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LESEPROBE

Bioanalytical Methods II

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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.

2 Inhaltsverzeichnis

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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.

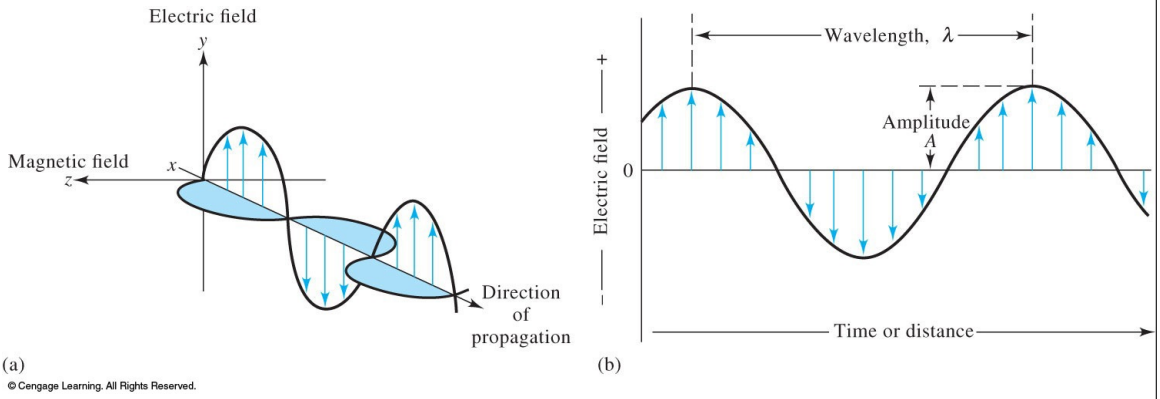


Fig. 1.1: a) Correlation of the magnetic field and the electric field of electromagnetic waves. b) Exemplary sketch of the wavelength λ and amplitude A of the electric field vector of an electromagnetic wave. (Source: Skoog, West, et al. 2014)

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**.

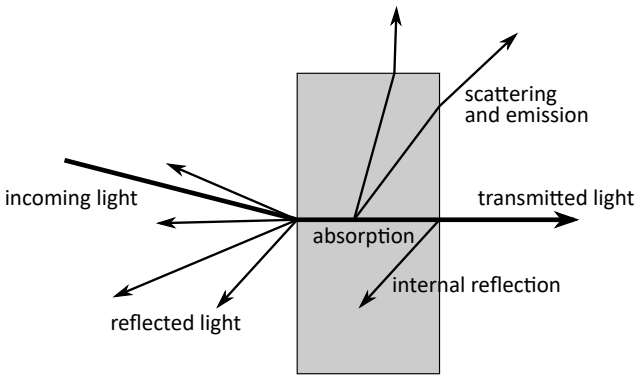


Fig. 1.2: Types of interaction of light with matter

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.

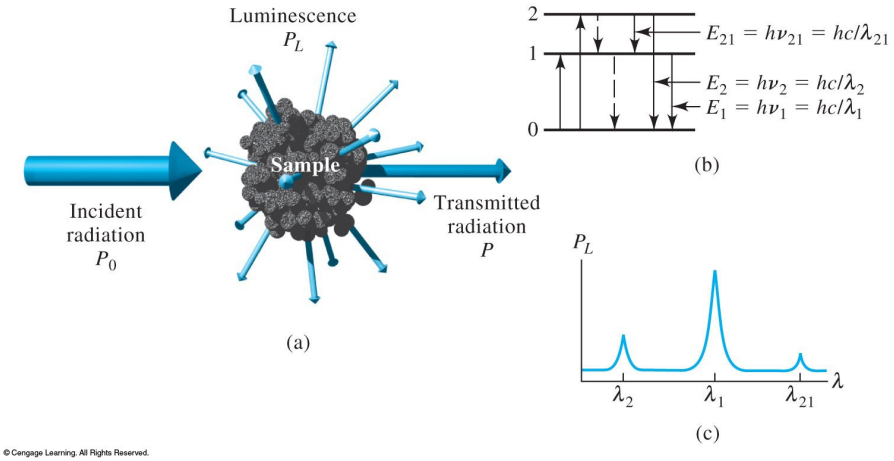


Fig. 1.3: a) Luminescence after photoexcitation of the sample b) relaxation processes c) resulting fluorescence spectrum (Source: Skoog, West, et al. 2014)

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**.

