Liquid chromatography - absorption spectrophotometry of a mixture of aromatic compounds

D. Sýkora, J. Fähnrich., K. Hájková

Basic principles of the elution liquid chromatography

A liquid chromatography is one of chromatographic methods used for separations. A chromatographic column (made of glass, metal or plastics) filled with appropriate particles, **sorbent/stationary phase**, is used for the separation of compounds, **analytes**. The stationary phase is surrounded with liquid (**mobile phase**, **eluent**) flowing through the column. The sample is usually injected in a small volume and it enters the column in the form of a narrow band. As the mobile phase flows, molecules of the sample distribute between the stationary and the mobile phase in effort to reach thermodynamic equilibrium. The extend of the retention of the separated compound (retarded by the stationary phase) is characterized by a **retention factor *k***, as indicated below

$$k = \frac{n_s}{n_m} \qquad (1)$$

n_s and n_m represent amounts of substance (number of moles) present in a stationary and in a mobile phase at equilibrium, respectively. If the sorption isotherm of the solute distributed between the phases is linear, the retention factor *k* becomes a characteristic feature of the separated compounds and it is independent on sample concentration and flow rate. As the solute contained in a mobile phase flows through a column, it can be retarded by interactions with the stationary phase. The described process is characteristic by the continuous effort of the system to reach an equilibrium which is disrupted by the flow of the mobile phase. The band of a solute flowing through the column is usually slower than a velocity of the mobile phase. This relationship can be represented as the amount ratio of the compound in a mobile phase *versus* its total amount injected to the column, $n_m/(n_m + n_s)=1/(1+k)$. Thus, the movement of the compound through the column is mainly affected by the retention factor *k* of the compound. The smaller the retention factor *k*, the faster is the elution of the compound from the column (shorter retention time) and *vice versa*.

A mobile phase from a column goes to a detector. Here, a presence of an eluted compound is indicated when physical or physico-chemical properties of the mobile phase containing the analyte are changing. As a result, a visual output from a detector appears in a form of a **chromatogram**, which depicts a dependence of a signal detected *versus* time (see Fig.1).

Furthermore, the zero point in the chromatogram depicts an injection of a sample to a column, as well as both peaks in the chromatogram depict specific separated compounds. The peaks should have a Gauss curve profile under ideal conditions. The top of the peak corresponds with the **retention time** t_R, which is the time necessary for a compound to flow through a whole column (from the injector to the detector). Another important parameter is the **retention volume** V_R, which can be calculated from a retention time and a volumetric flow rate F_m.

$$V_R = F_m t_R \tag{2}$$

retention volume V_R is a volume of a mobile phase necessary to flow through a column to elute an analyte. A compound that is not retained by a stationary phase at all ($k = 0$) goes through a column with the same speed as a mobile phase and it is eluted as a first peak. Its corresponding retention parameters have index M (or zero) and they are called:

t_M – dead or void time

V_M – dead or void volume

The dead/void volume is approximately equal to a total volume of a mobile phase in a column.

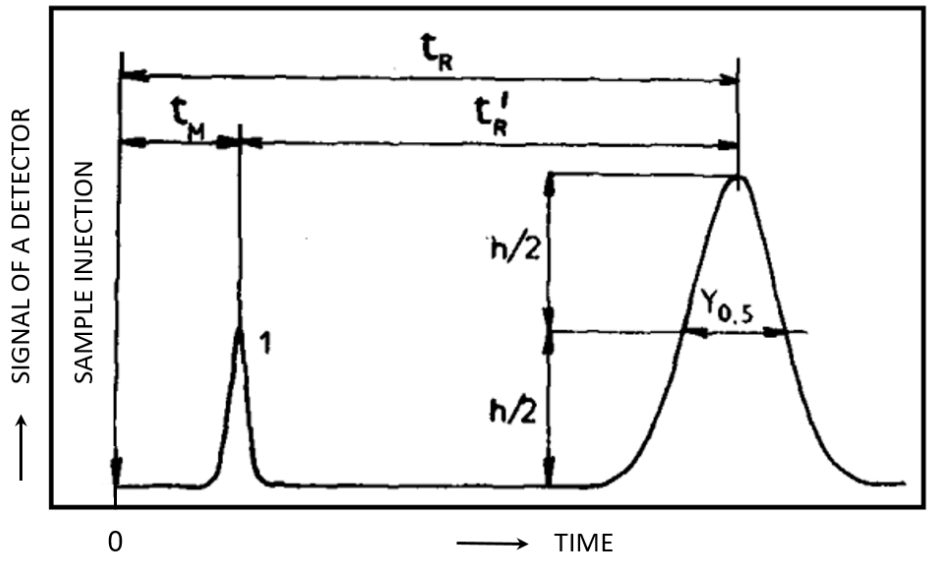


Figure 1: Parameters of a chromatogram (an elution curve). 1 - a non-retained compound

If a dead/void time/volume is subtracted from a retention time/volume the adjusted parameters are obtained (to differentiate adjusted retention parameters from retention parameters the comma is used) indicated **adjusted retention time *t′*** and **adjusted retention volume *V′***

$$t'_R = t_R - t_M \tag{3}$$

$$V_R' = V_R - V_M \tag{4}$$

The dead/void time is a time necessary for a mobile phase to flow through a column that has a length of *L*. Then, a linear velocity of a mobile phase *u* is

$$u = \frac{L}{t_M} \tag{5}$$

The speed of an elution zone of a compound with a retention time t_R is proportionate to a ratio $n_m/(n_m + n_s)$, so then it can be written as follows:

$$\frac{L}{t_R} = \frac{n_m}{n_m+n_s}u = \frac{1}{1+k}u = \frac{1}{1+k}\frac{L}{t_M} \tag{6}$$

The relation between retention parameters and a retention factor can be expressed as follows:

$$k = \frac{t_R-t_M}{t_M} = \frac{V_R-V_M}{V_M} = \frac{t_R'}{t_M} = \frac{V_R'}{V_M} \tag{7}$$

