CHM 317H1S
Winter 2018

Section A - HPLC

A: High-Performance Liquid Chromatography

1. List of Experiments

1. Separation of Aromatic Hydrocarbons by Reverse-Phase HPLC
2. Determination of Caffeine in Drinks by Reverse-Phase HPLC
3. Determination of something in something

2. Locker Inventory

✔	Quantity	Common items for all experiments (2 drawers)
	6	50 mL beakers
	4	100 mL beakers
	2	250 mL beaker
	1 each	10, 25, and 100 mL graduated cylinders
	2 each	1, 2, 5, and 10.00 mL transfer pipettes
	2 each	5.00 and 10.00 graduated (Mohr) pipettes
	8	10.00 mL volumetric flasks and stoppers
	2	Short-stemmed funnel
	3	3-Way bulb pipette filler
	2	Wash bottles for deionized water
	1 each	Small and large spatula

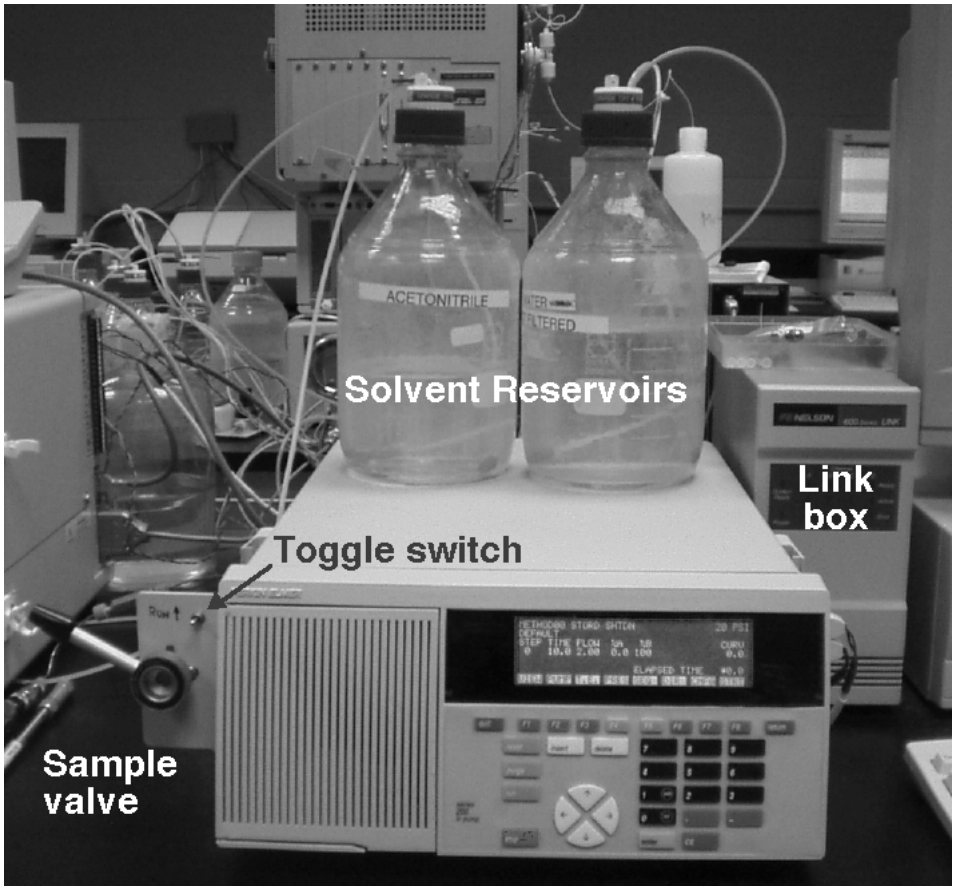
✔	Quantity	Additional items for Experiment A2 (2 drawers)
	6	25.00 mL volumetric flasks with stoppers
	2	50.00 mL volumetric flasks with stoppers
	1	250.0 mL volumetric flasks with stopper
		Pack of SPE cartridges
	6	50 mL Falcon tubes with screw caps
	6	13 mm diameter glass test tubes
	2	25 mL transfer pipettes

✔	Quantity	Additional items for Experiment A3 (2 drawers)
	3	50 mL volumetric flasks with stoppers
	5	25 mL volumetric flasks with stoppers
	5	1.00 mL transfer pipettes

3. Instrumentation

During this set of experiments, you will be using systems equipped with variable wavelength and diode array absorbance detectors.

- HPLC-Varwav, HPLC-DAD
  Perkin-Elmer series 200 LC pump with four eluent (mobile phase) reservoirs for a quaternary gradient capability. Detection using either the Perkin-Elmer Model 785A programmable absorbance or Shimadzu dual wavelength UV-visible detector,



HPLC Pump, valve, and solvent reservoirs.
The column is just visible on the left-edge of the picture.

The LCs are typically fitted with a 15 or 25 cm long × 4.5 mm internal diameter column, packed with 5 μm particles coated with a C8 or C18 stationary phase. A guard column is placed in-line between the sample injection valve and the column, which consists of the same packing material and a 0.45 μm filter to prevent particulate matter from contaminating or clogging the column. The injection valves on both LCs are fittedwith a 20 μL fixed volume sample loop. Both pumps and detectors are interfaced to computers by means of a Perkin-Elmer 600 and/or 900 LINK serial communication boxes. Instrument control and data collection is performed using Perkin-Elmer Client/Server TotalChrom Navigator software version 6.1.2.

4. General Operating Instructions

The following pages provide general information on using the HPLC-Var instruments in the Analest laboratory. Please take time to read through these instructions carefully **before coming to the laboratory.**

4.1 Starting the Instrument:

(a) Make sure that the Perkin-Elmer 600 and/or 900 LINK boxes between the LC and the computer are turned on, and that all the LEDs on their front panels are **not** flashing simultaneously. (The latter indicates an error condition: if this happens, turn these boxes off, wait at least 5 seconds, then turn them back on again.) Now turn on the LC pump and detector.

(b) If the computer adjacent to the LC is not on, start it up. Once it has booted, press the Ctrl+Alt+Del keys simultaneously to call up the login window. Login to the Analest network using the user name and password for the particular instrument you are assigned to use:

username chm317hplcvarwav and password hplcvarwav

username chm317hplcdad and password hplcdad

username chm317ic2 and password ic2

Once Windows has finished starting, double-click on the TCNav shortcut icon on the desktop to launch the TotalChrom software.

Note: Laboratory staff may have already performed the following operation. If the pump is already running when you check the instrument, you will *not* need to perform this step; it is, nonetheless, important to know that this is part of the standard operating procedure for using any HPLC system.

