Student Instructions

Lipid Residue Analysis of Archaeological Pottery: An Introductory Laboratory Experiment in Archaeological Chemistry

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Unglazed ceramics provide an excellent repository for organic molecules, which deposit within the porous matrix during cooking, food storage, or ceremonial usage. When a ceramic is buried these absorbed molecules are subject to chemical and microbial degradation and leaching. Only the most robust chemical species thus survive long-term internment. Molecules that have shown unsurpassed resiliency include lipids (triglycerides and fatty acids), sterols, and terpenoids.[1] Pottery residue analysis can provide archaeologists insight into the possible use of the original ceramic ware and assist in reconstructing regional technologies and economic and cultural practices.

Ceramic fragments, or sherds, are prolific throughout the archaeological record, permitting a destructive extraction method to maximize the residue available for analysis. Before crushing, each sherd must be photographed and documented based on archaeological methodology. Absorbed molecules are then extracted using a solvent mixture appropriate for the molecules thought to have been contained within the vessel. It is imperative to understand the possible sources of organic compounds within and on the surface of a sherd. These residues include those associated with the use of the ceramic vessel, molecules associated with contamination introduced during the excavation process, and finally contamination introduced during sample processing in the laboratory.[2]

Lipids have garnered attention in pottery residue analysis as they are robust and can provide direct evidence of diet, trade and exchange patterns, and status within a culture.[3–5] Lipids are nonpolar, medium sized molecules comprised of linear, branched, or cyclic hydrocarbon chains. Lipids commonly recovered from archaeological ceramics include fatty acids, triglycerides, long-chain ketones, wax esters, *n*-alkanols, and *n*-alkanes.[4] In arid conditions, lipids have been recovered from excavated pottery dating to the eighth millennium B.C.[6] Triglycerides and fatty acids, which arise from the processing of plants and animals in ceramic vessels, are the most prolific lipids found in the archaeological record and can serve as key biomarkers.

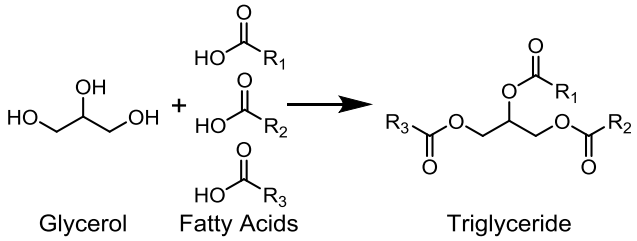
Fatty acids are long, hydrocarbon chains terminated by a carboxylic acid group (-COOH) that contain double bonds in unsaturated fatty acids, while those without double bonds are called saturated fatty acids. These lipids are referred to by either their common name or shorthand notation where the first number indicates the total number of carbons, including the carboxylic acid carbon, while the second number indicates the number of double bonds. Coordination about the double bond is indicated next (*cis* vs. *trans*) followed by the double bond's location found by counting from the carboxylic acid as carbon 1 (**Table 1**). Naturally occurring fatty acids predominately take on the *cis* configuration, which means that the carbon chain extends on the same side of the double bond to give a non-linear configuration.

Table 1. Names, Shorthand notation, and Structural Formulas of Common Biological Fatty Acids and Associated Fatty Acid Methyl Ester (FAME)

Name	Shorthand	Structural Formula	Name of Fatty Acid Methyl Ester	FAME MW
Capric	10:0	$CH_3(CH_2)_8COOH$	Decanoic acid, methyl ester	186.29
Lauric	12:0	$CH_3(CH_2)_{10}COOH$	Dodecanoic acid, methyl ester	214.34
Myristic	14:0	$CH_3(CH_2)_{12}COOH$	Tetradecanoic acid, methyl ester	242.40
Palmitic	16:0	$CH_3(CH_2)_{14}COOH$	Hexadecanoic acid, methyl ester	270.45
Palmitoleic	16:1 cis-9	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	9-Hexadecenoic acid (Z)-, methyl ester	268.43
Palmitelaidic	16:1 trans-9	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	9-Hexadecenoic acid (E)-, methyl ester	268.43
Stearic	18:0	$CH_3(CH_2)_{16}COOH$	Octadecanoic acid, methyl ester	298.50
Oleic	18:1 cis-9	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	9-Octadecenoic acid (Z)-, methyl ester	296.49
Elaidic	18:1 trans-9	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	9-Octadecenoic acid (E)-, methyl ester	296.49
Linoleic	18:2 cis-9,12	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COOH$	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	294.47
Linolenic	18:3 cis-9,12,15	$CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6COOH$	9,12,15-Octadecadienoic acid (Z,Z,Z)-, methyl ester	292.46

Fatty acid shorthand notation: 16:1 indicates a 16-carbon chain with one double bond (palmitoleic acid) and cis-9 indicates that the double bond is between carbon 9 and carbon 10 with a *cis* geometry.

