Chemistry 231 – Fall 2013
Analysis of Carbonyl Compounds or Fatty Acids by Derivatization and HPLC or GC

Overview

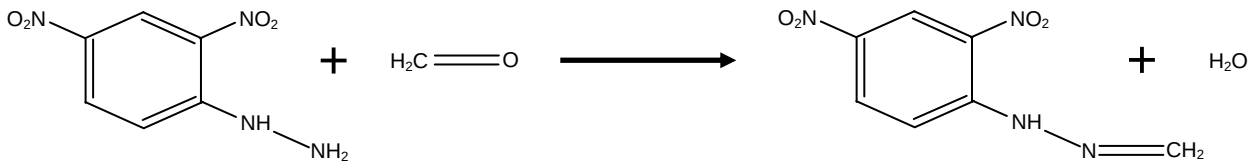
Both gas chromatography (GC) and high performance liquid chromatography (HPLC) have limitations for the analysis of certain compounds. For GC, only compounds that are volatile and do not decompose at high temperatures are acceptable analyte molecules. Derivatization in GC is commonly undertaken to increase the volatility or decrease the polarity of the analytes of interest, to change the elution time of a compound to move it away from interferences in the chromatogram, or to increase the sensitivity of a particular detector to specific analytes (for instance by halogenating compounds for detection by electron capture). With HPLC, which typically uses light absorption detectors, only analytes that have a significant chromophore are detected. Derivatization is usually carried out to produce products that are strongly absorbing. In some cases, the derivatization will also change the polarity in such a way as to improve separation. Derivatization also is commonly used with fluorescent agents to produce products that can be detected with outstanding sensitivity using fluorescence detectors.

Introduction

There are three parts to this lab: 1) derivatization and analysis of a set of standards, 2) derivatization and analysis of an unknown, and 3) derivatization and analysis of a sample of your choice. First you must determine if you will be analyzing carbonyl compounds by derivatization with 2, 4-dinitrophenylhydrazine (DNPH) to form hydrazones that will be quantified by HPLC, or if you will analyze fatty acids by derivatization with BF_3 in methanol to form fatty acid methyl esters to be quantified by GC. If you used GC to perform the previous lab, then you will use HPLC for this lab and vice versa. For step 3 of this lab, possible samples to analyze for carbonyl compounds include various types of air samples (indoor, outdoor, car exhaust or other source), decomposition products of alcohols in spirits, and lipid decomposition products (see some examples in the reference section). Good samples for analysis of fatty acids are oils, fats, or fatty food. These samples will require saponification of triglycerides to fatty acids before analysis (see an example in the reference section). For either lab, you may also obtain another type of sample, but you should consult with the instructor first. Step 3 of this lab will require more work on your part to identify possible samples and to look up past work in analyzing those types of samples using the methods described here.

Analysis of Carbonyl Compounds

While carbonyl compounds can be separated by HPLC and detected by absorption of UV light, they are not strongly absorbing and cannot be detected with good sensitivity. Carbonyl compounds react quickly with DNPH in acidic solutions as shown below for formaldehyde:



Once formed, DNPH derivatives can be separated by reversed phase HPLC and detected at a wavelength of 360 nm. As with most derivatizing agents, **DNPH is reactive and should be treated with care, so wear protective safety gear when preparing or using solutions.** Also check out the MSDS for DNPH before starting work.

Preparation of DNPH

Since DNPH reacts readily with carbonyl compounds and because formaldehyde and other carbonyl compounds are common indoor air pollutants, the reagent grade DNPH cannot be assumed to be very pure. In the first step, you should recrystallize DNPH to purify it. Recrystallization can be performed by dissolving DNPH in hot HPLC grade acetonitrile to form a saturated solution. Following dissolution, the solution should be cooled to a little above room temperature and capped in a brown vial for storage overnight in a refrigerator for further crystallization (label the vial). These crystals then will need to be collected by vacuum filtration. Often for research purposes, two recrystallizations are needed and a glove box is used to avoid absorption of carbonyl compounds during the recrystallization procedure. However, it is expected that a single recrystallization done in laboratory air should be sufficient for work undertaken here (this may not be true if you are trying to measure carbonyl compounds at low concentrations for the third part of the experiment). To avoid contamination, make sure there are no open reagent bottles of carbonyl standards nearby. One way to avoid cross contamination from carbonyl compound vapors is to work with concentrated carbonyl standards only in the hood.

