Instructor Notes

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

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Pre-Laboratory Questions

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

   An excellent summary of the history, culture, and archaeology of Minoan civilization can be found at http://ancient-greece.org/history/minoan.html (accessed on 5-31-17). Plants and animals exploited by the Minoan civilization include olives, grapes, fish, squid, cows, goats, pigs, deer, peas, wheat, barley, and safflower. Safflower is a lesser known commodity that traces its roots to ancient Egypt and has been found throughout antiquity around the Mediterranean Ocean. Historically, safflower was used for the synthesis of dyes, for cooking, and for medicinal applications.

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

   Possible residues include olive oil, cow fat (tallow), fish oil, safflower oil, and grapeseed oil.

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

   Residue analysis can allow archaeologists to determine the use(s) of a ceramic vessel which can help in reconstructing regional subsistence patterns, technologies, and economic and cultural practices.

Experimental Notes

Replicate Potsherd Preparation

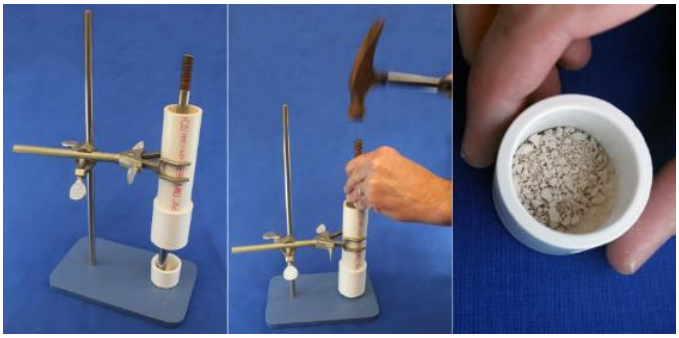
- Fired, unglazed pottery can be obtained from pottery clubs or high school/college art departments
  - If the above options are not available, fired, unglazed ceramic bowls can be purchased from multiple online vendors on eBay. Another option are unglazed terracotta flower pots. We have not tried this though the porous earthenware should facilitate oil absorption.
- The ceramic is broken into 3-5 g sherds and labeled in pencil with a unique identifier.
- Sherds are soaked in safflower oil, olive oil, or a 50/50 safflower and olive oil blend for 24 hours.
  - **<u>Check labels - 50/50 blend requires high linoleic rather than high oleic safflower oil. Do not use olive oil that says 'Mediterranean Blend' as these products are not 100% pure.</u>**
  - An alternative to safflower oil is grapeseed oil.
- Excess oil is wiped off and the sherd is left to dry for 1 hr.
- To add a patina, sherds can be rolled in fine soil, rinsed with water, and left to dry or one side can be held over a candle to blacken the surface.
- Sherds are stored in plastic bags labeled with unique serial numbers. Each bag is filled with a combination of three sherds (safflower, olive, 50/50 blend, or not soaked in oil)
  - Inclusion of a sherd not soaked in oil adds a unique analytical challenge and is true to the field in that not all surfaces of a ceramic vessel may contact food products thus absorb plant or animal oils.

- The placement of the three sherds in one bag is indicative of how archaeological chemists often receive samples. Pottery fragments are prolific throughout the archaeological record thus are often cataloged using one number and stored in bulk. The image to the right is how my laboratory received sherds for a study we are conducting on an Arikara Indian site from North Dakota (all seven bags were logged under one catalog number).

- The use of tweezers when handling the sherds is to reduce the introduction of new contaminants during sample preparation such as oils from the skin or residues from the gloves.