(c) If you are the first to use the LC in your lab session, you should purge the solvent reservoirs with helium gas; this prevents air bubbles from forming in the column and/or detector, which would adversely affect your results. The main He cylinder is located in the prep room, and is normally left on, so you should not need to adjust this. If you experience problems, ask your demonstrator for assistance.

Each HPLC station has a small needle valve and pressure gauge mounted behind the HPLC on the back of the benches. Open the needle valve by turning the knob counter-clockwise. The gauge should read a pressure of between 5–10 psi (the red scale) and you should see the Helium bubbling through the solvent reservoirs. Open the valves on the caps of HPLC solvent containers to be used in the experiment, and allow the He to purge any dissolved gases for about 15 minutes. You should hear a hissing sound as the gas escapes. Close the valves on the solvent reservoir caps, and then close the needle valve by turning it clockwise. **Do not** over-tighten the valve, as this will break the valve seal.

4.2 Setting up the TotalChrom Software:

(a) On the main screen, locate the **Instruments** box listing all the instruments currently running under TotalChrom. If the button corresponding to your instrument is not already selected, click on it.

(b) Select **CAM Administration** from the **Admin** menu. In the **CAM Admin Tool** window, double-click on **TotalChrom Servers** and click on instrument to be used (e.g. HPLCVAR) to select it. Click on the first icon in the toolbar (the padlock) to unlock the instrument and close the **CAM Admin Tool** window.

(c) Linking the computer and instrument: In the main window, click on the **Run** box and select **Attach** from the popup menu. Wait until the **Status** box in the main window indicates that the instrument/interface has been successfully attached, and then select **Take Control** from the same popup menu.

(d) Setup and Method: click on the large **Setup** button in the main window. In the dialog box that opens, make sure that the **Method** radio control at the top is selected. There are a number of line items with text entry areas beside them. The **Method:** line should show one of the following:

C:\TC4\CHM317\hydrocarbons.mth

C:\TC4\CHM317\caffeine.mth

C:\TC4\CHM317\parabens.mth

If not, you can either type this in or click on the button with the folder icon at the end of the line to browse to the appropriate file. Similarly, make sure that the **Data Path:** item contains the text: C:\TC4\CHM317\

Enter a suitable name for the **Base File Name:** – the software will automatically add a sequential number to this name for each chromatographic run.

Under the **Processing** heading, make sure that **Suppress processing** is *unchecked* and that **Suppress reports/plots** is *checked*. Click on the **Bind** button – this makes sure that the operating conditions transferred to the instrument cannot be changed accidentally once you have started your experiment. Finally, click on the **Vial list** button.

(e) Vial List: the vial list lets the software know how many samples you intend to run, and enables you to provide a title for each one. The sequence editor opens the vial list in a spreadsheet-style window. Enter a name for each one. Select **Insert** from the **Edit** menu or type a **ctrl–A** to add at least 20 extra lines. You can change the name for each run to be something more meaningful. Once you are done, save the vial list and exit the sequence editor.

(f) Now click the **OK** button in the **Setup Instrument** dialog box. Information will be transferred from the computer to the instrument; when setup is complete, all the lines in the **Status** box will be in green text and read "**Ready**". If you want to review the settings or check current values at any time after this, click on the large **Details** button in the main window.

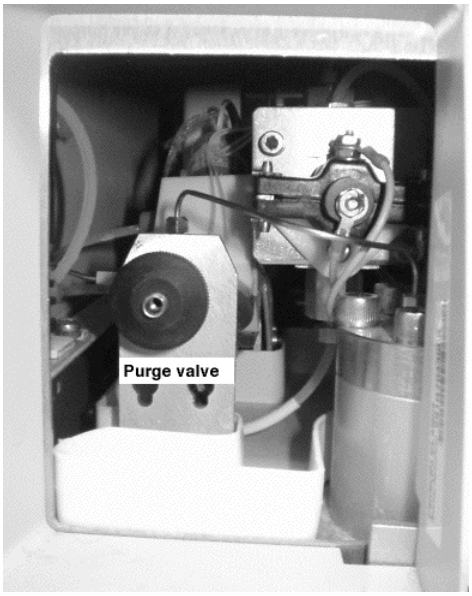
To print the setup parameters, click on the large **Method** icon in the main window. In the resulting window, select **Print** from the **File** menu; make sure that only those options you require are checked in the dialog box that comes up before printing (i.e. instrument parameters.) A summary of the relevant method parameters is provided in the individual experiment protocols following this section.

(g) Setting the PE Variable Wavelength Detector: you must manually select the detector wavelength by using the screen on the instrument panel of the UV-Vis Detector. To set the detector wavelength to the required value (*e.g.* 254 nm): push **F1**, type the value specified for your experiment, and press **Enter**. Now set the detector range to the required maximum (*e.g.* 0.2 units): push **F2**, type the value specified for your experiment, and press **Enter**.

Note: the detector outputs the absorbance reading as a voltage; this is converted to a digital reading by the LINK box, which then passes the data to the computer.

(h) Purging The Pump And Tubing: it is important to make sure that there are no air bubbles in the LC pump, and that the lines have all been purged with the mobile phase (solvent mixture) appropriate to your particular experiment. Check with the specific experimental protocol in the following sections to determine the correct mobile phase for your experiment. Once you have determined this, locate and make a careful note of the identifying letters on the correpsonding solvent reservoirs.

Note: if the pump is already running when you come to check the instrument, then this step has already been performed by laboratory staff; it does not need to be performed twice!



Open the door of pump compartment, and then open the wheel-nub valve by turning it counter-clockwise. In the main TotalChrom software window, make sure that the green status text is on, and then click on the large **Hands On** icon. In the **LC Hands On** window, select the **Purge** tab, set the **Flow** to 10 mL/min, and click the **Set Pump** button. Now click the **Start Pump** button, and let the solvent flow for 15 – 20 seconds, then click on **Stop Pump** and close the wheel-nub valve (turn it clockwise). Now click on the **Pump** tab and click on **Start the Pump**, then close the **TC Hands On** window.

4.3 Running the Samples:

(a) Make sure that all the items in the **Status** panel are green and show "**Ready**". You can check the various current settings for the different parts of the instrument by clicking on

the large **Details** icon. Allow some time for the column to equilibrate with the mobile phase: it is probably a good idea to get the LC running before making up your solutions.

