Add 0.5 ml of methyl orange stock solution to one of the portions of each of the buffer solutions. These are the sample solutions to be measured. The other portions of buffer have to be used for the reference measurements.

5 The following two steps have to be repeated for each corresponding pair of the buffer and the sample (same pH values):

- Measure a reference on the pure buffer.
- Measure the spectrum of the corresponding sample solution in the wavelength range from 250 to 650 nm.

Evaluation

Experiment “Influence of pH — Buffered Methyl Orange Solutions” on page 64 showed that the assumed two forms of methyl orange do have different spectra. This measurable spectral difference is one of the requirements for UV-visible multicomponent analysis.

In addition we assume that Beer’s law is obeyed by the protonated form A (component A) and the deprotonated form B (component B).

Then the absorbance at a given wavelength is the sum of the absorbance of component A and the absorbance of component B. In contrast to the single component analysis of the previous experiment, we cannot calculate the two concentrations by solving a single equation. We need at least as many equations as components.

The measured absorbance A_{jk} of the k^{th} sample at the j^{th} wavelength is:

$$A_{jk} = d \cdot \left(\sum_{i=1}^{m} c_{ik} \cdot \varepsilon_{ij} \right) \qquad i = 1, \ldots, m \qquad j = 1, \ldots, n \qquad k = 1, \ldots, p$$

where:

- m = number of components
- n = number of wavelengths
- p = number of samples
- d = path length [cm]
- ε_{ij} = extinction coefficient of the i^{th} component of the j^{th} wavelength [mol/l]
- c_{ik} = concentration of the component of the i^{th} component of the k^{th} standard [mol/l]

Such a set of equations can be written in matrix notation:

$$A = \varepsilon \cdot C$$

where:

- A = the matrix of sample data
- ε = the calibration coefficients matrix
- C = the sample concentration modules matrix

To simplify the equation above we have already assumed concentration modules. A concentration module is a concentration value multiplied by its path length.

The above equation can be solved for the calibration matrix:

$$\varepsilon = A \cdot C^{T} \cdot (C \cdot C^{T})^{-1}$$

In the calibration of an MCA analysis these coefficients are determined by measuring the standards and calculating the coefficients.

1 Create the concentration matrix C assuming a concentration of 1 for component A at a pH of 2.2 and component B at a pH of 5.2.

Evaluation Table 4.4. Concentration Matrix C

Component A	Component B	
		Standard (pH 2.2)
		Standard (pH 5.2)

2 Multiply the concentration matrix by its transposed form:

$$C \cdot C^{T}$$

Evaluation Table 4.5. Matrix Product

i = 1	i = m	
		i = 1
		i = m

3 Invert the concentration matrix product $C \cdot C^{T}$:

$$(C \cdot C^{T})^{-1}$$

Evaluation Table 4.6. Inverted Matrix

i = 1	i = m	
		i = 1
		i = m

4 Multiply the transposed concentration matrix by the inverted the concentration matrix product $(C \cdot C^T)^{-1}$:

$$C^T \cdot (C \cdot C^T)^{-1}$$

Evaluation Table 4.7. Matrix Product

i = 1	i = m	
		i = 1
		i = m

5 Set up your absorbance calibration data matrix A:

Evaluation Table 4.8. Calibration Data Matrix A

Component A (pH 2.2)	Component B(pH 5.2)	
		430 nm
		440 nm
		450 nm
		460 nm
		470 nm
		480 nm
		490 nm
		500 nm
		510 nm
		520 nm

6 Multiply your calibration data matrix A by the matrix product mentioned before.

$$\varepsilon = A \cdot C^{T} \cdot (C \cdot C^{T})^{-1}$$

Evaluation Table 4.9. Calibration Coefficient Matrix

Component A	Component B	
		430 nm
		440 nm
		450 nm
		460 nm
		470 nm
		480 nm
		490 nm
		500 nm
		510 nm
		520 nm

Based on the above calibration, concentration results can be calculated for measured sample data.

To be able to calculate the MCA results we go back to the basic equation:

$$A = \varepsilon \cdot C$$

Now we have to solve this equation for the unknown concentration matrix C:

$$C = (\varepsilon^{T} \cdot \varepsilon)^{-1} \cdot \varepsilon^{T} \cdot A$$

The matrix A is now the sample data matrix.

In preparation for the calculation of the concentration results the following steps have to be performed in advance:

7 Transpose the calibration coefficient matrix ε:

$$\varepsilon^T$$

Evaluation Table 4.10. Transposed Calibration Coefficient Matrix

430 nm	440 nm	450 nm	460 nm	470 nm	
					Component A
					Component B

480 nm	490 nm	500 nm	510 nm	520 nm	
					Component A
					Component B

8 Multiply the transposed calibration coefficient matrix ε^T by the calibration coefficient matrix ε:

$$\varepsilon^T \cdot \varepsilon$$

Evaluation Table 4.11. Matrix Product

1	2	
		1
		2

9 Invert the calibration coefficient matrix product $\varepsilon^T \cdot \varepsilon$:

$$(\varepsilon^T \cdot \varepsilon)^{-1}$$

Evaluation Table 4.12. Inverted Matrix

1	2	
		1
		2

10 Multiply the inverted the calibration coefficient matrix product by the transposed calibration coefficient matrix ε^T:

$$(\varepsilon^T \cdot \varepsilon)^{-1} \cdot \varepsilon^T$$

Evaluation Table 4.13. Result Matrix

430 nm	440 nm	450 nm	460 nm	470 nm	
					Component A
					Component B

480 nm	490 nm	500 nm	510 nm	520 nm	
					Component A
					Component B

Now we are able to analyze our sample data. We perform the final steps:

11 Set up your sample absorbance data matrices A:

Evaluation Table 4.14.

Absorbance [AU] of Sample (pH 3.0)	
	430 nm
	440 nm
	450 nm
	460 nm
	470 nm
	480 nm
	490 nm
	500 nm
	510 nm
	520 nm

Evaluation Table 4.15. Measured Absorbance Values at a pH of 3.8

Absorbance [AU] of Sample (pH 3.8)	
	430 nm
	440 nm
	450 nm
	460 nm
	470 nm
	480 nm
	490 nm
	500 nm
	510 nm
	520 nm

Evaluation Table 4.16. Measured Absorbance Values at a pH of 4.6

Absorbance [AU] of Sample (pH 4.6)	
	430 nm
	440 nm
	450 nm
	460 nm
	470 nm
	480 nm
	490 nm
	500 nm
	510 nm
	520 nm

12 Multiply the result matrix (Evaluation Table 4.13) by your sample measurement data matrices A (Evaluation Tables 4.14–4.15):

$$C = (\varepsilon^T \cdot \varepsilon)^{-1} \cdot \varepsilon^T \cdot A$$

Evaluation Table 4.17. Calculated Concentrations at a pH of 3.0

Sample (pH = 3.0)	
	Component A
	Component B

Evaluation Table 4.18. Calculated Concentrations at a pH of 3.8

Sample (pH = 3.8)	
	Component A
	Component B

Evaluation Table 4.19. Calculated Concentrations at a pH of 4.6

Sample (pH = 4.6)	
	Component A
	Component B

13 How many data points per standard/sample measurement are required in the above example?

14 What is the advantage of more than the minimum required wavelength wavelengths?

15 How many standards have to be used as a minimum in the example for calibration?

16 What is the advantage of using more than the minimum number of standards?

17 What is the major difference to single component analysis?

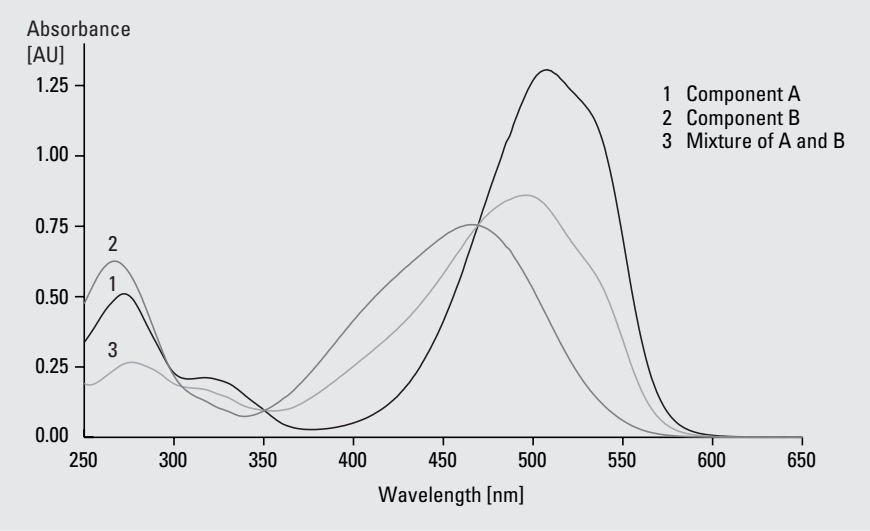
Example Results and Discussion

Samples: stock solution: methyl orange in distilled water (0.5 g/l)
buffered solution at pH values of 2.2, 3.0, 3.8, 4.6, 5.2 preparation

