High Performance Liquid Chromatography

HPLC MEASUREMENT OF POLYCYCLIC AROMATIC HYDROCARBONS IN CIGARETTE SMOKE

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INTRODUCTION

Even though cigarette smoking is stated as a known health hazard by the Surgeon General, it still remains a problem in America, and is actually on the rise in many foreign countries. The addictive nature of nicotine in tobacco is well known, as is its link to respiratory disorders, such as emphysema, due to tar and other compounds. The increased risk of cardiovascular problems from smoking has helped make heart disease the number one killer in the United States. There are even radioactive species within tobacco smoke. But the major health concern due to smoking for most people is the increased risk of cancer from the carcinogenic compounds present, and these are what will be monitored and discussed in this experiment.

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous components of diesel exhaust, wood smoke and cigarette smoke.[4] Many of them are mutagenic and/or carcinogenic. This experiment is designed to qualitatively identify as many PAH as possible in cigarette smoke using high performance liquid chromatography (HPLC). A particular focus is placed on quantifying benzo[a]anthracene because it is well-resolved in HPLC analysis.

The HPLC is equipped with a diode array detector (DAD). Absorption spectra for the PAH to be measured will be compared to standard spectra for identification and are also on reserve in the library under this experiment. Quantitatively, the amount of benzo[a]anthracene in a cigarette will be determined, using a set of standards for comparison. Solutions containing known concentrations of PAHs of interest will be used as standards.

BACKGROUND

I: General Description of Chromatography

Chromatography is the most powerful and widely-used separation technique for complex mixtures. All chromatographic methods use a *Stationary Phase* (solid or liquid) and a *Mobile Phase* or *Eluent* (gas or liquid) that carries the analyte through the *column*. The nature of the mobile phase determines the category of chromatography:

       1) Liquid Chromatography (LC) uses a liquid mobile phase

       2) Gas Chromatography (GC) uses a gaseous mobile phase

Traditionally, in Column Chromatography, the stationary phase is attached to small silica beads that are held in a column, which is essentially a long narrow tube. The mobile phase is forced through the column using pressure. This particular lab uses High Performance Liquid Chromatography (HPLC), sometimes called High Pressure Liquid Chromatography. In HPLC, pressures up to several hundred atmospheres can be used to force the mobile phase through the column in order to achieve chromatographic runs in reasonable times.

The HPLC instrument in our lab uses *reversed-phase partition chromatography*, in which the stationary phase liquid is nonpolar and the mobile phase liquid is polar. The stationary phase is usually a packing made of porous silica particles with a nonpolar liquid coating chemically bonded to them (commonly siloxanes). The mobile phase is commonly a mixture of solvents that can be combined in certain ratios to give an eluent with the desired effective polarity.
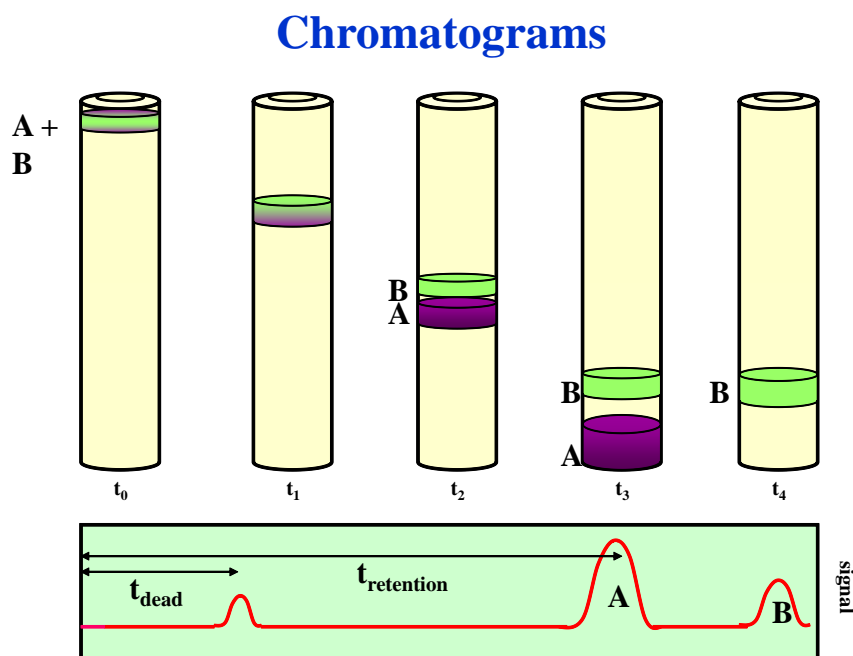
II: Chromatograms

