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LESEPROBE

Bioanalytical Methods I

Prof. Dr. Boris Mizaikoff, Angelika Holzingerand Julian Haas

Institute of Analytical and Bioanalytical Chemistry
Faculty of Natural Sciences
Ulm University

1 Modulinhalt

Modulnummer	4.3
Modultitel	**Bioanalytical Methods – Basics and Advanced**
Modulkürzel	BMB
Studiengang	Biopharmazeutisch-Medizintechnische Wissenschaften (M.Sc.)
Ort der Veranstaltung	Universität Ulm
Modulverantwortlichkeit	**Prof. Dr. Boris Mizaikoff**
Lehrende	Prof. Dr. Boris Mizaikoff
Voraussetzungen	
Verwertbarkeit	Das Modul ist im Masterstudiengang Biopharmazeutisch-Medizintechnische Wissenschaften, aber auch für andere naturwissenschaftliche Studiengänge, vor allem im Bereich der Biophysik, Biochemie, Biopharmazie und Biotechnologie anwendbar.
Semester (empfohlen)	2
Max. Teilnehmerzahl	25
Art der Veranstaltung	☐Präsenzveranstaltung(en) ☐Präsenzveranstaltung(en) mit E-Learning-Elementen ☐Präsenzveranstaltung(en) im Labor mit E-Learning-Elementen ☒reine E-Learning-Veranstaltung(en)
Veranstaltungssprache	☐Deutsch, ☒Englisch, ☐Weitere, nämlich:
ECTS-Credits	6 Credits
Prüfungsform und –umfang	☐Klausur, ☐Referat, ☐Kolloquium, ☐Posterpräsentation, ☐Podiumsdiskussion, ☐Mündliche Einzel-/ Gruppenprüfungen, ☒Essay, ☐Forumsbeitrag, ☒Übungen, ☐Wissenschaftspraktische Tätigkeit, ☐Bachelor- und Masterarbeit ☐Haus-/ Seminararbeit, ☐Einzel-/Gruppenpräsentation, ☐Portfolio, ☐Protokoll, ☐Projektarbeit, ☐Lerntagebuch/ Lernjournale <u>Umfang der Prüfung:</u> Die Teilnahme an den Übungen ist Voraussetzung für die schriftliche Ausarbeitung (Essay). Prüfungssprache wird mit Studierenden gemeinsam festgelegt.
Lernziele	**Fachkompetenz** Die Studierenden können bioanalytische Methoden und Verfahren (inkl. Chemo-/Biosensoren) grundlegend erklären.

	Die Studierenden können verschiedene Anwendungsgebiete identifizieren. Die Studierenden können analytische Ergebnisse bewerten. Die Studierenden können Methoden zur Strukturaufklärung, bildgebende Verfahren, sowie weitere fortschrittliche Methoden erklären. Die Studierenden erkennen den fachlichen Zusammenhang zwischen bioanalytischen Methoden und verschiedenen Anwendungsgebieten. **Methodenkompetenz** Die Studierenden verfügen über die Fertigkeit bioanalytische Fragestellungen zu analysieren und lösen zu können. Die Studierenden können selbstständig eine Datenanalyse durchführen. **Selbst- und Sozialkompetenz** Lernbereitschaft und Belastbarkeit helfen den Studierenden Anwendungsaufgaben zu analysieren und Lösungen zu erörtern.
Lehrinhalte	**Basics:** - Grundlagen und Kenngrößen der Analytischen Chemie - Probenvorbereitung (Zellaufschluss, Fällung, Zentrifugation, Dialyse, Filtration, Extraktion, Gelfiltration, Präzipitation) - Spektroskopische Methoden (Wechselwirkung Licht-Materie, UV-Vis-, Fluoreszenz-, IR-, Raman-, SPR-Spektroskopie, FRET) - Elektrophoretische Verfahren (Wanderung geladener Teilchen in elektrischem Feld, Gel-, Zonen-, Disk-, Kapillarelektrophorese, SDS-PAGE, nativ, isoelektrische Fokussierung, Elektroblotting, 2D) - Chromatographische Trennmethoden (Verteilung zwischen mobiler und stationärer Phase, RP, HIC, HILIC, IEXC, SEC, AC) - Massenspektrometrie (Trennung von Ionen, MALDI, ESI, TOF, Quadrupol, Ionenfalle, SEV, Nachweis, Identifizierung) - Assays (Prinzip, Enzym-, Immuno-Assays) - Chemo- und Biosensoren (Aufbau, elektrochemisch, optisch, radiochemisch) - Weitere Methoden (DNA Sequenzierung, PCR) **Advanced:** - Methoden zur Strukturaufklärung (CD-, NMR-Spektroskopie, Röntgenstrukturanalyse, SAXS, Sequenzanalyse, MS) - Bildgebende Verfahren (Licht-, Fluoreszenz-, Elektronen-, Raster-sondenmikroskopie, Probenpräparation)

	- Kopplungs- und Hochdurchsatzverfahren: LC-MS, MS-MS, Sensorarrays, etc. - Miniaturisierte Chemo- und Biosensoren - Lab-on-a-chip - Weitere Methoden (Ultrazentrifugation, Mikrokalorimetrie, etc.)
Literatur	- F. Lottspeich, J. W. Engels: Bioanalytik, 3. Auflage, Springer Spektrum, 2012 - S. R. Mikkelsen, E. Cortón: Bioanalytical Chemistry, Wiley-Interscience, 2004 - M. H. Gey, Instrumentelle Analytik und Bioanalytik, Springer Berlin Heidelberg, Berlin, Heidelberg, 2. Auflage, 2008. - Cammann, Instrumentelle Analytische Chemie, Spektrum Akademischer Verlag, Heidelberg, 1. Auflage, 2010. - M. Hesse, H. Meier and B. Zeeh, Spektroskopische Methoden in der organischen Chemie, Georg Thieme Verlag, Stuttgart, 7th edn., (2005). - D. A. Skoog, D. M. West, F. J. Holler and S. R. Crouch, Fundamentals of Analytical Chemistry, Cengage Learning, Brooks/Cole, 9th edn., (2014). - Skoog, F. J. Holler and S. R. Crouch, in Principles of Instrumental Analysis, Cambridge University Press, Cambridge, (2007), vol. 9.

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Basics of analytical chemistry

1.1 Introduction

The concept of *chemical analysis* was first developed by R. Boyle (1627 – 1691). However, analytical chemistry was readily used before, e. g. by Paracelsius (1493 – 1541) for water analysis. Even alchemy can be viewed as the allocation of materials into individual compounds. First *quantitative experiments* were executed by Antoine Lavoisier, who is known as the "father" of analytical chemistry. In 1801, the subject of analytical chemistry was first mentioned in a textbook leading to the establishment of **Analytical Chemistry** as an independent scientific discipline.

Fig. 1.1: Antoine Lavoisier (Public Domain, details on wikimedia commons)

Nowadays, analytical chemistry is used in many different areas ranging from clinical tests for blood or saliva samples to monitoring of critical parameters in food analysis. Likewise, concepts derived from analytical chemistry are essential in biomedical analysis, environmental analysis, and quality monitoring in production processes. Additionally, analytical tools are also used in security and forensic scenarios, and for the investigation of ancient art or archaeological artifacts, as well as for counterfeit screening.

