Gas Chromatography (headspace samples) using Thermo Trace1300 machine

Hazards to be aware of before starting:

- **Hydrogen gas is very flammable**. If you don't set up the GC correctly, it is possible for unburnt H_2 gas to come out of the detector or (worse) accumulate in the oven compartment, posing a risk of fire or explosion. Ensure GC columns are installed properly (this is the job of theboss of the lab), and the detector is lit properly before commencing work.

- Other **GC gases such as nitrogen and helium are asphyxiants** (cause suffocation). Ensurethat these gases are all plumbed into the GC correctly, and that there are no leaks.

- **Pressurised gas cylinders (H_2, N_2, air, helium) are hazardous,** especially if mishandled or dropped. There are two major risks here. Firstly, if the top of the cylinder is knocked off by a fall,the cylinder can launch like a rocket and go thru walls etc. Serious injury risk! Secondly, if the cylinder ruptures in a confined space, there is an asphyxiation and/or fire risk, depending on thegas. Don't handle gas cylinders without first having hands-on training from the lab boss !

- **The top of the GC is hot**, especially the injector port and the detector. Don't get burned !

- **Needles used for injecting samples can cause an injury**. Needlestick injuries pose an infection risk in a lab setting, especially if there are microbes or soil etc on the needle.

- Many of the **hydrocarbons or chlorinated hydrocarbons** that we analyse using the GC are either flammable or toxic or both. Learn the risks of these chemicals before starting work.

Background information

The principle of gas chromatography is that different compounds bind with different affinities to the column and can be eluted off the column at different temperatures. The retention time (RT) is the time at which a compound is eluted off the column. Once the compound is eluted it passes through the hydrogen/air/nitrogen flame which breaks down organic compounds and produces ions. The ions are collected on an electrode which produces an electrical signal. The software presents the run as a series of peaks which indicate the retention time (this can be used to infer the identity of the compound) and also the area of the peak. NB. It is the peak area that is proportional to the amount of analyte present, not the peak height.

Before starting, figure out if you need to use sterile needles or not. For monitoring live growing cultures, you should be using sterile needles, ie. a new needle for each sample. You will need two small glass beakers, one for sterile needles (foil-covered, autoclaved), and one for used needles. These needle beakers will rotate duties between clean and used. We use removable needles with point-style 5 (side-port). They are expensive, so take care of them. Don't flame them. For resting cell suspensions or cell extracts or standard curves it's OK to use a non-sterile needle, ie. the same needle for multiple samples.

During times of heavy use, the GC column should be cleaned approx. once per week (raise temperature to 240°C for 1 hour to burn off gunk). Also the septum in the injector port needs to be changed approx weekly if under heavy use, or perhaps monthly for light use.

Setting up the GC

1. This is an expensive and delicate lab instrument. You must be trained in person by the lab boss or lab manager in how to use this machine. Otherwise, do not go any further !
2. Check with lab boss or lab manager that the correct column is installed for the type of analysis that you need to do. Our default column in the Coleman lab is a TG-BOND Q+ ; this is good for analysis of gas samples of small hydrocarbons and chlorinated hydrocarbons.

3. Turn on all the gases that are needed to run the instrument. For our GC set up in the LEES lab, there are three gas cylinders to turn on: helium (carrier gas), nitrogen (makeup gas for FID*), and air (air supply to FID). Note that we have a hydrogen generator which supplies hydrogen to the FID, and this is constantly on, so no action needed for this gas.

   * Flame ionisation detector

   Only turn on the main valve to the gas cylinder (on/off valve) – this is the black plastic wheel that is closest to the cylinder. One complete turn of this valve is enough. Don't touch the pressure regulator valve (controls amount of gas pressure) – this is the one that is further from the cylinder.

4. Switch the GC machine power on. There is a large up/down toggle switch on the back of the machine at the top on the right.

5. Log in to the computer attached to the GC, and make sure "Instrument Controller" is on. You can access this by clicking on the arrow on the lower right of the screen.

6. Open the Chromeleon 7 software (there should be an icon on the desktop), and "Chromeleon console" window will open. On the "Instrument" page in the "Instrument Home" tab, click the "Connect" button to connect the PC to the GC. This should go green. If it does not, give the GC machine a bit more time to switch on, then try again.

7. Go to the "Oven" tab to set the temperature of the GC oven to whatever temperature is used for your analysis. Our standard method for short chain alkanes and alkenes is 150ºC, but this may vary for different analysis. Larger hydrocarbons or more chlorinated hydrocarbons will stick to the column more, and may need higher temperatures to elute. A temperature gradient may be needed to separate mixtures (this is set up in the Method).

