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Experiment #5: Identification of Phospholipids,Fatty Acids in Oils and Their Oxidation Products Using Supercritical Fluid Extraction (SFE), Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry and Electrospray Ionization Mass Spectrometry (ESI-MS)

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INTRODUCTION

The ability to sensitively detect and analyze large molecules and their reaction products is important for the analysis of biological materials, including medical samples, plants and foods. Such analyses present special challenges in terms of the need to selectively extract the compound or class of compounds in order to separate them from the complex mixture found in biological samples. In addition, conventional techniques for separating and identifying relatively small molecules are not applicable to high molecular weight materials where the volatility is low and they are often viscous liquids or solids at room temperature.

This experiment is designed to illustrate several facets of the analysis of high molecular weight compounds found in biological samples. In the first part of the experiment, you will identify a phospholipid that is a common component of the human body, e.g. lipid bilayers (see any basic book on biochemistry), and of the pulmonary surfactant that is found in the alveolar region of the lung where gas exchange occurs. In the alveoli, it reduces the surface tension of the fluid lining the lung to allow facile expansion and contraction during breathing. A deficit of the phospholipid is associated with hyaline membrane disease in newborns and adult respiratory distress syndrome in adults (e.g. associated with the hanta virus).

One particular phospholipid and its ozonolysis products will be identified using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The principle of MALDI, shown in Figure 1, is a combination of laser vaporization and ionization via interaction of the analyte with an added "matrix" molecule. The matrix donates a proton to the analyte, generating the $[M + H]^+$ ion which is detected. As you will see in this experiment, other ions such as Na^+ can also provide the ionizing adduct ion. A major advantage of this method is that it leads to very little fragmentation, making it ideal for observing parent peaks of large molecules. This is particularly useful for single compounds or relatively simple mixtures.

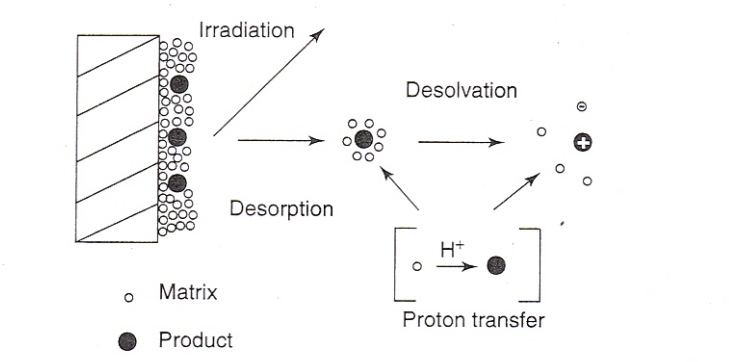


Figure 1. Principle of MALDI (from "Mass Spectrometry: Principles and Applications", DeHoffmann and Stroobant, Wiley, (2002)

In addition, you will use electrospray ionization mass spectrometry (ESI-MS) to identify the parent phospholipid and its oxidation products. ESI-MS, shown in Figure 2, involves forming droplets of the analyte in a solvent or solvent mixture and these droplets are ionized by a high voltage at the tip of the capillary as they are formed. As they travel through a vacuum, the solvent evaporates and the build-up of charge on the decreasing droplet size eventually causes it to fragment to many smaller pieces containing one charge. This generally gives $[M + H]^+$ or $[M + Na]^+$ peaks. ESI-MS is extremely useful for polar compounds and high molecular weight materials that are not amenable to analysis by techniques such as GC-MS that require some volatility. MALDI-MS is useful primarily for compounds with mass > 500 amu while ESI-MS can also be used for smaller masses.