Chromatograms show the peaks of each component vs. elapsed time traveling along the column (Figure 1). The migration rates of analytes down a column differ based on the time spent in the stationary phase vs. the mobile phase, allowing separation of the components in a mixture. If the mobile phase is polar, then the more polar the analyte, the faster it is eluted due to it having greater attraction to the mobile phase than the nonpolar stationary phase.

The *retention time* (time for an analyte to pass through the column) allows for qualitative identification and peak areas allow for quantitative analysis. Each molecule will have a unique retention time in a given column and for a given solvent. In the sample chromatogram shown in

Figure 1, two different molecules A and B have distinct retention times, t_A and t_B. Dead time, t_{dead}, is the time it takes for an unretained (solvent) molecule to go through the column.

Figure 1: Schematic representation of separation of two compounds in an HPLC column and the resulting chromatogram.

A key factor in chromatography that determines the rate of migration of a solute down a column is the *Partition Ratio* (or Partition Coefficient, K), which is the ratio of the concentration of the analyte in the stationary phase to the mobile phase ($K = c_s/c_m$). The larger the value of K, the longer the solute resides in the stationary phase, and thus the longer it takes to go through the column.

The peaks tend to broaden as they pass along the column, decreasing the column efficiency and resolution. The resulting peaks have approximately Gaussian shapes, which arise from random motions of molecules as they migrate down a column, passing in and out of the stationary phase, leading to a range of elution times for each analyte.

III: Column Efficiency and Resolution

The ability of a particular column to give distinct, separate peaks for each analyte in a reasonable period of time is based on column efficiency and resolution. Two main factors affect the efficiency and resolution of a column: The Partition Coefficient, K, (covered in Part II) and the *Selectivity Factor*, α, which relates to the relative migration rates between two solutes, A and B, providing a measure of how well the column separates A from B:

$$\alpha = \frac{K_B}{K_A} = \frac{(t_B - t_{dead})}{(t_A - t_{dead})}$$

There are several experimental factors that can be altered to increase column resolution and/or efficiency, as summarized below:

1) Decrease the flow rate of the mobile phase.
2) Alter the composition of the mobile phase
3) Increase the surface area of the stationary phase.
4) Use a smaller column diameter.
5) Increasing column length

To increase the resolution between peaks, the easiest way is to increase the length of the column. Unfortunately, this may lead to elution times that are too long and the peaks become too broad (this is called the General Elution Problem).

The solution for this is to experimentally alter the mobile phase composition as elution takes place via *gradient elution* or solvent programming. Once the faster moving components are eluted, the mixing ratio of solvents used in the mobile phase is altered to maximize the resolution and elution rate of the next components, and so on.