1.2 Definition

In general, analytical chemistry combines and embraces several scientific disciplines. Accordingly, analytical chemistry deals with a variety of challenges including, e. g., guidelines for inter-laboratory comparisons or sample (pre)treatment (i. e., sample collection, preparation, storage, and handling). Additionally, methods and critical data evaluation routines are defined and optimized for maximizing efficiency and accuracy. Analytical methods can be classified into **classical analytics** and **instrumental analytics**, whereby the classical methods include separation or extraction of

different components via so-called **wet-chemical methods**. This includes techniques like titration, gravimetry, extraction, distillation, filtration or chemical pulping.

Since the 20th century **instrumental methods** became increasingly important, and the technological progress along with advancements in computers and software have enabled the detection and determination of increasingly smaller compounds or traces of analytes within complex matrices. Nowadays, modern analytical methods deal with challenges including miniaturization (i. e., detection at micro- and nano-scales, but also devices that are small (e. g., on-chip) yet remain manageable), combination of techniques (i. e., so-called "hyphenated-techniques"), on-line detection scenarios, increasingly smart and reliable analytical tools (i. e., control systems and automation of processes), and the optimization of qualitative and quantitative results toward more precision, accuracy, reliability, and repeatability.

Different types of instrumental methods have been defined, which can be divided into several groups. The physical and chemical parameters used for the characterization and determination of analytical species are divided into radiation, electrical properties, mass and charge of the analytes, thermal or kinetic characteristics, radioactivity, etc. resulting in different demands and in consequence different instrumental methods. Hence, analytical chemistry is not just aimed towards the detection of analyte species, but also with the optimized application of physical stimuli followed by evaluation of the obtained response of the sample, i. e., analytical information and data. Analytical methods can also be divided with respect to the collected information. There are four "W-questions", which can be answered by analytical methods:

What's in the sample?	→	Qualitative analysis
What's the amount of analyte?	→	Quantitative analysis
Where is the analyte located?	→	Surface or distribution analysis
What's the structure of the sample?	→	Structural/chemical composition of the analytes

1.3 Criteria for selecting analytical methods

There are numerical criteria for selecting suitable analytical methods, and for assessing the obtained analytical result to its trueness and accuracy. In the following, some statistical descriptors are briefly explained, which are commonly used in analytical chemistry.

1.3.1 Population vs. sample

The **population** represents the entire sample unit (e. g., an entire lake, river, etc.). The entire population is therefore usually not available for evaluation due to restrictions in time, cost and appropriate effort. Hence, a so-called **sample** is collected, which should be a representative amount of the unknown species representing the entire system. By definition, statistical terms, which pertain to the entire population are written in *Greek letters*, and terms describing a sample are written in *Latin letters*.

	population	sample
Mean value	$\mu = \lim_{N\to\infty} \frac{\sum_{i=1}^{N} x_i}{N}$	$\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}$
Standard deviation	$\sigma = \sqrt{\lim_{N\to\infty} \frac{\sum_{i=1}^{N}(x_i-\mu)^2}{N}}$	$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i-\bar{x})^2}{N-1}}$
Variance	$\sigma_t^2 = \sigma_1^2 + \sigma_2^2 + ... + \sigma_n^2$	$s_t^2 = s_1^2 + s_2^2 + ... + s_n^2$
Relative standard deviation	$RSD = \frac{\sigma}{\mu} \times 10^z$	$RSD = \frac{s}{\bar{x}} \times 10^z$
Coefficient of Variation (z=2)	$CV = \frac{\sigma}{\mu} \times 100\,\%$	$CV = \frac{s}{\bar{x}} \times 100\,\%$

With: number of measurement (N), the signal value (x_i, with $i = 1 - N$) and $z = \frac{(x-\mu)}{\sigma}$.

Fig. 1.2: Population vs. sample

1.3.2 Trueness vs. precision vs. accuracy

The **trueness** of a result describes the level of agreement of the measured mean value with the *true value*. This value is in general unknown, however, the goal remains to get as close as possible to the true value. Therefore, every systematic error has to be eliminated. This can be realized by using *reference materials* and performing a method or instrumental *validation*. By repeating the measurement ≥ 30 times and taking the average, one may statistically assume normally distributed values close to the true value (in most cases; not always), if there is no systematic error. It should be noted again that meaningful average values are only obtained, if the underlying data points are normally distributed.

The **precision** describes the coincidence of individual data points, and the reproducibility of the obtained results using the same method (i. e., same technique, same instrumental set-up, etc.). In other words, the scattering of the data points around the average value is described using the absolute/relative standard deviation, variance, and the coefficient of variation. As a statistical parameter, precision can be divided into *reproducibility* of a result investigated by different laboratories, *repeatability* of consecutive measurements, and repeated measurements over a period of time (*intermediate precision*).

The **accuracy** describes the correctness of the result and combines trueness with the precision of an experimental value. It comprises all errors within the method or the system.

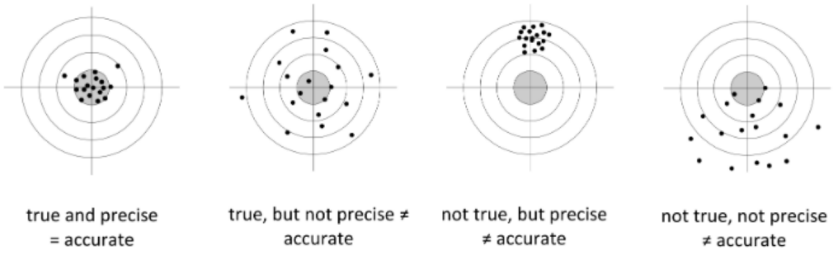

Fig. 1.3: Trueness vs. precision vs. accuracy

1.3.3 Differences in errors

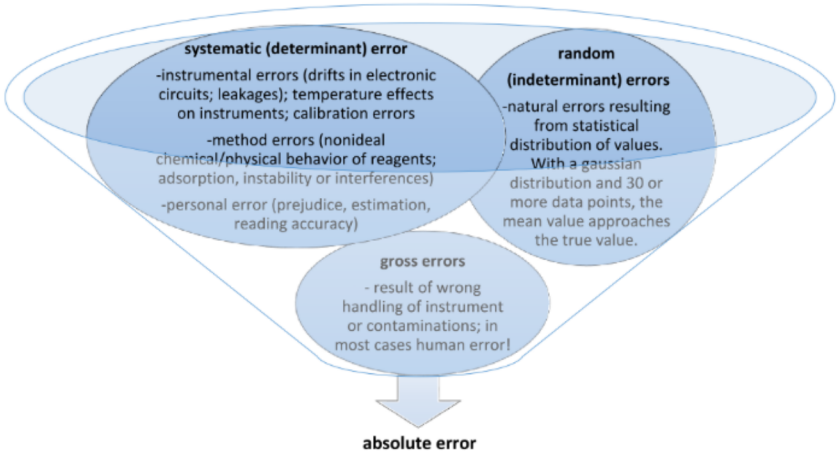

Fig. 1.4: Differences in errors

In an experiment with a large number of individual data points and the assumption that no systematic errors occur, the distribution of the values is safely assumed as "normal" (i. e., Gaussian). This implies the symmetrical distribution of values around the mean value, which has the maximum frequency of data points. Additionally, the behavior of the data points should show an exponential decrease with increase of the deviation from the mean value. This can be expressed by a *normal Gaussian distribution*.