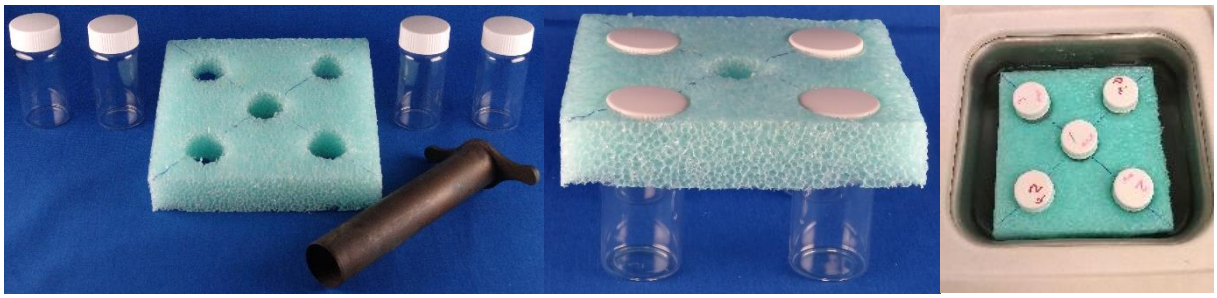
Sherd Crushing

- A homemade sherd crusher can be constructed using a 1-1/4" PVC pipe coupler and 6" length of 1-1/4" PVC pipe. Join the two pieces together (glue not required) and clamp to a ring stand.
    - A standard mortar and pestle can be used though it takes considerably longer to process the sherds and it is significantly messier.
- Ceramic samples are held in a 1" PVC endcap. A rotary tool or drill press may be required to smooth the bottom of the PVC endcap as some contain a raised button on the inside surface.
- To crush a sample, place the sherd in the endcap and lower the unit over the cup. With a metal rod (½" x 11") and hammer, pulverize the sherd to fragments smaller than 0.3 cm.
    - Remind students that the sample does not need to be pulverized to a fine powder for the extraction to work. Doing this will save considerable time.
    - The metal rod we use came from a broken porcelain titration stand.

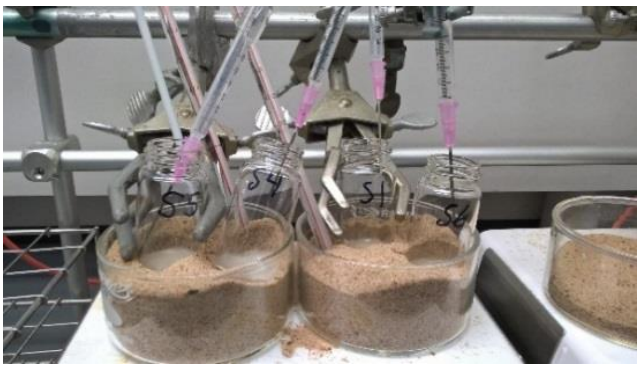


Lipid Extraction

- If an ultrasonic bath is not available, a rocker-table, magnetic stir plate and stir bar, or periodic agitation by a vortex mixer can be used.
- Vials are suspended in the ultrasonic bath using a homemade foam float. The foam floats are constructed from a 12x12x2.2 cm piece of closed-cell packing foam. Holes that matched the diameter of the extraction vials are cut into the foam using a #15 (2.2cm) cork punch.



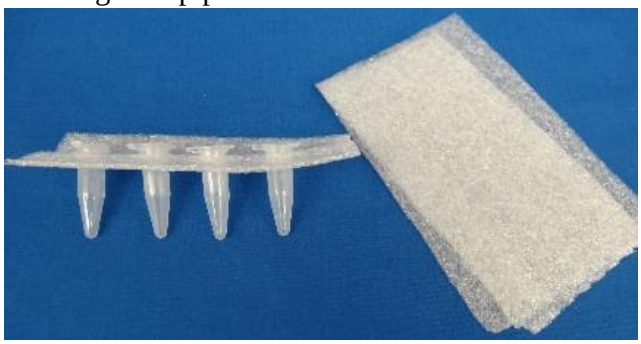
- A temporary nitrogen line is constructed using tubing, plastic T-connectors, and 1 ml syringes with the tops cut off. The syringes are inserted into each sample line and capped with 18 gauge needles that have the tips filed off. The needles are exchanged and methanol washed after each sample to prevent cross-contamination.



    - The nitrogen purge is used to prevent oxidative degradation of the polyunsaturated fatty acid chains.
    - Due to the high oil mass absorbed by each sherd (~0.5 g oil/3-5 g sherd), we have found that the nitrogen purge can be skipped without observing substantial degradation of the polyunsaturated fatty acid chains though this substantially increases the solvent reduction time.
- To assist students in gauging when 0.5 mL is left in the vial, a vial filled with 0.5 mL of water is placed next to the hotplates.

FAME Synthesis

- Before starting, students are given an introduction to using auto pipettes.
- Sample tubes are suspended in the ultrasonic bath using thin sheets of closed-cell packaging foam with holes punched in it.