Retention parameters and/or retention factors can be used for the identification of analytes since they are characteristic for them within a given separation system. The method of the identification of a specific compound might be based on a conformity in retention parameters of that compound with a set of known standards (frequently composed of structural homoloques). Five basic aromatic standards with growing number of fused aromatic rings are used in the liquid chromatography for that purpose: benzene (number of aromatic rings z=1), naphthalene (z=2), phenanthrene (z=3), benz[*a*]anthracene (z=4), and benzo(b)chrysene (z=5). The definition of retention index method is based on fact that logarithms of a standard's adjusted retention time (volume) is proportional to a number of its aromatic rings *z*. The retention indexes of standards are defined as $I_z = 10^z$ (or $\log I_z = z$). To determine a retention index (its logarithm) of a compound A, it is necessary to find coordinates connected to its adjusted retention time logarithm $\log t_{R,A}'$ and to an axis $\log I$ (see **Fig. 2**). A linear interpolation (or extrapolation in some cases) is necessary within the calculation.

$$\log I_A = z + \frac{\log t_{R,A}' - \log t_{R,z}'}{\log t_{R,z+1}' - \log t_{R,z}'} \tag{8}$$

Where $t_{R,z+1}'$ and $t_{R,z}'$ are adjusted retention times of two consecutive standards with values similar to an adjusted retention time of a compound A (ideally, $t_{R,z}' < t_{R,A}' < t_{R,z+1}'$).

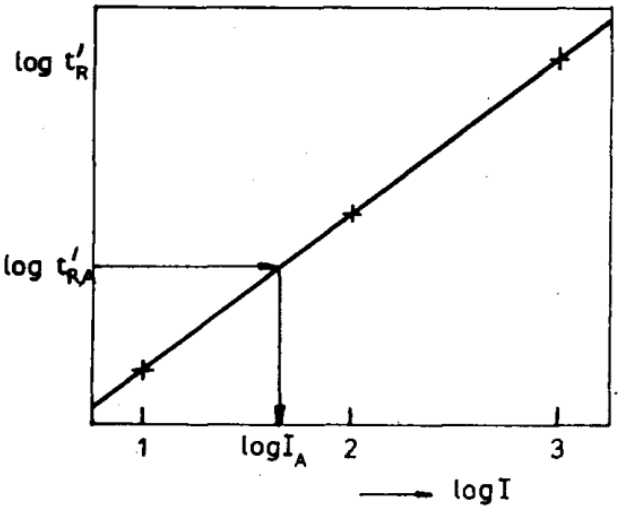


Figure 2: A calculation of retention indexes in a liquid chromatography

Different chromatographic columns packed with sorbent provide, in general, different elution zones, i.e. peaks with different widths are obtained. The narrower the peaks at the same conditions, the better the efficiency of the column. To evaluate an efficiency of a column, a parameter called **number of theoretical plates of a column** *n* (or shortly number of plates) can be used. For a given compound A, *n* can be expressed based on its retention time and its peak width at certain peak height (both has to be in the same units). A width at half height of a peak $Y_{0.5}$ (see Fig.1) is used most often

$$n = 5.54\left(\frac{t_R}{Y_{0.5}}\right)^2 \quad (9)$$

Number of theoretical plates of a column *n* is proportional to a length *L* of a column. Thus, to evaluate a quality of a column bed, a **height equivalent to a theoretical plate** *H* is often used.

$$H = \frac{L}{n} \quad (10)$$

Where *L* is a length of a column and n is a number of theoretical plates of a column. The efficiency of a column depends on the chromatographic conditions as for example, linear velocity of the mobile phase.

Basic principles of the absorption spectrophotometry in a visible and ultraviolet region

The method of the absorption spectrophotometry is based on the dependence of a sample absorption on the incident wave lengths and its ability to absorb ultraviolet and/or visible light.

As the beam of a monochromatic light goes through a sample (e.g., a solution of absorbing compounds in a cuvette – see Fig. 3), the incident radiant flux Φ_0 drops due to the absorption to a lower value transmitted radiant flux Φ. A ratio of both radiant fluxes (if reflexion and/or scattering losses are not considered) is called **transmittance** τ. The transmittance is not dependent on a quantity of an incident radiant flux.

$$\tau = \frac{\Phi}{\Phi_0} \tag{11}$$

A decimal logarithm of an inverse value of the transmittance is called an **absorbance** *A*:

$$A = -\log \tau \tag{12}$$

A dependence of the transmittance and/or of the absorbance on a wavelength is expressed as a **sample absorption spectrum**.

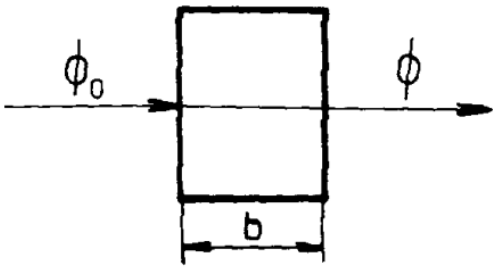


Figure 3: A sample transmittance measurement; Φ_0, Φ – incident radiant flux and transmitted radiant flux; b – a thickness of an absorbing medium

The absorbance of an absorbing compound in a solution is proportional to a mass concentration ρ and to an optical path length (a thickness of a measured substance) b. This relation is also called **Lambert-Beer Law**

$$A_\lambda = a_\lambda b \rho \tag{13}$$

Where a_λ coefficient is called an **absorption coefficient**. If the unit of a mass concentration is in $g.L^{-1}$ and the unit of a path length is in cm, then the unit of an absorption coefficient is $L.g^{-1}cm^{-1}$. If a concentration is expressed as $mol.L^{-1}$, then a **molar absorption coefficient** is expressed in $L.mol^{-1}.cm^{-1}$. The absorption coefficient is characteristic for each compound measured in a specific environment (solvent) and it depends on a chosen wavelength.