(b) Injecting The Sample: Sample injection is achieved by means of a 6-port rotary valve with a fixed volume sample loop. This valve has two positions: **load** and **inject**. The **load** position allows the fixed sample loop to be flushed and filled with sample solution while the mobile phase is pumped directly to the column. The valve is in the load position when the valve handle is pointing down and to the right. The **inject** position (handle pointing up) diverts the flow of mobile phase from the pump through the sample loop, flushing the contents of the loop on to the column.

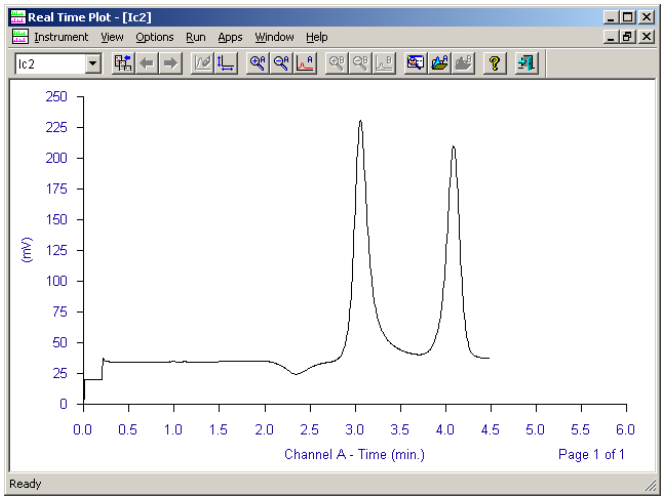
ⓘ There is an animation showing how the valve works on the analsci web site

http://www.chem.utoronto.ca/coursenotes/analsci/chrom/hplc03.html

With the valve in the load position, fill a 50 μL syringe with the solution to be injected, and insert the syringe into the port in the front-centre of the valve. Depress the plunger to displace the contents of the sample loop with your solution, leaving the syringe **in the valve**. Note the small toggle switch located just above the injection valve. Now smoothly and firmly switch the valve to the **inject** position by rotating the handle up in a clockwise direction whilst **simultaneously** flicking the toggle switch. This lets the computer know that an injection has been made, and will start data collection.

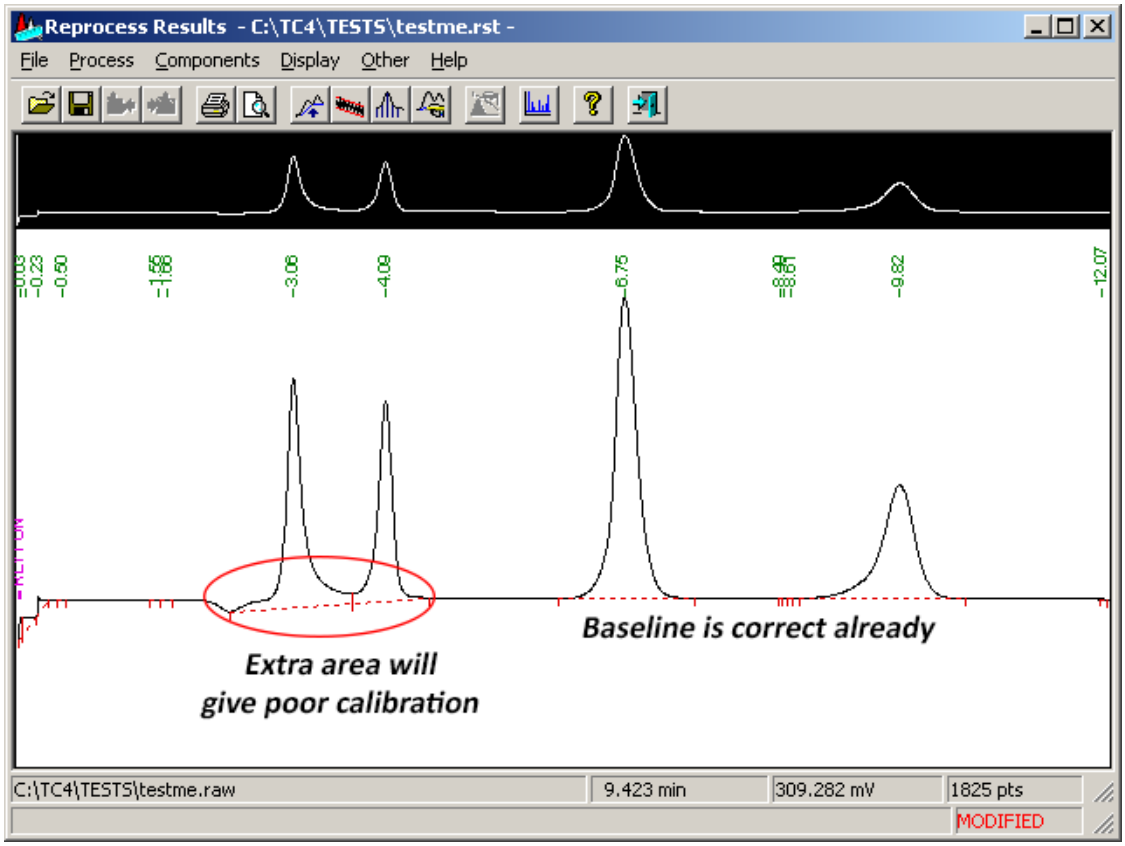
Note: The key to obtaining good LC results is to perform the sample injection as reproducibly as possible. A rapid injection is preferred; turning the valve slowly will temporarily stop the mobile phase flow, resulting in a pressure surge when the valve reaches the **inject** position. Note also that, once the sample has been injected from the loop into the column, the sample loop will be full of mobile phase; this must be fully displaced by your solution when you next load the sample loop or you will get sample dilution and contamination. Use a volume equal to about 2–3× the loop volume when loading the sample loop.

(c) Monitoring the chromatogram: Click on the large **Real-Time Plot** icon in the main TotalChrom window. This will show the raw data during collection by the computer (below). You can use the **Option** menu in this window to rescale the display if necessary.



(d) Processing the Chromatogram: This can be done at any time once a particular chromatographic run has been completed, i.e. you can start the next run, then go back and analyse the data from the preceding one. Minimize the real-time plot window, and click on the large **Results** icon in the **Reprocess** area of the main window. Open the file corresponding to the experiment you have just run: this will have the base file name you set in the method, followed by a time-stamp, with a .rst suffix.