Triglycerides are composed of three fatty acids linked to a glycerol backbone through three ester bonds (**Scheme 1**).



Scheme 1. Both fats and oils are comprised of three fatty acids linked through an ester bond to a glycerol backbone. $\mathbf{R_n}$ represents a unique saturated or unsaturated hydrocarbon chain between 9-17 carbons long.

Triglycerides comprised predominately of saturated fatty acids are referred to as fats as they are solid at room temperature, while those predominantly composed of unsaturated fatty acids are referred to as oils as they are liquid at room temperature. In oils and fats, the three fatty acid chains are never the same within a triglyceride molecule and the fatty acid chains typically range between 9 to 17 carbons with 0 to 3 double bonds. Each plant and animal species synthesizes triglycerides using a unique combination of fatty acids (**Table 2**).

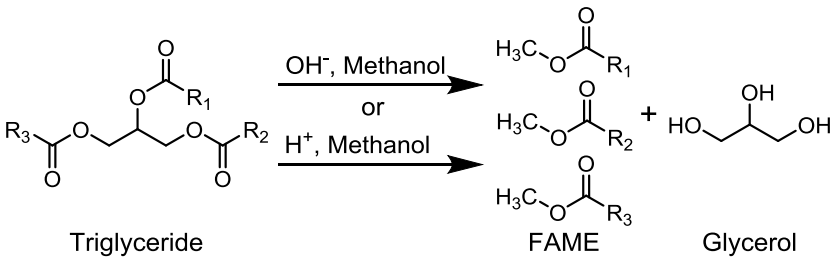
Table 2. The Major Fatty Acids Found in Several Edible Fats and Oils

	Relative % abundance				
Edible Fat/Oil	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**
Safflower Oil	4.3	1.9	14.4	74.6	0.0
Maize Oil	15.6	2.9	34.7	43.6	1.6
Cow Fat (tallow)	24.9	18.9	36.0	3.1	0.6
Olive Oil	11.3	2.0	71.3	9.8	0.8
Rapeseed Oil	4.3	2.1	61.7	19.0	9.1
Soybean Oil	10.5	4.4	22.6	51.0	6.8
Grapeseed Oil	6.7	2.7	15.8	69.9	0.1
Salema Fish Oil*	31.4	6.31	10.1	1.1	0.6

Data from the United States Department of Agriculture (USDA) Nutrient Database for Standard Reference: http://www.nal.usda.gov/fnic/foodcomp/search/ and *Prato, E.; Biandolino, F. *Food Chemistry* **2012**, 131, 1233–1239

As seen in Table 2, unsaturated fatty acids (18:1, 18:2, & 18:3) are more prominent in plant oils, whereas saturated fatty acids (16:0 & 18:0) are more prominent in animal fats. These unique fatty acid combinations are what allow archaeologists to associate recovered residues with specific plant oils and animal fats. To experimentally determine the relative fatty acid ratios using gas chromatography (GC) or gas chromatography coupled to mass spectrometry (GC/MS), the fatty acid components of the triglyceride are first converted to fatty acid methyl esters (FAMEs), a simple volatile derivative.

Methylation of the ester linkage to synthesize FAMEs can occur using either a base or acid catalyzed transesterification reaction (**Scheme 2**).[7]



Scheme 2. Fatty acid methyl esters (FAMEs) can be synthesized using either a base or acid catalyzed transesterification reaction. Both methods yield three FAMEs and glycerol molecule. $\mathbf{R_n}$ represents a unique saturated or unsaturated hydrocarbon chain between 9-17 carbons long.

Both catalytic methods yield three FAMEs to one glycerol molecule per triglyceride. The differences between acid and base catalyzed methylation are the reaction time and their ability to methylate free fatty acids, which can form due to hydrolysis, microbial degradation, or thermal degradation of the triglyceride. Base catalyzed transesterification occurs rapidly under moderate conditions (heat and stirring) but is limited to the methylation of triglycerides only. Acid catalysis can methylate both triglycerides and free fatty acids though longer reaction times and higher temperatures are required. The common names of prominent FAMEs found in methylated oils and fats can be seen in Table 1.