IV: Qualitative and Quantitative Analysis

Chromatography by itself is great if the components of the mixture are already known, and it is used to test for the presence or absence of these species, but for identification of unknowns in a mixture, the exit of the chromatographic unit must be linked to a suitable detection technique. In HPLC, the most common detectors are UV absorption and fluorescence.
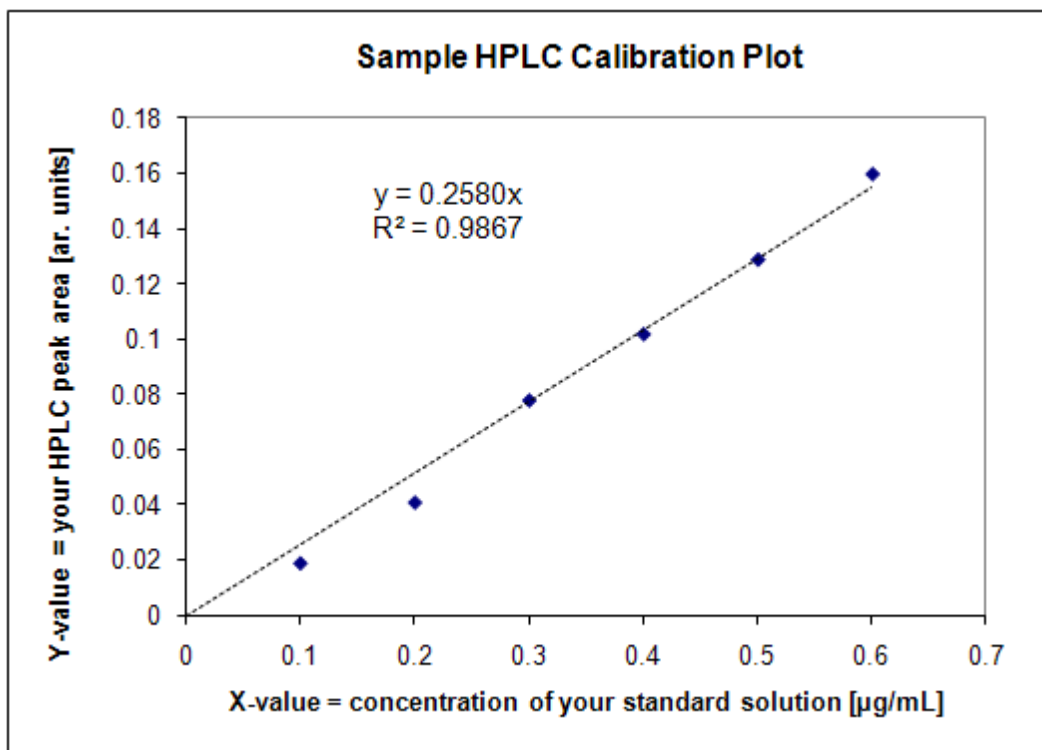
The premise for the quantitative analysis in HPLC is that the area under the peak of the eluting compound is directly proportional to the concentration of this compound:

$$peak\ area = slope \times concentration + intercept \qquad (y = mx + b)$$

The peak areas can be measured using automatic peak integration software. The calibration slope for the compound of interest is usually determined by graphing the HPLC response to standard solutions against the known concentrations of those solutions. For increased precision, not just one but several standard solutions are injected in the HPLC, and the slope and intercept values are determined from a least squares analysis as shown in Figure 2. Unknown sample concentrations can then be determined from this calibration curve.

$$concentration = (peak\ area - intercept) / slope$$

Figure 2: Sample calibration plot for an HPLC quantitative experiment. In your calibration, you will be using three standard solutions with different concentrations. Therefore, your calibration plot will have three points instead of the six shown here. In the sample calibration, the intercept value is zero.

Sample HPLC Calibration Plot

$y = 0.2580x$
$R^2 = 0.9867$

X-value = concentration of your standard solution [µg/mL]

Y-value = your HPLC peak area [ar. units]

EXPERIMENTAL

Note: An overview of the instrument (Figure 3) and safety instructions will be provided by your T.A.. More specific instructions for operation of the HPLC instrument and data analysis are provided in the CHEM 152 HPLC Operational Guide, which is always near the instrument.

Figure 3: Schematic diagram of an HPLC instrument and an LC injector.
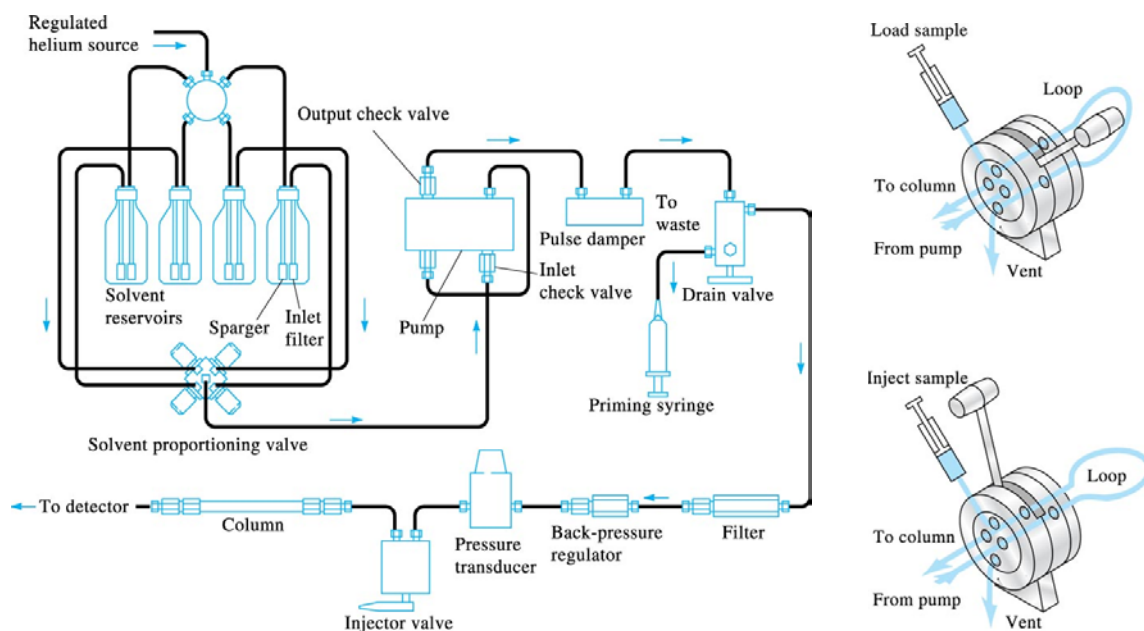
Part I: Instrument Set Up