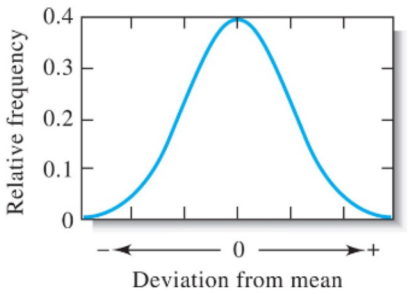

Fig. 1.5: Normal Gaussian distribution (ISBN: 9780495558286)

In general, a set of data is normally distributed – especially if a large number of data points is collected – and the sample can be viewed as a population. In most experiments, it is not possible however to repeat a measurement that frequently. Therefore, the mean value and a data range can be estimated and assuming that with a sufficiently high probability the true value is within this region. This is called the **confidence interval P**. The interval that is not taken into account, is called the **significance level α**. The calculation of an appropriate interval is done using the *t-test* (or student distribution).

It should be noted that next to a normal distribution, there are also other possibilities for data distributions, e. g., *Bernoulli* distribution, *Poisson* distribution, etc.

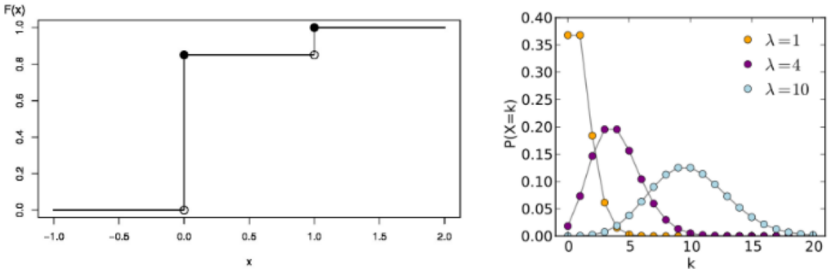

Fig. 1.6: Bernoulli distribution (left, https://media.wsb.wisc.edu/data/act_sci/Frees/ActMathII/InterestRateRisk/InterestRateRisk04Nov2012/bernoulli_distribution.html) and Poisson distribution (right, by Skbkekas, Poisson pmf, CC BY 3.0)

Hence, it is obvious that the mean value ± the standard deviation is not always the most suitable representation of the experimental values. Another possibility to characterize the measurement is the *median*. This is the value, which is in the middle of all data, i. e., divides all data points into 50 % to the left and right of the median. This method is more resistant towards outliers. The *mode* of a data set is the most frequently occurring value within all determined values, and is also rather insensitive to outliers.

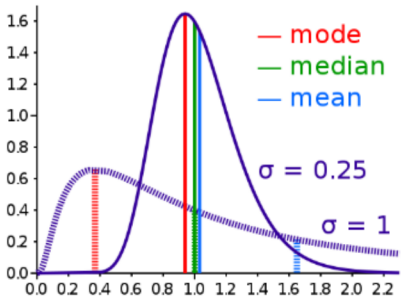

Fig. 1.7: Comparison mean median mode (by Cmglee, Comparison mean median mode, CC BY-SA 3.0)

1.3.4 How to find outliers?

The statistics and assumptions discussed above are only reliable, if there are no extreme individual values considered within the data range, i. e., which have a substantial deviation from the mean value. This may occur due to errors, but may also be in fact real values with a more pronounced deviation vs. other data points. Such data can significantly affect/distort a result that is usually based on calculating an average value, it is preferable to test an experimental data set for these **outliers**.

The most frequently used tests for outliers in analytic chemistry include:

- **Dean and Dixon:** An outlier x_1^* can be distinguished for data sets with $n \leq 25$ by this method also known as *Q-test*. Thereby, the values are sorted in increasing or decreasing order (dependent on the outliers with respect to the mean value) and calculated as

$$\widehat{M} = \frac{|x_1^* - x_b|}{|x_1^* - x_k|}$$

  with $b = 2$ $(3 \leq n \leq 10)$ or $b = 3$ $(11 \leq n \leq 25)$ and $k = n$ $(3 \leq n \leq 7)$, $k = n - 1$ $(8 \leq n \leq 13)$ or $k = n - 2$ $(14 \leq n \leq 25)$.

- **Graf and Henning:** For $n \geq 25$ the outlier can be determined by calculation of the mean value and the standard deviation without consideration of the suspected outlier. If this value is within $\bar{x} \pm 4s$, the discrepancy of this value is still acceptable.

- **Grubbs:** This test can be used for an undefined number of values to investigate the lowest and/or the highest value x^* in a data series

$$\widehat{G} = \frac{|\bar{x} - x^*|}{s}$$

  with the mean value $(\bar{x})$ and the standard deviation (s).

The critical values for $\widehat{G}$ are calculated using tabulated values for the *student distribution (t)* with N values and the *significance level* α:

$$\widehat{G} > \frac{(N-1)}{\sqrt{N}} \cdot \sqrt{\frac{(t_{\frac{\alpha}{2N},(N-2)})^2}{N-2+(t_{\frac{\alpha}{2N},(N-2)})^2}}$$

- **Nalimov:** The extension of the Grubbs outlier test is the Nalimov test taking the number of data points into account by

$$\widehat{G} = \frac{|\bar{x} - x^*|}{s} \cdot \sqrt{\frac{n}{n-1}}$$

1.4 Calibration in quantitative analysis

There are different possibilities for quantifying an unknown amount of a specific analyte within a sample. Accordingly, analytical methods can be divided into two groups: *absolute* and *relative* quantitative methods.

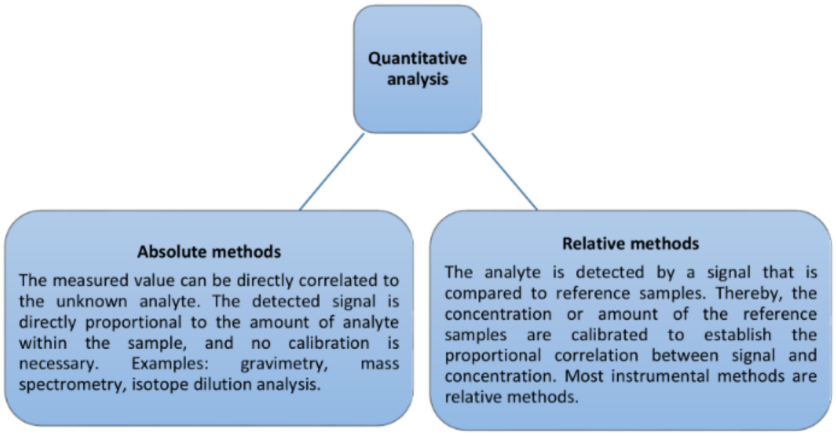

Fig. 1.8: Absolute and relative quantitative methods

Hence, an important aspect for the detection of a reliable and true value is an appropriate **calibration** routine providing an analytical signal as a function of the concentration or amount of the analyte in samples prepared with known concentrations. Important parameters associated with calibration are described in the following:

- **Sensitivity** describes the ability to discriminate between small differences in concentrations of the analyte, and can be calculated as the slope of the regression function in a calibration. A quantitative definition (i. e., following IUPAC) is

the calibration sensitivity, which is the slope at the concentration of interest. For a linear correlation between concentration and detection signal, the signal can be described by

$$S = mc + S_{blind}$$

with the signal (S) and the signal of the blind measurement (S_{blind}), the concentration (c), and the slope of the linear regression (m). The analytical sensitivity γ takes also the precision into account, and can be expressed by the standard deviation of the measurement as

$$\gamma = \frac{m}{s_S}$$

- **Selectivity** of the calibration or the analytical methods is described by the ability of discriminating between the analyte of interest, and other species within the sample matrix.

