HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING PHOTO DIODE ARRAY DETECTOR (HPLC-DAD) (2015)

Introduction
In all chromatographic experiments the components of a mixture are separated from one another by differences in their partition between a moving phase (in this case a liquid) and a stationary phase (in this case a solid). In contrast to standard bench top column chromatography where the mobile phase percolates through the stationary phase under the influence of gravity, the liquid phase is forced to flow under very high pressures through a much more densely packed solid phase. This results in much higher column efficiencies, i.e. better and faster separation of mixtures.

The partition of components between the two phases is strongly temperature dependent, a fact commonly used in gas chromatography where the column temperature is often varied in order to effect a separation. In liquid chromatography, however, the temperature is generally kept constant and changes in the partition ratios are obtained by changing the composition of the mobile liquid phase instead.

In this experiment a mixture of polyaromatic hydrocarbons (PAHs) are separated on a 4.6 x 250 mm column containing a 5 μm particle size non-polar solid (Eclipse XDB-C18, a resin with chemically bonded C18 alkyl chains) using a variable proportion mixture of polar solvents (acetonitrile and water). This is called reverse phase chromatography. The PAHs are weakly attached by Van der Waal’s forces to the C18 chains and are successively eluted as the organic nature of the mobile phase is increased. The material eluting from the column is detected using a photodiode array detector set to monitor a set number of selected wavelengths in the uv/vis range.

Procedure
Before beginning the analysis, the student will be given an overview of the different modules in the HPLC instrument and an introduction to the HPLC software.

For the analysis of the samples, the following parameters will be used:

- **Column**: Agilent ZORBAX Eclipse XDB-C18
- **Stationary phase**: ODS (octadecysilane), 5 μm particles
- **Volume flow rate**: 1 mL/min
- **Sample size**: 5μL
- **Mobile phase**: 70% acetonitrile/30% water to 100% acetonitrile in 15 min.
- **DAD wavelengths**: 254 nm, 270 nm, 285 nm

The three standards and two unknowns for analysis have been provided. Refer to the following...
table for the composition of the standards.

### PAHs Standard Solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/L) in acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard 1</td>
</tr>
<tr>
<td>Naphthalene*</td>
<td>100</td>
</tr>
<tr>
<td>2-chloronaphthalene</td>
<td>40</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>30</td>
</tr>
<tr>
<td>Anthracene</td>
<td>40</td>
</tr>
<tr>
<td>2,6-dimethylnaphthalene</td>
<td>50</td>
</tr>
<tr>
<td>9-methylanthracene</td>
<td>30</td>
</tr>
</tbody>
</table>

*Internal Standard

1) Inject 5 \( \mu \)L of Standard Solution 1 and analyze using 4110.m method. At the end of the run, with the aid of the spectra library supplied (black binder), identify the retention time for each PAH compound in the mixture. Repeat for the other two standards. This information will be used to identify the PAH compounds in the unknown solutions.

2) Select one standard solution to analysis in triplicate, i.e. run one of the standards twice more for a total of 3 runs. These results will be used in further calculations.

3) Analyze the two unknown samples for quantification of PAHs.

**Results**

1) For better visualization of the chromatogram, the stability of the baseline and the differences between the signals from monitoring three different wavelengths, print the chromatograms in merged format. Print one chromatogram per solution. Label peaks. [1.5 marks]

2) Determine the best results for each compound from the three detector wavelengths analyzed. Plot internal and external standard calibration curves using the best results for each compound. Show the regression analysis equations of the calibration curves. Include a summary data table for standards and unknowns of the compounds including retention times, wavelength selected, areas and ratios (as applicable). [2 marks]

3) Calculate the concentration of the PAHs in the two unknown solutions using the results from the standards for both internal and external standard calibration methods. Select the results from the same wavelengths as used for the standards. [2 marks]

4. For the triplicate analysis of the standard, calculate averages, standard deviations (N=3) and relative standard deviations of the retention times and the chromatographic areas. Tabulate and
discuss the results. [2 marks]

5. Discuss the differences between the internal and external standard calibration methods (theoretically and experimentally). [2 marks]

6. Mention at least one application of the analysis of PAHs by HPLC. Include the journal reference of the source (must be within the last 5 years) [0.5 mark]