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Laboratory Experiment

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

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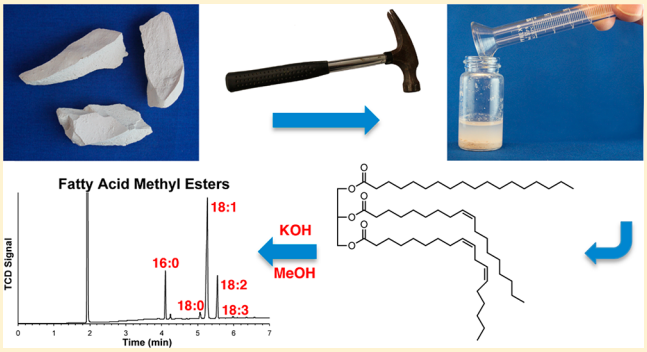
Supporting Information

ABSTRACT: In this research-based experiment, students are introduced to the interdisciplinary field of archaeological chemistry by extracting and analyzing lipid residues absorbed in pottery. Reproduction archaeological pottery sherds are prepared by soaking ceramic fragments in individual or combinations of vegetable oils. Students crush and extract the absorbed residues, transesterify the recovered lipids to fatty acid methyl esters, and analyze the product by GC or GC/MS. Recovered residues are characterized by analysis of the major fatty acid peaks, as identified using reference standards or mass spectral databases. An archaeological context that links the sherds to the Minoan civilization is provided to students and must be considered to correctly identify the absorbed residue(s). The laboratory has been used in a topical archaeological instrumentation course that has attracted second to fourth year students majoring in chemistry, biochemistry, and anthropology, and museum studies minors. Pedagogically, the laboratory introduces students to techniques currently used in the field of archaeological chemistry while reinforcing fundamental concepts in sample isolation and preparation, derivatization, gas chromatography, mass spectrometry, and multicomponent sample analysis.



KEYWORDS: *Second-Year Undergraduate, Analytical Chemistry, Biochemistry, Interdisciplinary/Multidisciplinary, Laboratory Instruction, Inquiry-Based/Discovery Learning, Applications of Chemistry, Gas Chromatography, Mass Spectrometry*

INTRODUCTION

Archaeology's goal to reconstruct humanity's past by analyzing material remains requires an interdisciplinary approach that can obtain the maximum amount of information from each unique and irreplaceable artifact. Since the end of the 18th century, chemistry has provided archaeologists a spectrum of chemical and instrumental methods to answer questions about composition, chronology, and authenticity while also contributing to the conservation of artifacts.[1,2] Chemistry's significant contributions to archaeology have been noted in this *Journal*[1,3−9] and other chemistry journals.[10−13] Recognition of chemistry's power to inform archaeological research led to the development of the specialized field of archaeological chemistry.

Nestled within the broader research discipline of archaeometry, archaeological chemistry applies chemical techniques and instrumental methods to the elemental, isotopic, and molecular analysis of artifacts and associated remains including bone, stone, soils, pigments, and organic residues.[2] It is believed that, in the future, archaeology's biggest discoveries will more likely occur in the laboratory than in the field.[2] This growing discipline provides an ideal platform to demonstrate research applications of chemical techniques and instrumentation and foster participation by students from outside the chemistry major. Recognition of this by others has led to the development of undergraduate accessible laboratory experiments that use scanning electron microscopy with energy dispersive X-ray spectroscopy[14] and flame atomic absorption spectroscopy[15] to study archaeological artifacts.

Undergraduate students benefit from engaging in practical and current research techniques when learning fundamental chemistry concepts[16−18] and when introduced to new instrumentation.[19] Faced with decreasing enrollment in our department's comprehensive instrumental analysis course and a campus-wide initiative to encourage interdisciplinary research, we split our instrumental analysis course in 2007 into a series of topical modules on forensic instrumentation, bioanalytical instrumentation, organic structure, microscopy, and archaeological instrumentation. Each module focuses on three or four key instruments and includes research-based laboratories that mimic current practice in the field. Before we implemented the topical modules, the comprehensive instrumental analysis course, which was offered biennially, averaged three students per course between 2002 and 2006. Student response to the topical modules, now offered three out of every four semesters, has been positive with enrollment averaging 13 students per