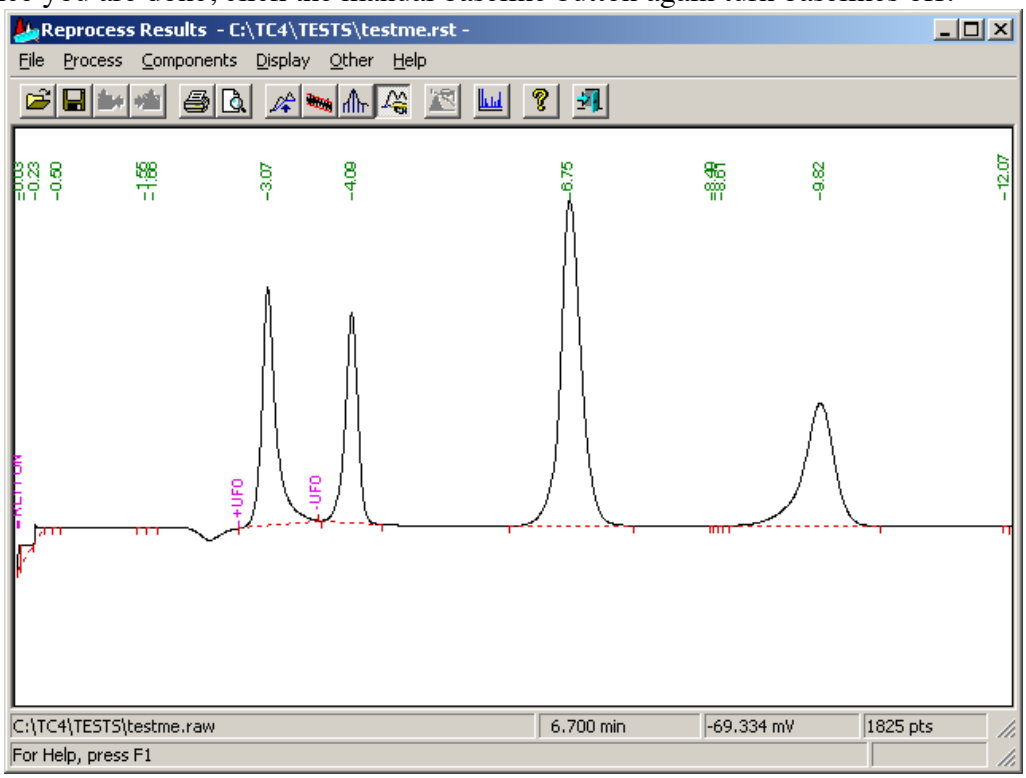
In the resulting chromatogram display, check that the software has correctly identified the various peaks in your sample, and that the baseline drawn across the bottom of each peak is reasonable. Also check that the software has not identified noise peaks as sample peaks: noise peaks are typically small and quite narrow, whereas your sample peaks will be broader and exhibit the expected Gaussian (or skewed-Gaussian) shape.



Results window showing a specimen chromatogram. Note that the baseline has been incorrectly assigned for the first two peaks (at 3.0 and 4.0 minutes) and should be corrected. Also note that baseline noise has been incorrectly identified as additional peaks in the chromatogram (e.g. the red vertical marks around 1.6 minutes)

- *To remove noise peaks*: click on the **Process** button and choose **Noise/Area Threshold** from the drop-down menu. Click and drag along a region of the baseline containing the noise peaks you wish to remove. A window will appear suggesting appropriate thesholds based on the size of these peaks; either change these values or accept them, then click **OK**.

- *To manually place a peak baseline*: click the manual baseline button in the toolbar to turn baselines on. Click and drag to draw a baseline across the bottom of a peak. Once you are done, click the manual baseline button again turn baselines off.



The same chromatogram with the baseline for the first peak corrected; the stray noise peaks still need to be removed.

When you are satisfied, print the results by selecting **Print** from the **File** menu; choose the appropriate formats for **Report** ("landscape") and **Replot** (checked), then click **OK**.

4.4 Shutting Down the HPLC:

(a) Make sure that the instrument is not running (all the status text items should be green.) Click on the large **Hands On** icon, select the **Pump** tab in the resulting window, click on **Stop Pump**, and then close the window.

(b) Click on the large **Run** icon on the main page, and select **Release Control** from the drop-down menu. Then click on the **Run** icon again and select **Detach**. When the status box text turns red, close the TotalChrom program, log off from the computer, and turn off the pump, detector, and 600/900 LINK boxes.

5. Experiment 1: Separation Of Aromatic Hydrocarbons By HPLC

This experiment is adapted from expt. 13-4 from "Chemistry Experiments for Instrumental Methods" by Sawyer, Heineman, and Beebe. You will be assigned *one* standard mixture for analysis (*either* set 1 *or* 2) and the corresponding HPLC unknown (1 or 2). Set 2 takes longer to elute, but requires you to make fewer solutions and run fewer chromatograms; both should take about the same time.

Set 1:

Contains a mixture of benzene, toluene, ethyl benzene, n-propylbenzene, and n-butylbenzene in methanol. Use a mobile phase of 30% by volume water in methanol, *i.e.* 70% methanol, 30% water.

Set 2:

Contains a mixture of benzene, cumene, and tetramethylbenzene in methanol. Use a mobile phase of 15% by volume of water in methanol, *i.e.* 85% methanol, 15% water.

After performing this experiment, you should:

- Understand the basis of qualitative analysis in liquid chromatography
- Understand the role of the solvent ratio in HPLC
- Start to develop an understanding of retention mechanisms in HPLC

IMPORTANT:

Most of the solutions you will be using in these experiments involve hydrocarbons. **Never use compressed air to dry your glassware**, as this will coat the interior with hydrocarbon contamination! See pages 24-25 of the introduction for general instructions.

Chemicals:

- HPLC "Unknowns" sets 1 and 2 (composition listed above)
- Individual samples of all the "unknown" components as pure liquids or solids
- HPLC-grade methanol

A. Instrument setup and solution preparation

1. Prepare separate standard solutions (10.00 mL) of each compound to be determined in your assigned set (1 or 2) to give a final concentration of about 1 mg/mL. Dispense the compounds into the volumetric flasks *by weight, not volume,* using the analytical balance. The total mass required is approximately 1 drop from a disposable Pasteur pipette (~10–20 mg, depending on the liquid). Dispense *one* drop, and record the actual mass as accurately as possible; do **not** try to get exactly the right mass in the flask! Dilute to the mark with HPLC-grade methanol.

Note: ordinarily, you would dissolve samples in the mobile phase; some of the larger hydrocarbons have borderline solubility in methanol-water at these concentrations,

however, and the difference in methanol content when combined with the sample volume is small enough not to affect the results of the analysis.