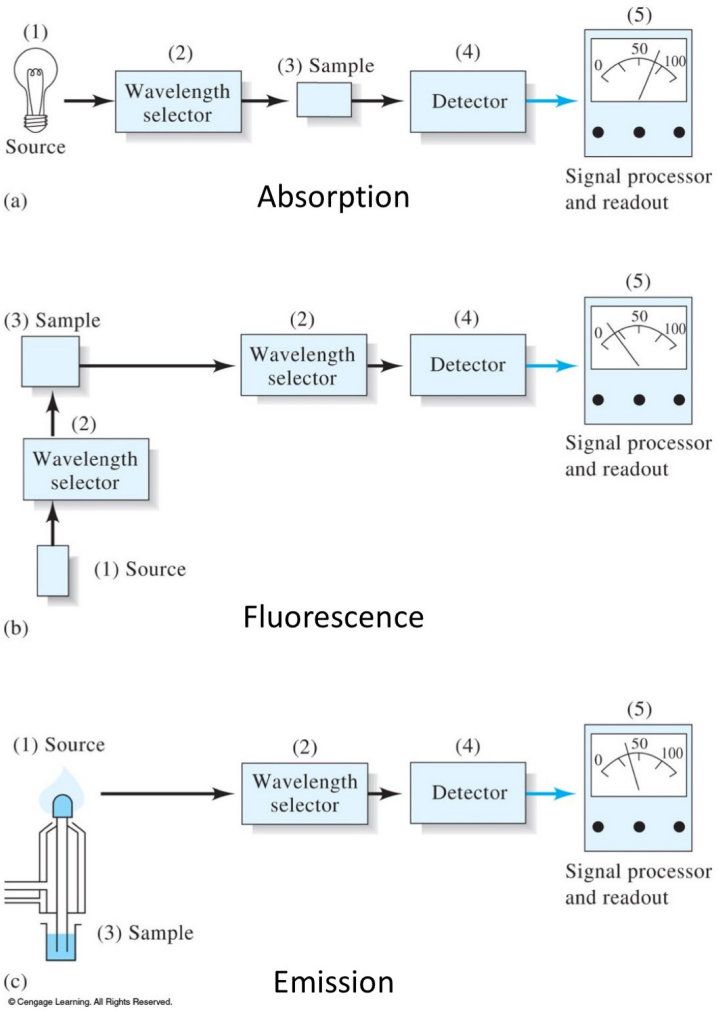


Fig. 1.4: Schematic spectrometry setup: Spectroscopic setups require light source, a certain interaction area, a wavelength selective element and a detector. (Source: Skoog, West, et al. 2014)

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.

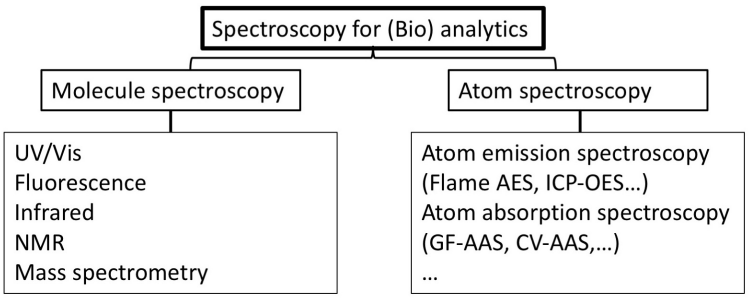


Fig. 1.5: Overview of spectroscopic tools used in bio analytics (Source: Gey 2008)

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**:

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

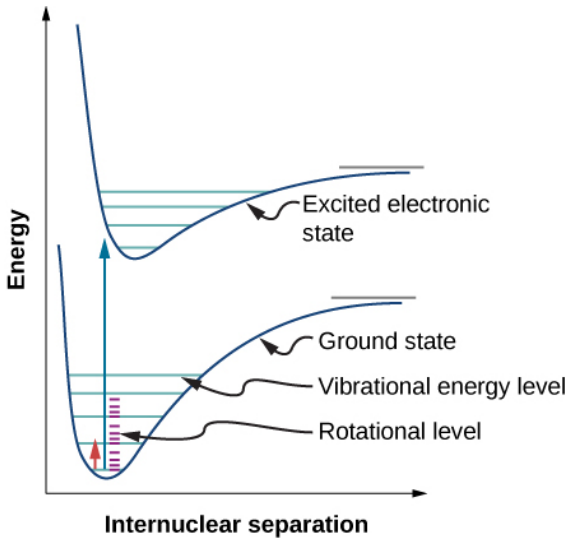


Fig. 1.6: Three types of energy levels in a diatomic molecule: electronic, vibrational, and rotational (Source: OpenStax University Physics, CC-BY 4.0)

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.

