High-Performance Liquid Chromatography

1. Preparation of the eluents

- Prior to the experiment any solvent eluent used must be degassed on the water jet pump in an ultrasonic bath. Why is this necessary?
- By watching the degassing procedure, the difference between degassing and boiling should become evident. Why should boiling be avoided in this process?

The following solvents are used:
A: This flask in use should be filled up to 500 mL, as it is frequently used; flasks B, C & D should contain 250 mL each; H_2O from the in-house supply is used (WEK water).

A	100%	MeOH
B	85%	MeOH / 15% H_2O v/v
C	75%	MeOH / 25% H_2O v/v
D	65%	MeOH / 35% H_2O v/v

2. Operating the device

Before starting, you should thoroughly familiarize with the HPLC, and its individual components and their function.

Make sure that the solvent filter is covered with solvent at all times, and that the waste container is not full. This will prevent undesirable flooding (especially in regard to the health damages caused by organic fumes).
When solvent A is connected, the pump is preliminarily set to a flow rate of 0.5 mL/min and after a few minutes the flow rate can be elevated to 2.0 mL/min. Generally, the flow should not be increased in steps greater than 0.5 ml/min. Does the actual flow rate deviate from the nominal value (set value)? Measure the pressure at various flow rates (2.0; 1.5; 1.0; 0.5 mL/ min) starting with the lowest value (0.5 mL/min) and plot the pressure vs. flow rate. What are your observations?

Apart from the solvent supply, the pressure gauge should be watched. When using pure methanol at a flow rate of 1.5 mL/min the pressure will be about 1000 PSI (70 bar); with other solvents, the pressure will be higher. The tolerable maximum for the pumps in use is 3000 PSI (210 bar). Too high a pressure will indicate that the filter is plugged (inform supervisor!).

When there are strong fluctuations in pressure (mostly accompanied by irregular pump noises), there is air in the device. In this case the pressure valve must be opened immediately and pumping is continued, in order to remove the air. Moreover, one should be aware of leaks (formation of droplets).
Then the detector is switched on (put in action) and the system is equilibrated at a flow rate of 1.5 mL/min. Make sure that the base line is stable.

3. Determination of dead time and dead volume

For use in HPLC in principle, only syringes with an obtuse needle are employed. Please do not force the piston (pistons which are not easily movable indicate contamination) but shift it preferably with two fingers and pull it up the same way. When applying pressure on the piston from above it will break or be kinked, rendering it unusable (as another piston will not fit in).

Rinse the syringes several times with a convenient solvent, eventually with methanol, after using them.

In order to determine dead time and dead volume a 50 mg/L solution of thiourea in water is prepared and injected, using solvent A as the eluent. For this experiment, the following questions should be answered:

- How long is the retention time (dead time)?
- How large is the dead volume?
- Is the dead time and/or the dead volume dependent on the flow rate?
- Is the dead time and/or the dead volume dependent on the quantity of the thiourea solution injected?
- Is the dead time and/or the dead volume dependent on the thiourea concentration?
- What are the requirements regarding chromatographic behaviour and processability, which a compound must satisfy in order to qualify for determining dead time/dead volume?

4. Determination of column data

A test mixture consisting of toluene, anisole, and phenol is injected into the HPLC using solvents A, B, C and D (take care to switch off the pump each time you change solvent, or air

could be sucked in). The flow rate in each case is set to 1.5 mL/min. Identify the peaks by comparing retention times and check by injecting individual substances.
Determine the following:

- the capacity factor k‘ of each individual compound;
- the theoretical number of plates N of each compound;
- the height of a theoretical plate HETP of each individual compound;
- the resolution R for each couple of peaks for every possible combination.

Plot in a graph the decadic logarithm of the capacity factor log k‘ versus the volume fraction of methanol in the mobile phase Ø. Is a linear relationship evident? If so, determine coefficients a and b according to

$$\log k' = a\,Ø + b.$$

What do these values mean? Which properties of the individual compounds are *a* and *b* related to? What are the consequences if the curves of the two compounds intersect if plotted on the same graph?

After the measurements have been taken, the whole device must be rinsed for 15 – 20 min. with pure methanol. Why is this necessary? During the rinsing pressure and absorbance have to be watched.

5. Separation of additional aromatic compounds

In addition to phenol, anisol and toluene, benzaldehyde, nitrobenzene, nitrobenzaldehyde and 2-(3-nitrophenyl)-1,3-dioxolane are to be separated with a binary methanol/water eluent. Is it possible to get a baseline separation for all compounds with reasonable k' values (e.g. if the dead time is 1.5 min, a k' value of 20 means a retention time of 31.5 min)? Keep a chromatogram of the best separation and label it carefully with the chromatographic conditions (column length, diameter, type of stationary phase, flow rate, eluent composition).

6. Method development for two compounds

With the same binary eluent system, a chromatographic method is developed for the compounds nitrobenzaldehyde and 2-(3-nitrophenyl)-1,3-dioxolane, which will be educt and product in an organic synthesis reaction later. Method development in this case consists of choosing an adequate value for the volume fraction of methanol in the mobile phase Ø.

Prelab-questions to HPLC-experiments

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(1) Look up and draw the chemical structure of all substances you will be analysing during the HPLC experiments, i.e.

   a. phenol, anisol, toluene
   b. benzaldehyde, nitrobenzene, nitrobenzaldehyde and 2-(3-nitrophenyl)-1,3-dioxolane

(2) Colour the structures according to the T-SAR colour coding scheme and use it for predicting the elution order of your analytes.

(3) Inform yourself about the safety data of the substances you are working with (especially methanol!). A collection of material safety data sheets (MSDS) will be available in the lab.