Introduction:

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous components of diesel exhaust, wood smoke and cigarette smoke (see Finlayson-Pitts and Pitts, 2000). Many of them are mutagenic and/or carcinogenic and hence are of concern with respect to health effects of these emissions.
This experiment is designed to identify and measure as many PAH as possible in cigarette smoke. The HPLC has two different types of detectors, a photodiode array (PDA) and a fluorescence detector. It is preferable (but not essential) that you do the separate fluorescence experiment in Chem 152 on quinine in tonic water separately before doing this experiment. Absorption and fluorescence spectra for many of the PAH you will be measuring are on reserve in the library under this experiment.

**Experimental:**

**Setup Software Method for Run:**

1. Turn on the three lowest HPLC modules and the fluorescence detector. Refer to Chem 152 HPLC Operational Guide for schematic and instructions.

2. Load the software by double clicking on the "**Instrument #1 Online**" icon.

   **NOTE:** Software and file loading on this instrument takes a while, indicated by a blue “Busy” in the lower right corner of the screen. Do NOT perform any software options while this busy signal is on!

3. Check to be sure the degasser is on (second module from top of stack, button on bottom left). Visually inspect the solvent lines from the solvent bottles to the degasser to be sure there aren't any air bubbles in any of the lines. If there are, call your TA and ask them to purge the lines.

4. Go to the **Instrument Menu** and choose: **Instrument... System On.**

5. Follow the equipment instructions in the operational guide to set up the conditions for your run.

The software will store each run under sequential numbers in this directory, e.g. as "SIG10001, SIG10002" etc for the first and second runs. This means you need to be sure to keep a good lab book record of your runs. Even if you end up not using one, make sure you record it so you know what run number corresponds to what sample injection.

Load the method called "pah-245". Check to be sure the following conditions are shown in this method by clicking on the **Method** menu and then under that, on **Edit Entire Method**:

1. **Pump Set-up:**

   **Starting conditions:**
   
   Flow rate: 1.00 ml/min  
   Stop time: 20 minutes  
   Post time: off

   **Solvents and Gradient Timetable:**
   
   50:40:10 H₂O:acetonitrile:tetrahydrofuran
4 min: 50:40:10  H₂O:acetonitrile:tetrahydrofuran
18 minutes: 90:10 acetonitrile: tetrahydrofuran

This starts the solvent mixture with the 50:40:10 mixture which stays constant to 4 minutes into the run. Then it changes the solvent mixture using a linear gradient until it reaches a 90:10 mixture at 18 minutes.

The pressure limits should be set to 250 bar maximum and 10 bar minimum.

2. FLD Signal (this is the fluorescence signal)

Check to be sure the fluorescence detector is selected to be on during the run and is set for excitation at 245 nm and monitoring the emission at 359 nm.

3. The column thermostat is not controlled in this experiment.

4. DAD Signal (this is the absorption signal using the photodiode array, PDA)

Check to be sure the photodiode array detector (called "DAD" in this software) is set to monitor at 241, 252, 269, 289 and 297 nm with bandwidths of 10 (reference off). Both the UV and visible lamps should be checked.

5. Signal Detail:

Check to make sure everything is OK, i.e. start time at 0, end time at 20, delay at 0.

6. Remaining Sub-menus:

For the remaining sub-menus, just say "OK" for each one.

7. After you close the window, be sure to use "File...Save"

8. Now click on the "RunControl" menu at the top, click on “Sample Info”. In the “subdirectory” box, type the date in the format 06JAN98. Then click “RunMethod” and click “OK” when prompted to create a new directory. You should see at the top left of the screen in bright pink a message that says “Run in progress, waiting for injection”. When you load the injector and then turn the valve to the Inject position, the run is now started automatically using the method you loaded and saved.

**Preparation and Initial Chromatography of Standards:**

1. Make up 20 mls of a 1:1 methylene chloride-methanol mixture, using the HPLC grade solvents provided. Clean the 10 µL HPLC syringe three times with the methylene chloride (CH₂Cl₂)-methanol mixture to be sure that you do not contaminate the reference mixture (NOTE: THESE ARE NOT INTERCHANGEABLE WITH GC SYRINGES!! DO NOT USE A GC SYRINGE HERE).
2. Make sure the injector is in the **LOAD** position. Use the 10 µL HPLC syringe to repeatedly inject 1:1 CH$_2$Cl$_2$-CH$_3$OH solvent. Watch the outlet of the stainless steel overflow tubes projecting out of the rear of the injector and keep injecting solvent until you see drops forming on the end of the overflow tube. This sweeps out the injector loop.

3. Inject the solvent by rotating the injector handle clockwise as far as it will go. Do this in a smooth, rapid motion so you get injection of the sample as a plug onto the column. Use "View...Online Signals" to watch all 6 signals. When the baseline on the DAD signals is stable, use “Balance” for **ALL** signals to “zero” the PDA. Once this is done, stop the run using **Run Control**...“Stop Run/Inject” sequence. **DO NOT USE THE “ABORT” COMMAND**... **THIS WIPES OUT ALL OF THE DATA TAKEN DURING THE RUN!** Do not return the lever to load until the run has finished.