Once crystals of DNPH have been prepared, they should be used to make a DNPH reagent solution. They should be transferred to a vial to make a saturated solution of DNPH in acetonitrile. The saturated solution should be of sufficient purity for use in derivatizing standards or samples. Over time, the DNPH solution may absorb carbonyl compounds from the air. The solution can be purified by pouring out the saturated DNPH and refilling the vial with fresh acetonitrile. Sufficient time should be given to allow for the solution to reach saturation. Possibly a better way to make the DNPH solution is to dissolve a known mass of DNPH in acetonitrile. This method makes it easier to estimate how much DNPH you have added to your reaction mixture (below). However, if this solution becomes contaminated, you will need to purify additional DNPH. **Be sure to dispose of DNPH in the proper waste container.**

Derivatization and separation of a carbonyl compound standard

Solutions will be provided containing the following carbonyl compounds each present at about 10 mM in water (all but benzaldehyde are in water):

formaldehyde
acetaldehyde
acetone
propionaldehyde
butaraldehyde
benzaldehyde

Dilute stock solutions to the 50 µM range for initial analysis. This solution should be reacted with the saturated DNPH by carefully transferring 2.0 mL of the carbonyl standard to a 5 mL volumetric flask, adding enough DNPH solution so that there is approximately 100 to 200% excess DNPH, adding 0.2 mL of 1.0 M HCl, and diluting to the mark with acetonitrile (dilution with acetonitrile is done because DNPH is not highly soluble in aqueous solutions). The

volumetric flask should be well mixed and then the contents transferred to HPLC vials. The solutions should be allowed to react for 40 minutes at room temperature before analysis or refrigeration. **Be sure to filter all solutions before analyzing by HPLC!**

Analysis of unknown solution

Unknown solutions will contain two of the six carbonyl compounds listed dissolved in water with concentrations similar to the suggested standard dilution mentioned above. Prepare your unknown for analysis in the same way as described for the carbonyl standard solution provided. After determining the identity of your unknowns, prepare a mixed standard containing the two unknown carbonyl compounds. This solution should be prepared for analysis in the same way as the other solutions. Make at least 4 quantitative standards for your analysis, and analyze your data in a similar manner as described in the first HPLC lab.

Analysis of your chosen sample

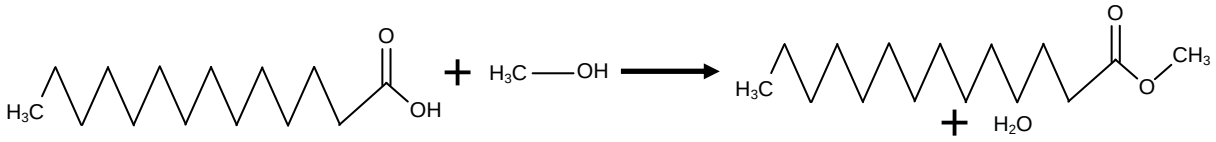
You will need to revise your method for analyzing carbonyls based on the type of sample you collect. Air samples generally are collected using C_{18} solid phase extraction (SPE) cartridges that have been treated with acidic DNPH before sample collection or by using a mist chamber initially filled with acidic DNPH. In this manner, derivatization occurs during sampling, and the analytes need only to be eluted from the SPE cartridge or removed from the mist chamber. The flow rate of sample air through the cartridge or mist chamber, and the sampling time need to be measured, or otherwise known for quantitative determinations. There is equipment available that could be used by one or two students. Additionally, it is critical that the addition of acid keeps the pH low (around 2). If possible, a recovery standard should be added to your sample (or a portion of your sample) to ensure that extraction and derivatization are complete. Although the accuracy of the quantitative analysis for this lab is not critical, you should do your best to quantify at least the carbonyls that appeared in your unknown. Other types of samples are spirits or possibly phospholipids following oxidation treatment (see example references). Other carbonyl compound standards may be available if you are interested in their analysis. Regardless of the type of sample that you choose, you should search the literature and see if you can find any hints as to how to properly analyze your sample. **It is especially important that you filter your sample before HPLC analysis for this part of the lab.**