The maxima and minima at the wavelengths in an absorption spectrum, the shape of the absorption spectrum, and values of the absorption coefficients depend on a structure of the

compound. The energy of absorbed photons in UV-VIS is spent on a transfer of electrons from molecule valence shells to shells with a higher energy.

To absorb in UV-VIS region (a common region for measurements $\lambda > 200$ nm), the molecules have to contain valence shell electrons with a low energy level of the excitation. If the molecule does not contain functional groups with electrons of low excitation level (i.e., chromophore) then the molecule cannot absorb in that specific region. The examples are as follows: water, saturated hydrocarbons, alcohols, ethers, esters, acids, etc. Because of that, all such compounds are suitable as solvents for spectra measurements of compounds which are able to absorb in the specific region. Aliphatic ketones and aldehydes have negligible absorbance in a region about 280 nm. A significant absorption in the region of 220 nm is common for compounds with conjugated double bonds and/or for aromatic compounds with π electrons of low excitation level.

The relation between the compound structure and its absorption spectra is applicable in a compound identification and/or in identification of a chromophore. Based on the comparison with spectra of a standard compound, an unknown compound can be identified when the same conditions of the measurements are used.

To quantify an absorbing compound in a solution, the LAMBERT-BEER law (13) has to be applied. If the absorption coefficient at a specific wavelength a_λ is known for a specific cuvette path length b, then a concentration ρ can be calculated from the absorption readings at same wavelength. To avoid errors from imprecise readings or from minor contaminants, the wavelengths should be chosen at absorbance maxima.

In systems where more compounds contribute to a total absorbance, the concentrations of absorbing compounds from the solution can be calculated from each absorbance addition (if the compounds do not influence each other). For example, when the absorbance A_λ consists of the contributions of two absorbing compounds A and B, then the calculation is as follows:

$$A_\lambda = \left(a_{\lambda,A}\rho_A + a_{\lambda,B}\rho_B\right)b \tag{14}$$

where $a_{\lambda,A}$, $a_{\lambda,B}$ are the absorption coefficients and ρ_A, ρ_B are the mass concentrations of compounds A and B. The equation can be applied for an absorbance in a specific wavelength λ, nevertheless the coefficients have to be of the same wavelength. If two equations for absorbances at two different wavelengths are constructed (14) for a system with two compounds, and the absorption coefficients at these wavelengths are known, then the unknown

concentrations ρ_A, ρ_B can be calculated from the system of two linear equations. An example of how to calculate the system of two linear equations will be shown in following sections.
The first wavelength should be chosen so that one compound is in its absorption maximum and the other compound is in its minimum. That means, one compound contributes to a total absorbance significantly more than the other at the specific wavelength. Then the second wavelength should be chosen exactly opposite, i.e., the compound strongly absorbing at the first wavelength now should absorb only weakly or at all and the second compound should absorb much more. The application of the described approach brings accurate results.

Laboratory instructions

Liquid chromatography - absorption spectrophotometry for the analysis of aromatic compounds

The aim of this practical laboratory work is to **identify** and **quantify two** compounds/**analytes** in **a specific sample**. The sample consists of two aromatic compounds dissolved in methanol. The preliminary identification is based on the liquid chromatography separation, where the found retention times (more exactly, calculated retention indexes) of the analyzed compounds are compared with values in **Table II**. The individual compounds are then obtained in the chromatographic run where they are collected in volumetric flasks for subsequent analysis, the measurement of their absorption spectra. Collected UV-spectra are then compared with reference spectra in a catalogue of neat standards to verify the previous tentative identification (based on the retention indexes) of compounds. Besides the identification, the spectra of compounds are also used for the quantification purposes. The final task is to acquire absorption spectrum of the received sample (containing both the compounds). The obtained spectrum is used to quantify both the compounds and to check the previously calculated results obtained by LC analyte separation and UV-spectrophotometry of the separated analytes.

Tasks:

1. Check the correct functioning of a liquid chromatography system by separation of a methanolic solution of three reference standard compounds (a mixture of benzene, naphthalene, and phenanthrene).
2. Separate a diluted sample received by an assistant in the laboratory.
3. Separate a mixture prepared by mixing of a diluted sample with a methanolic solution of the three above mentioned standards.
4. Isolate and collect two compounds from the received sample. (Inject the concentrated sample and collect each compound separately to the volumetric flasks.)
5. Acquire UV-spectra of both the isolated compounds on a CARY 50 spectrometer. Acquire an absorption UV spectrum of the diluted (non-separated) received sample.
6. Process the results obtained (calculate the retention indexes of the analytes; determine characteristics of the liquid chromatography system; identify the compounds of the received sample using a comparison of the acquired and catalogue spectra; quantify the concentration levels of compounds from the acquired absorption spectra and recalculate their concentrations taking into account dilution factor before UV-spectrofotometry).

Instrumentation and equipment

Liquid chromatograph and how to use it

The liquid chromatograph is described in Fig. 4. In our laboratory practise, a reverse phase liquid chromatography is used. The column is filled with **Eclipse Plus C18** sorbent, which contains spherical particles of 5 μm size. The sorbent is made of porous silica gel, which is chemically modified by hydrophobic octadecyl chains' bonding. As a mobile phase, a mixture of methanol/water (75:25, v/v) is used. The retention volume/time of hydrocarbons grows with the number of carbons in a molecule.

An electronically driven proportioning valve of the liquid chromatograph blends a pure methanol and a pure water together so that the appropriate mobile phase is produced on-line. A HPLC pump provide flow rate 300 uL min^{-1}. The sample is introduced to a flow of the mobile phase through an injection loop in a sampler. First, the mobile phase flows to a pre-column with dimensions of 12.5 × 2.1 mm, and then, it continuous to a column of 50 × 2.1 mm filled with the same sorbent (thus, the overall length of the separation system is 62.5 mm). Each constituent of a sample creates an elution zone that moves through the column at specific speed. The speed of an elution zone is affected by a value of a retention factor *k* of a given compound. The eluted analytes are then detected by a photometric detector connected at the end of the column. A signal from the detector is acquired by a computer and processed in a chromatographic SW Clarity.