**WARNING:** Make sure the injector handle is moved fully over as far as it will go and the same when you move it back to the **LOAD** position. **DO NOT LEAVE IT HALF WAY BETWEEN THE TWO POSITIONS!!**

4. You are provided a mixture of 16 PAH to use for identification and calibration purposes. However, it must be diluted first. To do so, measure 60 µL of the standard mixture into a clean vial. Use the larger syringe to add 1.0 mL of the 1:1 CH$_2$Cl$_2$-CH$_3$OH solvent. Put the vial cap on and swirl well to mix.

5. Fill the sample loop by injecting 10 µL of your diluted standard. Start the run by rotating the injector handle clockwise as far as it will go. Do this in a smooth, rapid motion so you get injection of the sample as a plug onto the column.

6. Use "View...Online Signals" to watch the peaks as they come out. Once the run is done, switch to the “Data Analysis” screen, “View” and “Data Analysis”, and take note of the file number of your run. Print the chromatograms using **File, Print, Selected Window**. You should get a printout with six chromatograms on one page (5 absorption plus 1 fluorescence).

7. Repeat all of this using the pah-263 method. This method has the same PDA monitoring but measures the fluorescence at 371 nm which has been excited at 263 nm. Print as above.

8. Repeat all of this using the pah-288 method. This method has the same PDA monitoring but measures the fluorescence at 405 nm which has been excited at 288 nm. Print as above.

9. **IMPORTANT:** Now clean the loop by repeatedly injecting clean solvent mixture until you see drops forming at the outlet of the overflow line.

**Data Analysis and Discussion:**

Use the PDA chromatograms to identify each PAH by comparison to the chromatogram (attached) provided by the manufacturer of the HPLC column, which was obtained using UV absorption at 254 nm. The order of elution of the compounds will be the same as shown by the
manufacturer of the column, but note that your standard does not contain all of the compounds shown in the manufacturer’s chromatogram (it shows 19 compounds, your PAH standard contains 16). Then by comparing your fluorescence chromatograms to the DAD, identify the species which are fluorescing.

In your lab report, discuss why the chromatograms using the PDA at the 5 different wavelengths look different, i.e. the peak heights for individual compounds differ. Also discuss why the fluorescence chromatograms using the three different excitation-emission combinations are different from each other and from the DAD.

**Calibrations Using Standard PAH Mixture:**

1. Measure 50 µL of the diluted standard into a vial. Prepare four different vials this way. To the first add 50 µL of the 1:1 solvent mixture (CH₂Cl₂:CH₃OH). To the second, add 100 µL, to the third 150 µL and to the fourth 250 µL. Put the lids on the vials and swirl to mix.

2. Using the pah-288 method, analyze each of these as you did for the undiluted mixture. (But don't use the other two methods, pah-245 and pah-263 on these).

3. Integrate all visible peaks for the 289 nm absorption signal and the FLD signal. Instructions for manual integration can be found in the operational guide in lab.

**What to print:** For each injection, print the set of six chromatograms (five absorbances plus the FLD signal). The 289 nm absorption signal and FLD signal should have all observable peaks integrated. You will have to make sure that the areas are legible, i.e., not overlapping, or take careful note of them.

**Data Analysis and Discussion:**

1. Plot the absorbance (manually integrated peak area) at 289 nm for the peak you identified as being due to benzo[a]anthracene as a function of the number of nanograms of this compound which were injected, and carry out a least squares analysis. (Hint: You need to know the volume of the injection loop on the HPLC.)

2. Calculate the limit of detection for benzo[a]anthracene using absorption by estimating the minimum peak area you could detect on the chromatogram and calculating how many ng of benzo[a]anthracene to which this would correspond.

3. Plot the fluorescence signal (peak area) for the peak you identified as being due to benzo[a]anthracene as a function of the number of nanograms of this compound which were injected, and carry out a least squares analysis.

4. Calculate the limit of detection for benzo[a]anthracene using fluorescence by estimating the minimum peak area you could detect on the chromatogram and calculating how many ng of benzo[a]anthracene to which this would correspond.
Preparation of Cigarette Smoke Particle Sample

1. Place the funnel with the fritted disc upside down over the cigarette, which is clamped to a retort stand. Using the Tygon tubing, connect the funnel to the vacuum line and turn the vacuum on.

Measure the length of the cigarette. Then light it using the rubber pipette bulb and let the smoke be drawn through the filter until about 20-30% of the cigarette has burned. Then extinguish the cigarette using a beaker filled with water. Turn off the vacuum before removing the filter funnel. Measure the fraction of the total burnable length of the cigarette which has been consumed so you can convert your results to amounts per cigarette.