2. Follow the instructions for the particular instrument assigned to you to obtain chromatograms for each substance in turn, followed by your unknown mixture. The relevant conditions contained in the hydrocarbons.mth file are ***typically***:

Mobile phase:	75% Methanol, 25%water (v/v)
Flow rate:	1.0–1.2 mL/min (varies with column)
Detector:	λ = 254 nm, 0.1 or 0.01 absorbance units
Column:	C18

Laboratory staff may make adjustments to the flow-rate and range based on the size and age of the column being used. **Please do not adjust any settings without asking first!**

➔ Since the make of column may vary from year to year, make sure you record the actual manufacturer, type, and dimenions (length, diameter, particle size) for the column connected to your HPLC pump and detector.

B. Characterization of Retention Times and Sample Identification

3. Start by running the unknown sample mixture assigned to your demo group; **note that you do *not* need to dilute this**. Record the unknown number and base file name in your lab notebook, inject your sample, and start the run (section 4.3(b)).

4. Once the sample run has completed, *immediately* start your first standard in the same way, recording its identity in your lab notebook. Once the run has been started, you can process the chromatogram from the previous run: open the file and check that all the peaks have been integrated correctly (section 4.3(d)). Once satisfied, print a single copy of the chromatogram and tabulate the retention times and areas of the peaks in your lab notebook.

5. Once the first standard has eluted from the column (check in the real-time plot window), you can stop that run and start the next standard. Make sure that you record the identity of each standard or sample in your lab notebook *as you run each chromatogram*. Once one each chromatogram is completed, inject the next sample, and then process the results from the preceding run (section 4.3(b)). As you process each run, compile a table the retention time and peak area for each pure substance in your lab notebook. Use this information to identify each of the components in your sample mixture.

➔ Be sure to note any unexpected peak shapes in your lab notebook. Print only one copy of the chromatograms for each of your standards for reference; you will *not* need to submit these with your formal reports.

Note that, in order to make a positive identification from your standards, the retention times should be in good agreement, but are not expected to be exactly the same; typically, a difference of ~0.1 minutes is acceptable. You may also observe more than one peak in a standard due to impurities in the original materials; in this case, the peak with the larger area is obviously the one you should record!

6. Use the peak areas and the concentrations of your standards to estimate the amount of each component in your unknown. This is effectively a *single-point* calibration in which you

are assuming a zero intercept, and does *not* require a calibration plot. (Correlation calculations are also meaningless for single-point calibrations – why is this?)

7. Perform ***at least*** one replicate injection of your assigned sample mixture, in order to obtain an estimate of the uncertainty in both retention times and peak areas as reported by the software.

C. Effect of Mobile Phase Composition

8. You will now perform several runs of the unknown mixture using *different* ratios of organic to aqueous mobile phase components, monitoring the effect on retention times, resolution, and overall run-time. **It is important that, each time you change the mobile phase composition, you allow the stationary phase time to equilibrate with the new mobile phase.** For small changes in composition, this is usually sufficient time for the entire mobile phase volume within the column to be exchanged 4–5 times, *i.e.* about 4–5 times the t_M (or t_0) for the column and flow rate used. You should be able to identity this value from any of your standard chromatograms.

9. Start by clicking on the **Modify** button in the main TotalChrom window, and selected **Downloaded Method** from the popup menu. Within the new window that opens, select **Instrument→Control Options→Pump Program**. This will open a dialog window containing a spreadsheet showing the current mobile phase gradient. Increase the methanol content at step 0 and step1 in the program by 5% (use the tab key to switch fields); the water content should automatically adjust accordingly. Once done, click **OK** and make sure you choose to save the modified method.

10. Now click on the **Hands On** button and, in the **LC Hands On** dialog, change the mobile phase composition to match that entered in the method in the preceding step. Check that the indicated pump flow rate is correct, and click on **Set Values** to update the pump. Confirm from the display on the front of the pump that these update correctly, and that the pump is still running. Once sufficient time has elapsed, autozero the detector and then inject your assigned unknown and record the new chromatogram.

11. Once the sample has finished running with the new mobile phase composition, repeat steps 9 and 10 to further increase the organic (methanol) content of the mobile phase and perform another run.

12. Once you have completed two or three runs with different methanol contents, restore the values in the method to the original ones, and use the Hands On feature to reset the pump program also.

D. Finishing Up

13. Once you have finished your experiment, follow the procedure outlined above to shut down the HPLC system *but leave the pump running*. Dispose of all your solutions in the proper waste container. Any used Pasteur pipettes should be rinsed thoroughly with methanol and then water before being disposed in the teal-coloured decontaminated glass bin. Clean all your glassware, using some alcohol on a KimWipe™ to remove any labels, and return it to the correct bench drawers.

➔ Please keep all the transfer and graduated pipettes in a different drawer to the rest of the glassware, in order to minimize accidental breakage

14. Check all areas where you have been working – balance, bench, fume hood, sink, and instrument – to make sure that they are clean and tidy. Return all chemicals to the correct shelves. When done, have your TA validate your lab notebooks before leaving.

E. Data Analysis

15. In addition to identifying the sample components in your mixture and estimating their concentrations by comparison to the standards, you should determine both the structures and basic physical properties of these substances. Compare these with the nature of the stationary phase, and attempt to answer the following:

    - What factors allow you to explain the elution order (*i.e.* sequence of peaks/retention times) of the different sample components?
    - Where would you expect the related compound *para*-xylene to elute relative to the other components of your sample?
    - How do the retention times change as the methanol content of the mobile phase increases?
    - What would happen if you *decreased* the methanol content instead?
    - Calculate values of log k (where k is the retention factor = $(t_R - t_M)/t_M$) and plot these against the methanol content (%volume) for your different isocratic elution runs. What does this tell you about retention behaviour?

6. Experiment 2: Determination of Caffeine

This experiment is adapted from expt. 13-1 from "Chemistry Experiments for Instrumental Methods" by Sawyer, Heineman, and Beebe. **You will need to provide your own black coffee, black or green tea, cola, or other samples for analysis.** Do ***not*** use any drinks containing milk fat or other proteins, as these require extensive additional sample preparation. The mobile phase employed in this experiment is a mixture of 30%v methanol and 70%v water acidified with 20 mM potassium dihydrogen phosphate. You will also need to analyse one of the provided "caffeine unknowns". You can find additional information about solid phase extraction (SPE) from both the course text and http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4538.pdf. The specific method used here is for the Phenomenex® Strata™ X SPE cartridges as described in their application note #14449.

Choose one of the following research questions to answer, or propose your own (but check with your TA!):

- Which has more caffeine: Starbucks or Tim Horton's coffee?
- Which has more caffeine: Green tea or black tea?
- How decaffeinated is decaffeinated coffee?
- Does root beer contain caffeine? Which brands?