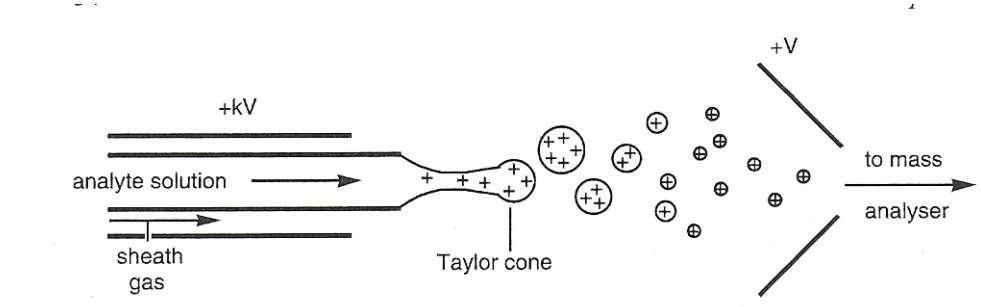
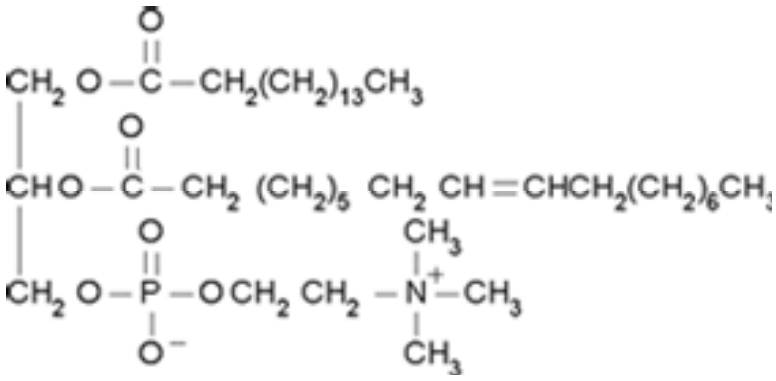


Figure 2. Cross-Section of an Electrospray Ionization Source (from "Mass Spectrometry, A Foundation Course", Downard, Royal Society of Chemistry (2004).

Note that HPLC-MS is a powerful method for separating complex mixtures of high molecular weight material and then identifying each component by MS as they elute from the column. ESI is commonly used as the ionization method for the column eluents. You will not be using the combination of HPLC with MS in this lab, but the principles are the same. The review article by Simal-Gandara in the reference list treats this subject in more detail and is provided for your information should you encounter HPCL-MS in your future work.

The particular lipid you will use in this experiment is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; see structure below). Phosphocholines have a glycerol-type backbone with fatty acids in the 1- and 2- positions. POPC has palmitic acid (16:0) in the 1- position and oleic acid (18:1) in the 2- position; the use of (*x:y*) denotes the number of carbon atoms as *x* in the fatty acid attached to the glycerol backbone and the number of double bonds in the fatty acid as *y*. The molecular mass of POPC is 760.08 and the molecular formula is $C_{42}H_{82}NO_8P$. It is important to recognize that with these mass spectrometric techniques, you are measuring the real masses of particular molecules. The ***molecular mass*** of POPC you find on a bottle of this chemical is an average that reflects the contribution of small amounts of the heavier isotopes such as ^{13}C. The ***exact mass*** or ***monoisotopic mass*** is that for a particular molecule with specific isotopes. The difference between the molecular mass and exact mass can be significant for large molecules that have large numbers of carbons, for example, where the minority isotopes start to contribute significantly.

While POPC might seem like a large compound, at least relative to the other compounds you analyze in this class, relative to most biological molecules of interest it is actually quite small.



Structure of POPC

Because POPC has a double bond, it reacts with ozone (O_3) to form a secondary ozonide, an aldehyde and a carboxylic acid. In this experiment, you will react POPC with ozone and identify these products using MALDI as well as ESI-MS.

The second part of the experiment involves a much more complex (but real) sample: soybeans. Soybeans are a major food crop world-wide. They contain significant amounts of oil which have a high concentration of fatty acids. Typically, the oil has ~ 25% oleic acid (18:1) and ~ 50% linoleic acid (18:2). In order to extract the oil, you will apply the technique of supercritical fluid extraction (SFE), which uses CO_2 above its critical point to dissolve the oils. An organic solvent is often added to improve the extraction of certain components. This can be done using an additional pump on the extraction apparatus. Another ("poor man's method") way to accomplish this is to soak the beans overnight in the organic solvent. Your faithful TA's will provide you with soybeans that have been soaked in ethanol overnight as well as soybeans that have not been exposed to ethanol. You will extract both samples to see if there are any differences. You will then react the soaked ones with ozone and analyze the reacted and the unreacted oils using ESI-MS. From this, you should be able to say something about the need (or lack thereof) of a cosolvent to extract the fatty acids and the identification of the ozonolysis products.

This experiment is under development (you are the lucky souls to try it first!) and we will not ask you to quantify compounds at this point. However, you will gain experience with three very important techniques for extracting and analyzing high molecular weight compounds.