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module and participation by physics, biology, geology, and anthropology majors and museum studies minors in addition to chemistry and biochemistry majors. The archaeological instrumentation module, which has been offered twice since 2014, focuses on chromatography, mass spectrometry, and X-ray fluorescence spectroscopy as applied to the analysis of archaeological residues and artifacts. The module has attracted second to fourth year majors in chemistry, biochemistry, and anthropology, and museum studies minors. Research literature is used to reinforce theory while laboratory experiments demonstrate current methodologies and best practice. This paper describes a pottery residue analysis experiment developed for the archaeological instrumentation module.

Unglazed ceramics provide an excellent repository for organic molecules, which deposit within the porous matrix during cooking, food storage, or ceremonial usage. When ceramic fragments, or sherds, are buried, absorbed molecules are subject to chemical and microbial degradation and leaching. Only the most robust chemical species survive long-term internment. Molecules that have unsurpassed resiliency include lipids, sterols, and terpenoids.[12] In arid conditions, lipids have been recovered from excavated pottery dating to the eighth millennium BC.[20] Lipid residue analysis can assist archaeologists in reconstructing regional subsistence patterns, technologies, and economic and cultural practices.[21−24] 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.[12] Since each plant and animal species synthesizes triglycerides using a unique combination of fatty acids (Table 1), the relative percent fatty acid abundances can be used to identify extracted lipid residues.

Table 1. Major Fatty Acids and Their Relative Percent Abundances Found in Several Edible (Nongenetically Modified) Fats and Oils

	Fatty Acid[c] Relative % Abundance by Fat or Oil Source				
Edible Fat/Oil[a]	16:0 (Palmitic)	18:0 (Stearic)	18:1 (Oleic)	18:2 (Linoleic)	18:3 (Linolenic)
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[b]	31.4	6.3	10.1	1.1	0.6

[a]USDA nutrient database for standard reference; see ref 25. [b]Prato et al.; see ref 26. [c]The fatty acid shorthand notation specifies the total number of carbons in the fatty acid and the number of double bonds. For example, 18:1 denotes an 18-carbon fatty acid with one double bond. The common fatty acid name is in parentheses.

In this experiment, students take on the role of an archaeological chemist tasked with analyzing sherds typical of those associated with the Minoan civilization from the island of Crete for evidence of vegetable oils and animal fats. Students prepare for the experiment by learning the basics of triglyceride structure, fatty acid nomenclature, and chromatography. The prelaboratory questions then ask students to research Minoan culture around 1500 BC and generate a list of animal fats and plant oils that could be associated with the sherds. The analyzed pottery sherds are prepared by soaking ceramic fragments in individual or combinations of plant oils. Students crush the sherds and extract the absorbed residues, transesterify the recovered lipids to fatty acid methyl esters (FAMEs), and analyze the product by GC or GC/MS using literature derived methods.[24,27] The only deviation from the literature methods is that a base catalyzed transesterification,[28] rather than an acid catalyzed[22] transesterification, is used to expedite the synthesis step. Extracted triglycerides are characterized by analysis of the relative fatty acid peak areas, as identified using reference standards or mass spectral databases. Students must consider the archaeological context to correctly identify the absorbed lipid(s) and elucidate the ceramic vessel's original use. Through this authentic research-based experiment derived from archaeological chemistry, students gain practical experience in sample isolation and preparation, derivatization, gas chromatography, mass spectrometry, and multicomponent sample analysis.

EXPERIMENTAL OVERVIEW

Replicate Pottery Sherd Production

Fired, unglazed ceramic can be acquired from college/high school art departments or local pottery clubs. The ceramic is broken into 3−5 g sherds, labeled in pencil with a unique identifier, and soaked in safflower oil, olive oil, or a 50/50 safflower and olive oil blend for 24 h. Olives were used extensively in Minoan cuisine while safflower was used for the synthesis of dyes, for coloring and flavoring food, and for medicinal applications. Excess oil is removed, and the sherds are left to dry for 1 h. Plastic bags labeled with unique serial numbers are prepared with a combination of three sherds (olive, safflower, olive/safflower blend, or not soaked in oil). Inclusion of a sherd not soaked in oil represents a ceramic vessel not used in food production or long-term food storage, which is common in the archaeological record.