1. Unless they are already on, turn on the four lowest HPLC modules (Degasser, Pump, ColComp and Diode Array Detector). Then turn on the computer and printer (if not already on). Refer to the Chem 152 HPLC Operational Guide for schematics and instructions.

2. Check to be sure the degasser is on (first 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.

NOTE: The solvent bottles should be at least ¼ full and there should be no leakage of solvent anywhere in the system. Make sure the switch lever of the Injector is in the LOAD position.

3. If already in the ChemStation program, load the appropriate software by double clicking on the "**HPLC 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! The program expects to interface with a fluorescence detector, which we are not going to use, so click "OK" when the software says it cannot find the FLD detector.

4. Go to *Instrument* and then *System ON* .

5. Set up the conditions for your run, which are shown below and are most likely already loaded. Do this under *Method* and then *Load Method* to scroll down to the desired method. Load the method called "PAH-288.M". 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*:

NOTE: 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.

Pump Set-up:

Starting conditions:

Flow rate: 1.00 mL/min

Stop time: 21 minutes

Post time: off

Solvents and Gradient Timetable:

A: 40.0 %   Acetonitrile

B: 50 %   Water

C: 0 %   Methanol

D: 10 %   THF

Time	B	C	D
4	50	0	10
18	0	0	10

This starts the solvent mixture with the 50:40:10 mixture which stays constant until 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 350 bar maximum and 10 bar minimum.

Column Thermostat:

The thermostat is set to 19°C.

DAD Signal:

This is the absorption signal using the diode array detector (DAD). Check to be sure that DAD 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.

Signal Detail:

Check to make sure everything is OK: Start time at 0, end time at 20, delay at 0.

Remaining Sub-menus:

For the remaining sub-menus, just say "OK" for each one. After you close the window, be sure to use "File...Save…Method."

Naming and Injecting the Sample

Now click on the "RunControl" menu at the top, click on "Sample Info". In the "subdirectory" box, type the date in the DDMONYY format 25JUN10. Set to sample name to "prefix/counter", name PAH and start at 0001. Then click "Run Method" and click "OK" when prompted to create a new directory. When you load the injector and then turn the valve to the *Inject* position, the run is started automatically using the method you loaded and saved.

<u>Part II: Preparation and Initial Chromatography of Standards</u>

1. Make up 20 mL of a 1:1 methylene chloride (CH_2Cl_2)-methanol (CH_3OH) mixture (solvent), by measuring 10 mL of each chemical using a graduated cylinder (chemicals are in the hood) into a 100 mL beaker. Condition a 100 µL HPLC syringe three times with the CH_2Cl_2-CH_3OH mixture.

2. Make sure the injector is in the **LOAD** position. Use the 100 µL HPLC syringe to repeatedly inject 1:1 CH_2Cl_2-CH_3OH solvent (about 4–5 times; there is a lot of solvent and the sample loop is only 20 µL). 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 to the **INJECT** position. 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 monitor a representative DAD signal (currently set to 252 nm). When the baseline on the DAD signals is stable, use "Balance" signal to "zero" the DAD. 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 the LOAD position 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!!**

Part III: Calibration Using the Standard PAH Mixture

1. 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 200 μL of the standard mixture, using the 200 µL pipette, into a clean 1 mL volumetric vial. Fill the rest of the vial to the 1.0 mL line with the 1:1 CH_2Cl_2-CH_3OH solvent. Put the vial cap on and swirl well to mix. Be sure to record the original concentration of the PAH compounds in the undiluted mixture (this will be needed later) here: _____

2. Measure 50 μL of the diluted standard into a small, clear vial with a screw cap. Prepare three different vials this way. To the first add 50 μL of the 1:1 solvent mixture (CH_2Cl_2:CH_3OH). To the second, add 150 μL and to the third add 250 μL. Put the lids on the vials and swirl to mix. It is a good idea to label these vials with tape.