- The **limit of detection (LOD)** is defined by the blank value and its standard deviation and describes the minimum signal that can be detected by an analytical method and discriminated from the background noise. Occasionally, the LOD is defined as approx. 5-times the standard deviation of the blank value. By statistical calculations and in agreement with literature the detection limit S_m is more precisely calculated as

$$S_m = \overline{S_{blind}} + 3 \cdot s_{blind}$$

with the mean blind signal ($\overline{S_{blind}}$) and its standard deviation (s_{blind}).

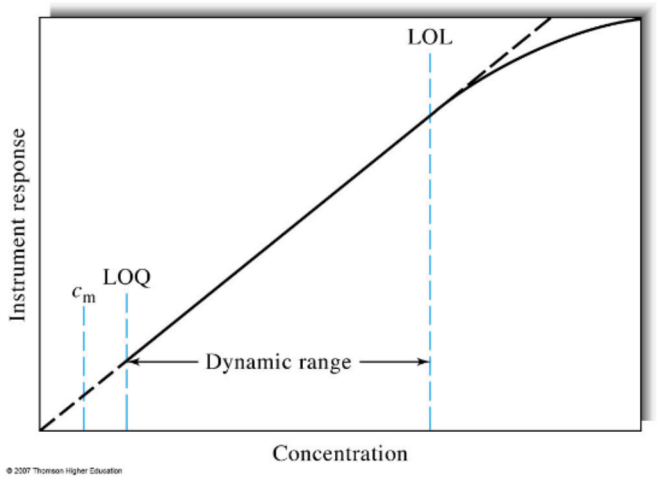

Fig. 1.9: Dynamic range (ISBN: 9781337468039)

- The **dynamic range** is defined by the lowest detectable value sufficient for quantifying the obtained analytical signal (limit of quantification – **LOQ**) and the highest concentration concluding the linear regime of the calibration (limit of linearity – **LOL**). The LOQ is defined as approx. 10-times the standard deviation of the blank value. In general, 5 % deviation from linearity is considered as the upper limit. This results from either chemical effects (e. g., molecular interactions) or non-ideal detection regimes at high concentrations.

1.4.1 Calibration methods

The easiest calibration routine is the **external standard calibration**. Thereby, the requirement for the sample is that the unknown concentration has to be within the *dynamic range* of the method, and above the *LOD*. Additionally, the *standard solutions* applied for the calibration routine have to be similar to the sample in their composition (i. e., also the background matrix and potential interference). To determine an adequate linear function for the relation between signal and concentration, **least squares regression method** is used based on the assumption that there is a linear correlation between signal and concentration, and that the deviation of the data points from this *linear regression* are due to errors of the experimental method.

In regression analysis, the deviation of individual data points (i. e., *residual*) is calculated, and the minimized sum for all residuals of x_i data points is determined. The square of this values is used neglecting the direction of deviation (i.e., smaller or larger than the regression value).

$$Minimum = \sum_{i=1}^{N} [y_i - (b + mx_i)]^2$$

with the detected signal (y_i), the slope (m) and the y intercept (b).

The *standard error of the estimate* (s_r) is then:

$$s_r = \sqrt{\frac{Minimum}{(N-2)}}$$

4 Beratung und Kontakt

Ansprechpartner

Dr. Gabriele Gröger
Albert-Einstein-Allee 45
89081 Ulm

Tel 0049 /31 – 5 03 24 00
Fax 0049 731 – 5 03 24 09

gabriele.groeger@uni-ulm.de
www.uni-ulm.de/saps

Geschäftsführender Direktor der SAPS: Prof. Dr.-Ing. Hermann Schumacher

Postanschrift

Universität Ulm
School of Advanced Professional Studies
Albert-Einstein-Allee 45
89081 Ulm

Der Zertifikatskurs „Bioanalytical Methods“ wurde entwickelt im Projekt CrossOver, das aus Mitteln des Ministeriums für Wissenschaft, Forschung und Kunst Baden-Württemberg und vom Ministerium für Soziales und Integration Baden-Württemberg aus Mitteln des Europäischen Sozialfonds gefördert wird (Förderkennzeichen: 696606).

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Lehrende	Prof. Dr. Boris Mizaikoff
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Veranstaltungssprache	☐Deutsch, ☒Englisch, ☐Weitere, nämlich:
ECTS-Credits	6 Credits
Prüfungsform und –umfang	☐Klausur, ☐Referat, ☐Kolloquium, ☐Posterpräsentation, ☐Podiumsdiskussion, ☐Mündliche Einzel-/ Gruppenprüfungen, ☒Essay, ☐Forumsbeitrag, ☒Übungen, ☐Wissenschaftspraktische Tätigkeit, ☐Bachelor- und Masterarbeit ☐Haus-/ Seminararbeit, ☐Einzel-/Gruppenpräsentation, ☐Portfolio, ☐Protokoll, ☐Projektarbeit, ☐Lerntagebuch/ Lernjournale <u>Umfang der Prüfung:</u> Die Teilnahme an den Übungen ist Voraussetzung für die schriftliche Ausarbeitung (Essay). Prüfungssprache wird mit Studierenden gemeinsam festgelegt.
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	Die Studierenden können verschiedene Anwendungsgebiete identifizieren. Die Studierenden können analytische Ergebnisse bewerten. Die Studierenden können Methoden zur Strukturaufklärung, bildgebende Verfahren, sowie weitere fortschrittliche Methoden erklären. Die Studierenden erkennen den fachlichen Zusammenhang zwischen bioanalytischen Methoden und verschiedenen Anwendungsgebieten. **Methodenkompetenz** Die Studierenden verfügen über die Fertigkeit bioanalytische Fragestellungen zu analysieren und lösen zu können. Die Studierenden können selbstständig eine Datenanalyse durchführen. **Selbst- und Sozialkompetenz** Lernbereitschaft und Belastbarkeit helfen den Studierenden Anwendungsaufgaben zu analysieren und Lösungen zu erörtern.
Lehrinhalte	**Basics:** - Grundlagen und Kenngrößen der Analytischen Chemie - Probenvorbereitung (Zellaufschluss, Fällung, Zentrifugation, Dialyse, Filtration, Extraktion, Gelfiltration, Präzipitation) - Spektroskopische Methoden (Wechselwirkung Licht-Materie, UV-Vis-, Fluoreszenz-, IR-, Raman-, SPR-Spektroskopie, FRET) - Elektrophoretische Verfahren (Wanderung geladener Teilchen in elektrischem Feld, Gel-, Zonen-, Disk-, Kapillarelektrophorese, SDS-PAGE, nativ, isoelektrische Fokussierung, Elektroblotting, 2D) - Chromatographische Trennmethoden (Verteilung zwischen mobiler und stationärer Phase, RP, HIC, HILIC, IEXC, SEC, AC) - Massenspektrometrie (Trennung von Ionen, MALDI, ESI, TOF, Quadrupol, Ionenfalle, SEV, Nachweis, Identifizierung) - Assays (Prinzip, Enzym-, Immuno-Assays) - Chemo- und Biosensoren (Aufbau, elektrochemisch, optisch, radiochemisch) - Weitere Methoden (DNA Sequenzierung, PCR) **Advanced:** - Methoden zur Strukturaufklärung (CD-, NMR-Spektroskopie, Röntgenstrukturanalyse, SAXS, Sequenzanalyse, MS) - Bildgebende Verfahren (Licht-, Fluoreszenz-, Elektronen-, Raster-sondenmikroskopie, Probenpräparation)