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 250–650 nm
absorbance range: 0.0–1.5 AU

Diagram: Pure Components A and B and Mixture of A and B (Buffered Solution of Methyl Orange, pH 3.8)



Calculations

1 Concentration matrix C:

Results Table 4.4. Concentration Matrix C

Component A	Component B	
1	0	Standard (pH 2.2)
0	1	Standard (pH 5.2)

2 Concentration matrix product:

$$C \cdot C^T$$

Results Table 4.5. Matrix Product

i = 1	i = m	
0	1	i = 1
1	0	i = m

3 Inverted concentration matrix product:

$$(C \cdot C^T)^{-1}$$

Results Table 4.6. Inverted Matrix

i = 1	i = m	
1	0	i = 1
0	1	i = m

4 Matrix product:

$$C^T \cdot (C \cdot C^T)^{-1}$$

Results Table 4.7. Matrix Product

i = 1	i = m	
0	1	i = 1
1	0	i = m

5 Calibration data matrix A:

Results Table 4.8. Calibration Data Matrix A

Compound A	Compound B	
0.186481	0.608564	430 nm
0.283442	0.663574	440 nm
0.412372	0.713896	450 nm
0.575434	0.748984	460 nm
0.766198	0.752545	470 nm
0.954226	0.714345	480 nm
1.126214	0.635997	490 nm
1.269060	0.526056	500 nm
1.302705	0.400055	510 nm
1.241117	0.280303	520 nm

6 Calibration coefficient matrix:

$$\varepsilon = A \cdot C^{T} \cdot (C \cdot C^{T})^{-1}$$

Results Table 4.9. Calibration Coefficient Matrix

Component A	Component B	
0.186481	0.608564	430 nm
0.283442	0.663574	440 nm
0.412372	0.713896	450 nm
0.575434	0.748984	460 nm
0.766198	0.752545	470 nm
0.954226	0.714345	480 nm
1.126214	0.635997	490 nm
1.269060	0.526056	500 nm
1.302705	0.400055	510 nm
1.241117	0.280303	520 nm

7 Transposed calibration coefficient matrix:

$$\varepsilon^{T}$$

Results Table 4.10. Transposed Calibration Coefficient Matrix

430 nm	440 nm	450 nm	460 nm	470 nm	
0.186481	0.283442	0.412372	0.575434	0.766198	Component A
0.608564	0.663574	0.713896	0.748984	0.752545	Component B

480 nm	490 nm	500 nm	510 nm	520 nm	
0.954226	1.126214	1.26906	1.302705	1.241117	Component A
0.714345	0.635997	0.526056	0.400055	0.280303	Component B

8 Calibration coefficient matrix product:

$$\varepsilon^{T} \cdot \varepsilon$$

Results Table 4.11. Matrix Product

1	2	
8.230179	4.538105	1
4.538105	3.877759	2

9 Inverted calibration coefficient matrix product:

$$(\varepsilon^{T} \cdot \varepsilon)^{-1}$$

Results Table 4.12. Inverted Matrix

1	2	
0.342551	-0.40088	1
-0.40088	0.727031	2

10 Matrix product:

$$(\varepsilon^{T} \cdot \varepsilon)^{-1} \cdot \varepsilon^{T}$$

Results Table 4.13. Result Matrix

430 nm	440 nm	450 nm	460 nm	470 nm	
-0.18008	-0.16892	-0.14493	-0.10314	-0.03922	Component A
0.367688	0.368812	0.353712	0.313853	0.239968	Component B

480 nm	490 nm	500 nm	510 nm	520 nm	
0.040501	0.130824	0.22383	0.285867	0.312776	Component A
0.136818	0.010909	-0.12629	-0.23138	-0.29375	Component B

11 Sample absorbance data matrices A:

Results Table 4.14. Measured Absorbance Values at a pH of 3.0

Absorbance [AU] of Sample (pH 3.0)	
0.28029	430 nm
0.36811	440 nm
0.47692	450 nm
0.61471	460 nm
0.76364	470 nm
0.90020	480 nm
1.01720	490 nm
1.10440	500 nm
1.10220	510 nm
1.02930	520 nm

Results Table 4.15. Measured Absorbance Values at a pH of 3.8

Absorbance [AU] of Sample (pH 3.8)	
0.42128	430 nm
0.49539	440 nm
0.58063	450 nm
0.67284	460 nm
0.75884	470 nm
0.81955	480 nm
0.85307	490 nm
0.85608	500 nm
0.80123	510 nm
0.70799	520 nm

Results Table 4.16. Measured Absorbance Values at a pH of 4.6

Absorbance [AU] of Sample (pH 4.6)	
0.56573	430 nm
0.62513	440 nm
0.68320	450 nm
0.73143	460 nm
0.75347	470 nm
0.73828	480 nm
0.68539	490 nm
0.60180	500 nm
0.49263	510 nm
0.37885	520 nm

12 Concentration result matrices:

$$C = (\varepsilon^T \cdot \varepsilon)^{-1} \cdot \varepsilon \cdot A$$

Results Table 4.17. Calculated Concentrations at a pH of 3.0

Sample (pH = 3.0)	
0.778232	Component A
0.222047	Component B

Results Table 4.18. Calculated Concentrations at a pH of 3.8

Sample (pH = 3.8)	
0.44404	Component A
0.55621	Component B

Results Table 4.19. Calculated Concentrations at a pH of 4.6

Sample (pH = 4.6)	
0.102103	Component A
0.897806	Component B

13 For a two component systems at least two data points are required. In our example above we used 10 data points. The system is redundant. Therefore the least squares algorithm was applied.

14 The advantage of a redundant system is that the precision of the result is improved. Under the assumption that the noise of the measurement data is normally distributed, redundancy in data points minimizes the standard deviation of the result.

15 As with the data points at least two standards must be used in calibration. In this example we used two “pure component” standards. Also two mixtures of the components A and B could have been used for calibration.

16 If more than the minimum number of standards is used in calibration, the precision of the calibration coefficient matrix can be improved. Statistical errors due to standard preparation like volumetric errors and weighting errors are minimized.

17 Instead of a single linear equation a set of linear equations has to be evaluated. According to this set of equations multiple data points and multiple standards have to be used. In addition the components of the mixture must have different extinction coefficients at the data points used in calibration and analysis.

4.3. Derivative Spectroscopy

Introduction

The number of aromatic amino acids in proteins can be determined by a method using 2nd order derivative spectroscopy.

To apply this method developed by Levine and Federici, only the concentration and the molecular weight of the protein must be known.

In this experiment we analyze Angiotensin III.

Reagents and Equipment

- ❏ N-acetyl-tryptophan-ethylester (MW = 274 g/mol)
- ❏ N-acetyl-tyrosine-ethylester (MW = 251 g/mol)
- ❏ N-acetyl-phenylalanin-ethylester (MW = 235.2 g/mol)
- ❏ guanidine hydrochloride
- ❏ angiotensin III (MW = 931.1 g/mol)
- ❏ 0.02 M phosphate buffer at a pH of 6.5
- ❏ distilled water

- ❏ 100-ml volumetric flask
- ❏ four 10-ml volumetric flasks
- ❏ disposable glass Pasteur pipettes (minimum 5)
- ❏ 10-mm path length quartz cell with a stopper

Experiment

Time: about 120–150 min

1. Prepare 100 ml stock solution of 0.02 M phosphate buffer at a pH of 6.5 with 6 mol guanidine hydrochloride dissolved in the buffer.
2. Prepare 10 ml of an N-acetyl-tyrosine-ethylester (Tyr) standard solution with a concentration of about $1 \cdot 10^{-3}$ mol/l in the stock solution.
3. Prepare 10 ml of an N-acetyl-tryptophan-ethylester (Trp) standard solution with a concentration of about $0.3 \cdot 10^{-3}$ mol/l in the stock solution.
4. Prepare 10 ml of an N-acetyl-phenylalanin-ethylester (Phe) standard solution with a concentration of about $10 \cdot 10^{-3}$ mol/l in the stock solution.
5. Prepare 10 ml of an Angiotensin III sample solution with a concentration of about $1 \cdot 10^{-4}$ mol/l in the stock solution.

6 Use the stock solution for the reference measurement.

7 Measure all standards (Tyr, Trp, Phe) using the 2nd order derivative of absorbance in a wavelength range from 220 to 400 nm.

8 Measure the sample (Angiotensin III) using the 2nd order derivative of absorbance in a wavelength range from 220 to 400 nm.