Having chosen on a research question, make sure your bring the appropriate samples with you to the lab! Hot drinks should be at room temperature by the time they are analysed, so ideally you should obtain these the day before. If you are analysing tea samples, be sure to *remove the bag(s) after allowing the tea to steep for a known time*. Carbonated beverages should ideally be flat before you bring them to the lab, so obtain bottled samples if possible and leave them open overnight.

Chemicals:

- Caffeine "Unknowns" in 30%v HPLC grade methanol-water
- 10.00 mg/L stock solution of caffeine in 30%v HPLC grade methanol-water
- HPLC-grade methanol and 30%v HPLC grade methanol-water

A. Instrument setup and solution preparation

1. Use the stock solution provided to start preparing prepare 10.00 or 25.00 mL each of five different calibration solutions covering the range 0.10 – 5.00 mg/L. Use serial dilution where necessary to obtain the lowest concentration solutions from more concentrated ones.
2. While the standards are being prepared, check that the assigned HPLC has the caffeine method loaded and the pump running to ensure column equilibration. Also check that a unique base file name has been set, and that there are a sufficient number of vials to complete your experiment (when viewing the current method, it should list vials 1-20 at least). **If in doubt about any software settings or configuration, please check with the lab instructor or your TA.**

3. While steps 1 and 2 are underway, another group member should start preparing your samples for analysis: see sub-section C for details.

➔ Save time! Although you would ordinarily want to run your standards in order from least to most concentrated, have one person start running one of the caffeine unknowns and then the standards *as they are prepared*.

B. Caffeine Characterisation and Calibration

4. Follow the instructions for the particular instrument assigned to you to obtain chromatograms for each standard in turn, followed by your assigned caffeine check sample. **Note that the check sample does *not* need to be diluted**. The relevant conditions contained in the caffeine.mth file are:

Mobile phase:	30%v methanol 70%v aqueous 20 mM KH_2PO_4
Flow rate:	1.50 mL/min (**but may vary depending on column)**
Detector:	λ = 254 nm, 0.010 absorbance units
Column:	*Record the type, stationary phase, and dimensions of the actual column you use in your lab notebook*

➔ Each run takes about 10 minutes to fully complete, however you may stop your **standards** once the caffeine peak has eluted. All **samples *must*** be run for the full time indicated in the method, in order to prevent any cross-sample contamination.

Make sure that you record the identity of each standard or sample in your lab notebook *as you run each chromatogram*. Once one run is completed, inject the next sample (section 4.3(b)), and then process the results from the preceding run (section 4.3(b)). As you process each run, compile a table the retention time and peak area for each pure substance in your lab notebook. You should then use this information to identify each of the components in your sample mixture.

➔ Be sure to note any unexpected peak shapes in your lab notebook. Print only one copy of the chromatograms for each of your standards for reference; you will *not* need to submit these with your formal reports. You should *each* have a copy of the chromatrogram for the assigned caffeine unknown.

5. Construct a calibration plot of peak area *versus* caffeine concentration *as you record the data*. Review this plot to determine if you need to re-check any peak areas or re-make any standards while you are still in the lab. Use this plot to determine the concentration of caffeine in your assigned unknown, based on the recorded peak area.

➔ The peak areas as reported are very large numbers, which will result in large *absolute* values of statistical parameters such as $s_{y/x}$. For computational simplicity (and to reduce rounding errors), it can be helpful to normalise the areas before performing regression analysis. A very simple normalisation scheme is to simply divide all recorded areas by the maximum value within the data, *i.e.* reduce all areas to a scale of 0 – 1.

C. Sample Extraction and Determination of Recovery

6. Prepare ***each*** of your drink samples by pipetting the volume specified into a clean, dry, 20.00 mL volumetric flask, and dilute to the mark with mobile phase:

   (a) Regular tea or coffee: filter the sample to remove larger suspended particles. Pipette 1.000 mL of the filtrate into the flask and dilute to volume.

   (b) Decaffeinated tea or coffee: filter as in (a) but pipette 10.00 mL of the filtrate into the flask and dilute to volume.

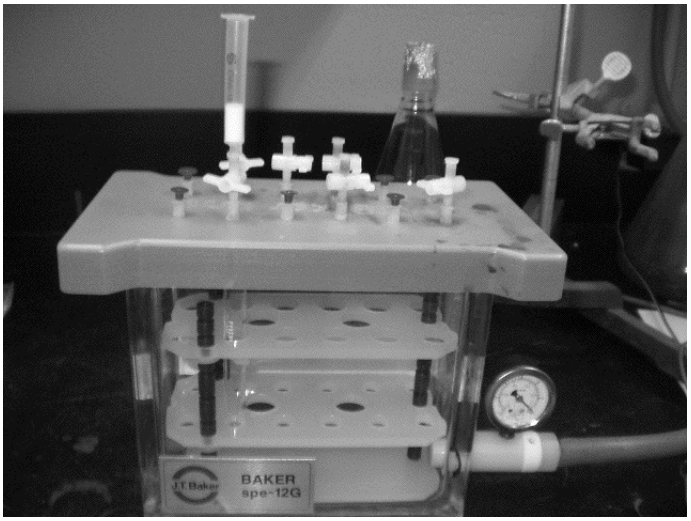
   (c) Carbonated drinks: first, decarbonate ~10–15 mL of the beverage by pouring it repeatedly between two small clean, dry beakers until the bubbling stops. Accurately pipette 10.00 mL of the flat beverage.

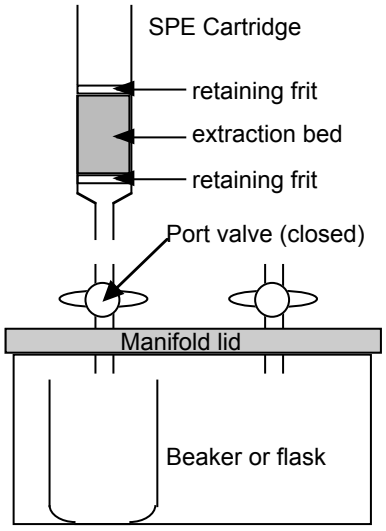
   (d) Other drinks: certain energy and rave drinks can also be analysed. Generally, these should be clear and not contain milk or protein.

7. Prepare an ***additional*** two samples that are "spiked" with a known amount of caffeine: for ***each*** sample you will be analysing, accurately dispense the same volume of your sample (see step 6 above) into a clean 25.00 mL volumetric flask; using a transfer pipette, add 1.00 mL of the caffeine stock solution into the flask before making to final volume.