	- Kopplungs- und Hochdurchsatzverfahren: LC-MS, MS-MS, Sensorarrays, etc. - Miniaturisierte Chemo- und Biosensoren - Lab-on-a-chip - Weitere Methoden (Ultrazentrifugation, Mikrokalorimetrie, etc.)
Literatur	- F. Lottspeich, J. W. Engels: Bioanalytik, 3. Auflage, Springer Spektrum, 2012 - S. R. Mikkelsen, E. Cortón: Bioanalytical Chemistry, Wiley-Interscience, 2004 - M. H. Gey, Instrumentelle Analytik und Bioanalytik, Springer Berlin Heidelberg, Berlin, Heidelberg, 2. Auflage, 2008. - Cammann, Instrumentelle Analytische Chemie, Spektrum Akademischer Verlag, Heidelberg, 1. Auflage, 2010. - M. Hesse, H. Meier and B. Zeeh, Spektroskopische Methoden in der organischen Chemie, Georg Thieme Verlag, Stuttgart, 7th edn., (2005). - D. A. Skoog, D. M. West, F. J. Holler and S. R. Crouch, Fundamentals of Analytical Chemistry, Cengage Learning, Brooks/Cole, 9th edn., (2014). - Skoog, F. J. Holler and S. R. Crouch, in Principles of Instrumental Analysis, Cambridge University Press, Cambridge, (2007), vol. 9.

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Spectroscopic methods

In the following chapter, a general overview of spectroscopic methods that are used in analytical chemistry will be given alongside with several application examples for the particular spectroscopic method. Since spectroscopy describes generation of analytical information through light-matter interaction, a brief introduction into general properties of light as well as light-matter interaction will be given in the first section. Furthermore, general principles and pre-requisites, both from an instrumental as from an analyte perspective are described that are required to perform spectroscopic investigations.

In the subsequent paragraphs, more detailed description of the used spectroscopic methods for (bio)medical and pharmaceutical demands is presented. First, an introduction to the topic is given by UV/Vis spectroscopy and fluorescence spectroscopy. Subsequently, IR and Raman spectroscopy are introduced and a context is given to application of those spectroscopy tools.

1.1 Interactions of light and matter

1.1.1 Introduction

Interaction of electromagnetic (EM) radiation, aka light, with matter has been extensively explored throughout history. However, not all aspects are understood completely yet, and high-class research is still carried out nowadays. Geometric optics, or ray optics, are taught in basic physics lessons and research dates back to the time of Newton, although some evidence about very basic geometric optics dates back to the time of the ancient Greeks. More recent research has led to the wave-particle dualism and quantum physics finally led to a consistent description of light-matter interaction. **Spectroscopy** is based on decomposing light of certain wavelength in small portions and evaluation of the response of a certain analyte of interest thereon. Based on the manifold interactions that are possible, like excitation of rotation, vibration or electronic transitions, lots of qualitative information on the molecular structure as well as quantitative information can be derived.

1.1.2 Historical development

Research on light and light-matter interaction lead to a lot of insight into physical mechanisms, principles or fundamentals. For example, investigation on thermal light sources lead to the description of **black body radiation** and – as a consequence – to the discovery of the **Plank constant**. Attempts to describe the nature of light lead to the formulation of **Ray optics** and a particle-based theory by Newton. An alternative theory was formulated by Huygens that described light as wave whereas Youngs double slit experiment requires waves and Einstein's photo effect requires photons to be light quants. Maxwell unified electromagnetism and lead to the description of light as electromagnetic wave. Nowadays, particle-wave dualism is widely accepted. However, wave functions in quantum mechanics (Schrödinger equation) solve this issue. What is more, particle-wave dualism has been expanded to matter such as matter waves as postulated by De Broglie.

1.1.3 What is light?

To understand how matter and light can interact, basic principles of light have to be considered first. Formulation of light as particle and wave, **photon** and **electromagnetic wave** respectively, requires definition of several properties of light.

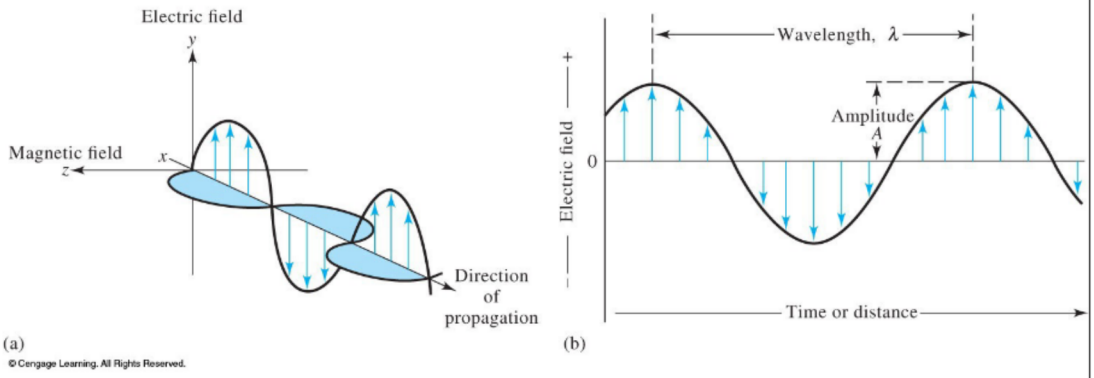


Fig. 1.1: a) Correlation of the magnetic field and the electric field of electromagnetic waves. b) Exemplary sketch of the wavelength λ and amplitude A of the electric field vector of an electromagnetic wave. (Source: Skoog, West, et al. 2014)

Electromagnetic radiation consists of an electric field portion and a magnetic field portion that are *perpendicular* to each other. Electromagnetic radiation does not need any medium to be propagated. The amplitude of each filled portion oscillates with a certain amplitude and frequency with respect to the direction of propagation.

Vacuum speed of light c has a constant value of

$$c = 2.99792 \times 10^8 \, \frac{m}{s}$$

Relation of the speed of light to its frequency ν and wavelength λ is given by

$$c = \nu \cdot \lambda$$

However, in spectroscopy wavenumber is more often used, since it scales with energy

$$\tilde{\nu} = \frac{1}{\lambda} = \frac{\nu}{c}$$

Correlation to the energy of a light particle, the photon is given by

$$E = h \cdot \nu = \frac{hc}{\lambda}$$

with the Planck constant

$$h = 6.62607 \times 10^{-34} Js$$

However, photons can be generated or destroyed upon light-matter interaction. (Cammann 2010)

1.1.4 Basic theory of light-matter interaction

Depending on the wavelength and what kind of matter it encounters, various types of interaction can appear: light can be **transmitted**, **reflected**, **refracted**, **diffracted**, **adsorbed** or **scattered**.