The photometric detection is based on the absorption of UV light by compounds dissolved in the mobile phase that elutes from the column. A deuterium lamp is used as a source of photons and the wavelength is set at 254 nm. A flow cell has an optical pathlength of 1 cm (and a total volume of 14 μL). A photodiode measures the UV radiation transmitted through the flow cell. The signal from the detector is equal to a light absorbed by a sample.

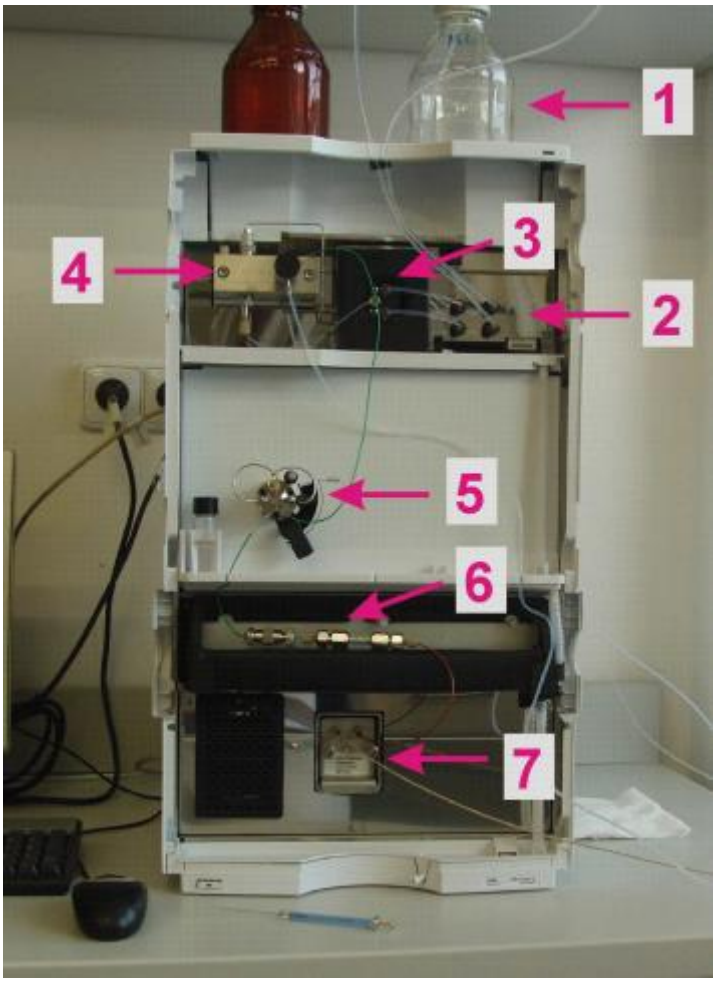


Figure 4. The liquid chromatograph

1 – mobile phase reservoirs, 2 – degasser of a mobile phase, 3 – proportioning valve for a mobile phase mixing (low-pressure gradient), 4 – high-pressure pump, 5 – injection valve (with sample loop), 6 – chromatographic column with a pre-column, 7 – detection cell in a UV-VIS detector

Chromatogram processing

The chromatography SW Clarity works under the operation system Windows. Saved data files/chromatograms can be retrieved whenever the user needs to process the data e.g., to obtain information on retention times, peak areas, and/or about a column efficiency. Just a small portion of the software features will be exploited in this laboratory work.

Switching on the chromatography system

Check the chromatography system and all its connections. Then, check if the chromatography system and the PC are switched off. Switch on the chromatography system (button placed bottom left), an orange LED diode (placed up right) will light up, what means that the instrument is in the stand-by mode. Switch on the PC. The Windows will be automatically loaded and a login dialog window will come up, user name will be automatically filled (Student) and for Password "Student" must be filled manually. Then switch on the Clarity SW by double-click on its icon. A scheme of a chromatograph will appear. Click on the scheme and a login dialog window will appear – "Enter user name", do not change anything and press "OK". As a result a several icons allowing for LC system control and operation will appear. There is also a sign "*No method sent*" and a next to it a green-white arrow. Click on the arrow and the status of the LC system will change from stand-by to operate, which means that a pump, a degasser and a deuterium lamp will be switched on. Then click on the icon "Device Monitor" in the main window. A new window showing an actual status of the instrument will appear. Then click on the icon "Single Analysis". A new window will be opened, where you can fill in the name of the incoming chromatogram. Fill only the "Chromatogram File Name", how to do that will be described thereinafter in chapter **How to create chromatogram names** in **Working instructions**. Let the other fields empty. Now, the system is ready for an injection of a sample.

Injection of a sample on a column and stopping the analysis

By moving a lever of the injector, an injector loop (20 μL volume) consisting of a stainless capillary is connected/disconnected from a flow-path of a mobile phase heading to a column. Move the lever to a position "Inject". Check, if there is a status "Waiting" in the main window of Clarity.

Draw 2 μL of a solution to a micro-syringe and insert the syringe needle into a middle port of the injector valve. By slight rotation, push the syringe through an inner insulation into the valve (finally, push the syringe gently to make sure that the syringe is fully inserted into the sampler port). Move the lever of the injector to a position "Load" and then press a piston of the syringe, so that you inject 2 μL of a solution to a loop. Then, move the lever back to a position "Inject". Now, a content of the loop is washed by a flow of the mobile phase to the column and the syringe can be pulled out of the valve. Wash the syringe several times with methanol and then, wash also the valve without moving the lever. The chromatography data start to be

acquired automatically when the lever is moved to a position “Inject”. It is possible to load and see the chromatogram *via* the main window of the Clarity, where the indicator change from “Waiting” status to “Running” status by clicking on icon “Acquisition”.

Important: Move the lever carefully but fast, because the flow of a mobile phase is partially blocked when the lever is moving. The flow interruption should be as short as possible.