Evaluation

1 Use the 2nd order derivative data of your standards for the analysis of the three aromatic amino acids: Tyr, Trp, and Phe. Use a wavelength range from 240 to 300 nm for the calibration. The algorithm for the calibration is the same as explained in the experiment “Multicomponent Analysis” on page 162

Analyze the Angiotensin III sample 2nd order derivative data using the above calibration:

Evaluation Table 4.20. 2nd Order Derivative Data

	Tyr [10^{-3} mol/l]	Trp [10^{-3} mol/l]	Phe [10^{-3} mol/l]
Angiotensin III			

2 Calculate the aromatic amino acid units in the Angiotensin III protein. The amino acid units per protein molecule are calculated:

$$X_{Prot} = c_{Acd} / c_{Prot}$$

where: X_{Prot} =aromatic acid units [units]

c_{Acd} =result amino acid concentration [mol/l]

c_{Prot} =protein concentration [mol/l]

Enter your calculation results in the table below.

Evaluation Table 4.21. Amino Acid Units

	Tyr [units]	Trp [units]	Phe [units]
Angiotensin III			

3 Why can the unit content of aromatic amino acids be calculated using the above method?

4 Why is derivative spectroscopy used for the MCA?

Example Results and Discussion

Standards: N-acetyl-tyrosine-ethylester ($0.93 \cdot 10^{-3}$ mol/l)
N-acetyl-tryptophan-ethylester ($0.27 \cdot 10^{-3}$ mol/l)
N-acetyl-phenylalanin-ethylester ($10 \cdot 10^{-3}$ mol/l)

Sample: Angiotensin III ($9.5 \cdot 10^{-5}$ mol/l)

Cell: 10-mm path length quartz cell

Instrument Parameters: wavelength range: 220–400 nm
absorbance range: 0.1–0.6 AU

Diagram: Measured Spectra of the Standards

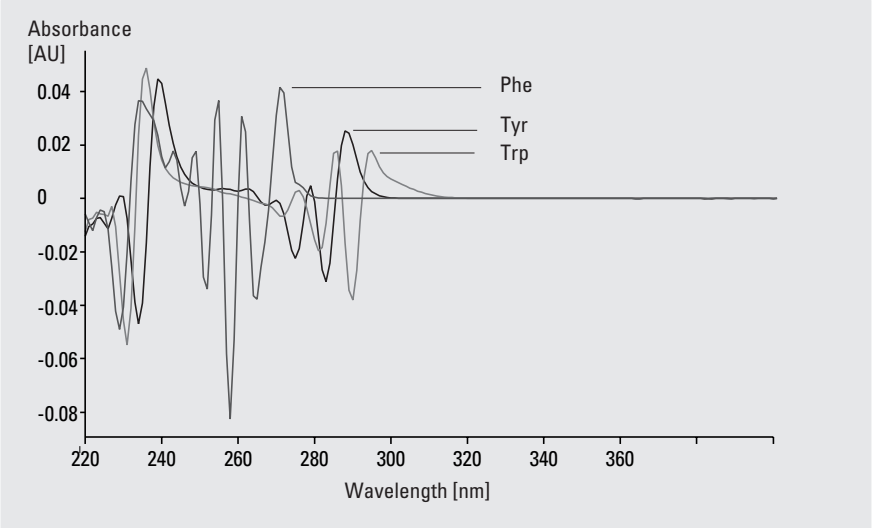
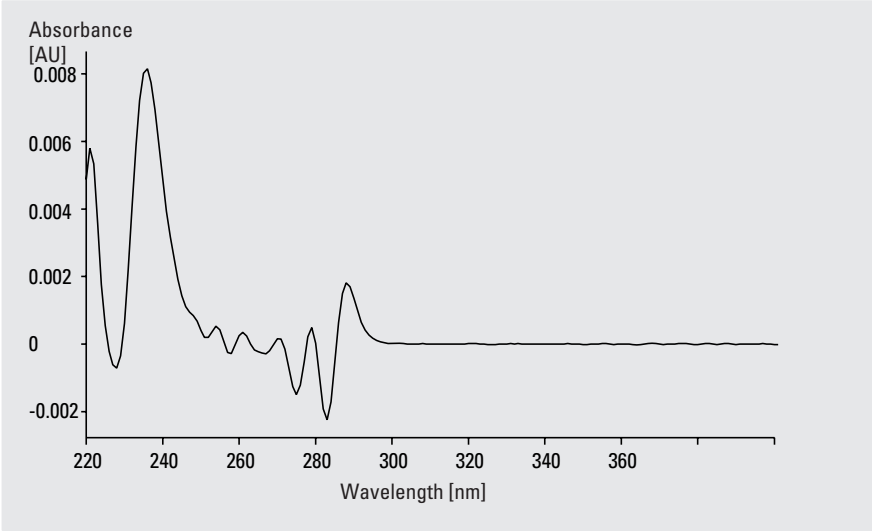


Diagram: Measured Spectrum of the Sample



1 The analysis results using 2nd order derivative data are shown in the table below.

Results Table 4.20. 2nd Order Derivative Data

	Tyr [10^{-3} mol/l]	Trp [10^{-3} mol/l]	Phe [10^{-3} mol/l]
Angiotensin III	0.081	0.002	0.092

2 The units of amino acids per protein molecule can be integers only. The calculated units in the Angiotensin III protein are given in the table below. The amino acid sequence of Angiotensin III is: Arg-Val-Tyr-Ile-His-Pro-Phe.

Results Table 4.21. Amino Acid Units

	Tyr [units]	Trp [units]	Phe [units]
Angiotensin III	1	0	1

3 The absorbance spectrum of the denatured protein can be approximated by the sum of the absorbance spectra of the constituent amino acids. Due to the buffer used for denaturation, only the spectra of the aromatic amino acids can be seen. They absorb in a range from 250 to 300 nm. These aromatic systems in the protein seemed to be not very much affected by the amino acid sequence. Therefore the aromatic amino acids can be treated as individual units in the calculation.

4 Derivative spectroscopy is applied to eliminate the scattering background due to light scattering of higher molecular weight proteins.

References

R. L. Levine, A. M. Federici, “Biochemistry 1982, 21”, page 2600ff

4.4. Kinetic Analysis - Studying Melting Temperatures of DNA

Introduction

The denaturation and renaturation of deoxyribonucleic acid (DNA) can be detected by UV-visible spectroscopy. We measure the denaturation profile and determine the melting temperature. In addition we calculate the percentage of the guanine/cytosine base pairs (% GC).

Reagents and Equipment

- ❏ highly polymerized calf thymus DNA (MW ≈ 8.6 MDa)
- ❏ citrate buffer at a pH of 7.0
  - $1.5 \cdot 10^{-2}$ M sodium chloride
  - $1.5 \cdot 10^{-3}$ M sodium citrate
- ❏ 0.5-ml pipette with adjustable volume
- ❏ 3-ml pipette with adjustable volume
- ❏ 10-mm path length quartz cell with a stopper

Experiment

Time: about 120 - 240 min

1 Fill the quartz cell with 2.7 ml of the citrate buffer.

2 Set the temperature of your cell holder to 40 °C.

3 Place the cell in the cell holder and allow to equilibrate to the temperature set.

4 Prepare a DNA stock solution of about 0.35 mg/ml DNA in the citrate buffer.

5 Measure the reference on the stock solution in the cell.

6 Add 0.3 ml of the stock DNA solution to your cell, gently stir the solution.

7 Start your measurements with the current temperature of 40 °C. Then increase the temperature in 5 degree steps up to 90 °C. For each temperature do the following:

- Allow to equilibrate to the temperature set.
- Measure the actual temperature of the DNA solution in the cell.
- Measure the absorbance value at 260 nm.