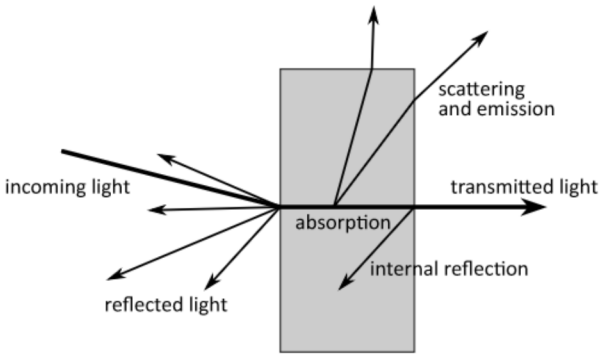


Fig. 1.2: Types of interaction of light with matter

The simplest interaction with light is transmission, which occurs when light passes through the object without interacting. As light is transmitted, it may pass straight through matter or it may be refracted or scattered as it passes through.

Electrons are situated in various energy levels in a molecule. It is possible for a photon, which is a **quantum of EM radiation**, to interact with an electron by causing its state to change, i. e. *causing it to occupy a different energy level*. When an electron absorbs a photon to move to a higher energy state that is available, it is called **absorption**. The difference in energies of the final and initial state is equal to the

photon energy. **Reflection** occurs when the incoming light hits a very smooth surface like a mirror and bounces off. **Diffraction** occurs when light hits an object that is similar in size to its wavelength. When light passes through a sufficiently-thin slit, it will diffract and spread. If it's visible light, this will also create a rainbow. **Scattering** occurs when the incoming light bounces off an object in many different directions. A good example of this is known as **Rayleigh scattering**, where sunlight is scattered by the gases in our atmosphere.

An electron in a higher energy state relaxes to a lower energy state by emitting a photon with an energy equal to the difference between the two states called **spontaneous emission**. Notice that it is exactly similar to the absorption process, except that the directions are reversed. It is also possible to have a different emission mechanism called **stimulated emission**. In stimulated emission, an electron in a higher energy state is stimulated to relax to a lower energy state with the energy difference $h\nu$ by an incident photon of the same energy. The incident and emitted photons *share all attributes* such as direction, phase and polarization. In other words, stimulated emission produces coherent photons. Emission of light by certain materials, when they are relatively cool, is called **luminescence**. As light emission does not result from the material being above room temperature, luminescence is often called **cold light**. Photoluminescence is one of many forms of luminescence and is initiated by **photoexcitation**. Photons are re-radiated after various relaxation processes.

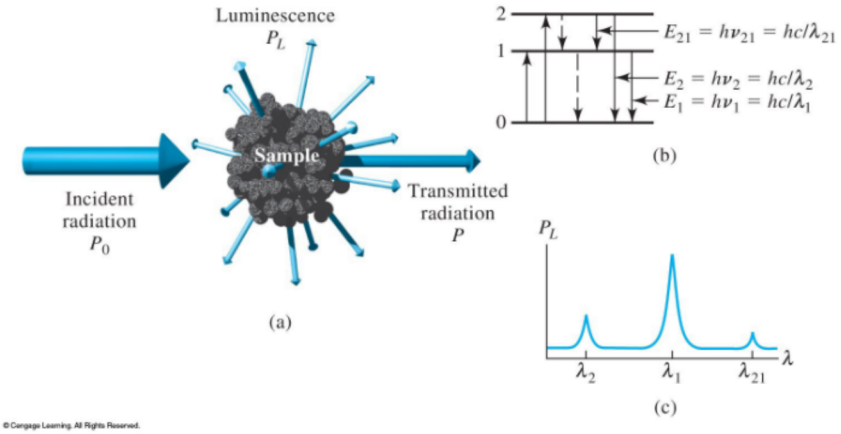


Fig. 1.3: a) Luminescence after photoexcitation of the sample b) relaxation processes c) resulting fluorescence spectrum (Source: Skoog, West, et al. 2014)

1.1.5 Basic spectrometer pre-requisites

A basic spectrometer setup is given in Figure 1.4. In brief, an **excitation light source** is required that emits radiation of the wavelength of interest. A **wavelength selective element** is mounted in front of the light source to further narrow down the wavelength of interest. Depending on the particular setup, spectra can be recorded

directly in transmission (Figure 1.4 a). If a radiative response of an analyte of interest is being probed, a 90° geometric can be beneficial to separate the excitation light from the emitted light. In this case a further wavelength selective element has to be introduced to split the emitted spectrum (Figure 1.4 b). Alternatively, directly exciting the analyte and using the emitted light as source is possible, too (Figure 1.4 c). For all experimental designs, some kind of **detector**, that is responsive to the wavelength of interest is required to transduce the optical signal into an electrical signal. In modern spectrometer setups, further processing, readout and storing of the acquired data is done with a **computer**.

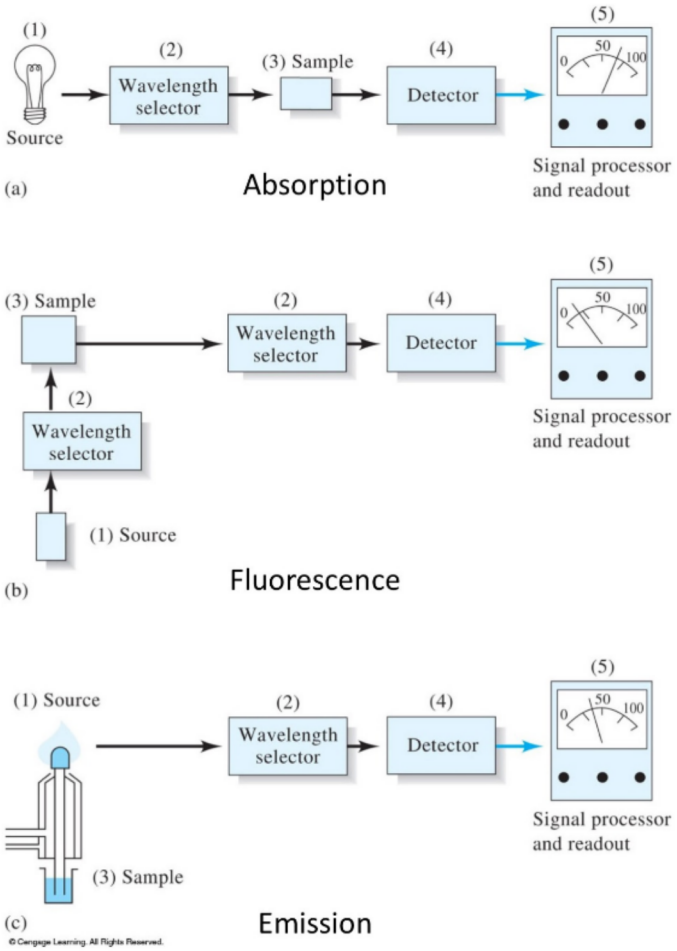


Fig. 1.4: Schematic spectrometry setup: Spectroscopic setups require light source, a certain interaction area, a wavelength selective element and a detector. (Source: Skoog, West, et al. 2014)

A brief overview of some spectroscopic methods that are used in (bio)analytics is given in Figure 1.5. A rough differentiation can be done between **molecular spectroscopy** and **atom spectroscopy**. Commonly, molecular spectroscopy can often be done *non-destructively*, while atom spectroscopy is often related to a *decomposition* of the molecules of interest into their atomic components for further analysis.