The acquisition of data can be ended by clicking on “STOP”. However, there is also automatic auto-stop set at 8 min, thus, the analysis is stopped automatically at that time when not stopped by clicking “STOP” icon. As soon as the analysis is finished, a chromatogram can be automatically opened in “Chromatogram Window”.

The absorption spectrometer and how to use it

The CARY 50 spectrometer continuously records an absorbance of a sample in dependence on the wavelength in the range from 190 to 1100 nm. A source of light is a xenon lamp working in a pulse mode. The upper limit of a scanning speed is 24000 $nm.min^{-1}$. The instrument is fully controlled by a PC with an operation system Windows and a Cary WinUV software.

Double-click on a folder “Cary WinUV” that is on the PC screen. When the folder is opened, find an icon “Scan” and double-click on it. The program will be opened in a new window. Click on a “File” and then on “Open method”, choose a **Uvhplcmethod.MSW** from a folder C:\UVHPLC. Now, the spectrometer is prepared for operation.

How to manipulate with cuvette

Touch the cuvettes only at the opaque sides. Do not touch the quartz parts. The cuvette which you are planning to use has to be clean. Clean the cuvette just by a solvent rinse. Let the cuvette dry on a filter paper or (to save time) dry larger drops by a filter paper edge.

Fill and pour out the liquid from the cuvette over the opaque sides otherwise you contaminate the quartz parts.

Working instructions

1. Check the characteristics of the liquid chromatography system

Use a methanol for a cleaning of a syringe and a valve. Clean it several times. Set the flow at 0.3 mL.min^{-1}. Then, inject **2 µL of a mixture of standards** (benzene, naphthalene, and phenanthrene) by the Hamilton syringe. Acquire and save a chromatogram (runtime is approximately 6 min). You should see three main peaks of the used standards (benzene, naphthalene, and phenanthrene). Except of the peaks of the standards, there should be present one small peak (eluted as the first baseline blip/peak from the column) at approximatelly 0.65 min. This baseline blip corresponds to the column dead/void time and will be used in calculations of adjusted retention times of compounds. Wash the syringe thoroughly by a methanol. Inject the standards in 3 replicates. If the chromatograms are reproducible, continue with the next task.

How to create chromatogram names

Chromatogram file names should have the following structure:

date_initials of student name_name of sample_injection number

name of samples are:
standard solution (benzene, naphthalene, and phenanthrene) => **std**
diluted sample number **X** (X is number of the received sample) => **sampleXdiluted**
standard solution+diluted sample number **X** => **mixX**
concentrated (not diluted) sample number X => **sampleX**

1st example

On **10**/**02**/2020, student **K**arel **N**ovák injected a solution of standard (**std**) for the first time (**1**).

- ⇨ File name: **1002_KN_std_1**

2nd example

On **11**/**02**/2020, student **J**an **V**oska injected concentrated **sample** number **2** for the second time (**2**).

- ⇨ File name: **1102_JV_sample2_2**

IMPORTANT: Do not forget to use the number of the injection at the end of the file name.

2. Separate the diluted sample

Add approx. 0.5 mL of methanol by a burette and approx. 10 μL of a sample to a plastic microtube, mix a solution and inject **2 μL** to the column. Acquire a chromatogram. Wash a syringe thoroughly by methanol. There should be two main peaks and one small peak at approx. 0.65 min (a dead time) in the corresponding chromatogram. The sample should be injected at least twice.

3. Separate the mixture of the standards and the diluted sample to determine retention indexes of both the analytes

Add approx. 20 μL of the standard (benzene, naphthalene, and phenanthrene) and approx. 20 μL of the **diluted** sample (prepared according to step 2) to a microtube. Mix the solution and inject 2 μL of the resulting mixture into the column. If necessary, adjust concentration of the compounds in the mixture as needed considering peak heights in the resulting chromatogram (so that the peaks were of similar heights) and reinject.

4. Separate and isolate both the analytes in the sample

Inject 4 μL of the concentrated (not diluted) sample in the column. Check when your analytes are eluted and be prepared to collect them in the next repeated run. Save a chromatogram. Consider that a) concentrations of some analytes can be so high that a detector can be over-saturated (peak tops can be cut), and b) there can be contaminants in the sample, thus, more peaks than just 2 main analytes can be observed (the other peaks correspond to impurities and they should not be collected instead of the main compounds/analyes). When you are prepared for a preparative/isolation run, inject **4 μL** of the concentrated sample. Now, be prepared to collect each analyte separately to **volumetric flasks of 5 mL** volume. Save the chromatogram after you collect the analytes. Fill the volumetric flasks up to the graduation mark with methanol.

By this, the chromatographic part of the work is finished.

Take **10 mL volumetric flask** and fill it nearly to the mark with methanol. Then, inject **10 μL** of the concentrated sample by a Hamilton syringe under a surface of the liquid. Finally, adjust the volumetric flask up to the graduation mark with methanol.

Shake the content of all three volumetric flasks (filled up to the graduation mark with methanol) to make homogenous solutions.

IMPORTANT: When you finish this part, wash the syringe and **the injection valve thoroughly by methanol**.

5. Acquire the absorption spectra of the isolated compounds and the diluted sample

Place the quartz cuvette filled with methanol to the holder of the UV-spectrometer. Press a button BASELINE on the left side of the main window of the SCAN. The baseline will be measured.

Pour out methanol from the cuvette and fill it with a small amount (about 300 μL) of your first analyte collected within a chromatographic part in 5 mL volumetric flask (in step 4). Pour out the solution and rinse the cuvette again in the same way. Refill the cuvette to a half (about 1 mL) with the same solution. Place the cuvette to the spectrometer and press START. A directory window will come up for saving a new file. Choose a directory according to your assistant advice. Fill the "File Name" at the bottom part of a window according to instructions **How to name UV-VIS files** provided beneath. Save the name to a Clipboard (CTRL+C) and then press SAVE. Paste (CTRL+V) the name to the field "Sample Name" in the next window. Press the OK and a spectrum will be acquired. Apply the same procedure to measure the UV-spectrum of your second analyte and the sample in 10 mL volumetric flask (mix of both the compounds).

