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ARTICLES

Optimization of an Acid-Catalyzed Direct Extraction-Methylation Method of Lipid Residues in Archaeological Vessels by Experimental Design and Response Surface Methodology

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Abstract—In this study, parameters of an acid-catalyzed direct extraction methylation method used for the determination of low-level lipid residues in the ceramic artifacts were investigated and optimized as a result of chemometric modeling techniques that enable comprehensive statistical data to be obtained with fewer analyses in the optimization studies. For this purpose, 25 amorphous unglazed ceramic artifacts found during the 1996–2021 excavations at the Küllüoba Höyük archaeological site were subjected to lipid residue determination. The ceramic samples extracted by the proposed method were analyzed by gas chromatography-mass spectrometry, and the lipid residues present in them were determined. According to the obtained fatty acid composition results, it can be said that the lipid residues in the analyzed ceramics are of both animal and plant origin. The ceramic artifact with the highest lipid residue concentration was selected, and the parameters of the extraction method were examined using the chemometric models. According to the response surface methodology, the extraction time and temperature should be high as long as the experimental conditions and environment allow for the extraction of the lipid residues in the ceramic artifacts, and the silylation process has a limited positive effect on the yield, but the extraction with methanol can be achieved successfully when the silylation process is not performed.

Keywords: archaeometry, chemometrics, GC-MS, Küllüoba Höyük, lipid residue

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Küllüoba which was settled uninterruptedly during the Early Bronze Age (**EBA**, roughly 3200–1950 BC) in western Central Anatolia [1, 2] reflects important stages such as the urbanization process [3, 4], emergence of complex societies, and formation of interregional trade networks which are the characteristics of the period [5]. A significant number of publications have been made about the excavations that have been going on since 1996.

The mound is located on a very important natural route, the western end of the Upper Sakarya basin, that connects Central Anatolia to the Marmara Region and thus to the North Aegean and the Balkans [6]. The Küllüoba excavations provided evidence that allowed reliable stratigraphy to be determined throughout the entire EBA in western Central Anatolia and to reveal the characteristics and development of the EBA architecture of the region [7]. In addition, evidence of relations that took place since 2500 BC between Syria-Cilicia and, therefore, Mesopotamia and the North Aegean (Troy) over the Eskişehir Region has been obtained [5]. The cemetery area discovered here in recent years is the only known example of this period in western Anatolia.

As a result of the excavations, ceramic repertoire samples reflecting the eating and drinking habits of the EBA and the change of these habits were unearthed, and various forms were found among them [8, 9] which are understood to have been brought to the region from outside and shed light on the relations with distant regions [3, 4, 10, 11]. When evaluated together with the other archaeological findings, the data obtained from Küllüoba are a clear indicator of the socio-economic change in this period. The organic residue (**OR**) assay made on the vessels found in the excavations in recent years constitute other important evidence of this change [12].

During processes such as cooking, transferring, and storing, the ORs of the food interact with the vessel, and they are absorbed in the pores on the walls of these vessels. In particular, the inorganic porous matrix of unglazed ceramic vessels can provide a protective environment for the decomposition products and biomolecules of the absorbed ORs, and thus the relevant organic compounds can be preserved for

thousands of years [13, 14]. Proteins, lipids, and carbohydrates appear as the largest group of ORs found in archaeological artifacts [15]. Edible oils in the lipid group and especially fatty acids which are the building blocks of these oils are considered the most important members of this OR group [16–18].

When the fatty acids come into contact with water while they are buried or during post-excavation processes, they are washed away from the ceramic pores to a negligible extent, thanks to their hydrophobic structure, and these properties make the fatty acids very durable and resistant to the environmental factors. In addition, even very low levels of fatty acids can be successfully detected by gas chromatography–mass spectrometry (**GC-MS**). Compared with other groups of ORs such as proteins and carbohydrates, fatty acids stand out with their specific stability [15].