Lipid Extraction

Groups of 2−3 students receive a labeled bag with three sherds. The bag's serial number and each sherd's unique identifier, dimensions, and masses are recorded. To prevent sample contamination, gloves are worn, and the sherds are handled with methanol washed tweezers. Each sherd is placed in a 1 in. PVC pipe end-cap and positioned under a homemade crusher constructed of a $1^1/_4$ in. PVC pipe coupler, 6 in. length of $1^1/_4$ in. PVC pipe, and 1/2 in. × 11 in. metal rod (Figure 1A).

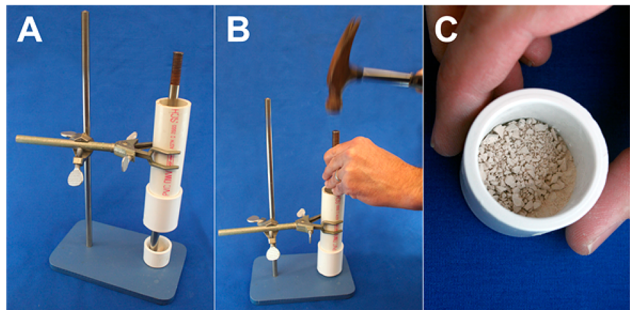

Figure 1. (A) PVC end-cap placement. (B) Sherd crusher operation. (C) Required coarseness of final product.

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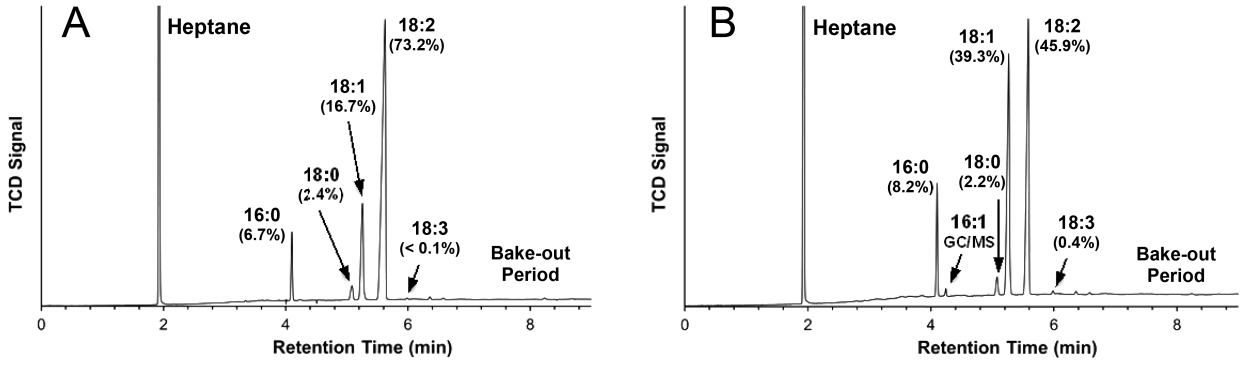

Figure 2. Chromatograms from (A) safflower oil and (B) blend of safflower/olive oil samples extracted from pottery sherds and converted to fatty acid methyl esters. Peak labels are determined by retention time comparison with a commercial FAME standard: 16:0, methyl palmitate; 18:0, methyl stearate; 18:1, methyl oleate; 18:2, methyl linoleate; 18:3, methyl linolenate. Oil type is determined by comparison of the relative peak percent areas (*x.x*%) against the relative percent abundances in Table 1. In part B, the peak at 4.2 min is due to the presence of 16:1 palmitoleic acid in olive oil as identified by GC/MS.

The pipe coupler is lowered over the end-cap, and the metal rod is struck with a hammer until the sherd is reduced to pieces smaller than 0.3 cm (Figure 1B,C). Sherds do not need to be pulverized to a fine powder. The metal rod is washed with methanol and dried after each use to prevent cross-contamination.