How to name UV-VIS files

The structure of the naming should be:

date_ initials of student name_name of sample_measurment number

the abbreviations of the measured liquids will be as follows:

1st collected peak/analyte (in 5 mL volumetric flask, labelled A) within the HPLC separation of the sample X (X is number of the received sample) => **sampleXA**

2nd collected peak/analyte (in 5 mL volumetric flask, labelled B) within the HPLC separation of the sample X => **sampleXB**

Diluted sample (in 10 mL volumetric flask) (containing both the analytes) number X => **sampleXC**

The examples of UV-VIS file names:

1st example

On **10**/**02**/2020, student **K**arel **N**ovák measured UV-VIS spectrum of a diluted **sample 1** (in 10 mL volumetric flask) for the **first** time.

⇨ File name: **1002_KN_sample1C_1**

2nd example

On **11**/**02**/2012, student **J**an **V**oska measured UV-VIS spectrum of **sample 3** of the first collected peak in chromatographic run for the **second** time.

⇨ File name: **1002_JV_sample3A_2**

6. How to process the results

a) Assign peaks (to standards and to unknown analytes in the received sample) in chromatograms acquired in steps 1-3. To select desired chromatograms, open a window "Chromatogram Window" and click on an icon "Open Chromatogram", where the chromatogram can be opened by a double-click. There is a possibility of overlaying several chromatograms in one window by activation a function Overlay by an icon "Overlay on/off". To determine retention times to calculate retention indexes, use preferably the chromatograms from Step 3, where the standards and analytes are eluted together in the same run. If the peaks are not integrated automatically, integrate them manually. To integrate peaks, use an icon "Add positive" on the left side of a chromatogram. By a mouse clicking, fix the start and the end of a peak. Save the changes by clicking on an icon "Save chromatogram". Results of your integration will be visible in a table bellow the chromatogram. The most important parameters in the table are: retention times and widths of peaks at a half of their height (w05). The retention times will be used in a calculation of the retention indexes. Compare the retention indexes calculated according equation (8) with values in **Tab. II** and tentatively identify your analytes.

b) To evaluate the chromatographic characteristics, use the last chromatogram from the Step 1. Sum up the information into two tables. The first table should contain: flow of a mobile phase (0.3 $mL.min^{-1}$), pressure on the column, dead/void time and dead/void volume. The second table should contain: the retention time and peak width at a half height of phenanthrene; corresponding calculated retention volume, retention factor, number of theoretical plates of the column *n*, and height equivalent to a theoretical plate *H* (a column length is 12.5+50 mm).

c) To unequivocally identify the analytes of the received sample, compare acquired UV-spectra of the separated and isolated analytes with the catalogue spectra (in a paper format

or in an electronic version). The electronic version of the catalogue spectra can be found in a folder C:\UVHPLC\kataloguvhplc. The identification can be considered correct only in case of very good matching of the acquired and the catalogue spectra, i.e., the maxima and minima in the spectra of the sample and in the catalogue have to match within 1 nm. Compare the results of the identification with related retention indexes found out within a liquid chromatography (above (step a)) and check if the tentative identification of the analytes provided the correct result. (The results obtained by UV-spectroscopy are decisive.)

d) To quantify concentrations of both the analytes in a solution after their identification read the absorbance value at maximum in the acquired spectrum of the first compound (move the cursor to an apex of a peak, read and write down a value of the absorbance and also the wavelength, which you will find at the bottom-right corner of the screen). Use the same approach for the corresponding catalogue standard in the PC spectra database. Beside the absorbance and the wavelength, the catalogue standard spectra also contain an information on the standard concentration. Based on the obtained data, the LAMBERT-BEER law (13) is used to calculate the absorption coefficient of a given compound at specific wavelength. The concentration calculations will be done as follows:

1. <u>The calculations related to the isolated fractions (two solutions in 5 mL volumetric flasks):</u> Use the spectra of the isolated compound. For calculations, use the LAMBERT-BEER law (13) at a single wavelength, which you chose at a suitable maximum within absorption spectrum.
2. <u>The calculations related to the mixture (the solution containing both the analytes in 10 mL volumetric flask):</u> Use the spectrum of the mixture of both the analytes and construct system of two equations (14). The system of two equations consists in equations for two suitable absorbances at two different wavelengths (left sides of the equations). An example of such calculation is provided below.

   For calculations, use wavelengths from the region above 230 nm, where the monochromatic light should not be subjected to inconsistencies (light dispersion) and the measurements should not be influenced by impurities from a sample (e.g., dissolved oxygen).

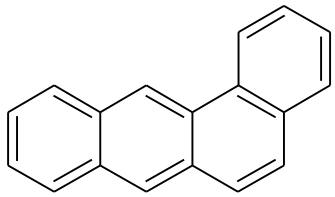
   In protocols, write down all used wavelengths, absorbances, concentrations from the spectra database, calculated absorption coefficients, absorbances from acquired spectra, and the constructed equation system.

Calculate the concentrations using both approaches (1. and 2., i.e., calculation based on isolated fractions and the mixture of the analytes), and consider dilution factor of the samples measured by UV-spectrometry. Finally, provide mass concentrations (in $g.L^{-1}$) for both the analytes **in the original sample**. Compare the results obtained for isolated fractions *versus* the mixture.

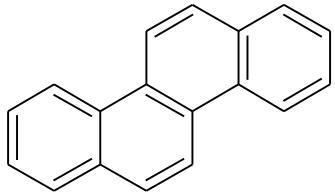
An example of how to calculate concentrations from the absorption spectra of the two-compound mixture:

Calculate concentrations of two aromatic hydrocarbon in a mixture,

benz[*a*]anthracene



chrysene



their absorption spectra are in Fig. 5a. The absorption coefficients for the calculations have to be taken from spectra of a benz[*a*]anthracene and of a chrysene (in the catalogue) in Fig. 5b.