Evaluation

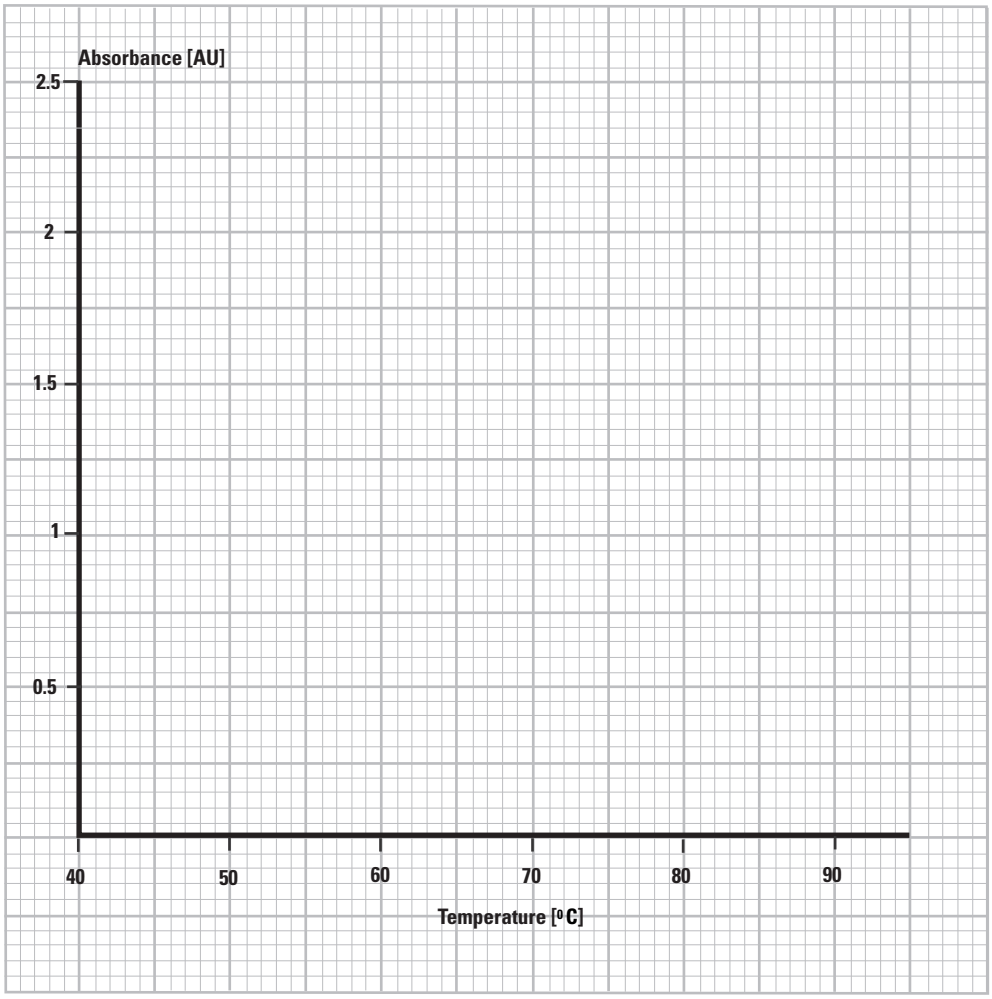
1 Enter your measured values in the table below.

Evaluation Table 4.22. Absorbance Measurements at 260 nm for Different Temperatures

Set Temperature [°C]	Sample Temperature [°C]	Absorbance at 260 nm [AU]
40		
45		
50		
55		
60		
65		
70		
75		
80		
85		
90		

2 Enter your measured absorbance values at 260 nm versus the sample temperature in the following diagram.

Diagram: Absorbance at 260 nm as a Function of Temperature



3 Determine the melting temperature T_m in the above diagram using the inflection point of the melting interval.

T_m =	°C

4 Calculate the percentage of guanine/cytosine base pairs (% GC) using T_m and the salt molarity M of the solvent using the equation below:

$$\% GC = 2.44 \cdot (T_m - 81.5 - 16.6 \cdot \log(M))$$

where: %GC =guanine/cytosine content [%]

T_m = melting temperature [°C]

M = salt molarity of the solution [mol/l]

Salt molarity:

M = mol/l

Logarithm log [M]:

Log(M) =

Result % GC:

% GC = %

Example Results and Discussion

Sample Stock: DNA in citrate buffer pH = 7 (0.195 mg/ml)

Sample: 1:10 dilution of sample stock solution

Cell: 10-mm path length quartz cell

Instrument Parameters:

wavelength:	260 nm
absorbance range:	0.0 - 1.0 AU

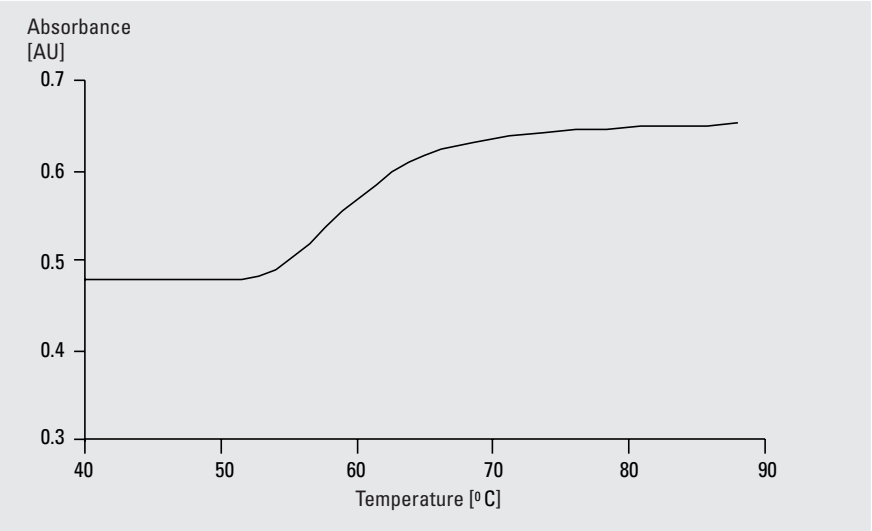
1 The following table shows the measured values.

Results Table 4.22. Absorbance Values at 260 nm for Different Temperatures

Set Temperature [°C]	Sample Temperature [°C]	Absorbance at 260 nm [AU]
40	38.9	0.477
45	43.6	0.477
50	49.0	0.478
55	54.0	0.491
60	58.9	0.555
65	63.8	0.608
70	68.6	0.632
75	73.7	0.643
80	78.3	0.647
85	83.4	0.650
90	88.0	0.652

2 The following diagram shows the measured absorbance versus temperature.

Diagram: Absorbance at 260 nm as a Function of Temperature



3 The melting temperature can be determined as:

$$T_m = 60\ ^{\circ}C$$

4 The percentage of guanine/cytosine base pairs (% GC) can be calculated as:

$$M = 1.65 \cdot 10^{-2}\ mol/l$$

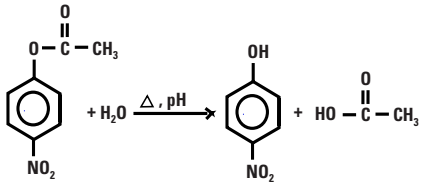
$$Log(M) = -1.7825$$

$$\%GC = 19.7\ \%$$

4.5. Isosbestic Points

Introduction

UV-visible spectroscopy is an ideal tool to study the kinetics of a chemical reaction. The measurement is fast and the acquired data can be used to understand the molecular mechanism as well as for quantitative calculation of reaction constants.



As an example here we use the hydrolysis of p-nitrophenolphenyl acetate. In principle it is a second order reaction, but with water in large excess it can be expected to show pseudo first-order kinetics. The reaction rate constant is sensitive to pH and temperature.

Reagents and Equipment

- ❏ p-nitrophenyl acetate
- ❏ dry acetonitrile
- ❏ 0.1 molar phosphate buffer at pH 8.5

- ❏ 3-ml pipette
- ❏ 50-µlsyringe or pipette with adjustable volume
- ❏ 10-mm path length quartz cell with stopper
- ❏ thermostattable cell holder

Experiment

Time: about 50–60 min

1. Prepare a stock solution of about 0.5 mg p-nitrophenyl acetate in about 2 ml dry acetonitrile.
2. Fill the quartz cell with 3 ml of the phosphate buffer.
3. Set the temperature of your cell holder to 60°C.
4. Place the cell in the cell holder and allow to equilibrate to the temperature set.
5. Measure the reference on your prefilled cell.
6. Prepare your system to acquire absorbance spectra every 2 minutes for 30 minutes using a wavelength range from 214 to 550 nm.

7 Add 30 µl of the p-nitrophenyl acetate stock solution to the cell, quickly stir or shake the solution and immediately start the data acquisition. Do not shake or stir too strong to avoid bubble formation.