    - If an ultrasonic bath is unavailable, samples can be placed in a 50°C sand bath. To increase the reaction efficiency, a micro stirbar can be added and the sample stirred at a moderate rate.
- After 30 min, a golden to light brown glycerol bottom layer should be observed in samples containing oil.
- As determined by FTIR, the synthesis conditions yield a 60-70% conversion efficiency.
- Acid catalyzed methylation of the triglycerides is more common in the field though requires synthesis times of 1+ hours. A base catalyzed methylation was chosen to allow the extraction and synthesis steps to fit within the first hour and half of the laboratory.
    - If a more, true to the field, method is desired, the Correa-Ascencio reference (Correa-Ascencio, M.; Evershed, R. P. *Anal. Methods* **2014**, *6* (5), 1330–1340) provides details for a one-step extraction and acid-catalyzed methylation procedure that works well.

GC and GC/MS Analysis

- The FAME reference standard can be extended by diluting the stock with heptane, splitting the sample into new ampules, and sealing them. We typically prepare eight ampules per purchased reference standard and found them to be good for up to two years if stored in the freezer.
- Samples were run on an Agilent 6850 Gas Chromatograph with a thermal conductivity detector and a Griffin Analytical 400 Gas Chromatograph Mass Spectrometer.
- The GC conditions in Table 4 from the Student Instructions yield a 9 min total run time and excellent baseline resolution.

- An initial oven temperature of 230°C with a 2 min hold followed by a 100°C/min ramp to 250 followed by a 4 min hold can reduce the run time to under seven minutes.
- GC/MS conditions listed in Table 5 from the Student Instructions yield a 6.5 min total run time.
- The USDA website hosts an outstanding database which students can use to determine the trace fatty acids found in the oils listed in Table 2 in the Student Instructions - (http://www.nal.usda.gov/fnic/foodcomp/search/). In addition to the 16:1 peak observed in olive oil, both safflower and olive oil contain trace 20:0 and 20:1 (peaks at 6.4 min and 6.6 min in Figure 2 from the paper). Evaluation of these peaks serve as an excellent extra credit or exam question.

Control Samples (requires two laboratory periods)

- If the experiment can be divided into two laboratory periods, two control samples, a sherd with no absorbed lipids and a sherd with a known absorbed oil such as canola or maize, can be added.
- Analysis of these two control samples may increase students' confidence in their observations especially with respect to analyzing a replicate sample with no dissolved lipids.
- The first laboratory period would be used to prepare the samples while the second period would be used for GC and/or GC/MS analysis.
- Optimization of the GC separation conditions could be conducted with the known oil control rather than the FAME standard. This would allow students to better understand the need for the bake-out period as the control sample would contain both long chain FAMEs and di- and monoglycerides.

Data Analysis – *sample data*

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

(Data collected using the GC conditions from Table 4 in the student instructions***).***

		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	*4.098*	*4.101*	*4.101*	*4.100*
18:0	*5.068*	*5.070*	*5.075*	*5.083*
18:1	*5.230*	*5.270*	*5.264*	*5.254*
18:2	*5.537*	*5.549*	*5.583*	*5.562*
18:3	*5.993*	*5.982*	*N/A*	*5.988*

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

(Data collected using the GC conditions from Table 4 in the student instructions***).***

	Relative Peak Area (% Area)		
FAME	**Sherd #1**	**Sherd #2**	**Sherd #3**
16:0	*12.0*	*7.7*	*8.5*
18:0	*1.8*	*1.6*	*1.9*
18:1	*69.4*	*16.2*	*41.9*
18:2	*10.6*	*74.1*	*44.7*
18:3	*0.6*	*N/A*	*0.5*

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.

   (For systems without an autosampler) Small differences in the retention times are observed due to the variations in the delay between injecting a sample and pressing the start button.

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 change in retention time and peak resolution between the four trials. Do your results agree with chromatographic theory?

   For upper level students, this question can be changed to include - "To assist in answering this question, calculate the capacity factor (k'), selectivity factor (α), plate number (N), plate height (H), and resolution (R_s) for the 18:0 and 18:1 peaks."

3. The relative peak ratios provide a way to determine the extracted residue's composition. Based on Table 2 and Table 7, what is the possible plant or animal lipid source of the extracted residue from the Minoan sherds?