2. Using the filter flask, wash the cigarette residue collected using 1 ml of the 1:1 solvent mixture, collecting the solvent into a vial. Use the Acrodisc filter to refilter this solution by drawing it up into the large glass syringe, attaching the Acrodisc filter and then forcing the solution through the filter into a vial.

3. Evaporate the sample to dryness in the hood using a gentle stream of He or N₂ directed into the vial. Add 50 µL of the 1:1 solvent and swirl to mix well.

NOTE: The sample will be deposited along the sides of the walls of the vial as well as on the bottom. To be sure you get all of it into solution, dispense the solvent along the walls of the vial to wash it into the bottom.

Analysis of the Sample:

1. Load and check the method pah-288.

2. Inject 10 µL of this sample into the HPLC and record the chromatogram using both the PDA and the fluorescence detector using the method pah-288. If your fluorescence spectra "top out", i.e. are so strong that the peaks are cut off at the top, quantitatively dilute it and reanalyze. You will have to estimate how much to dilute it by based on how much the fluorescence signal has been cut off.

By comparison to both the absorption and fluorescence spectra and retention times obtained for the standard solution, identify as many of the PAH in cigarette smoke as you can.

3. IMPORTANT: Clean out the loop by injecting the 1:1 solvent mixture repeatedly until the overflow is clear, not yellow. Do this with the injector in BOTH the Load and the Inject positions. That is, flush the loop while the injector is in the Inject position while the sample is running. Then switch the injector to the Load position and flush the loop thoroughly.

What to print: Print out a full page FLD signal chromatogram of your cigarette sample with all observable peaks integrated. This is done by clicking All Loaded Signals from the upper left
area of the screen, then choosing \textit{FLD signal}. Also, print out a full page chromatogram of the 289 nm absorbance chromatogram of your cigarette sample with all observable peaks integrated. Finally, print out one page of all signals (five absorbances plus the FLD signal) for identification of peaks.

\textbf{Data Analysis:}

1. Use your calibration from above for the fluorescence to calculate how many ng of benzo[a]anthracene are in the smoke sample. Then using the fraction of the cigarette you burned, convert this to the number of ng of benzo[a]anthracene per cigarette.

\textbf{NOTE:} The PAH fluorescence peaks are on top of a broad “hump” of junk from the cigarette. You need to draw a baseline across the bottom of the individual peaks, along the top of the hump and use the peak heights or areas from that baseline rather than from the true “zero”.

2. Pick the peak due to benzo[a]anthracene using the 289 nm absorbance and calculate, based on your calibration for this, how much was in the portion of the cigarette you burned. Then using the fraction of the cigarette you burned, convert this to the number of ng of benzo[a]anthracene per cigarette. How does this compare to the results you obtained using fluorescence?

3. Identify as many PAH in cigarette smoke as you can using the five absorbances and the fluorescence signal.

4. Choose one additional PAH that can be identified in your fluorescence chromatogram of the cigarette sample as well as in your calibration solutions. Plot the integrated FLD peak area for the peak you have identified as a function of ng of this compound injected. Then use this calibration curve to calculate ng of the compound per cigarette.

\textbf{Issues to Discuss in Your Report:}

1. Compare the relative peak intensities of the PAH for the five different wavelengths used in this experiment. Explain, based on the absorption spectra, why the highest peaks are observed for each wavelength chosen.

2. Why are the relative peak intensities for each of the PAH you observed with the calibration mixture not the same when you changed methods from pah-245, pah-263, and pah-288? Choose at least two peaks to \textbf{quantitatively} illustrate your answer.

3. Use your calibrations to calculate how much more sensitive fluorescence detection is compared to the absorbance measurement; express this as a factor, e.g. it is twice as sensitive etc. Show all reasoning clearly.
**Extraction of PAH with surrogate and internal standards on Bondapak C18**

- **Column:** Bondapak C18
- **Dimensions:** 125 x 3.2 mm
- **Part Number:** 08E-3029-R0
- **Mobile phase:** Equilibrate column with 50:40:10 H2O:CH3CN:THF, run isocratic under this condition for 4 minutes following injection. Then run a linear gradient to 90:10 CH3CN:THF over 14 minutes (elapsed time 18 minutes).
- **Flow rate:** 1 mL/minute
- **Detection:** UV at 254 nm

**Sample Peaks:**
- Extracted component PAH mixture plus 2 surrogates and 1 internal standard (EPA 8330) &
- **Naphthalene**
- **Acenaphthylene**
- **Acenaphthene**
- **Fluorene**
- **Phenantrone**
- **Anthracene**
- **Fluoranthene**
- **Pyrene**
- **Benz[a]anthracene**
- **Chryene**
- **Benzo[b]fluoranthene**
- **Benzo[k]fluoranthene**
- **Benzo[a]pyrene**
- **Benzo[h,k]fluoranthene**
- **Indeno(1,2,3-cd)pyrene**

**Surrogate standards, Internal standard**