8. Go to the "Back detector" tab. Click all the buttons here to switch on the flows of all the gases that are needed to make the detector work (hydrogen, air, nitrogen), if these are not already on. Also switch on the button to ignite the flame. Give it a little time (10-20 sec), and you should hear a 'pop' from the back detector as the FID flame ignites. You can confirm the FID is properly ignited because the 'Signal' box will display a signal >10 pA.

9. On the same tab ("back detector"), check the flow rates of the different gases. You can change them here if you prefer. Our typical flow rates are: H_2 at 35.0 mL/min, air at 350 mL/min, and makeup gase (N_2) at 10.0 mL/min.

Injecting samples on the GC

1. Start by creating a new sequence. The software has two components, the "Console" interacts with the GC, while the "Studio" lets you play with data.

2. In the Console window, follow this sequence to create a new file: "Create" → "Sequences" → "Next" → "Injection Valve" (Because we will not be using the autosampler).

3. After this, a window called "New sequence wizard" should pop up. You should select the "Instrument method" and "Analysis method" that best suits your samples, click through the local files in the file explorer window that pops up to find it. These should have been set up by your lab boss, (See step 5 below). You can set the number of vials here if you'd like, or you can add these later. Use concise and informative sample names eg. 12janE1 might be the first sample for ethene on January 12th.

4. In the same window, you can also determine what the generated report will look like, but this can be kept as "default". When the file explorer window pops up, click through to the older GC files to find the "Default" option.

5. If the options are not automatically filled in, click through the options and find the “Default” option for each of them. The lab boss or lab manager should have already set up the “Instrument Method” and “Analysis Method”; check these are correct.

6. Once you see the window that shows the “Injection list”, press “Start”. The panel should go green. Now watch the lights on the GC. The “Ready” light will flash orange, then it will then flash green, then it will become a steady green light.

7. Once the green light is steady, inject your sample, as follows. Usually this would be 250 µL of gas, injected with a gas tight syringe that has a ‘side-port’ needle.

   Suck up about 300 µl of sample from the sample vial/bottle, pull the needle out* of the vial, expel gas down to 250 µl, then lock in the gas by pushing in the red knob on the syringe.

   * when you pull out, hold the top of the needle where it joins the syringe, rather than the body of the syringe itself. This helps to stop the needle coming off the syringe barrel.

   Push the needle through the septum into the back right injector port (this has a silver knob on top with a central hole). Push the needle in as far as it will go, then unlock the syringe, and inject the gas in one rapid and smooth motion.

   Immediately push the “start” button on the GC. The machine should beep and the ready light should go blue. Leave the syringe there for 2 to 3 seconds then pull out (again hold the nut at the top of the needle, not the syringe barrel, to avoid these coming apart…your sample will leak out of the machine if they do!)

8. You can watch the chromatograph developing over the course of the analysis. This can be found in the “Console” window in the “data” section. The machine will beep when the run is over.

9. Before you inject the next sample, make sure to check the “Ready” light on the GC. You must wait until the steady green light comes back before you can inject the next sample. If your sequence contains multiple injections, you shouldn’t need to ‘start’ them again on the computer, you only need to start the sequence once. Each individual injection start is controlled by the start button on the GC machine.

10. Repeat steps 6-8 above until all your samples are done.

11. Double click on each chromatogram and you should see the integrated area under the peak calculated. Check that the software has integrated the peak correctly (ie. It has correctly estimated the start and end of the peak) – if it has not, you need to manually correct this. Note down the area values in your lab book, then into Excel.

12. Apply a calibration curve (below) to calculate the amount of substrate in each sample.

Turning the GC off

1. When you’re done, set the oven temperature to 50ºC in the “Oven” tab of the “Console” window’s “Instruments” tab (Lower left of the “Console” window). It is bad for the column to be very hot with no carrier gas running through it, so we want to cool the machine down somewhat before switching it off.

2. After the oven has cooled to 50ºC, go back to the “Instrument Home” tab and hit the green ‘connect’ button to disconnect the PC from the GC. This should go red.

3. Turn the GC off at the back, **and turn the gases off at the cylinders!**

Making calibration standards

Things to consider before making calibration standards:

- These must be kept at the same temperature as your experimental samples
- These must be in the same size bottle with same volume of liquid as your experimental samples
- The experimental samples should be run within approx. 3-6 months of the standards (detector response and other factors change over time, and a new std curve is required)
- Don't throw out your standards! If you look after them, they should stay good for many months, and are valuable positive controls if you have concerns about your syringe function or any other aspects of your GC performance. Best practice would be to keep your calibration standards wrapped in foil, at 4°C, and inverted (so that liquid covers the lid). Make sure to let them equilibrate at your sample temperature before use however (at least 1 hr if they were at 4°C).

Method for making calibration standards

1. Figure out an appropriate range of concentrations of substrate to use for your experiment. This will depend on the activity of your cells, the volume of the reactions etc. A good starting range might be 0 µmoles to 10 µmoles of substrate in a 16 ml vial, or 0-100 µmoles substrate in a 120 ml serum bottle.