Once methylated, isolation of the FAME product is simplified by the fact that glycerol is polar, whereas FAMEs are nonpolar. The products create two distinct layers that can easily be separated for purification. Isolated FAMEs are then diluted in a volatile solvent and analyzed by GC. Utilizing a polar column, FAMEs separate based on carbon number as predicted by boiling points and the degree of unsaturation within each carbon number since dipole-induced dipole interactions between the FAMEs and the polar column are stronger with increased unsaturation. Under optimal temperature ramping conditions, FAMEs will traverse the column at different rates thus creating distinct peaks. Peak retention times are then used to identify each FAME by comparison to a FAME standard (such as Restek – FAME #1) that includes the five most prominent fatty acids found within plant oils and animals fats (**Table 3**).

Table 3. Restek FAME #1 Reference Standard Composition and Retention Order

Shorthand	Common Name	%	Retention order
16:0	Methyl palmitate	20	1
18:0	Methyl stearate	20	2
18:1	Methyl oleate	20	3
18:2	Methyl linoleate	20	4
18:3	Methyl linolenate	20	5

1=shortest retention time, 5=longest retention time

Note that 18:1 migrates faster on a polar column than 18:3 as predicted by dipole-induced dipole interactions. Peaks can also be analyzed by coupling the GC to a mass spectrometer (GC/MS). Each peak generates a unique mass spectrum that can be identified using a searchable database such as the National Institute of Standards and Technology (NIST) spectral database. FAMEs are listed in this database according to their formal names found in Table 1. Once peaks are identified, peak areas can be calculated and used to determine the relative percent abundance for each FAME. Table 2 can then be used to determine the origin(s) of any dissolved lipids within each sherd.

In this laboratory experiment, you will take on the role of an archaeological chemist tasked with analyzing pottery fragments typical of those associated with the Minoan civilization from the island of Crete for evidence of vegetable oils and animal fats. Dating of the associated remains place the sherds around 1500 BC. Before crushing, each sherd must be weighed and documented in your notebook. Any absorbed molecules will then be extracted and a base catalyzed methylation will be used to convert the recovered residues to FAMEs. FAMEs will be identified based on their GC retention times in comparison to a FAME standard. It is critical that *both the standards and samples are analyzed under the same conditions for this to be valid*. If there are peaks that cannot be identified using the GC and FAME standards then GC/MS will be utilized, so save your prepared samples. Based on the relative percent peak areas, you will attempt to identify any extracted lipids from the sherds and place them into archaeological context.

Pre-Laboratory Questions

1. Briefly summarize what food sources were exploited by the Minoan civilization around 1500 BC.

2. Based on your answer to question 1, predict what fats or oils found in Table 2 may be absorbed within the sherds.

3. What question(s) will you try to answer by studying these sherds? Why is this of interest to archaeologists?

Procedure

Each group will analyze three sherds provided in a labeled plastic bag. Please note the bag's serial number in your notebook and laboratory report. As noted above, to maximize the lipid extraction a destructive method will be utilized. Before crushing, draw a picture or photograph, measure the thickness, width, and length, and weigh each sherd. Note any structural features (thickness, curvature, and carbonization) that may indicate the sherd's location in the original pot. So as not to contaminate the sherds with oils from your hands or cross-contaminate the samples, ***gloves should be worn throughout the experiment and the sherds only handled with methanol washed tweezers***.

Lipid extraction

Place each sherd in a clean 1" PVC end cap. Place the end cap on the sherd crusher stand, lower the unit onto the end cap, and pulverize the sample (smaller than 0.3 cm) using a hammer and metal rod (**Figure 1**). The sherd does not need to be pulverized to a fine powder for the extraction to be successful. Wash the metal rod with methanol and dry after crushing each sherd to prevent cross-contamination.

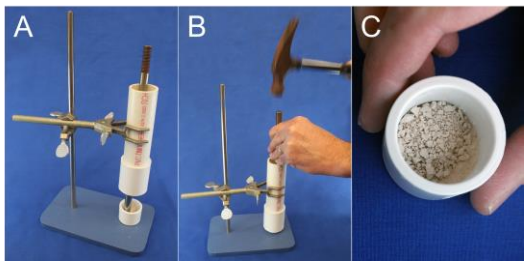


Figure 1. (A) PVC end cap placement. (B) Sherd crusher operation. (C) Required coarseness of final product.

Transfer each crushed sample to a labeled 20 mL scintillation vial using a wide-mouth funnel. Wash the funnel with methanol and dry before each transfer.