Analysis of fatty acids

Fatty acids are important components of many biologically important molecules such as triglycerides (fats or oils) and phospholipids. The length and number of double bonds in the fatty acids are also important for health reasons in the consumption of fats and also affect physical properties (such as melting point temperatures) of triglycerides. Fatty acids occur frequently in the environment and can be used as tracers (e.g. in atmospheric aerosols). Most of the triglycerides and phospholipids cannot be analyzed well in their natural state. However, it is relatively easy to convert fats or phospholipids to fatty acids through base catalyzed hydrolysis.

Fatty acids cannot easily be analyzed by GC, but may be derivatized to fatty acid methyl esters (FAMEs) that are volatile and stable enough for analysis by GC. There are a few commonly used methods for alkylation of fatty acids. Diazomethane and methanol are commonly used reagents. Diazomethane is generally considered to be unsafe due to the potential

for explosions. Methanol can be used with BF_3, HCl, and other catalysts. We will be using BF_3 in methanol for derivatization. The derivatization reaction is as follows:



Derivatization and separation of a fatty acid standard

Standard solutions are to be derivatized for the purpose of determining the FAME retention times and for the purpose of determining response factors for quantitation. Solutions will be provided containing the following fatty acids each present at about 10 mM in a methanol/dichloromethane mixture:

lauric acid (C12)
myristic acid (C14)
palmitic acid (C16)
heptadecanoic acid (C17)
stearic acid (C18)
oleic acid (C18:1)

Dilute standards to the 1 mM range for initial analysis. This solution should be reacted with the BF_3 in methanol by carefully transferring 3.0 mL of the fatty acid standard to an 18 x 150 mm test tube, adding 5 mL of BF_3 in methanol, and placing the test tube in a hot water bath so that the solution boils for 3 minutes (a glass bead should be present in the bottom to avoid bumping). This allows the formation of FAMEs. After cooling, quantitatively transfer the solution to a separatory funnel containing 25 mL of hexane and 20 mL of saturated NaCl(aq). After shaking and allowing the layers to separate, the water layer should be saved for a second extraction with hexane while the hexane layer should be transferred to a 50 mL volumetric flask. Extract the water layer a second time with hexane and combine the hexane extracts. An internal standard will need to be added to use the standard for quantitative purposes. Then, fill to the mark on the volumetric flask. There may be a FAME standard available of one the above fatty acids that can be used for estimating the derivatization yield. An alternative method is to use a surrogate standard of known concentration. Use GC to analyze the FAMEs.

Analysis of unknown solution

Unknown solutions will contain two of the fatty acids listed (except not heptadecanoic acid and not a mix of stearic and oleic acid), present in the 1mM range. The derivatization procedure should be the same as for the mixed standard. Use an internal standard to quantify the fatty acids in your unknown. You also can add heptadecanoic acid as a recovery standard. Be sure to make multiple analyses and report the precision of your measurements.

Quantification by GC

You must use an internal standard for this lab. Be sure to choose a compound as your standard that can easily be separated from the analytes in your sample, and is completely soluble in the solvent you are using. The following lists the steps necessary to use an internal standard for quantitative analysis.