8. Many beverages contain additional flavour and/or colour components that, over time, adversely affect the chromagraphic column. To prevent this from occurring, as well as reduce the number of extraneous peaks in the chromatogram, you will need to perform a solid-phase extraction (SPE) procedure. You can run several SPEs at the same time: perform the following procedure using *both* of your samples and the samples spiked with caffeine standard.

   (a) Obtain four octyl (C8) SPE columns, four 15 mL Falcon tubes, and four glass test tubes, and locate the SPE apparatus in the fume hood in LM6. Position the empty tubes inside the manifold, and then place the columns into the valves directly over the four Falcon tubes (to collect liquid passing through the column). Make sure that all the valves are closed (valve arm perpendicular to direction of flow).





The SPE manifold, with an SPE cartridge connected to one of the ports and all the port valves closed.

(b) Following your demosntrator's instructions, open the vacuum valve on the side of the fumehood just enough to obtain a reading of about –20 psi on the gauge located on the side of the manifold box. **Note:** the white collar just below the gauge allows you to release the vacuum; make sure it is closed when you set the initial vacuum reading.

(c) *Activation:* to activate each SPE cartridge, dispense about 1 mL of HPLC-grade methanol into the upper part of the cartridge. Open the port valve to draw the methanol slowly through the SPE cartridge; close the valve once the liquid meniscus is touching the upper frit. Now dispense about 1 mL of ultrapure deionised water into the upper part of the cartridge; open the port valve and slowly draw the water through the cartridge, closing the valve as soon as the liquid meniscus is touching the upper frit.

(d) *Loading:* to load each sample onto its cartridge, accurately dispense 1.00 mL of the sample solution into the upper part of the cartridge using a transfer pipette. **Slowly** open the port valve to draw the sample gently through the SPE cartridge; leave the vacuum applied for ~3-5 minutes after the sample has been pulled into the cartridge.

(e) *Elution:* move each SPE cartridge to a port directly over a clean, empty glass test tube. Dispense ~ 1 mL of either ethyl acetate or dichloromethane into the upper part of each cartridge. Open the port valve to draw the solvent slowly through the SPE cartridge; close the valve once the liquid meniscus is touching the upper frit. Repeat this step with a further 1 mL portion of the same solvent, collecting the eluent from the column in the *same* container; leave the vacuum applied for a few minutes to ensure all the solvent has been drawn through the SPE column.

(f) *Final processing:* Release the vacuum and remove the tubes from the manifold. For each sample in the glass test tubes, evaporate any residual solvent using a gentle stream of nitrogen gas using the heated nitrogen manifold provided. Redissolve the remaining solids in 1.000 mL of HPLC grade methanol, and transfer to a small glass screw-cap vial until you are ready to run the extract on the HPLC.

(g) Turn off the main vacuum valve on the side of the fume hood and dispose of the Falcon tube contents in the HPLC waste container for this experiment.

D. Analysis of Research Question Samples

9. Run a small portion of your sample through a 0.45 μm syringe filter, and then perform duplicate injections of the filtered sample. Use the peak retention time from your caffeine standard to identify the corresponding caffeine peak in your samples. Use the calibration curve obtained earlier to determine the caffeine concentration of your drink sample. Don't forget to take the sample dilution in the preparation step into account.

10. If time allows, perform replicate injections of as many of your samples and/or calibration standards as necessary to improve the quality of your data.

E. Finishing Up

11. Once you have finished your experiment, follow the procedure outlined above to shut down the HPLC system. Dispose of all your solutions in the proper waste container. Any used Pasteur pipettes should be rinsed thoroughly with methanol and then water before being disposed in the teal-coloured decontaminated glass bin. Clean all your glassware, using some alcohol on a KimWipe™ to remove any labels, and return it to the correct bench drawers.

➔ **Please keep all the transfer and graduated pipettes in a different drawer to the rest of the glassware, in order to minimize accidental breakage**

12. Check all areas where you have been working – balance, bench, fume hood, sink, and instrument – to make sure that they are clean and tidy. Return all chemicals to the correct shelves. When done, have your TA validate your lab notebooks before leaving.

F. Data Analysis

13. Make sure that you calculate *all* the relevant statistical parameters for your calibration curve (r, $s_{y/x}$, limits of detection and quantitation) and calculate the uncertainties in the interpolated values for your assigned unknown, research samples, and the spiked research samples.

14. Use the difference between the caffeine content of each research sample and the corresponding spiked sample to calculate the recovery of the SPE process: this is sample the ratio of the amount of analyte extracted to the amount initially loaded onto the SPE column, typically expressed as a percentage. Use this information to determine if any caffeine from your samples was 'lost' by irreversibly binding to the SPE column; if so, use this to adjust the caffeine content of your original samples before attempting to answer your research question. Remember to perform error propogation when determining the concentrations of caffeine in the actual samples, taking into account the various dilution factors that occurred at each state of the procedure.

15. In comparing your research samples, make sure you apply the appropriate statistical tests when attempting to draw conclusions. Some of the things you should consider include:

    - Did you have sufficient data to answer your research question? If not, what additional information would you require?
    - Is there a significant difference in caffeine content between your two samples? Is the result what you expected to find?
    - What variables might affect the caffeine content of your two samples? How might this affect your conclusions?

7. Experiment A3: Use of Dual Wavelength Detection in HPLC

This experiment is adapted from Remcho, McNair and Rasmussen "HPLC Method Development with the Photodiode Array Detector" in *J. Chem. Ed.*, **1992**, *69(4)*, A117-119. You will be using an HPLC with a UV detector that can be configured to monitor at two different wavelengths essentially simultaneously. The compounds used in this experiment are a homologous series of *para*-hydroxybenzoate esters (parabens), which are found in various products as preservatives and UV blocking agents. A related compound included in the experiment is a phthalate diester: these are widely used as plasticisers in the production of flexible plastics, and are the subject of considerable debate regarding their safety.

After performing this experiment, you should:

- Understand how substituent side chain length affects retention behaviour
- Understand the role of the solvent ratio in reverse phase HPLC
- Understand the usefulness of so-called "2-dimensional" techniques

IMPORTANT:

Most of the solutions you will be using in these experiments involve hydrocarbons. **Never use compressed air to dry your glassware**, as this will coat the interior with hydrocarbon contamination! See pages 24-25 of the introduction for general instructions.