EXPERIMENTAL

Supercritical Fluid Extraction of Soybean Oils

The supercritical fluid extractor uses high pressure, high temperature carbon dioxide for the extraction of oils from various media. Care should be taken to tighten all fittings on the instrument and when touching hot areas of the extractor.

1. Before you get to lab, your TA should have set the heater for 40ºC. Check the digital readout to ensure that the instrument is at the correct temperature. Also ensure that the chiller (located to the left of the instrument) is on and cold.

2. Weigh out 80 grams of soybeans. Load your soybeans into the extractor and screw on the top fitting to close the extraction chamber; you may have to pack them in with a wrench to get them to fit. Attach the CO_2 inlet and outlet lines to the extractor. Finger tighten the nuts. Ask your TA to be present to supervise the final tightening. In general, a good seal can be obtained by tightening the nuts 1/8 turn beyond finger tight with the wrenches provided. Do not over-tighten them. Ask your TA if you are unsure.

3. Close the valve labeled "static/dynamic" valve. The restrictor valve should be open only three turns (all the way closed is all the way toward "increase").

4. Open the CO_2 tank. Make sure that the compressed air is attached to the supercritical fluid extractor and that all valves are open. The pressure reaching the extractor should be at least 100 psi.

5. Open the air supply valve and the CO_2 supply valve. Use the vessel pressure regulator to set the pressure to 2000 psi. Open the door to the extractor and listen for any leaks in the top and bottom fittings; tighten as necessary.

6. Increase the pressure to 4000 and then 6000 psi and leave it there for 45 minutes to complete the static extraction.

7. After 40 minutes, open the restrictor valve by 13 more turns (open 16 turns total) and open the static/dynamic valve to initiate the dynamic extraction. Make sure there is a small, clean flask underneath the extraction outlet and that the extraction outlet valve is open. The dynamic extraction process lasts until there is no more oil coming out of the instrument; this should take about 15 minutes.

8. After the dynamic extraction, close the CO_2 tank and open the restrictor valve all the way to let the pressure decrease. Turn the vessel pressure regulator all the way down. Once the pressure reaches around 1000 psi, the instrument will not be able to decrease it further; open one of the vessel fittings a very small amount to allow the rest of the CO_2 to escape. Remove all the soybeans from the extractor and clean it as well as you can using soapy water followed by thorough rinsing with water and then an organic solvent such as isopropanol or acetone.

9. Repeat the extraction using the soybeans that have been soaked in ethanol overnight.

10. Use a stream of N_2 to dry off any excess ethanol that has been extracted from the soybeans and dissolve the remaining oil in the flask in 4 mL of a 9:1 hexane/ethanol solution. Divide the liquid sample into two parts, one of which will be reacted with ozone and one of which will be a blank.

11. Dry both samples by blowing nitrogen gas over each sample using a glass pipette in a nitrogen gas line. Put caps on both vials after evaporating off the solvent. Be sure to label the vials, one as unreacted and the other as reacted (i.e. this is the one you will expose to ozone; see below)

Preparation of the POPC

1. Weigh out 5.0 mg of POPC and dissolve it in 2 mL of a 9:1 (v:v) mixture of hexane and ethanol.

2. Pipette 1 mL of this solution into a vial to be used as the unreacted sample and 1 mL into a second vial that will be reacted with O_3. Be sure to label the vials accordingly!

3. Dry both samples by blowing nitrogen gas over each sample using a glass pipette in a nitrogen gas line. Put caps on both vials after evaporating off the solvent.

4. You will carry the samples over to the Mass Spectrometry facility in this form of dry samples in the vials. At the MS Facility, you will add 1 mL of of a 1:1 (v:v) acetonitrile-water solvent mixture to each of these vials before diluting further and then carrying out MALDI-MS and ESI-MS.

Ozonolysis of the POPC and Soybean Extracts

1. When all of your samples (POPC and soybean extracts) are ready to be reacted with ozone, your TA will take you to another lab to flow concentrated O_3 into the vials to do the ozonolysis. Expose the contents of the vial to O_3 for 10 minutes, then immediately cap the vial and let sit for an additional 20 minutes.

2. After ~ 30 min, place the vials containing the samples and O_3 in the hood and flow N_2 into the vials to displace any remaining O_3.