1. Let X = the unknown, and S = the standard.
2. $\frac{A_X}{A_S} = F\frac{C_X}{C_S}$, where A is the area, C is the concentration, and *F* is a 'response factor' between X and S.
3. A solution is made that contains known amounts of X and S, this solution is used to determine *F*.
4. Add a known amount of S to any solution that contains an unknown amount of X. Realize that this will dilute the solution, and this must be accounted for when calculating the final concentration of X.
5. After obtaining a chromatogram of the solution, use the equation in '2' above, to determine C_X. Be sure to account for the dilution that occurred when adding a known amount of S.

Analysis of chosen sample

You will need to research what steps are normally taken for analysis of various types of real samples. The *J. Chem. Ed*. article referenced below provides a method for the analysis of fats and oils (these should be the easiest types of samples to analyze, although you could also chose seeds or nuts if you are more ambitious). The goal is to show that the method can be used for qualitative and quantitative analysis in real samples. However, you should just try to quantify concentrations of fatty acids for which you have standards. Realize that overlap with similar peaks may limit both quantitative and qualitative analysis. It also may be possible to use GC-MS. This may allow detection of unsaturated fatty acids even if they overlap with saturated fatty acids. There may be an odd numbered fatty acid (e.g. C_{17}) or triglyceride containing fatty acids that could be used as a recovery standard (e.g. can be added to the sample to estimate the % recovery to a FAME).

Report

Your report should be double spaced, with 12 pt Times New Roman font and 1 inch margins. Include your name, a title, a 2-3-sentence abstract, a short introduction, experiment description, results and discussion, and a short conclusion. In the introduction, be sure to document past analyses mentioned in the literature for this type of sample. In the experiment description be sure to include (in addition to the operating parameters of the HPLC or the GC), a description of the derivatization procedure. The results and discussion should include a discussion of the sample that you chose for analysis, as well as the unknown. As always, be sure to follow the guidelines for report writing that were handed out early in the semester.

Oral Report:

In addition to the written report, you will give a brief (10 min.) oral presentation of your results, focusing on the samples of your choice. The presentation should be done in a Powerpoint format and we will schedule class time for the presentations. This presentation should have an introduction, in which you discuss why analysis of the type of sample you chose is important. This will rely heavily on the literature search that you have carried out. Next you should describe methodology for collection and treatment of the sample with a focus on any differences or additions versus the standard/unknown sample procedure. Then, you should show your results. If some part did not work well, this should be explained. The data also should be

interpreted in terms of what you were sampling. For example, if you measured formaldehyde in the air of your apartment, you should give an air concentration (in appropriate units) and compare the concentration to what others have found. You may want to give profiles of carbonyl compounds or fatty acids in this part of the presentation. You can have one slide for concluding remarks including how the experiment could have been improved if you had a chance to do it again.

References:
Some uses of DNPH derivatives:
Cardoso**,** D. R., S. M. Bettin, R. V. Reche, B. S. Lima-Neto and D. W. Franco, (2003), HPLC–DAD analysis of ketones as their 2,4-dinitrophenylhydrazones in Brazilian sugar-cane spirits and rum, *Journal of Food Composition and Analysis*, **16**(5), 563-573.
K. Müller, S. Haferkorn, W. Grabmer, A. Wisthaler, A. Hansel, J. Kreuzwieser, C. Cojocariu, H. Rennenberg and H. Herrmann, (2006), Biogenic carbonyl compounds within and above a coniferous forest in Germany, *Atmospheric Environment*, **40**, 81-91.
G.A. Cordis, D. K. Das and W. Riedel, (1998), High-performance liquid chromatographic peak identification of 2,4-dinitrophenylhydrazine derivatives of lipid peroxidation aldehydes by photodiode array detection *Journal of Chromatography A*, **798(1,2)**, 117-123.

For fatty acid derivatization:
Rubinson, J. F., and J. Neyer-Hilvert, (1997), Integration of GC/MS Instrumentation into the Undergraduate Laboratory: Separation and Identification of Fatty Acids in Commercial Fats and Oils, *J. Chem. Ed.*, **74,** pp 1106-1108.