Evaluation

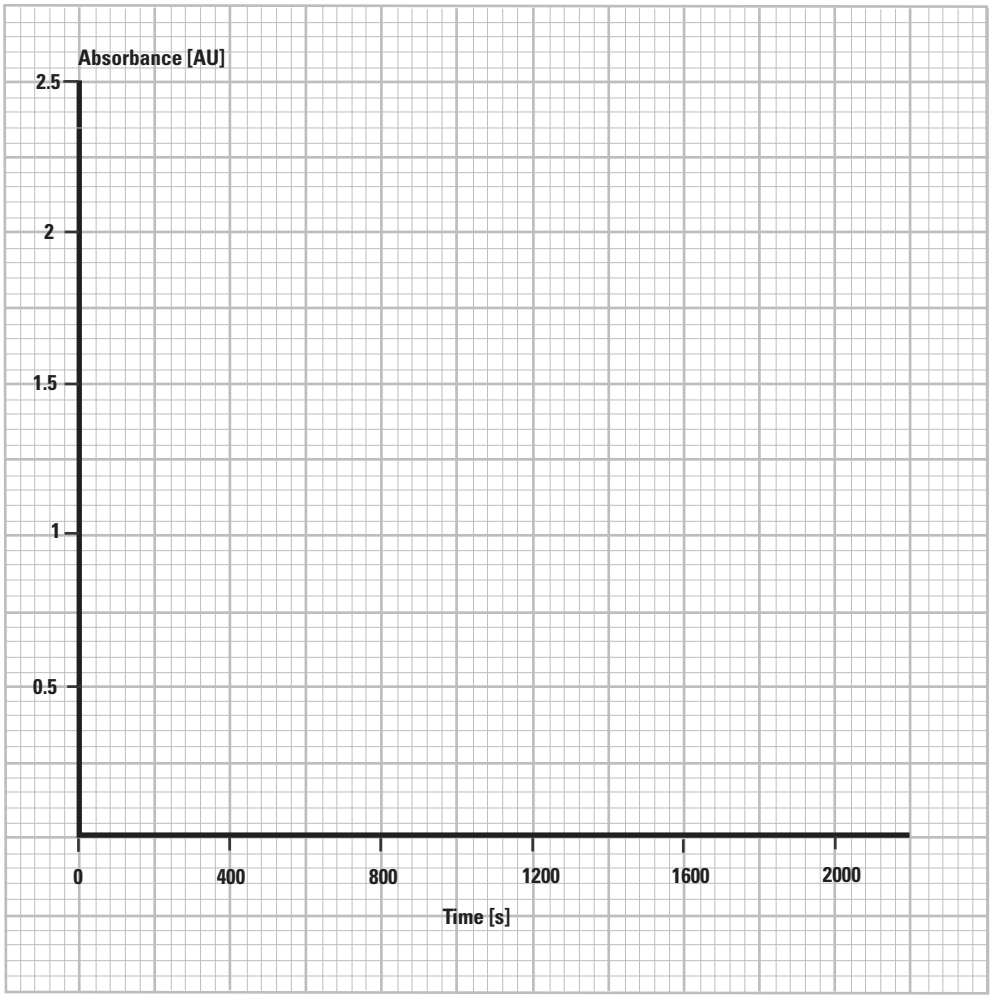
1 Determine the isosbestic points of the measured absorbance spectra:

Evaluation Table 4.23. Wavelengths of Isosbestic Points

Name	Wavelength [nm]
Isosbestic Point 1	
Isosbestic Point 2	
Isosbestic Point 3	
Isosbestic Point 4	
Isosbestic Point 5	

2 Enter your measured absorbance values at 400 nm versus reaction time in the diagram below.

Diagram: Absorbance at 400 nm as a Function of Time



3 What type of time dependence does the above diagram show?

4 Calculate the rate constant k. Assuming a pseudo first-order reaction the following equations can be used:

$$A(t) = A_\infty + (A_0 - A_\infty)^{e\text{-}kt}$$

where: $A(t)$ =absorbance at time t

A_0 = initial absorbance at time 0

A_∞ = absorbance at infinite time

k = first order rate constant

t = reaction time

For the evaluation the above equation can be expressed as:

$$\ln(A_t - A_\infty) = kt + \ln (A_0 - A_\infty)$$

Calculate a linear regression with offset for the above equation. Use the calculation formula given in the appendix for $y = ax + b$. Here the x,y pairs are the reaction time (x) and the logarithms of the absorbance differences $\ln(A_t - A_\infty)(y)$.

Determine your $A_{\infty:}$ value at 400 nm using the data value at 1800 seconds:

Simplification:

$$A_{400,\infty} = A_{400,1800}$$

$A_{400,\infty}$ =	[AU]

Enter the values for linear regression calculation in the table below.

Evaluation Table 4.24. Linear Regression Calculations

Reaction time t [s]	$A_{400,\tau}$ [AU]	$A_{400,\tau}-A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau}-A_{400,\infty}\rvert)$
0			
120			
240			
360			
480			
600			
720			
840			
960			
1080			
1200			
1320			
1440			
1560			
1680			
1800			

Calculate the linear regression and enter the results in the table below.

Evaluation Table 4.25. Calculation Results

Name		400 nm, T = 60 °C
Slope	a	
Intercept	b	
Correlation coefficient	R	

Enter the pseudo first order rate constant:

k = s^{-1}

5 How does the rate constant depend on the wavelength used for rate constant evaluation?

6 What is the result if one of the isosbestic points' wavelengths is used for evaluation?

7 At which wavelength can the most precise rates constant results be expected?

Advanced Experiment

Time: 90–120 min

1 Repeat the above experiment

- using a set temperature of 50 °C
- using a set temperature of 55 °C

2 Measure spectra every 2 minutes for half an hour.

Advanced Evaluation

1 Isosbestic points of the measured absorbance spectra:

Evaluation Table 4.26. Wavelength Measurements of Isosbestic Points at 50 °C

Name	Wavelength [nm]
Isosbestic Point 1	
Isosbestic Point 2	
Isosbestic Point 3	
Isosbestic Point 4	
Isosbestic Point 5	

Evaluation Table 4.27. Wavelength Measurements of Isosbestic Points at 55 °C

Name	Wavelength [nm]
Isosbestic Point 1	
Isosbestic Point 2	
Isosbestic Point 3	
Isosbestic Point 4	
Isosbestic Point 5	

2 Enter your measured absorbance values at 400 nm at 50 °C and 55 °C versus reaction time in the following diagrams.

Diagram: Absorbance at 400 nm and 50 °C as a Function of Time

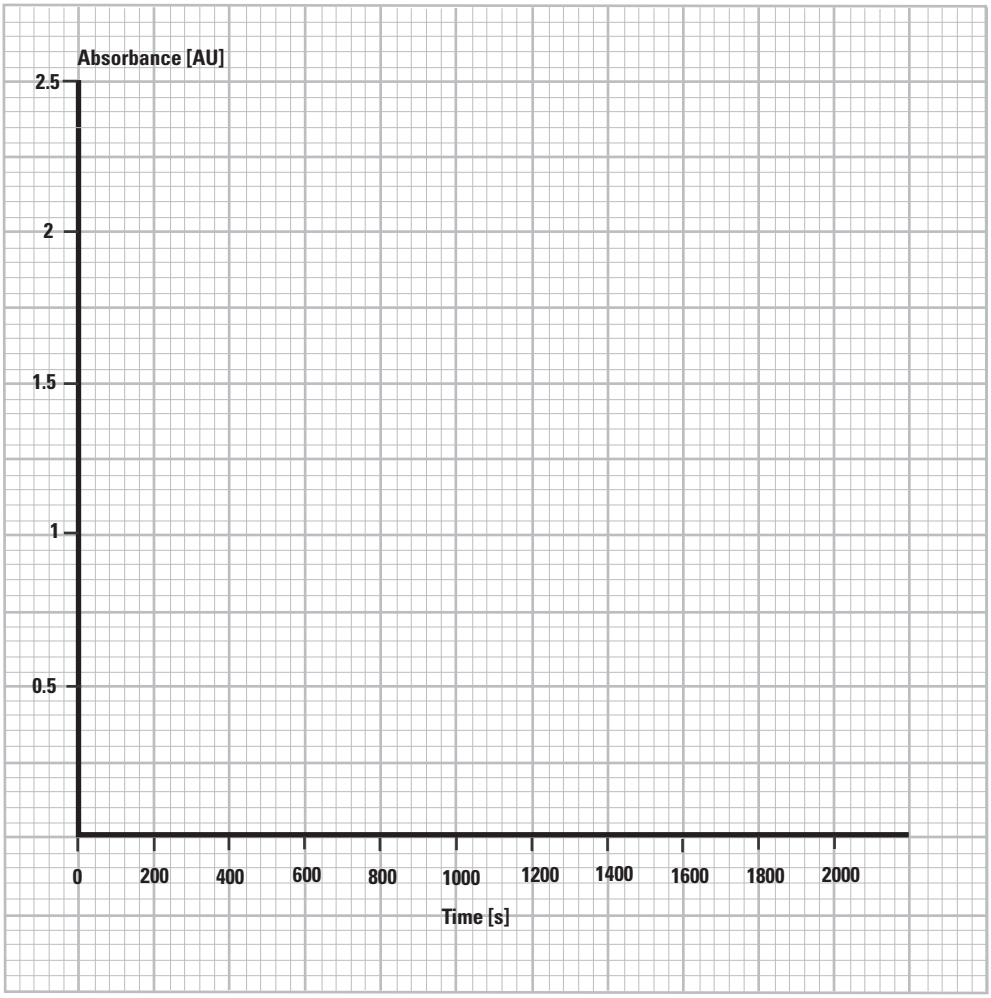
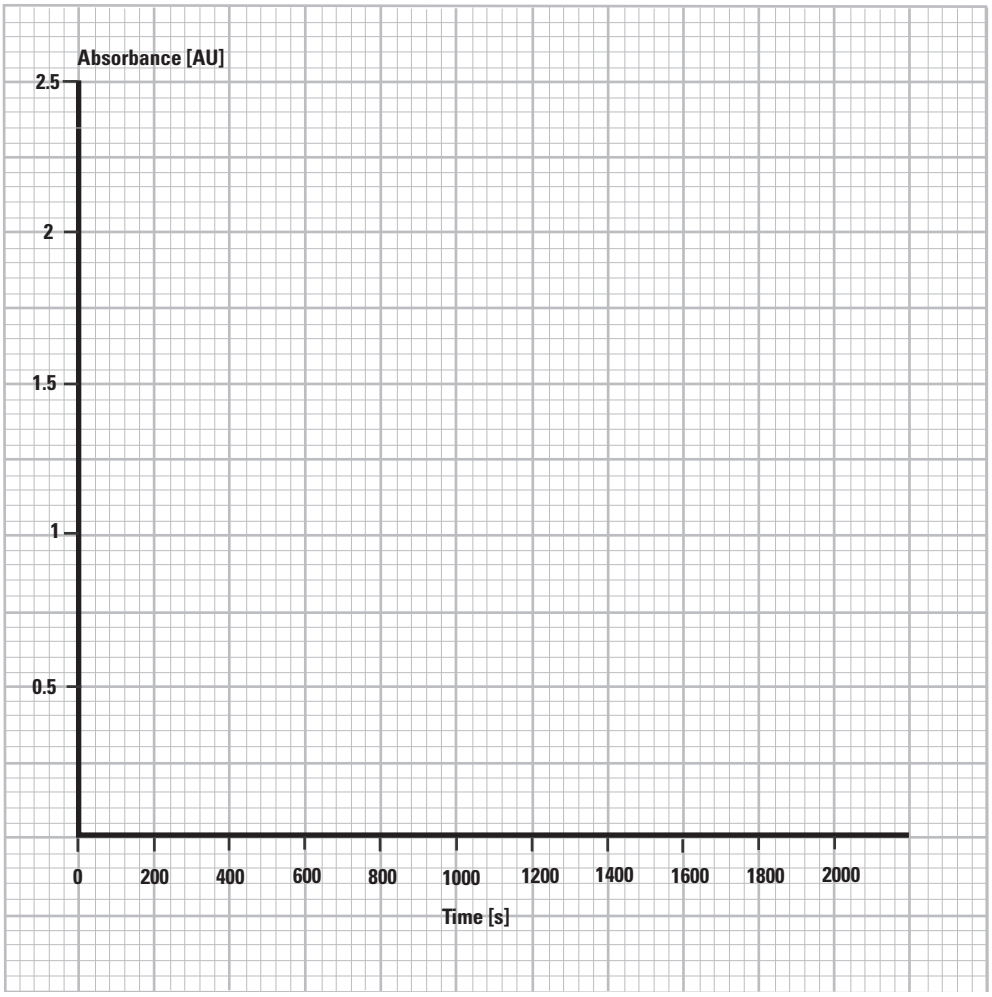