   Sherd 1 – olive oil, Sherd 2 – safflower oil, Sherd 3 – The ratios most closely match those of maize oil though this is not a possible source due to the archaeological context.

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.

   Adjusting the percent compositions in Table 2 (green boxes) to 52% safflower oil and 48% olive oil results in a match (as indicated by sherd 3's data boxes turning yellow in Table 1) for four of the five key fatty acids. Note that the 18:3 peak area does not fall within the 15% deviation window thus is not highlighted by the conditional formatting. This is the closest match for this data set. Deviations from 10-20% in the relative peak areas have been observed and can be traced to the conditions under which the oils were grown/processed and natural degradation.

Table 1. Raw GC percent area data.

	Relative % peak areas				
	16:0	18:0	18:1	18:2	18:3
Example	8.0	3.5	39.2	35.0	8.2
Sherd 1	12.0	1.8	69.4	10.6	0.6
Sherd 2	7.7	1.6	16.2	74.1	0.0
Sherd 3	8.5	1.9	41.9	44.7	0.5

Table 2. Variable composition relative percent area calculator.

		Fatty acid relative % abundance				
	%	16:0	18:0	18:1	18:2	18:3
Grapeseed Oil	0	*6.7*	*2.7*	*15.8*	*69.9*	*0.1*
Safflower Oil	52	*4.3*	*1.9*	*14.4*	*74.6*	*0.0*
Maize Oil	0	*15.6*	*2.9*	*34.7*	*43.6*	*1.6*
Soybean Oil	0	*10.5*	*4.4*	*22.6*	*51.0*	*6.8*
Cow Fat (tallow)	0	*24.9*	*18.9*	*36.0*	*3.1*	*0.6*
Olive Oil	48	*11.3*	*2.0*	*71.3*	*9.8*	*0.8*
Salema Fish Oil*	0	*31.4*	*6.3*	*10.1*	*1.1*	*0.6*
Rapeseed Oil	0	*4.3*	*2.1*	*61.7*	*19.0*	*9.1*
Total	100	**7.7**	**1.9**	**41.7**	**43.5**	**0.4**
		Calculated relative % areas				

5. Do your results agree with your pre-laboratory predictions?

   The most common response is no since grapeseed oil is often predicted over safflower oil. Determining how safflower oil was used in antiquity may require some additional research by student.

Chemicals and Equipment

Chemicals	CAS Number	Sigma-Aldrich Number
Methanol (anhydrous)	67-56-1	322415
Dichloromethane (ACS Reagent)	75-09-2	320269
Heptane	142-82-5	H2198
Potassium Hydroxide	1310-58-3	P1767
Acetic Acid	64-19-7	695092
Safflower Oil	**Make sure the label says "high linoleic" not "high oleic"**	
Olive Oil	Make sure the label does not say 'Mediterranean Blend' as these products are not 100% pure olive oil.	

Equipment	Company	Product Number
GC Column - Polyethylene glycol, crosslinked and bonded (30 m x 0.25 mm x 0.25 µm)	GS-Tek	2025-3002
Gas Chromatography	Agilent	6850-TCD
Gas Chromatography – Mass Spectrometer	Griffin Analytical (FLIR)	G400
Sherd crusher 1" PVC end cap 1-1/4" PVC pipe coupler 1-1/4" PVC pipe (6") 1/2" x 11" metal rod	PVC parts can be purchased from a local home supply or hardware store while the metal rod can be borrowed off of a ring stand.	
Ultrasonic Bath	Branson	1210
Microcentrifuge Tubes (2.0 mL)	Sigma-Aldrich	Z717533
20 mL Scintillation vials	Sigma-Aldrich	Z190527
Pyrex Centrifuge Tubes (15 mL)	Sigma-Aldrich	CLS9950215
Phenolic caps (15-415)	Sigma-Aldrich	CLS9999915
Microcentrifuge	Labnet International, Inc.	Spectrafuge 24D
Autopipets (20-200 µL, 2-20 µL)	Gilson	Pipetman Classic
Glass Pasteur pipets	Corning	7095D-5X
2 mL Glass Vials w/ caps	Supelco	29141-U
1 mL plastic syringes	Sigma-Aldrich	Z683531
18 ga. needles (1.5")	Sigma-Aldrich	Z118044