JACOBS UNIVERSITY

LAB COURSE ANALYTICS

Handout Fall 2012 – **HPLC**
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Operating the device

It is important to wear safety glasses and lab.coat while working with the HPLC – System.

Inform yourself about the safety data of the substances you are working with (especially methanol and acetonitrile!)

A collection of material safety data sheets (MSDS) will be available in the lab

Before starting you should thoroughly familiarize with the HPLC and the 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. When solvent A is connected, the pump is preliminarily set to flow rate of 0.5 ml / min and after a few minutes the flow rate can be elevated to 1.0 ml/min.Generally, the flow should not be increased in steps greater than 0.5 ml/min.

Apart from the solvent supply the pressure gauge should be watched. When using pure methanol

the pressure will be about 1000 PSI (70 bar) ; with other solvents, the pressure will be lower.The tolerable maximum for the pump in use is 210 bar. Too high pressure will indicate that the column is blocked (inform the lab. Instructor. When there are strong fluctuations in the pressure there is air in the device. In this case also inform the lab. instructor.

Then the detector is switched on and the system is equilibrated at flow rate of 1.0 ml/min for at least 15 min. Make sure that the base line is stable. (Choose monitore baseline in the software to start your methode.)

Determination of column data

A test mixture consisting of caffeic acid , ferulic acid and sinapic acid is injected into the HPLC using the following solvent mixtures.

A. 100 % Methanol
B. 65 % Methanol / Water
C. 45 % Methanol / Water

For changing the solvent mixture you need to create a new method.

Column : Phenomenex C-18 – 250 mm length / 4,6 mm ID / 5 µm particle size

The flow rate in each case is set to 1.0 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

$$\text{Log } k` = a \, Ø + b$$

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 measurement have been taken , the whole device must be rinsed for 15-20 min. with pure methanol.

From here decide by yourself with solvent conditions to use to get almost baseline separation.

Determination of dead time and dead volume

For use in HPLC in princible, 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 pressure on the piston from above it will break or be kinked, rendering it unusable (as another piston will not fit in) .

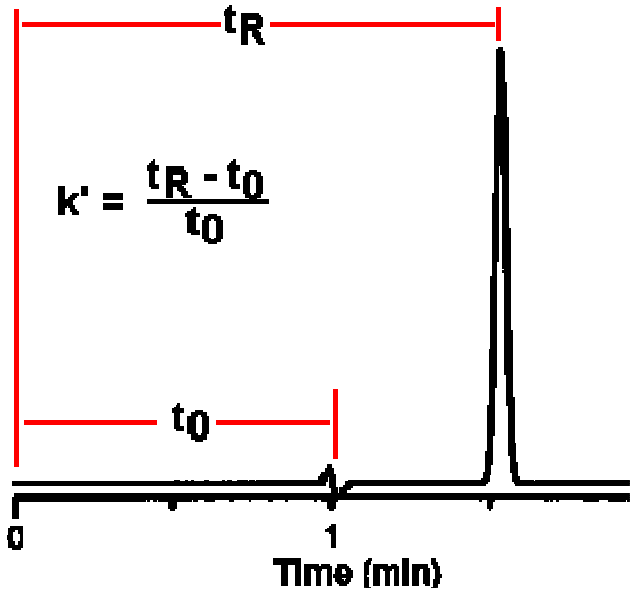
Rinse the syringes several times with convenient solvent, eventually with methanol , after using them.

In order to determine dead time and dead volume a prepared solution of 50mg/L thiourea is 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 or dead volume dependent on the quantity of the Aceton solution injected ?
- Is the dead time and/or the dead volume dependent on the Acetone concentration ?

- What are the requirements regarding chromatographic behavior and processability,which a compound must satisfy in order to qualitify for determining dead time/dead volumne ?

Calculations :



Calculations and Explenations :

The **dead time** **to** is the time required by an inert compound to migrate from column inlet to columne end without any retardation by the stationary phase. Consequently the dead time is identical with the residence time of the sample compound in the mobile phase.

The net retention time (t`r1 or t`r2) is the difference between total retention time and dead time. That is the time the sample component remains in the stationary phase.

t`r1 = t`r1 – t`o

Dead volume : Flow rate / Dead Time (in minutes.)

The **number of theoretical plates** **n** characterizes the quality of a column packing and mass transfer phenomena. Large values for n qualify the column to separate complex sample mixtures.

$$N = 5.54 \times \left(\frac{t_R}{w_{(50\%)}}\right)^2$$

The **capacity factor** **k`** is a measure of the position of a sample peak in the chromatogramm.
It is specific for a given substance. k` depends on the statinary phase , the mobile phase , the temperature , quality of the packing etc.

$$k' = \frac{t_R - t_0}{t_o}$$

The **height equivalent** of a theoretical plate h. HETP , is the length , in which the chromatographic equilibrium between mobile and stationary phase has been adjusted once.

HETP = L / n

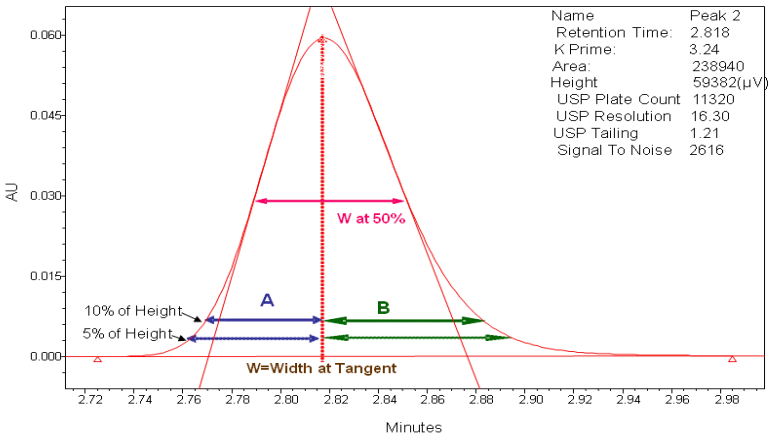


Figure 5: System Suitability measurements on a single peak

Influence of different number of plates in HPLC