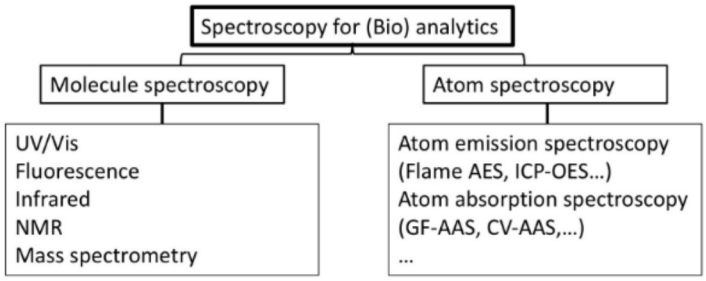


Fig. 1.5: Overview of spectroscopic tools used in bio analytics (Source: Gey 2008)

Atomic absorption: The passage of polychromatic ultraviolet or visible radiation through a medium that consists of monoatomic particles results in the absorption of a few well-defined frequencies. Such spectra are very simple due to the small number of possible energy states for the absorbing particles.

Molecular absorption: Absorption spectra for polyatomic molecules are considerably more complex than atomic spectra because the number of energy states of molecules is generally enormous when compared with the number of energy states for isolated atoms.

During absorption of light, molecules undergo changes in electronic transitions. These electronic transitions tend to accompany both rotational and vibrational transitions. These are often portrayed as an electronic potential energy curve with the vibrational level drawn on each curve. Additionally, each vibrational level has a set of rotational levels associated with it.

The energy E of a molecule is made up of **three components**:

$$E = E_{electronic} + E_{vibrational} + E_{rotational}$$

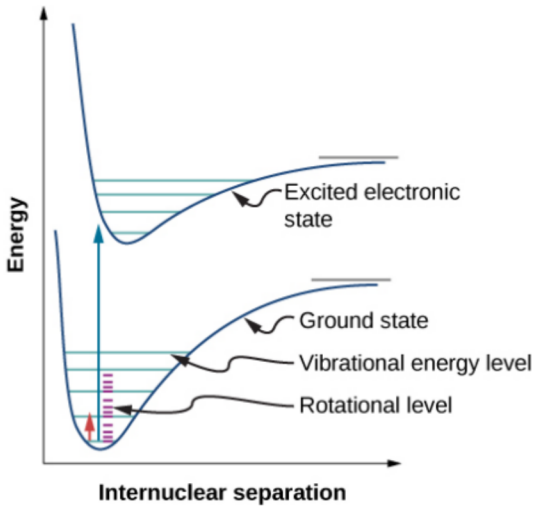


Fig. 1.6: Three types of energy levels in a diatomic molecule: electronic, vibrational, and rotational (Source: OpenStax University Physics, CC-BY 4.0)

1.1.6 The Franck-Condon principle

According to the **Born-Oppenheimer approximation**, the motions of electrons are much more rapid than those of the nuclei (i. e. the molecular vibrations). Promotion of an electron to an antibonding molecular orbital upon excitation takes about 10^{-15} s, which is very quick compared to the characteristic time for molecular vibrations ($10^{10} - 10^{-12}$ s). This observation is the basis of the *Franck-Condon principle*: an electronic transition is most likely to occur without changes in the positions of the nuclei in the molecular entity and its environment. The resulting state is called a **Franck-Condon state**, and the transition is called **vertical transition**, as illustrated by the energy diagram of Figure 1.7 in which the potential energy curve as a function of the nuclear configuration (internuclear distance in the case of a diatomic molecule) is represented by a **Morse function**.

At room temperature, most of the molecules are in the lowest vibrational level of the ground state (according to the *Boltzmann distribution*). In addition to the 'pure' electronic transition called the $0-0$ transition, there are several vibronic transitions whose intensities depend on the relative position and shape of the potential energy curves.

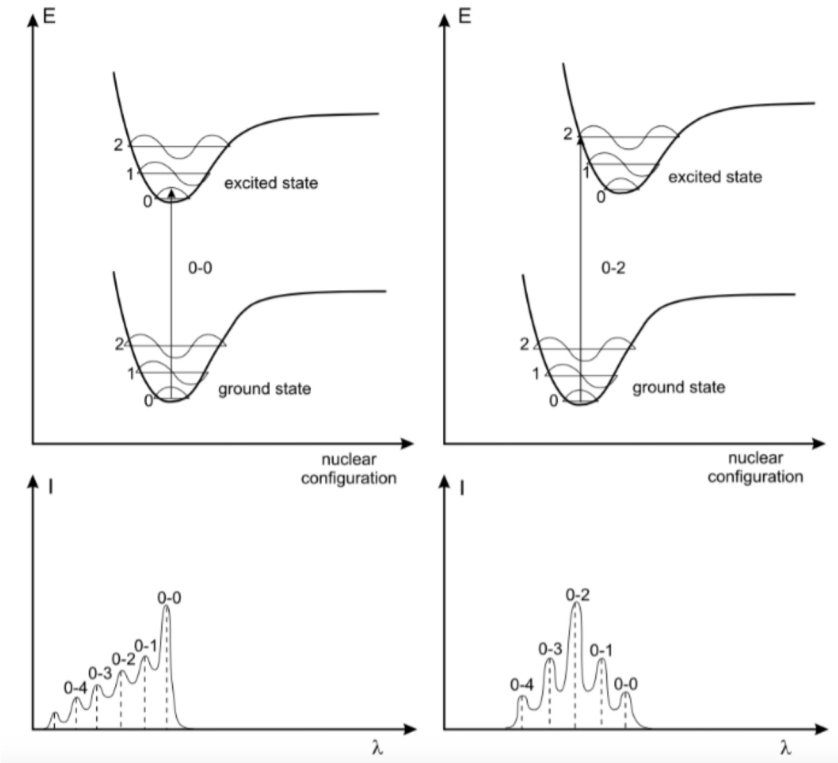


Fig. 1.7: Top: Potential energy diagrams with vertical transitions (Franck-Condon principle). Bottom: shape of the absorption bands; the vertical broken lines represent the absorption lines that are observed for a vapor, whereas broadening of the spectra is expected in solution (solid line). (Source: Bernard Valeur (2001): Molecular Fluorescence: Principles and Applications. Wiley-VCH Verlag GmbH, ISBNs: 3-527-29919-X (Hardcover); 3-527-60024-8 (Electronic)

1.1.7 Various types of spectroscopy

The large number of different types of spectroscopy can be arranged most clearly according to the wavelength regions of the incident "light". In optical spectroscopy, it must be distinguished whether absorption, reflection, scattering or luminescence is measured.

Spectroscopy in the ultraviolet and visible spectral range (**UV/Vis spectroscopy**), sometimes also called *electron spectroscopy*, has been a standard method used for many decades to obtain information about the substances present in the analyte sample.

The advantage of **IR spectroscopy** is the recognition of molecular structures and to achieve good quantitative results.