3. Set up the software for each run as before at the end of Part I and inject each standard (be careful to use smaller amounts to flush the loop since there is limited solution in the vials). When the run is finished, open up an Offline version of the software to view the chromatogram and integrate peaks in the "Data Analysis" screen. Do not use the Online version to view data because if the run is active, you cannot get out of the Data Analysis window to view the run progress. Take note of the file number of your run.

It may be more time efficient to jump ahead to Part IV and start preparing the cigarette sample while running the chromatograms for last few standard solutions.

4. In order to identify the benzo[a]anthracene peak, compare your chromatogram to the reference chromatogram (given on the last page) in each of the three standard solutions. You will need to know the initial concentration in the undiluted solution of the standard and account for all dilutions made.

5. Examine the instrument data in the "Data Analysis" screen. Take note of the integrated peak areas for the compounds of interest for each DAD signal and record them in your lab book. Your TA will assist you with navigating through the "Data Analysis" screen.

Create a calibration table as follows to record the integrated peak area for the peak you identified as the benzo[a]anthracene.

Sample (conc. in µg/mL)	241nm	252 nm	269 nm	289 nm	297 nm
STD 1 (_____µg/mL)					
STD 2 (_____µg/mL)					
STD 3 (_____µg/mL)					
Cigarette (unknown conc.)					

Part IV: Preparation and Analysis of the Cigarette Smoke Sample

SAFETY CONSIDERATIONS: Do not burn the cigarette anywhere near the flammable solvents!!!! The TA should set up a separate area with adequate air suction for the cigarette burning, which is away from all the organic solvents.

1. Place the funnel with the fritted disc upside down in the hood (it will go over the cigarette). Using the Tygon tubing, connect the funnel to the yellow vacuum line in the hood and turn the vacuum on. Place the small filter on the fritted disc (the vacuum should make it stay in place). Measure the length of the cigarette, then light it with a match near the hood. Suck on the cigarette with a rubber pipette bulb to keep it burning under the funnel and filter.

2. Let the smoke be drawn through the filter until the entire cigarette has burned if it is a filtered cigarette (have a beaker ready to catch any falling ashes). If it is an unfiltered cigarette, burn as much as possible, and measure the lengths of a new cigarette and the leftover amount. The percent burned can be calculated. Then extinguish the cigarette using a beaker filled with water. Turn off the vacuum before removing the filter funnel.

3. Using the filter flask connected to the vacuum, wash the cigarette residue collected with 2 mL of the 1:1 solvent. Turn off the vacuum.

4. Filter the extract with the 0.2 uL syringe filter (this may be easiest by pulling out the plunger and pouring the sample into the syringe, then push the plunger so that the solution goes through the filter) and dispense the filtered brownish liquid into a small sample vial.

5. Evaporate the sample to dryness using a gentle stream of N_2 or dry air directed into the vial (a dry air nozzle should be just below the vacuum line in the hood). Connecting a glass pipet to a rubber hose from the N_2 (or air) line would give more control.

6. Using the 200 μL pipette, add 50 μL of the 1:1 solvent along the walls of the vial to wash the sample into the bottom and swirl to mix well.

7. When ready to run the sample in the HPLC, load the software, check the method and name the file as before.

8. Inject around 10 μL of the cigarette sample into the HPLC (usually twice) with the Injector in the LOAD position to flush the sample loop (be careful…you only have 50 μL of solution!). Add another 20 μL and rotate the sample loop into the Inject position.

9. Record the chromatogram.

10. Record the Peak Areas of benzo[a]anthracene, but use the DAD wavelength you used to make the Standard Curve when calculating the amount of benzo[a]anthracene present in the cigarette.

11. 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 *Load* position.