Solution:

First, choose wavelengths, for which you intend to construct a system of two linear equations (14). Here, one suitable wavelength is 286 nm, as benz[*a*]anthracene has at this wavelength maximal absorbance and chrysene absorbs minimally. The concentration of a mixture at that wavelength is mainly dependent on a concentration of benz[*a*]anthracene. Second, choose a wavelength, at which a chrysene absorbs dominantly but a benz[*a*]anthracene minimally. Optimal wavelength is 267 nm, where chrysene has a maximum. Although the absorption

coefficient of a benz[*a*]anthracene at 267 nm is relatively high, the contribution of benz[*a*]anthracene to a total absorbance at other wavelengths is even higher. As a result, the calculations are subjected to very limited inaccuracy. Higher inaccuracies can occur at wavelengths, where absorbance of a compound is rapidly growing or decreasing. Because of that, it is better to avoid such wavelengths.

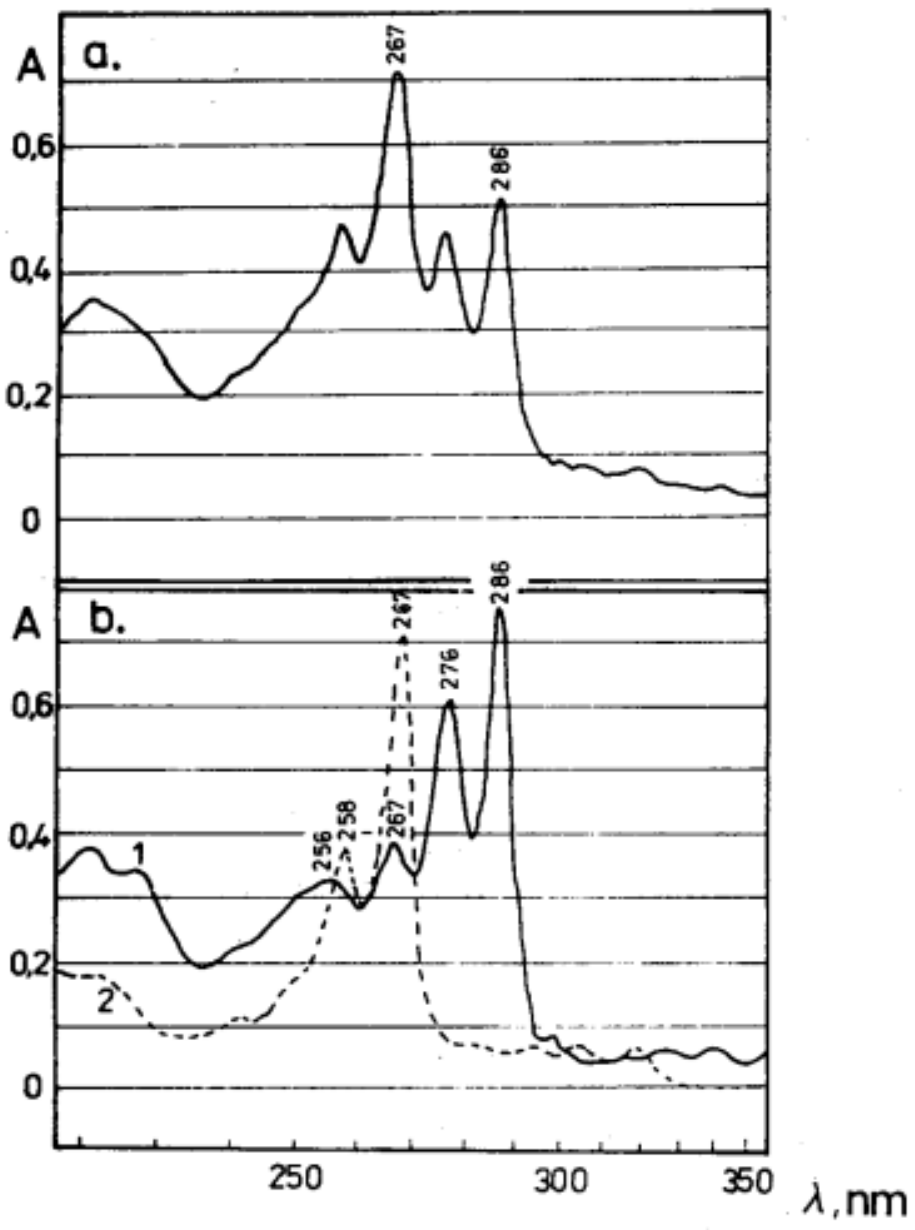


Figure 5:

A. Absorption spectrum of a mixture of a benz[*a*]anthracene and of a chrysene in a methanolic solution, a path length b=1 cm;

B. Absorption spectrum of a benz[*a*]anthracene and of a chrysene, a path length b=1 cm

1. Benz[a]anthracene, with a concentration 2.1 mg.l^{-1}
2. Chrysene, with a concentration 2.6 mg.l^{-1}

Read the absorbances of both compounds at chosen wavelengths – 286 nm and 267 nm – from the spectrum at Fig. 5b (values for benz[a]anthracene are 0.758 and 0.387, and for chrysene are 0.054 and 0.710). To calculate absorption coefficients from equation (13), use absorbances from above, concentrations (2.1 mg.L^{-1} for benz[a]anthracene, 2.6 mg.L^{-1} for chrysene), and a cuvette path length (1 cm). The final results are summarized in **Tab. I**.

Table I: Values of absorption coefficients for benz[a]anthracene (BA) and chrysene (CH) calculated from Fig. 5b.

λ, nm	A_λ, L.g^{-1}.cm^{-1}	
	BA	CH
286	361.0	20.8
267	184.3	273.1

To construct a system of equations (14), choose absorbances A (0.517 and 0.720) at same wavelengths as were used above from the spectrum of a mixture on Fig. 5a.

$$0.517 = 361.0\,\rho_{BA} + 20.8\,\rho_{CH} \qquad (15)$$

$$0.720 = 184.3\,\rho_{BA} + 273.1\,\rho_{CH}$$

The solution of the system of equations is as follows: a concentration of benz[a]anthracene $\rho_{BA} = 1.33\,mg.L^{-1}$ and a concentration of chrysene $\rho_{CH} = 1.74\,mg.L^{-1}$. Verify the results by a substitution of results to the original equations. Finally, do not forget that the original sample was diluted during its measurement, thus, **it is necessary to recalculate the concentrations of the found analytes to get the correct results**.