MALDI-MS analysis of POPC Samples

1. The following steps will be done in the Mass Spectroscopy Facility under the supervision of your TA. All of the sample preparation steps above must be done before proceeding to the Mass Spectroscopy Facility.

2. Each of your vials started out with 2.5 mg of POPC. One of them is unreacted and still has this 2.5 mg POPC, while the one that has reacted with O_3 will have some unreacted POPC remaining in addition to at least three oxidation products formed in the ozonolysis. To each vial, add 1 mL of the 1:1 (v:v) acetonitrile-water solvent mixture. Cap the vials and use the vortex mixer for 30 s to 1 min to assist in the dissolution of the compounds into the solvent.

3. MALDI-MS gives better signal at low concentrations. For this reason, prepare a 1:10 dilution of your samples by mixing 100 microliters of the sample with 900 microliters of the acetonitrile-water solvent mixture. Be sure to save the remainder of the sample as you will use it for ESI-MS analysis. Use the small vials provided for this.

4. Matrix preparation:

    a. First you will need to prepare a solution of the matrix; the matrix used will be 2,5-dihydroxybenzoic acid (DHB), one of the most common MALDI matrix compounds as it readily transfers a proton to analytes. To make the solution, pipette 500 microliters of acetonitrile and 500 microliters of water in a small vial and add an excess of the matrix. Mix the solution well and allow the excess solid to settle.

    b. Pipette 0.5 microliters of the various samples and 0.5 microliters of the matrix (i.e. the clear liquid above the solid) onto the selected wells of the MALDI plate provided. Record which samples are placed on which spots. It is important to pipette the sample on the spot first and then pipette the matrix directly on top of the sample droplet for proper crystallization to take place.

    c. To take a spectrum of the matrix itself, you will need a spot where there is only matrix and no sample. Use 0.5 microliter of matrix solution for this spot.

5. Proceed to the MALDI instrument. Turn on the television monitor and the oscilloscope on top of the mass spectrometer.

6. Open the mass spectrometry program (“Voyager Control Panel”) on the instrument’s computer.

    a. All the indicators at the bottom of the program screen should be green after a short wait. If they are yellow, let your TA or the facility manager know.

b. Press the yellow lightning bolt icon to turn off the high voltage to the instrument.

c. Press the "hand" button and then press "eject" to bring the sample holder out of the instrument.

7. Insert the sample plate with spot number one in the upper front corner, ensuring that all the sample spots are dry before you do so. The sample plate is fully inserted when you feel the first click.

8. Take spectra of the various sample spots one at a time.

   a. Once the sample plate is in the holder, click the "hand" button again.

   b. From the "Plate ID" pulldown menu, select the first number 100. Click "Load". The sample spots should start to become visible on the TV screen as the plate is loaded into position. Turn the high voltage back on.

   c. The parameters for the scans should be as follows:
      i. Mass range – 500 to 2000 Da.
      ii. Low mass gate – 500
      iii. 500 shots per spectrum
      iv. 20,000 V accelerating voltage
      v. Grid – 94
      vi. Guide Wire – 0.0001
      vii. Delay time – 100 nsec.

   d. Select a sample spot from the "Active Position" pulldown menu. The laser intensity should be between 2300-2400.

   e. Enter a filename for your scans. The program will automatically add the suffix "_00N" (where N is the scan number) to your filename, so just pick one that identifies your group. Save it in the appropriate folder on the computer's hard drive.

   f. To begin sampling, press the button on the left of the top left corner of the joystick controller. Look at the oscilloscope and use the joystick to move the laser until the signal peaks are maximized. Note that the spectrum is inverted so the larger the peak, the more towards the bottom of the screen it is. When you are happy with the peak size, press the start/stop button again to stop the scan. Press the other button on the top left corner of the controller to save the spectrum.

   g. When you are finished with your samples, clean the MALDI plate by rinsing it with water and then with isopropanol.

9. Be sure to take spectra of the unreacted POPC, the reacted POPC, the matrix AND a blank spot on the plate. MALDI-MS is so sensitive that contaminants

may be present on the plate and appear in your spectra. You need to examine the spectra of your samples carefully and compare them to both that of the matrix and that of a blank spot on the sample plate to be sure which peaks are due to POPC and its oxidation products.