Diagram: Absorbance at 400 nm and 55 °C as a Function of Time



3 Calculate the rate constants k according to the basic kinetics experiment equations at 50°C and 55°C. Due to the much slower speed, the absorbance value A_{1800} is no longer a good approximation for the A value A_{∞}. Try to extrapolate the value out of the time trace diagram. If not possible, use the value at 60°C as an approximation.

$A_{400,\infty}(50\ °C)$ = AU	$A_{400,\infty}(55\ °C)$ = AU

Enter the values for linear regression calculation in the tables below.

Evaluation Table 4.28. Linear Regression Calculations at 50 °C

Reaction time t [s]	$A_{400,\tau}$ [AU]	$A_{400,\tau} - A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau} - A_{400,\infty} \rvert)$
0			
120			
240			
360			
480			
600			
720			
840			
960			
1080			
1200			
1320			
1440			
1560			
1680			
1800			

4 Calculate the linear regressions and enter the results in the table below:

Evaluation Table 4.29. Linear Regression Calculations at 55 °C

Reaction time t [s]	$A_{400,\tau}$ [AU]	$A_{400,\tau} - A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau} - A_{400,\infty} \rvert)$
0			
120			
240			
360			
480			
600			
720			
840			
960			
1080			
1200			
1320			
1440			
1560			
1680			
1800			

Calculate the linear regression and enter the results in the table below.

Evaluation Table 4.30. Calculation Results

Name		400 nm, T = 50 °C	400 nm, T = 55 °C
Slope	a		
Intercept	b		
Correlation coefficient	R		

Enter the pseudo first order rate constants:

k(50 °C) = s^{-1}	k(55 °C) = s^{-1}

5 How are the isosbestic wavelength affected by temperature changes?

6 How are the rate constants affected by temperature changes?

Example Results and Discussion

Sample: p-nitrophenyl acetate (244 mg/l)
(dry acetonitrile)

Assay: 30 µl sample injected in 3 ml
0.1 molar phosphate buffer at pH 8.5

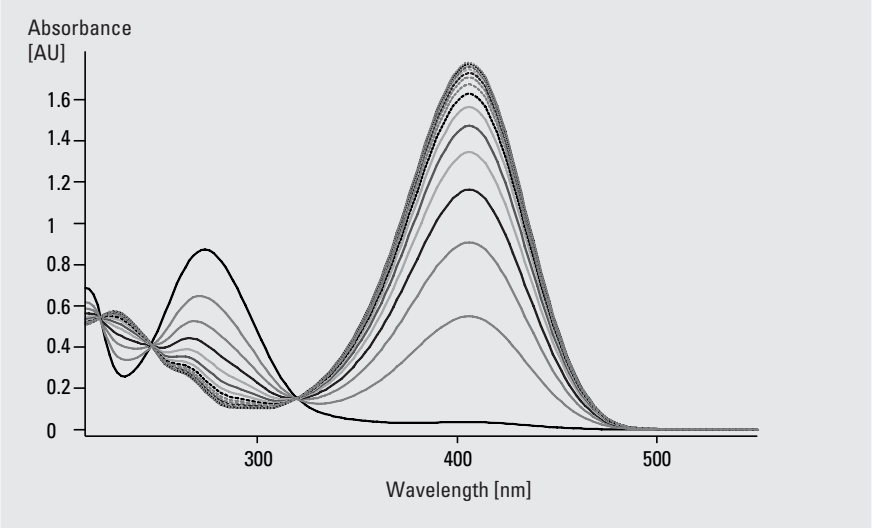
Cell: 10-mm path length quartz cell with a stopper

Instrument Parameters:

run time:	30 min
cycle time:	2 min
wavelength range:	214–550 nm
absorbance range:	0.0–2.0 AU

Temperature: temperature set: 60 °C

Diagram: Reaction Spectrum at 60 °C



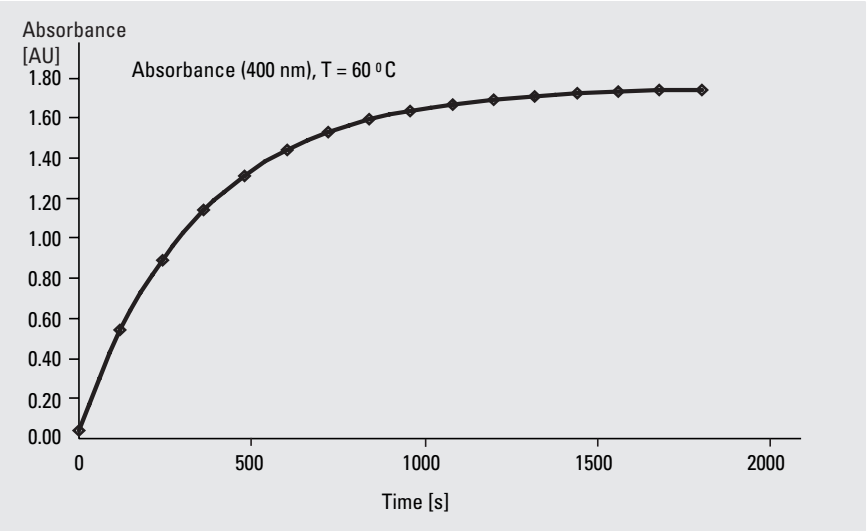
1 The table below shows at which wavelengths the isosbestic points can be found.

Results Table 4.23. Wavelengths of Isosbestic Points

Name	Wavelength [nm]
Isosbestic Point 1	221
Isosbestic Point 2	247
Isosbestic Point 3	320

2 The following diagram shows the absorbance at 400 nm as a function of time:

Diagram: Absorbance at 400 nm as a Function of TimeReaction Spectrum at 60 °C



3 The shape of the curve indicates an exponential relationship. This shape of time traces is an indication for a simple, mono-molecular reaction mechanism.

4 Estimated absorbance value at infinite time:

$A_{400,\infty}$ = 1.752 AU

The table below shows the values for linear regression calculation.