Chemicals:

- Methyl, ethyl, propyl and butyl paraben (*para*-hydroxybenzoate esters)
- Diethyl phthalate
- HPLC-grade methanol
- 50%(v/v) HPLC methanol in ultrapure deionised water

A. Instrument setup and solution preparation

1. Prepare separate stock solutions by accureately dispensing between 1.0 and 2.0 mg (0.0010–0.0020 g) of each compound into separate 25.00 mL volumetric flasks, and diluting to final volume with 50%(v/v) methanol–water. Sonicate the solutions for a few minutes to ensure that the compounds are fully dissolved (*especially* the diethyl phthalate, which is an oily liquid.)

➔ **Be careful *not* to exceed the upper mass limit, as higher concentrations may cause problems with the HPLC column.**

2. Check that the instrument assigned for this experiment is on, and that the pump is running with the correct initial mobile phase composition (80% methanol–20% water at 1.5 mL/min.) The detector should be preset to monitor at 225 and 255 nm. The column for this experiment is a 10 cm × 4.6 mm ∅ × 5 μm particle size C18 column.
    - Method: Parabens.mth
    - Detector sensitivity: 0.0500 absorbance units full-scale
3. Prepare a mixture of all five compounds by dispensing 1.00 mL of each one into the same 50.00 mL volumetric flask, and diluting to final volume with 50%(v/v) methanol–water.

4. Prepare two additional samples in order to obtain the UV-visible absorption spectra of representative compounds: dispense 1.00 mL each of methyl paraben and diethyl phthalate into *separate* 50.00 mL volumetric flasks, and dilute to final volume with 50%(v/v) methanol–water.

B. Determining the Optimum Wavelength for Measurement

5. Following the instructions from your lab TA, record the UV absorption spectra of methyl paraben and diethyl phthalate in 50%(v/v) methanol–water, using the same solvent mixture as the reference and blank. Use quartz cells, and scan from 400 nm down to 200 nm. Identify the wavelengths of maximum absorption for both compounds (ignoring the solvent cut-off) and print copies of the spectra for your lab notebooks. Clean and return the cells to your TA once you are finished.

C. Exploring the Effect of Solvent Composition on Separation

6. Run the mixture of compounds on the HPLC using the initial 80:20 methanol–water mobile phase, and compare the chromatograms obtained at the two different wavelengths.

7. Modify the running method so that the mobile phase composition is altered: you can try methanol:water ratios 75:25, 70:30, and 65:35. Allow the column to equilibrate with the new mobile phase composition for 3 minutes before running your mixture again. Note the resulting changes in the chromatogram, and identify the peaks based on (a) their UV absorption spectra and (b) their expected order based on the increasing length of the alkyl side chains. Make a note of the retention times of the peaks corresponding to the analytes in the mixture.

To modify the running method:

(a) Start by clicking on the **Modify** button in the main TotalChrom window, and selected **Downloaded Method** from the popup menu. Within the new window that opens, select **Instrument→Control Options→Pump Program**. This will open a dialog window containing a spreadsheet showing the current mobile phase gradient. Increase the methanol content at step 0 and step1 in the program by 5% (use the tab key to switch fields); the water content should automatically adjust accordingly. Once done, click **OK** and make sure you choose to save the modified method.

(b) Now click on the **Hands On** button and, in the **LC Hands On** dialog, change the mobile phase composition to match that entered in the method in the preceding step. Check that the indicated pump flow rate is correct, and click on **Set Values** to update the pump. Confirm from the display on the front of the pump that these update correctly, and that the pump is still running. Once sufficient time has elapsed, inject your sample mixture and record the new chromatogram.

8. Modify the running method a second time to a final mobile phase composition of 60:40 methanl–water, allow the column to equilibrate, and run the mixture a third time. Again, compare peak retention times for the five compounds in your mixture.

D. Gradient Elution for Chromatographic Separation

9. Change the method being used to ParabensGrad.meth, reset the base file name and file list, and allow the column ~4 minutes to equilibrate to the new settings. This method switches the mobile phase composition over a 5-minute period: make a note of the gradient being used (click on the large **Details** button in the main window.) Once ready, run your parabens mixture a fourth time; compare the chromatograms observed at the two wavelengths. How does the gradient affect the retention times for each component? What happens to the phthalate compared to the parabens?

10. If time allows, discuss with your lab TA ideas for modifying the gradient: predict what you think will happen, then test your theory by running the new gradient. Make sure that, if you change the method file, you change it back to the original settings before you leave.

E. Finishing Up

11. Once you have finished your experiment, follow the procedure outlined above to shut down the HPLC system. Dispose of all your solutions in the proper waste container. Any used Pasteur pipettes should be rinsed thoroughly with methanol and then water before being disposed in the teal-coloured decontaminated glass bin. Clean all your glassware, using some alcohol on a KimWipe™ to remove any labels, and return it to the correct bench drawers.

➔ **Please keep all the transfer and graduated pipettes in a different drawer to the rest of the glassware, in order to minimize accidental breakage**

12. Check all areas where you have been working – balance, bench, fume hood, sink, and instrument – to make sure that they are clean and tidy. Return all chemicals to the correct shelves. When done, have your TA validate your lab notebooks before leaving.

F. Data Analysis

13. Compile the data from the different conditions used in this experiment. Why does increasing the chain length of the alkyl substituent affect the retention order of the parabens in the way it does? In this experiment, you used a reversed phase (C18) coumn; if we used a normal phase (silica) column instead, would you expect the elution order to be the same or different? Why?

14. What are the primary advantages and disadvantages of gradient elution in HPLC, as compared to isocratic (i.e. constant mobile phase composition) elution?

8. Elements for Report Discussion

Your discussion for this report should address both the specific analysis results and the general lessons to be learned about HPLC from these experiments. In particular, you should discuss the factors governing elution order in HPLC and the factors governing the choice of column for any given sample separation. In doing so, make sure you compare and contrast the different mobile and stationary phases used in the various experiments, along with the properties of the sample components being separated. Also consider the following questions in respect to the specific experiments you have performed:

1. With compounds like caffeine, it is common to acidify the aqueous component of the mobile phase using either acetic or phosphoric acid in some form. Why is this? What difference does the presence or absence of the acid make to the shape of the sample peak? In general terms, how might you determine when you need to use such a modifier in HPLC analysis?
2. Could an ion exchange column be used instead of the C18 column for any of these experiments? Why or why not? What would you expect the effect of switching to a polar column (such as silica) to be?
3. Would constructing a calibration curve on the basis of peak height (rather than peak area) give accurate results for the determination of caffeine? In general terms, what factors have the greatest influence on accuracy and precision in quantitative HPLC?
4. What are some of the advantages and disadvantages of using a single wavelength *versus* multiple wavelengths for detection? What factors would you need to take into account when choosing what was appropriate for a particular sample?