10. Print your mass spectra for later analysis. Be sure to print out all the spectra you will need.

ESI-MS of Samples

Your TA will work with you on this part of the experiment as this is a top-notch research grade instrument not normally available to classes. You will do ESI-MS in the positive ion mode for POPC and its oxidation products, but for soybean oils and their oxidation products, you will use the negative ion mode. Be sure to run a "blank" of the solvents used for comparison.

Since you will have done the MALDI first, your POPC samples will already be in a CH_3CN:H_2O solvent mixture. However, the soybean oil samples will be dry. You will need to dissolve them in 1 mL of the 1:1 (v:v) CH_3CH:H_2O and again, vortex mix them. If one or both of the solutions look like they have formed an emulsion and not dissolved completely, you will need to centrifuge to try to deposit out any undissolved material. Use the supernatant liquid for analysis in this case.

DATA ANALYSIS AND DISCUSSION

POPC and Its Oxidation Products: Calculate how many moles of the unreacted POPC you put on the MALDI plate for analysis. This will be a good indicator of the sensitivity of this technique.

Use the MALDI mass spectra for the unreacted POPC and reacted POPC samples to identify what changes, if any, were caused in POPC by ozone.

Important: Remember the difference between molecular mass and exact or isotopic mass in assigning your peaks. Also remember that the matrix you used adds a proton to the molecule so that it is the $[M + H]^+$ peak that is generally seen. **BUT**, as it turns out, Na^+ is a very common contaminant that is present in virtually all samples. For example, it leaches out of glass so that it will be present due to your use of glass vials. Potassium is also a common contaminant, generally present at smaller levels than sodium. You should consider the possibility that some of your peaks may represent addition of Na^+ or K^+ rather than H^+ to the parent compound or its oxidation products.

Also be sure to compare your sample spectra to that of a "blank" of the matrix alone and to a blank spot on the plate to be sure that the peaks you are assigning are truly due to POPC and its oxidation products and not to the matrix or to contaminants on the plate.

Describe the mass difference between the reactants and products, and what kind of reaction might cause this difference. Include a discussion of the mechanism that was responsible for creating your observed products. Comment on the ways in which ozone

could react with the POPC molecule, and whether or not your mass spectra provide evidence for such a reaction.

Now identify the major peaks in your ESI-MS spectrum for the POPC and its oxidation products. Again recognize that Na^+ and K^+ are likely to be present. Also, if you added CH_3OH to the sample to do ESI-MS, you need to know that methanol can add to an aldehyde to give a peak that is at (parent + 32 amu + ionizing species),or $[M + CH_3OH + H]^+$.

Compare the MALDI and ESI-MS spectra of the oxidation products and comment on any differences you see in the peaks present and/or their relative intensities.

Soybean Oils and Their Oxidation Products: For the ESI-MS of soybean oil and the oxidation products, focus on the mass region between about 150 and 350 amu. Unlike MALDI, ESI-MS can be used to look at masses below 500 amu.

Given the high content of oleic and linoleic acids in soybean oil, can you identify peaks that are due to these fatty acids in the ESI-MS spectra? Remember that in negative ion mode, these fatty acids will lose a proton to form the anion $[M - H]^-$. Also remember to compare to the spectrum of the solvent alone to be sure that the peaks are due to your analyte and not the solvent itself.

Comment on the differences, if any, between the unreacted soybean oils that were from ethanol-soaked beans and those from beans that had not been soaked in ethanol. Which technique is preferable for the fatty acid extractions?

Compare the ESI-MS spectrum of the unreacted (but ethanol soaked) soybean oils to that of the comparable ozonized soybean extract. What peaks decrease and what peak(s) increase? Again, be careful to take into account what peaks are present in the solvent. You should see some peaks that do not appear to change; you can use these to help assess which peaks are increasing and which are decreasing.

It will be helpful to recognize that palmitic acid (16:0) is a very common component of biological systems but since it does not contain a double bond, it is not reactive to O_3. There are usually also smaller amounts of stearic acid (18:0). You can use these as a sort of "internal standard" to assess the impacts of ozonolysis since they will not decrease due to reaction and any differences in absolute ion counts are due to variability in the amount of sample that the MS sees.

Based on this, which fatty acid predominates, and at what location in the molecule is the double bond that is breaking during ozonolysis? What product is being formed that you see by ESI-MS?