The coarse powder is transferred to a 20 mL glass scintillation vial, and 5 mL of 2:1 v/v dichloromethane/methanol is added. The vial is capped, sonicated in an ultrasonic bath for 5 min, allowed to stand for 2 min, and sonicated for another 5 min. The solution is transferred to a 15 mL glass centrifuge tube using a glass pipet and centrifuged for 5 min at 3500 rpm. Using a glass pipet, the liquid is transferred to a 20 mL glass scintillation vial. The volume is reduced to ~0.5 mL in a 50 °C sand bath with a 2 psi nitrogen gas stream directed over the surface using a blunt-tip 18 gauge needle. The nitrogen stream prevents any unsaturated fatty acids from undergoing oxidative degradation. Gas needles are methanol washed and dried after each use.

FAME Synthesis and Sample Preparation

The extracted residue (200 μL) and anhydrous methanol (40 μL) are pipetted into a 2 mL plastic microcentrifuge tube. Then, 5 M KOH in anhydrous methanol (4.3 μL) is carefully added to the top methanol layer to catalyze the transesterification (6 g KOH/L of extract). The tube is placed in an ultrasonic bath and sonicated for 30 min. Next, 0.1 M acetic acid (200 μL) is added to neutralize the base catalyst. The solution is gently rocked to mix and centrifuged for 5 min at 6000 rpm. A golden brown glycerol bottom layer should be observed if the extract contained lipid residues. The top layer (40 μL) is then transferred to a 2 mL glass GC vial and diluted with heptane (40 μL). The vial is capped for later GC analysis.

GC Analysis

Samples are analyzed using a GC equipped with a thermal conductivity detector (TCD) and a polyethylene glycol cross-linked and bonded column (30 m × 0.25 mm × 0.25 μm). A 0.2 μL injection of a commercial FAME standard composed of the five most common fatty acids found in plants and animals (16:0, 18:0, 18:1, 18:2, and 18:3) is first analyzed using a preprogrammed method (conditions provided in the Supporting Information). The FAME standard is analyzed again under three unique sets of conditions where only one parameter (column pressure, initial oven temperature, or oven temperature ramp) is adjusted. The four chromatograms are used to determine the optimal separation conditions to resolve the FAME standard's five components in the shortest time. Finally, 0.2 μL injections of the methylated extraction solutions are analyzed. The final method should include a 4 min bake-out period to ensure the complete migration of any long chain FAMEs or di- and monoglycerides due to incomplete transesterification. Chromatograms are evaluated by comparing peak retention times to the FAME standard and integrating the FAME peak to determine the relative percent peak areas.

GC/MS Analysis

Peaks not present in the commercial FAME standard require analysis using a GC/MS equipped with a polyethylene glycol, cross-linked, and bonded column (30 m × 0.25 mm × 0.25 μm). A 0.2 μL injection is analyzed using a preprogrammed method (conditions provided in the Supporting Information) and evaluated using a mass spectral database.

■ HAZARDS

Personal protective gear including safety glasses and gloves should be worn. KOH (5 M) is caustic. Methanol and dichloromethane are toxic by inhalation and flammable and should be handled with care in a fume hood. Remaining extraction solution should be disposed of in a halogenated organic waste container due to the trace presence of dichloromethane. Vials should be tightly sealed during sonication and centrifugation.

■ RESULTS AND DISCUSSION

The experiment is designed to be completed in a 3 h laboratory period. Preparation of the three sherds for FAME synthesis takes ~1 h. To ensure uniform class progress, groups prepare one sherd at a time rotating through each of the preparation steps. Once all the sherds are processed and the transesterification reactants added, the 30 min sonication step is started as a class. While samples are sonicating, students begin determining the optimal GC separation conditions. This step can be conducted in small groups or as a class depending on instrument availability and class size. Students acquire four total chromatograms, each with a unique set of conditions to explore the relationship between initial oven temperature, oven temperature ramp, total separation time, and resolution. A total of 45 min is required, which can be split between the 30 min transesterification step and the last 90 min of the

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laboratory. Students spend the remaining laboratory time obtaining chromatograms of their extracted samples.