   The range depends on the purpose of the GC monitoring. If you are following substrate depletion in a growing culture, this might have very high gas conc's (10% of headspace) while in a more 'analytical' situation like testing enzyme activity, a lower gas concentration is more appropriate. You need to match the calibration curve to the experimental situation.

   For gases, you can use the gas equation PV= nRT to calculate what volume corresponds to what number of moles, or you can "cheat" if you assume the reactions are taking place at Normal Temperature and Pressure (NTP), where 1 mole of gas will occupy 22.4 litres of volume (i.e. 1 µmol = 22.4 µl).

2. Before you make your calibration standards, you need to know how much liquid will be in each vial in your experimental samples, then match the calibration standards to these. For a resting cell assay we would usually use 1 mL liquid in a 16 mL vial, while for growing cultures this would typically be 30 ml in a 120 ml vial. Its important that the liquid/headspace ratio in your calibrations is the same as your experimental vials. Use the same solution or medium in calibrations and experimental vials too (e.g. KP buffer or minimal medium).

3. Crimp seal your vials and inject your gas (or volatile liquid) substrate using a gas-tight glass/teflon syringe attached to a side-port needle. Try to find a syringe that matches the amount of substrate delivered, e.g. a 250 µl syringe is good for volumes approx. 50-250 µl, but if you had 20 µl, you should be using a 50 µl syringe etc.

4. If you need to inject really tiny volumes, consider making a dilution and injecting that instead. You can easily make dilutions of gases in air in a crimp-sealed vial (can add some plastic beads to shake around to mix the gas), while for volatile liquids you can dilute in water or buffer or an organic solvent. Check solubility and toxicity issues first !

5. We would typically prepare a 5-point calibration curve e.g. 0,1,2,5,10 µmol per vial. If you want to be rigorous, you should do multiple injections of each standard, or even set up multiple standards at each calibration level.

6. Once you've set up your calibration standards, you need to let your vials equilibrate to the temperature you are going to do your experimental assay at (e.g. 30C or 37C). This allows the volatile substrates to equilibrate (partition) between the gas and liquid phase in the vial. Give them a good shake by hand first (~5 seconds) then put them in the shaking incubator. About 10 minutes is enough. Note that things equilibrate faster in shaking waterbaths compared to regular shaking incubators.

Making the calibration curve

1. Open the Chromeleon Chromatography Studio window by clicking “studio” in the “data” section of the “Console” window, then go to the “Injection List” and set your injections to “Calibration Standard”. Then, in each “Level” cell, assign a “Level” for each calibration standard (e.g. 1,2,3,4,5)
2. Now go to “Data processing” on the bottom left, then click “Panes”, then “Data processing”. A “Compartment Table” window should open and there should be cells for you to fill in the amount of µmol for each calibration Level. For each standard, enter the amount of µmol.
3. On the lower left of the “Studio” window, return to “Injection list”, and press “Start”. Then follow the “injecting samples” method above to gather the data for the calibration curve. You should be able to get less than 5% peak area difference between replicates of the same calibration standard. If your errors are much more than 5% then you are doing something wrong, or the equipment is faulty (most commonly the syringe plunger is not gas-tight).
4. A report should’ve been generated, and on the top left, click the chameleon icon to export the data as an Excel file or a PDF.
5. The curve will not have an equation displayed, but you can easily work it out using y = mx+b with the values given to you (Gradient = m, and offset = b). The line of best fit should be linear, and you should be able to get r^2 values of at least 0.99 if you are careful.

GC Troubleshooting – no peaks / variable peak areas:

- the needle may be blocked, usually with rubber material from the septum; you can use a very fine piece of wire to clean it - these are found in the wooden drawer of GC stuff.
- gas may be escaping because the white Teflon plunger is not tight. Test this by sucking in some air, pushing in the red locking button and depressing the plunger most of the way in, then letting go. If the plunger bounces back some of the way, you have a good seal. If not, unscrew the inner plunger and tap the white Teflon tab several times (quite hard) on the bench surface and retest. When tapping the plunger, support the metal shaft with several fingers so it doesn’t bend. Its good practice to do this plunger-tapping before starting each GC session.
- gas may be escaping due to foreign matter (grit, dust, soil etc) in the syringe body. You can clean by squirting water through into syringe, but ensure it is fully dry before re-using.

GC Troubleshooting – wrong or variable retention times

- This often happens after many injections have been done through the same septum on the GC; the hole in this septum gets bigger and bigger until eventually carrier gas (and samples!) start to come back out the hole. The usual symptoms here may be that the retention time changes or that peaks are smaller than they should be. The solution is to replace the rubber septum in the injector port. This task should be done with the GC switched off and the injector port cooled to room temp, otherwise you may burn yourself !