Table II:

Retention indexes of aromatic compounds (mobile phase methanol/water 75/25, v/v).

Compound	log I[a]	Molecular formula
Phenol	- 1,25	C_6H_6O
Indole	0,28	C_8H_7N
2-Nahtol	0,55	$C_{10}H_8O$
1-Naphtol	0,74	$C_{10}H_8O$
Benzene	1,00	C_6H_6
Benzo[b]thiophene	1,24	C_8H_6S
Carbazole	1,56	$C_{12}H_9N$
Naphtalene	2,00	$C_{10}H_8$
4-Azaphenanthren	2,03	$C_{13}H_9N$
1-Chlornaftalene	2,24	$C_{10}H_7Cl$
Acenaphtylene	2,27	$C_{12}H_8$
Biphenyl	2,59	$C_{12}H_{10}$
1-Methylnaftalene	2,61	$C_{11}H_{10}$
Acenaphten	2,92	$C_{12}H_{10}$
Fluorene	2,93	$C_{13}H_{10}$
1,3,5-Trimethylbenzene	2,93	C_9H_{12}
Diphenylenoxide	2,95	$C_{12}H_8O$
Dibenzothiophene	2,96	$C_{12}H_8S$
Phenanthrene	3,00	$C_{14}H_{10}$
Tetraline	3,04	$C_{10}H_{12}$
p-Isopropyltoluene	3,08	$C_{10}H_{14}$
Antracene	3,13	$C_{14}H_{10}$
Fluoranthene	3,45	$C_{16}H_{10}$
1-Ethylnaphtalene	3,51	$C_{12}H_{12}$
Pyrene	3,60	$C_{16}H_{10}$
3,3-Dimethyldiphenyle	3,64	$C_{14}H_{14}$
Triphenylene	3,86	$C_{18}H_{12}$
Benz[a]antracene	4,00	$C_{18}H_{12}$
Chrysene	4,18	$C_{18}H_{12}$
Benzo[e]pyrene	4,66	$C_{20}H_{12}$
Benzo[a]pyrene	4,71	$C_{20}H_{12}$
Benzo[k]fluoranthene	4,88	$C_{20}H_{12}$

Test questions

1. A value of the absorbance ***A*** of a sample that completely absorbs light (at given wavelength), is equal to

a. Infinity

b. 1

c. 0

2. A definition of a retention factor ***k*** of a compound

a. $k = \frac{t_R - t_M}{t_M}$

b. $k = \frac{t'_R + t_M}{t_M}$

c. $k = \frac{t'_R - t_M}{t_M}$

3. The absorption spectrum shows dependence of

a. Absorbance (or absorption coefficient) *versus* wavelength of the incident light

b. Transmittance *versus* cuvette path length

c. Absorbance *versus* concentration of an analyte

4. In this laboratory work, the identification of compounds by a liquid chromatography will be based on

a. Peak height

b. Retention volumes

c. Retention indexes

5. Why are the volumetric flasks used in this laboratory practice?

6. The separation of compounds by a liquid chromatography is based on differences in

a. Values of diffusion coefficients of the analytes in a mobile phase

b. Values of a boiling point of the analytes

c. Values of retention factors ***k*** of the analytes

7. The absorbance A is defined by a radiant flux Φ emitted by a monochromatic source that passes through a cuvette and by a comparing radiant flux Φ_0

a. $A = (\Phi_0 - \Phi)/\Phi_0$

b. $A = -\log(\Phi_0/\Phi)$

c. $A = -\log(\Phi/\Phi_0)$

8. A photometric detector in a liquid chromatography is dedicated to detect

a. A change in the pressure of a mobile phase in a cuvette

b. A change of the absorbance of a monochromatic light in a cuvette

c. A change of a mobile phase refractive index in a cuvette

9. The higher the retention factor ***k*** of a compound A, the

a. Faster the compound A flows through a column

b. Slower the compound A flows through a column

c. Column efficiency is better

10. **Number of theoretical plates** express

a. A compound retention on a column

b. A column efficiency

c. A size of a separation system

11. The Lambert-Beer law is defined as a dependence of the absorbance on

a. Concentration of an absorbing compound and wavelength

b. Transmittance and wavelength

c. Concentration of an absorbing compound, its absorption coefficient, and cuvette path length

12. In this laboratory practice, the following light source is used in absorption spectrometer CARY 50

a. A xenon arc lamp
b. Mercury-vapor lamp
c. A tungsten light bulb

13. In this laboratory practice, absorption coefficients used in concentration calculations

a. Are calculated from catalogue spectra information
b. Are calculated from spectra of isolated compounds
c. Can be found in tables of a manual

14. A retention volume of a compound A is

a. A volume of a solution, in which a compound A is injected to a column
b. A volume of a mobile phase, which flows through a column from the beginning to the end of a peak A
c. A volume of a mobile phase, which flows through a column from its beginning to the apex of a peak A

15. A **shape** of an absorption spectra of a compound is influenced by

a. A chemical structure of a compound
b. A cuvette path length
c. A concentration of a compound A

16. A non-retained compound is commonly used

a. To find suitable wavelengths of a detector
b. To find a total volume of a column
c. To find a dead/void volume" of a column

17. To quantify a compound in an absorption spectrometry, it is necessary to choose a wavelength, for which

a. A substance to be determined and an interfering compound have approximately equivalent absorbances
b. A substance to be determined absorbs strongly and an interfering compound does not absorb
c. A substance to be determined absorbs minimally

18. A value of the absorbance A of a sample, which does not absorb light, is equal to
a. Infinity
b. 1
c. 0