Results Table 4.24. Linear Regression Calculations

Reaction time t [s]	$A_{400,\tau}$ [AU]	$A_{400,\tau} - A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau} - A_{400,\infty} \rvert)$
0	0.037	-1.7154	0.53962
120	0.539	-1.2134	0.19339
240	0.889	-0.8628	-0.14763
360	1.139	-0.6131	-0.48918
480	1.316	-0.4363	-0.8294
600	1.442	-0.3104	-1.16976
720	1.531	-0.2210	-1.50964
840	1.593	-0.1592	-1.83778
960	1.640	-0.1125	-2.18462
1080	1.671	-0.0814	-2.50789
1200	1.693	-0.0588	-2.83293
1320	1.710	-0.0422	-3.16486
1440	1.723	-0.0304	-3.49233
1560	1.731	-0.0212	-3.85517
1680	1.738	-0.0142	-4.25381
1800	1.741	-0.0113	-4.48384

The table below shows linear regression results.

Results Table 4.25. Calculation Results

Name		400 nm, T = 60°C
Slope	a	-0.99992
intercept	b	0.529323
Correlation coefficient	R	-0.00282

The pseudo first-order rate constant can be calculated as:

$$k = 2.8 \cdot 10^{-3}\ s^{-1}$$

5 The rate constants are independent of the wavelength chosen for evaluation. Dependent on the precision of the data, the algorithm used for data evaluation and the total absorbance difference, the precision of the rate constant determination varies. In our example the absorbance value at infinite time (due to the algorithm used) and the total absorbance difference have most impact on the precision of the rate constant results.

6 The isosbestic point is characterized by its invariance with reaction time. Therefore no rate constant can be calculated due to the lack of a measurable absorbance difference.

7 The most precise results can be expected at a wavelength where the absorbance change with reaction time is high. In our example it is in the absorbance maximum of the growing absorbance band at about 400 nm.

Advanced Example Results and Discussion

Sample: p-nitrophenyl acetate (244 mg/l)
(dry acetonitrile)

Assays: 30 µl sample injected in 3 ml

Cell: 10-mm path length quartz cell with stopper

Instrument settings:

run time:	30 min
cycle time:	2 min
wavelength range:	214–550 nm
absorbance range:	0.0–2.0 AU

Temperature:

set temperature:	50 °C
set temperature:	55 °C

Diagram: Reaction Spectrum at 50 °C

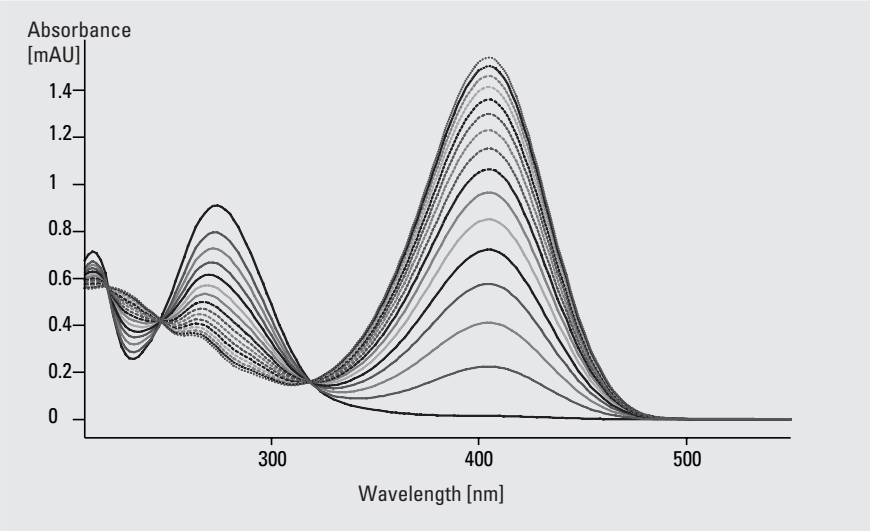
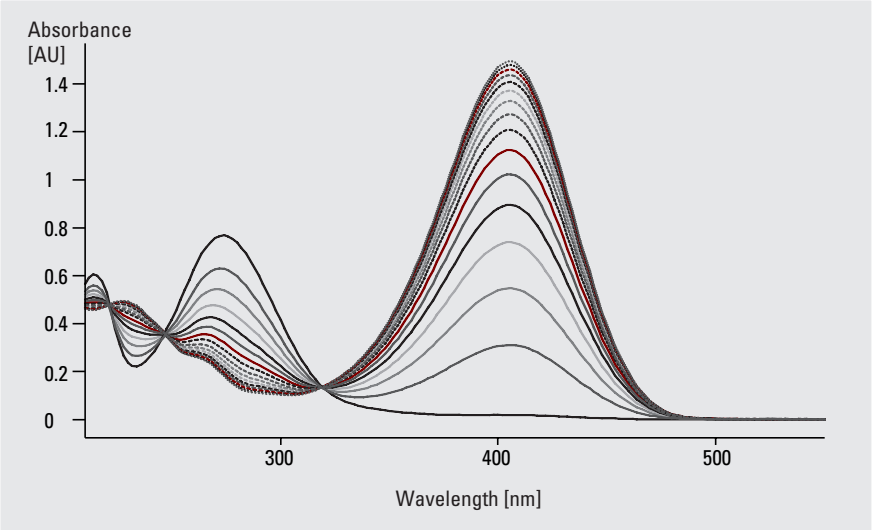


Diagram: Reaction Spectrum at 55 °C



1 The following tables show the wavelengths of the isosbestic points.

Results Table 4.26. Wavelengths of Isosbestic Points at 50 °C

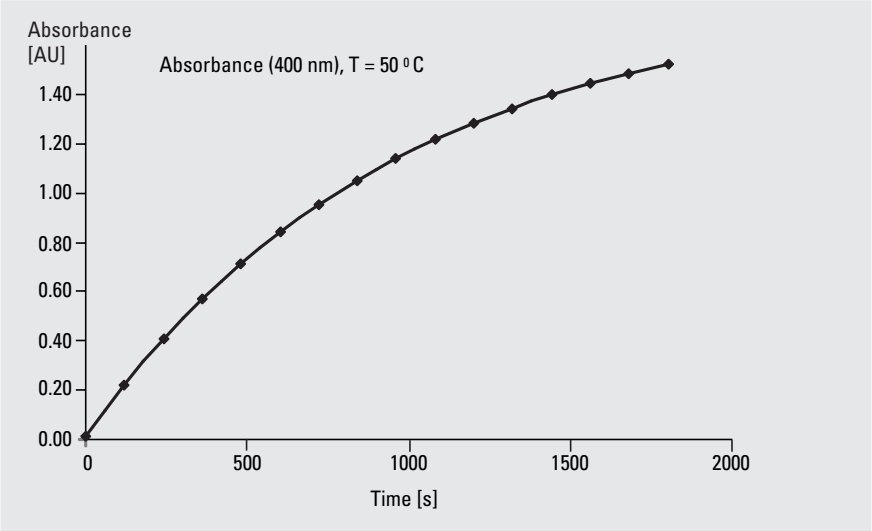
Name	Wavelength [nm]
Isosbestic Point 1	221
Isosbestic Point 2	247
Isosbestic Point 3	320

Results Table 4.27. Wavelengths of Isosbestic Points at 55 °C

Name	Wavelength [nm]
Isosbestic Point 1	221
Isosbestic Point 2	247
Isosbestic Point 3	320

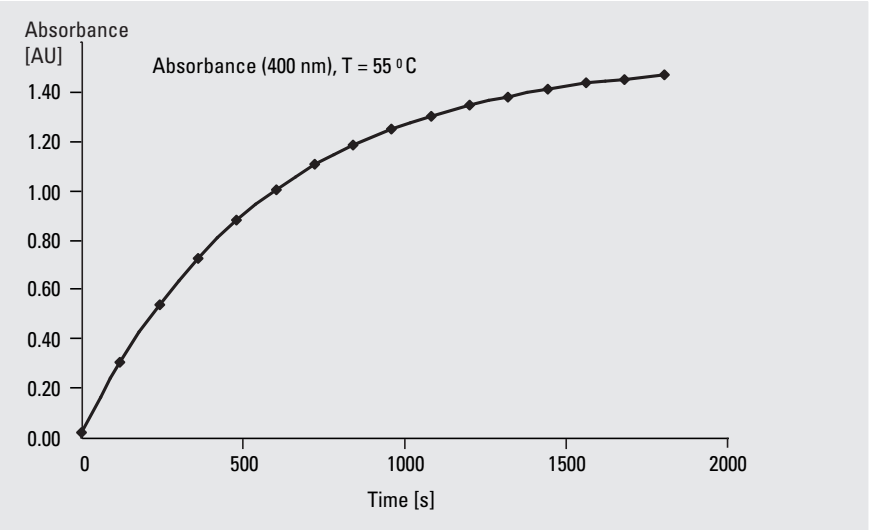
2 The following diagram shows the absorbance at 400 nm and 50 °C as a function of time.

Diagram: Absorbance at 400 nm and 50 °C as a Function of Time



The following diagram shows absorbance at 400 nm and 55 °C as a function of time.

Diagram: Absorbance at 400 nm and 55 °C as a Function of Time



3 The estimated absorbance values at infinite time are:

$A_{400,\infty}(50\ °C)$ =	1.75 AU	$A_{400,\infty}(55\ °C)$ =	1.56 AU

The linear regression calculation data for the measurements at 50 °C listed in the table below.