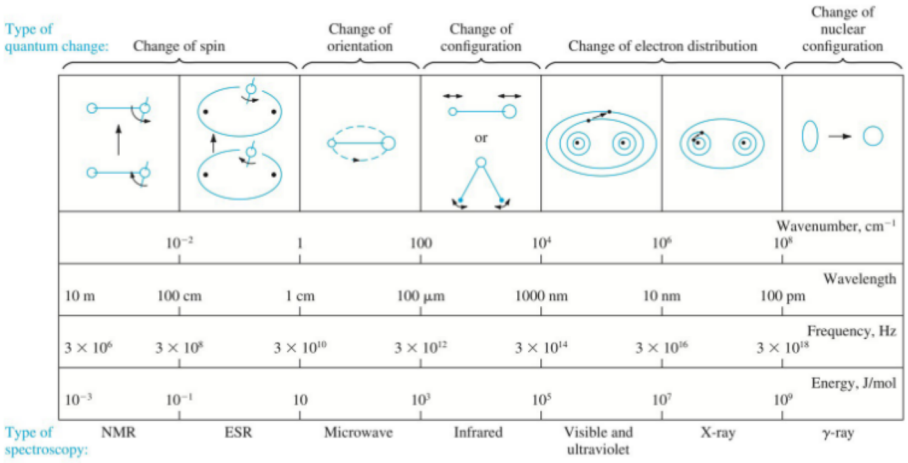
Luminescence comprises fluorescence, phosphorescence, photoacoustics and atomic emission. The fluorescence of matter that is irradiated with light in the UV/Vis range has the greatest significance.

Continuum Sources for Optical Spectroscopy

Source	Wavelength Region, nm	Type of Spectroscopy
Xenon arc lamp	250–600	Molecular fluorescence
H_2 and D_2 lamps	160–380	UV molecular absorption
Tungsten/halogen lamp	240–2500	UV/visible/near-IR molecular absorption
Tungsten lamp	350–2200	Visible/near-IR molecular absorption
Nernst glower	400–20,000	IR molecular absorption
Nichrome wire	750–20,000	IR molecular absorption
Globar	1200–40,000	IR molecular absorption

Fig. 1.8: Types of Spectroscopy (Source: Skoog, West, et al. 2014)

Depending on the respective wavelength, various kinds of spectroscopy can be distinguished:



(From C. N. Banwell, *Fundamentals of Molecular Spectroscopy*, 3rd ed., New York; McGraw-Hill, 1983, p. 7.)

Fig. 1.9: Spectrum of electromagnetic waves: Ranging from radio waves to gamma rays, different molecular or atomic transitions can be excited and various spectroscopic tools have been developed to analyze the light matter interaction at the respective wavelength. (Source: Skoog, West, et al. 2014)

1.1.8 Quantitative spectroscopy

The spectroscopic techniques described herein are perfectly suited and commonly used for **either qualitative or quantitative analysis**.

Quantitative analysis can be defined as the determination of the absolute or relative abundance of one, several or all substances present in a sample. The quantity may be expressed in terms of mass, concentration, or relative abundance of one or all components of a sample.

For this, a calibration is established (see *calibration methods, BM I chapter 1*), and the analyte solutions are sampled in a cuvette with known length (d) and irradiated with light (I_0) of the respective wavelength(s). After interaction with the analyte solutions, the light (I) is detected and produces the measured signal (Figure 1.10).

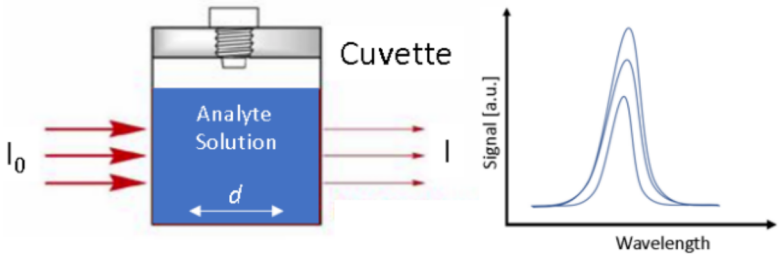


Fig. 1.10: Simplified scheme of a spectroscopic measurement which produces a quantitative usable signal

Prerequisite for a quantitative measurement is a mathematical relationship between the measured signal and the analyte(s).

1.1.8.1 Beer Lambert's Law

In principle, the measured variables in quantitative spectroscopic methods are expressed as **absorption (A)**, **transmission (T)** or **intensity** in case of Raman spectroscopy. For simplicity we will focus on absorption and transmission. The dependency of both on the concentration is given by Beer-Lambert's law:

$$\log \frac{I_0}{I} = A = \varepsilon \cdot c \cdot d$$

with:

I_0 = Incident light
I = Detected light
ε = Absorption coefficient
c = Concentration
d = Pathlength

According to this, absorption shows a *linear dependency* on concentration (c in mol/L) and the irradiated pathlength or cuvette length (d in cm) (see Figure 1.11 a). The constant ε is called the molar *absorption coefficient* (in $L \cdot mol^{-1} \cdot cm^{-1}$). It should be noted that this coefficient is characteristic for the measured substance and dependent on the wavelength of the absorbed light.

The mathematical relationship for transmission can be established as:

$$T = \frac{I}{I_0} = e^{-(\varepsilon \cdot c \cdot d)}$$

Hence, transmitted light follows an exponential decay function upon rising concentrations (Figure 1.11 b).

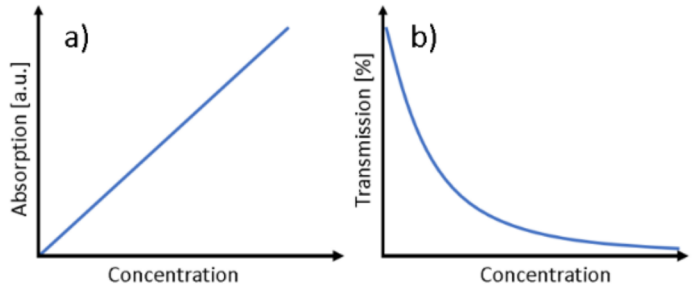


Fig. 1.11: Behavior of absorption and Transmission upon concentration according to Beer Lambert's Law

1.1.8.2 Limitations of Beer Lambert's Law

Prior to setting up quantitative measurement, it is important to first reflect the restrictions of Beer Lambert's Law. It is only applicable for:

- Absorption spectroscopy
- Monochromatic light (molar absorption coefficient is wavelength dependent)
- Clear solutions (not opaque)
- Dilute solutions

Hence, Beer's law can only be applied to clear dilute solutions and in this sense is a limiting law. At concentrations exceeding a certain value, the average distances between ions or molecules of the absorbing species are diminished to the point, when they can interact with each other and effect the charge distribution and thus alter the absorption of their neighbors. Hence, this concentration-dependent effect causes deviations from the linear relationship of Beer's Law.

Additionally, chemical deviations from Beer's law are also possible. For example, analyte analytes can undergo association, dissociation, or reaction with the solvent to give products that absorb differently from the analyte.

4 Beratung und Kontakt

Ansprechpartner

Dr. Gabriele Gröger
Albert-Einstein-Allee 45
89081 Ulm

Tel 0049 /31 – 5 03 24 00
Fax 0049 731 – 5 03 24 09

gabriele.groeger@uni-ulm.de
www.uni-ulm.de/saps

Geschäftsführender Direktor der SAPS: Prof. Dr.-Ing. Hermann Schumacher

Postanschrift

Universität Ulm
School of Advanced Professional Studies
Albert-Einstein-Allee 45
89081 Ulm

Der Zertifikatskurs „Bioanalytical Methods“ wurde entwickelt im Projekt CrossOver, das aus Mitteln des Ministeriums für Wissenschaft, Forschung und Kunst Baden-Württemberg und vom Ministerium für Soziales und Integration Baden-Württemberg aus Mitteln des Europäischen Sozialfonds gefördert wird (Förderkennzeichen: 696606).