Add 5 mL of 2:1 dichloromethane/methanol, cap, and place the vial in a foam float. Sonicate the mixture in an ultrasonic bath for 5 minutes, let the sample sit for 2 minutes, and sonicate for another 5 minutes. Transfer the liquid to a 15 mL glass centrifuge tube using a glass pipet and centrifuge for 5 minutes at 3,500 rpm.

Using a glass pipet, transfer as much liquid without disturbing the solid pellet at the bottom into a clean and labeled 20 mL scintillation vial. Reduce the vial volume to ~0.5 mL by placing in a 50°C sand bath and directing a 2 psi nitrogen gas stream over the surface using a blunt-tip 18 gauge needle (**Figure 2**). The nitrogen stream prevents any unsaturated fatty acids in the extracted sample from undergoing oxidative degradation. When done, to prevent cross-contamination, remove the needle and place it in the used needle beaker for cleaning.

Figure 2. Extraction liquid reduction setup using a sand bath and 2 psi nitrogen stream.

FAME synthesis and sample preparation

Pipette 200 µL of the extraction solution and 40 µL of anhydrous methanol into a 2 mL plastic microcentrifuge tube. To the top methanol layer, carefully add 4.3 µL of 5 M KOH in anhydrous methanol to catalyze the transesterification. Place each sample in a foam float and sonicate for 30 minutes. Next, add 200 µL of 0.1 M acetic acid to neutralize the base catalyst, gently rock the solution to mix, and centrifuge for 5 min at 6000 rpm. When removing the tube note the color of the two layers. Extract 40 µL from the top layer and place in a labeled 2 mL glass vial. Add 40 µL of heptane to the vial, cap, and store for GC analysis.

GC Analysis

The GC is equipped with a polyethylene glycol, crosslinked and bonded column (30 m x 0.25 mm x 0.25 μm, GS-Tek, GsBP-Innowax). The maximum temperature for the column is 260°C. To get started, select the 'FAME' method under the method pull down tab. The initial method conditions are listed in **Table 4**.

Table 4. Initial *FAME* Method Conditions and User Adjustable Conditions

Condition	Set point	Adjustable
Injector Port Temperature	250°C	No
Injector Port Split ratio	100:1	No
Detector Temperature	300°C	No
Final Column Temperature	250°C (hold at least 4 min)	No
Column Pressure	18 psi	Yes
Initial Oven Temperature	180°C	Yes
Oven Temperature Ramp	20°C/min to 250°C – hold 5.5 min (9 min analysis time)	Yes

Ideally, the method should facilitate the separation and detection of all the sample components and do it in the shortest time possible so as to minimize peak broadening. The difficulty when analyzing real samples is that there may be dozens of other molecules in the mixture in addition to the molecules of interest. To accommodate for this, it is critical the injector port and final column temperatures are set high and the oven temperature ramp program includes a final bake-out and hold step. Since, the methylated extracted samples may contain di and monoglycerides, the final temperature ramp should reach a maximum column temperature of 250°C and be held there for at least five minutes.

To explore the relationship between total separation time, resolution, and the oven temperature ramp, analyze a 0.2 μL injection of the provided FAME standard (**Table 3**) using the initial 'FAME' conditions. Based on your evaluation of the initial chromatogram, investigate the FAME standard under three unique sets of conditions. Remember to only adjust one parameter (column pressure, initial oven temperature, or oven temperature ramp) for each trial. Upon completion of each trial, change conditions, and immediately start the next separation. Note the file name, conditions (**Table 4**), and peak details (retention times, heights, and widths) for each trial in your notebook. Based on these data, select the best conditions to evaluate your samples and analyze 0.2 μL injections of each.

To analyze the sample chromatograms, go to the 'Data Analysis' window in the Agilent Chemstation software. The program will automatically load the last chromatogram and locate and analyze all the present peaks. Delete the solvent peak from the integrated peaks table as the 'Area %' column will be used to determine the relative FAME concentrations. Be sure that all of the FAME peaks (even those not identifiable using the FAME standard) are integrated to ensure an accurate relative percent peak area calculation. Print the final/optimized standard and sample chromatograms for inclusion in the laboratory report.

GC/MS Analysis (optional)

The GC/MS is equipped with a polyethylene glycol, crosslinked and bonded column (30 m x 0.25 mm x 0.25 μm, Restek, Stabilwax). The maximum temperature for the column is 260°C. To get started, select the 'FAME' method under the method list. The initial conditions are listed in **Table 5**.