Results Table 4.28. Linear Regression Calculations at 50 °C

Reaction time t [s]	$A_{400,\tau}$[AU]	$A_{400,\tau} - A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau} - A_{400,\infty} \rvert)$
0	0.015	-1.7350	0.55101
120	0.223	-1.5270	0.42331
240	0.407	-1.3430	0.29491
360	0.570	-1.1800	0.16551
480	0.714	-1.0360	0.03537
600	0.841	-0.9090	-0.09541
720	0.954	-0.7960	-0.22816
840	1.052	-0.6980	-0.35954
960	1.139	-0.6110	-0.49266
1080	1.216	-0.5340	-0.62736
1200	1.283	-0.4670	-0.76143
1320	1.343	-0.4070	-0.89894
1440	1.396	-0.3540	-1.03846
1560	1.443	-0.3070	-1.18091
1680	1.485	-0.2650	-1.32803
1800	1.520	-0.2300	-1.46968

The linear regression calculation data for the measurements at 55 °C are listed in the table below.

Results Table 4.29. Linear Regression Calculations at 55 °C

Reaction time t [s]	$A_{400,\tau}$ [AU]	$A_{400,\tau} - A_{400,\infty}$ [AU]	$\ln(\lvert A_{400,\tau} - A_{400,\infty} \rvert)$
0	0.020	-1.5400	0.43178
120	0.306	-1.2540	0.22634
240	0.538	-1.0220	0.02176
360	0.727	-0.8330	-0.18272
480	0.881	-0.6790	-0.38713
600	1.005	-0.5550	-0.58879
720	1.105	-0.4550	-0.78746
840	1.188	-0.3720	-0.98886
960	1.251	-0.3090	-1.17441
1080	1.305	-0.2550	-1.36649
1200	1.348	-0.2120	-1.55117
1320	1.383	-0.1770	-1.73161
1440	1.412	-0.1480	-1.91054
1560	1.436	-0.1240	-2.08747
1680	1.454	-0.1060	-2.24432
1800	1.469	-0.0910	-2.3969

The linear regressions results are listed in the table below.

Results Table 4.30. Calculation Results

Name		400 nm, T = 50 °C	400 nm, T = 55 °C
Slope	a	-0.00112	-0.00159
Intercept	b	0.569364	0.382845
Correlation coefficient	R	--0.99985	-0.9995

The pseudo first order-rate constants are:

k[50 °C] = $1.1 \cdot 10^{-3}$ s^{-1}	k[55 °C] = $1.6 \cdot 10^{-3}$ s^{-1}

4 The wavelengths of the isosbestic points are not affected by changes of the set temperature as long as the molecular mechanism does not change.

5 The rate constants are increasing with temperature. This increase in reaction speed with temperature is due to the increasing amount of molecules at higher energy levels. More molecules have sufficient energy to undergo the chemical reaction.

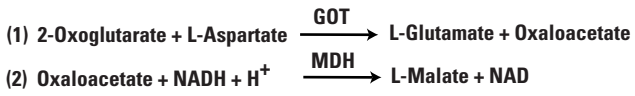
4.6. Biochemical Spectroscopy

Enzyme Activity

Introduction

The enzyme activity is a measure of the quality of an enzyme. During purification steps the activity is monitored.

The activity of glutamate-oxaloacetate transaminase (GOT) can be measured by coupling the production of Oxaloacetate by GOT to the oxidation of NADH using Malate dehydrogenase (MDH):



Reagents and Equipment

- ❏ 0.1 molar phosphate buffer at pH 7.4
- ❏ 10 ml of 0.19 molar L-aspartate in phosphate buffer
- ❏ 1 ml of 0.6 molar 2-oxoglutarate in distilled water
- ❏ 1 ml of 0.012 molar NADH
- ❏ 1 ml of malate dehydrogenase (MDH) in 3.2 molar ammonium sulphate solution (about 1200 U/mg)
- ❏ glutamate-oxaloacetate transaminase (GOT)
- ❏ 0.1 M phosphate buffer at pH 7.4

- ❏ 3-ml pipette
- ❏ 50-µland 100-µlsyringe or pipette with adjustable volume
- ❏ 10-mm path length quartz cell with a stopper
- ❏ thermostattable cell holder

Experiment

Time: about 30–50 min

1 Prepare the assay in the cell:

- add 3 ml of the L-aspartate in phosphate buffer solution
- add 100 µl of the 2-oxoglutarate solution
- add 50 µl of the NADH solution
- add 10 µl of the MDH solution

2 Set the temperature of your cell holder to 37 °C.

3 Place the cell in the cell holder and allow to equilibrate to the temperature set.

4 Dilute your GOT enzyme suspension (about 10 mg/ml) 1:2000 with ice-cold phosphate buffer

5 Measure the reference on your cell.

6 Prepare your system to acquire absorbance values in 5 second intervals for 100 seconds (at 340 nm).

7 Add 20 µl of your diluted GOT suspension to the cell, quickly stir or shake the solution and immediately start the data acquisition. Do not shake or stir too strongly to avoid bubble formation.

Evaluation

1 Calculate the rate constant k. Assuming a zero-order reaction the following equation can be used:

$$A(t) = k \cdot t + A_0$$

with:

$A(t)$ = absorbance at time t

A_0 = initial absorbance at time 0

Calculate a linear regression with offset for the above equation. Use the calculation formula given in the appendix for $y = ax + b$. Here the x, y pairs are the reaction time (x) and the absorbance values A_t at time (y).

Enter the absorbance values for linear regression calculation in the table below.

Evaluation Table 4.31. Absorbance at Different Reaction Times

Reaction Time t [s]	Absorbance [AU] at 340 nm
0	
5	
10	
15	
20	
25	
30	
35	
40	
45	
50	
55	
60	
65	
70	

Calculate the linear regression and enter the results in the table below:

Evaluation Table 4.32. Calculation Results

Name		340 nm, T = 37 °C
Slope	a	
Intercept	b	
Correlation coefficient	R	

Enter the zero-order rate constant:

k = s^{-1}

2 Use the absolute value of the zero-order rate constant and use a conversion factor of 7200 U · s/mg to convert to the specific activity.

Specific activity = U/mg

3 Why are two enzyme reactions used to monitor the activity of GOT?

4 Why are not all data acquired used for the rate constant calculation?

Example Results and Discussion

Sample: GOT enzyme suspension (10mg/ml)
(diluted 1:2000 with phosphate buffer)

Assay: 20 µl sample injected in
- 3 ml L-aspartate
- 100 µ2-oxoglutarate
- 50 µNADH solution
- 10 µMDH solution

Cell: 10-mm path length quartz cell with a stopper

Instrument Parameters:

integration time:	0.1 s
run time:	100 s
cycle time:	5 s
wavelength:	340 nm
absorbance range:	0.0–1.0 AU

Temperature: set temperature: 37 °C

1 The measured absorbance values are listed in the table below.

Results Table 4.31. Absorbance at Different Reaction Times

Reaction Time t [s]	Absorbance [AU] at 340 nm
0	0.991
5	0.926
10	0.863
15	0.798
20	0.738
25	0.684
30	0.623
35	0.562
40	0.501
45	0.439
50	0.378
55	0.318
60	0.258
65	0.197
70	0.135

The linear regression results are listed in the table below.

Results Table 4.32. Calculation Results

Name		340 nm, T = 37 °C
Slope	a	-0.01215
Intercept	b	0.985808
Correlation coefficient	R	-0.99995

The zero-order rate constant is:

$k = -1.2 \cdot 10^{-2}\ s^{-1}$

2 The calculated enzyme activity is:

Specific activity = 86.4 U/mg

3 The second enzyme reaction (MDH) is used to monitor the first reaction. It must be much faster, such that its reaction time is negligible compared to the GOT. In this indicator step (2), NAD^+ is formed, which has its absorbance maximum at 340 nm. This absorbance does not interfere with any of the other components of the assay and its extinction coefficient is quite high. This allows to detect even small amounts of the product (oxaloacetate) formed.

4 The conditions for the linear relationship of absorbance change with time are only valid, if the substrate is available in large amounts. Therefore, the rate is usually determined as an initial rate only. In our example the data acquired in the time interval from 0 to 70 s is by far sufficient for the rate calculation.

Appendix

Linear equation: $y = ax + b$

Least square method: $\sum_{i=1}^{n} (y_i - (ax_i + b))^2 \rightarrow \text{minimum}$

$$a = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}$$

$$b = \frac{\sum_{i=1}^{n} x_i^2 \sum_{i=1}^{n} y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i}{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}$$

$$R = \frac{\sum_{i=1}^{n} x_i y_i - \frac{1}{n}\sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{\sqrt{\left(\sum_{i=1}^{n} x_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} x_i\right)^2\right)\left(\sum_{i=1}^{n} y_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} y_i\right)^2\right)}}$$