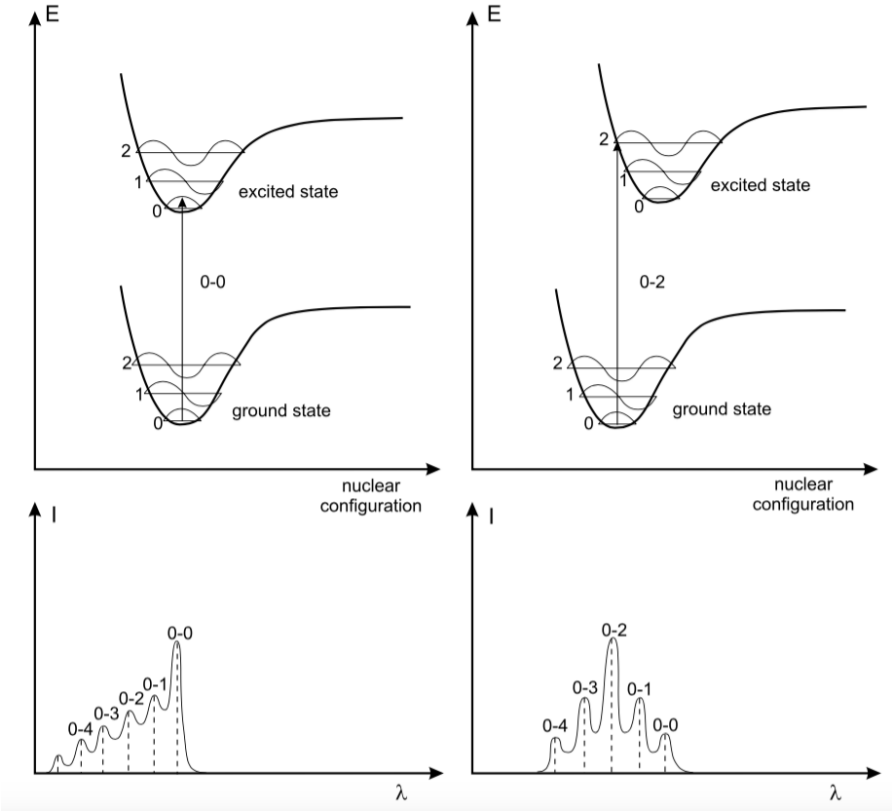


Fig. 1.7: Top: Potential energy diagrams with vertical transitions (Franck-Condon principle). Bottom: shape of the absorption bands; the vertical broken lines represent the absorption lines that are observed for a vapor, whereas broadening of the spectra is expected in solution (solid line). (Source: 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.

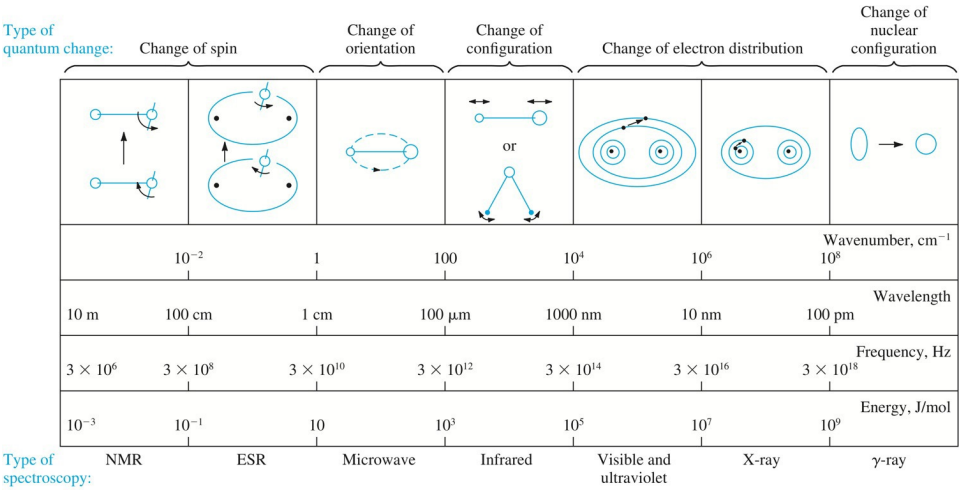
Luminescence comprises fluorescence, phosphorescence, photoacoustics and atomic emission. The fluorescence of matter that is irradiated with light in the UV/Vis range has the greatest significance.

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).

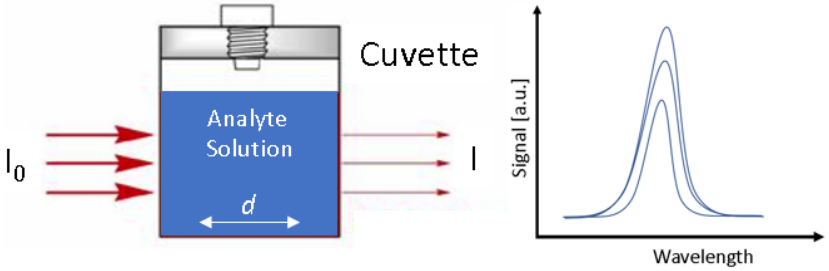


Fig. 1.10: Simplified scheme of a spectroscopic measurement which produces a quantitative usable signal

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).

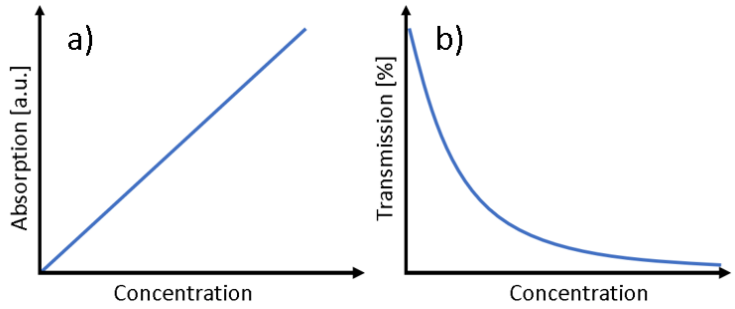


Fig. 1.11: Behavior of absorption and Transmission upon concentration according to Beer Lambert's Law

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

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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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