12. Shut down the software and instrument according to directions from the 152L instrument manual.

Data Analysis:

Benzo[a]anthracene Quantification

1. From your recorded DAD integrated peak areas, you only need data at one representative DAD wavelength to perform calibration in order to quantify the amount of benzo[a]anthracene in cigarette smoke.

Plot the measured absorption peak area vs. concentration for the standard solutions at the representative wavelength. Carry out a least squares analysis as shown in Figure 2. Instructions for using Microsoft Excel to do this are in the Appendix.

2. Now use your linear fit to calculate the concentration of benzo[a]anthracene in the unknown sample obtained by dissolving the cigarette smoke.

3. Finally, calculate how many ng of benzo[a]anthracene there was in the cigarette. Correct for any unburned lengths of the cigarette if it was not completely burned.

References:

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5. C. L. Gerlach, "New Instrument Brings PAH Analysis to the Field", *Env. Sci. Technol.* **30**, 252A (1996).
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7. See Web site: http://www.phenomenex.com/phen/Products/HplcAppBrand.asp?Code=13

Appendix: Reference Chromatogram of an PAH mixture
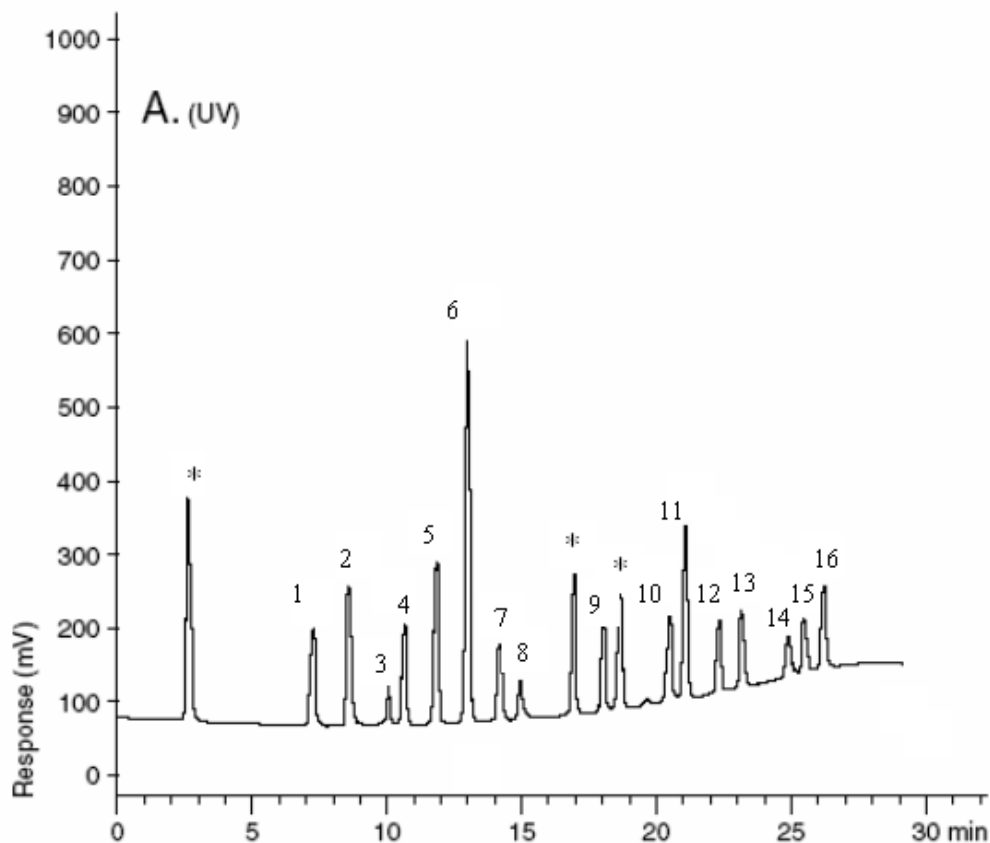
1. Naphthalene	9. Benzo[a]anthracene
2. Acenaphthylene	10. Chrysene
3. Acenaphthene	11. Benzo[b]fluoranthene
4. Fluorene	12. Benzo[k]fluoranthene
5. Phenanthrane	13. Benzo[a]pyrene
6. Anthracene	14. Dibenzo[a,h]anthracene
7. Fluoranthene	15. Benzo[g,h,i]perylene
8. Pyrene	16. Indeno[1,2,3-cd]pyrene

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