First, analyze a 0.2 μL injection of the provided FAME standard. This will be used to determine the retention time of the five main FAMEs. Next, analyze all of your samples that contain unidentifiable peaks using 0.2 μL injections. To analyze the data, press the 'Results Library' button in the Griffin System Software and select the appropriate data file. Right click on each unknown peak, select 'target', and 'add'. Once a peak is added to the target list, under the 'spectral results' tab on the bottom left box, press the 'spectral search' button. This command will compare your unknown mass spectrum against the NIST spectral database and produce a list of potential matches. Clicking on a potential match will cause its mass spectrum to appear below your unknown peak's mass

spectrum. When a match is made, right click on the compound and select 'set target to selected compound'. Repeat this for all of the unknown peaks. Print the results for inclusion in the laboratory report.

Table 5. GC/MS *FAME* Method Conditions

Condition	Set point	Adjustable
Injector Port Temperature	250°C	No
Split Flow	50.0 %	No
Column Head Pressure	25 psi	No
GC Inlet and Outlet	250°C	No
Transfer 1 and 2	250°C	No
Manifold Adapter	250°C	No
Initial Column Temperature	230°C (hold 4 min)	No
Oven Temperature Ramp	100°C/min to 250°C – hold 2 min (6.5 min analysis time)	No

Data Analysis

Evaluate the FAME standard chromatogram to determine the retention times for the five major FAMEs expected to be found in methylated edible fats and oils. Summarize the results in **Table 6**.

Table 6. Peak Retention Times for FAME Standard and Extracted Samples

		Is FAME peak present in sample? If yes, list retention time (min)		
FAME	**Retention time in standard (min)**	**Sherd #1**	**Sherd #2**	**Sherd #3**
16:0				
18:0				
18:1				
18:2				
18:3				

Use the standard FAME retention times to evaluate the peaks found in the sample chromatograms. Determine what FAMEs are present and summarize the results in **Table 6**. Are there any peaks in the sample chromatograms that cannot be explained using the standard? If time allows, run the optional GC/MS procedure on the sample(s) and use the NIST database to identify the peak(s) based on the MS profile.

The relative peak area ratios provide a means to elucidate the extracted lipid residue's source(s). Summarize the relative peak percent area data from the three sample chromatograms in **Table 7**.

Table 7. Relative Peak Areas (%) for Extracted Samples.

	Relative Peak Area (% Area)		
FAME	**Sherd #1**	**Sherd #2**	**Sherd #3**
16:0			
18:0			
18:1			
18:2			
18:3			

Use Table 2 and Table 7 (Table 1 if GC/MS was conducted) to determine what lipid residues were contained in the sherds, if any. Be sure to note the presence of other peaks and see if you can identify them utilizing the known peaks and the USDA website to determine the other fatty acids that may be present in your samples (http://www.nal.usda.gov/fnic/foodcomp/search/)

Laboratory Questions

1. Are the peak retention times for the samples exactly the same as the retention time of the standards? If not, what about the experimental method might cause the deviation? Remember that the time starts the instant the needle goes through the septum.
2. What parameters were changed when determining the optimal separation conditions for the FAME standard? What effect did these changes have on the separation? Note specifically the changes in retention time and peak resolution between the four trials. Do your results agree with chromatographic theory?
3. The relative peak ratios provide a way to determine the extracted residue's source(s). Based on Table 2 and Table 7, what is the possible plant or animal lipid source of the extracted residue from the Minoan sherds?
4. If there is not a clear single oil or fat source, is it possible that there is a mixture of oils? Use the Excel based multicomponent sample analysis program to determine what combination of oils or fats would yield the observed relative peak percent areas.
5. Do your results agree with your pre-laboratory predictions?

Laboratory Report

Based on your answers to the prelaboratory and laboratory questions, you will write a formal laboratory report. The laboratory report will consist of four parts: an introduction that clearly defines the archaeological question(s) to be answered through the analysis of the pottery sherd residues within the archaeological context (particularly that of fatty acid residues), an experimental section that describes the extraction, FAME conversion, and GC (and GC/MS if applicable) conditions, a results section that includes labeled chromatograms and pertinent tabulated separation results, and a conclusion that clearly summarizes your interpretation of the results. Provide clear evidence for your conclusion based on the data summarized in the results section and the sherds' date and excavation site. If the extracted residue source(s) cannot be identified based on the acquired data, note what additional data could be acquired to assist in the evaluation.

References

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