Since groups receive a random combination of three sherds, each group generates a unique data set. When a sherd not soaked in oil is processed, students should note the lack of a glycerol layer and connect this with the lack of peaks in the associated chromatogram. Chromatograms of samples extracted from sherds containing safflower and olive oil reveal a distinctive combination of FAME peaks and relative percent peak areas (Figure 2).

Students first use the FAME standard peak retention times to identify the sample FAME peaks. Samples containing safflower oil (Figure 2A) contain four main FAME peaks (16:0, methyl palmitate; 18:0, methyl stearate; 18:1, methyl oleate; 18:2, methyl linoleate), which are all identifiable using the FAME standard. Olive oil containing samples (Figure 2B) have trace levels of palmitoleic acid (16:1, 4.2 min), which is not often included in commercial FAME standards designed for the analysis of plant oils and animal fats. This key olive oil biomarker, though, can be identified by GC/MS analysis.

When a single oil is present, the extracted residue can be identified by comparing the relative peak percent areas to the fatty acid percent abundances found in Table 1. The relative peak percent areas in Figure 2A most closely resemble safflower oil's profile but show similarities to grapeseed oil due to the higher than predicted 16:0 percentage of 6.7% and 18:0 percentage of 16.7%. Since both plants are associated with Minoan culture, this creates a discussion point and encourages further statistical analysis of the class data. A similar single component percent peak area comparison of Figure 2B would predict the extracted residue is maize oil due to the comparable percent abundance of 18:1 (39.3%) and 18:2 (45.9%). On the basis of the location and associated date, this is not plausible as maize is a New World crop and had yet to be introduced. The olive and safflower oil blend requires students to integrate the sherds' archaeological context into the analysis. It also emulates lipid residue analysis of real sherds as ceramic vessels can have complex use patterns that bring them in contact with multiple oil and fat types. Students use an Excel spreadsheet (included in the Supporting Information) to calculate the relative peak percentages for a multicomponent system. The program compares the calculated peak percentages against the sample data and highlights FAME peaks that match using conditional formatting. The program indicates that the relative peak area percentages in Figure 2B correspond to a 45/55 blend of olive and safflower oil.

Upon completion of the laboratory experiment, students compile their data in tables as described in the student instructions and answer a series of guided questions on chromatographic theory and practice (1−2) and extracted residue identification (3−5). Question 5 asks students to reflect on the archaeological context researched in the prelaboratory questions when identifying the residues. Groups present their results in a formal laboratory report that includes an introduction that clearly defines the archaeological question to be answered and the archaeological context.

STUDENT LEARNING

There were 24 students in two sections of the archaeological instrumentation module who conducted the laboratory experiment. Students worked in pairs, though they answered the pre- and postlaboratory questions and wrote the laboratory report independently. Of the 24 students, 23 correctly identified the sherds soaked in a single oil while 18 of the 24 students correctly identified the safflower/olive oil blend. Maize oil was the most common incorrect answer given and occurred predominantly in groups that did not include a student majoring in anthropology or minoring in museum studies. This unintended consequence helped reinforce the need for interdisciplinary research especially in the field of archaeological chemistry. Students were found to have the most trouble analyzing data from the unsoaked sherds. Many attributed the lack of results to poor technique rather than the possibility that the original ceramic vessel had not been used in the processing or long-term storage of plant or animal products. Gaining confidence that they did do the derivatization and analysis correctly can be an important learning goal.

SUMMARY

A research-based lipid residue analysis laboratory experiment has been developed and tested with second to fourth year undergraduate students in an archaeological instrumentation course. The laboratory experiment includes an introduction to triglycerides and fatty acid nomenclature and is suitable for nonmajors that have taken general chemistry. Students gain practical experience preparing and analyzing GC samples through the analysis of lipid residues extracted from reproduced archaeological sherds. The experiment is designed such that the sherds' archaeological context must be considered to correctly identify the extracted lipids. Pedagogically, the laboratory introduces students to techniques currently used in archaeological chemistry while reinforcing fundamental concepts in sample isolation and preparation, derivatization, and analysis by GC and GC/MS.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00225.

Student instructions (PDF, DOCX)
Instructor notes with a materials list (PDF, DOCX)
Multicomponent analysis program (XLSX)

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Notes

The authors declare no competing financial interest.

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