Lipid residues have been extracted using chloroform/methanol or dichloromethane/methanol mixtures in many studies [17, 19–25]. However, these approaches are effective for the extraction of wax esters and triglycerides on various archaeological ceramics in different geographical and burial conditions, but they are not effective for fatty acids in the structure of very well-absorbed ceramic pores [26–28] and are found to be able to extract measurable amounts of fatty acids from only a very limited number of ceramics [29]. These methods which involve solvent extraction followed by saponification can reveal lipid residues in 50 or 60% of unglazed ceramics from the UK and Northern European regions [19, 30–33], whereas it has a lower yield for artifacts originating from Southeast Europe and the Middle East. Evershed et al. [34] in their extensive study of ORs in more than 2200 unglazed ceramics obtained from 23 different archaeological sites in the Balkans, Greece, Turkey, and the Eastern Mediterranean have been able to detect lipid residues in only 11.5% of the existing artifacts with a similar extraction method. It has been suggested that this low rate in Southeastern Europe and the Middle East may be due to a combination of factors such as the extraction method used, older pottery, seasonal temperatures, changes in precipitation, and the characteristic calcareous soil structure of many archaeological sites in the region [17].

The one-step acid-catalyzed direct extraction-methylation method emerges as a relatively high-yield (ORs could be detected in approximately 30% of the total samples) alternative for the extraction of low levels of lipid residues, especially in ceramic artifacts from Southeastern Europe and the Middle East, and has recently been successfully applied by many researchers in studies on the lipid residues in the ceramic artifacts [29, 35–38]. However, these and other extraction methods consist of many experimental stages using various solvents, and all studies in the literature apply these processes without examining the effects of these processes on the analysis results.

Within the scope of this study, the parameters of the acid-catalyzed direct extraction methylation method used for the determination of low-level lipid residues in the ceramic artifacts were investigated and optimized as a result of chemometric modeling techniques that enable comprehensive statistical data to be obtained with fewer analyses in the optimization studies. In addition, it is aimed to reveal important archaeological data by interpreting the lipid residues in the ceramics unearthed from an area that hosted one of the important ancient civilizations in Anatolia, such as Küllüoba Höyük.

EXPERIMENTAL

Ceramic samples. The unglazed ceramics found during the 1996–2021 excavations at the Küllüoba Höyük archaeological site were used in the study. 25 ceramic samples which are amorphous and do not have any museum value were selected and subjected to the acid-catalyzed direct methylation extraction method available in the literature, and the lipid residue determination was carried out by GC-MS. The images and information of the archaeological ceramics subjected to the lipid residue determination are given in Fig. 1 and Table 1, respectively. As a result of the analyses carried out, the lipid residues of the related ceramics were interpreted. The artifact with the highest lipid residue concentration was used as the stock ceramic in the planned experimental design analysis.

Sampling from the ceramics is of primary importance in obtaining the highest possible concentrations of well-preserved lipid residues. Due to their low-density molecular structure, lipids accumulate in the relatively upper parts of the ceramic vessels, and their concentrations decrease from top to bottom according to the vessel profile [16, 39]. For this reason, as in many studies [19, 32, 40–45], the samples taken from the rim and body regions of the related ceramics which are more likely to contain lipid residues were subjected to lipid residue determination.

Due to the complexity of the interpretation of the archaeological lipid residues, it is crucial to determine whether the lipid residues present are the result of soil deposition or post-excavation contamination. For this purpose, the outer surfaces of the ceramics were mechanically cleaned with an engraving hand drill to remove lipid residues that might have been contaminated by the soil or by the workers during the excavation operations [21, 23, 24, 35, 36, 38, 46]. Then, approximately 1 g of powder sample was obtained from the cleaned surface with the drill. All solvents and chemicals used obtained from Sigma-Aldrich (Darmstadt, Germany) were of analytical and chromatographic grades and were employed without further purification. Glassware was washed three times in a solvent, and nitrile gloves were always worn.

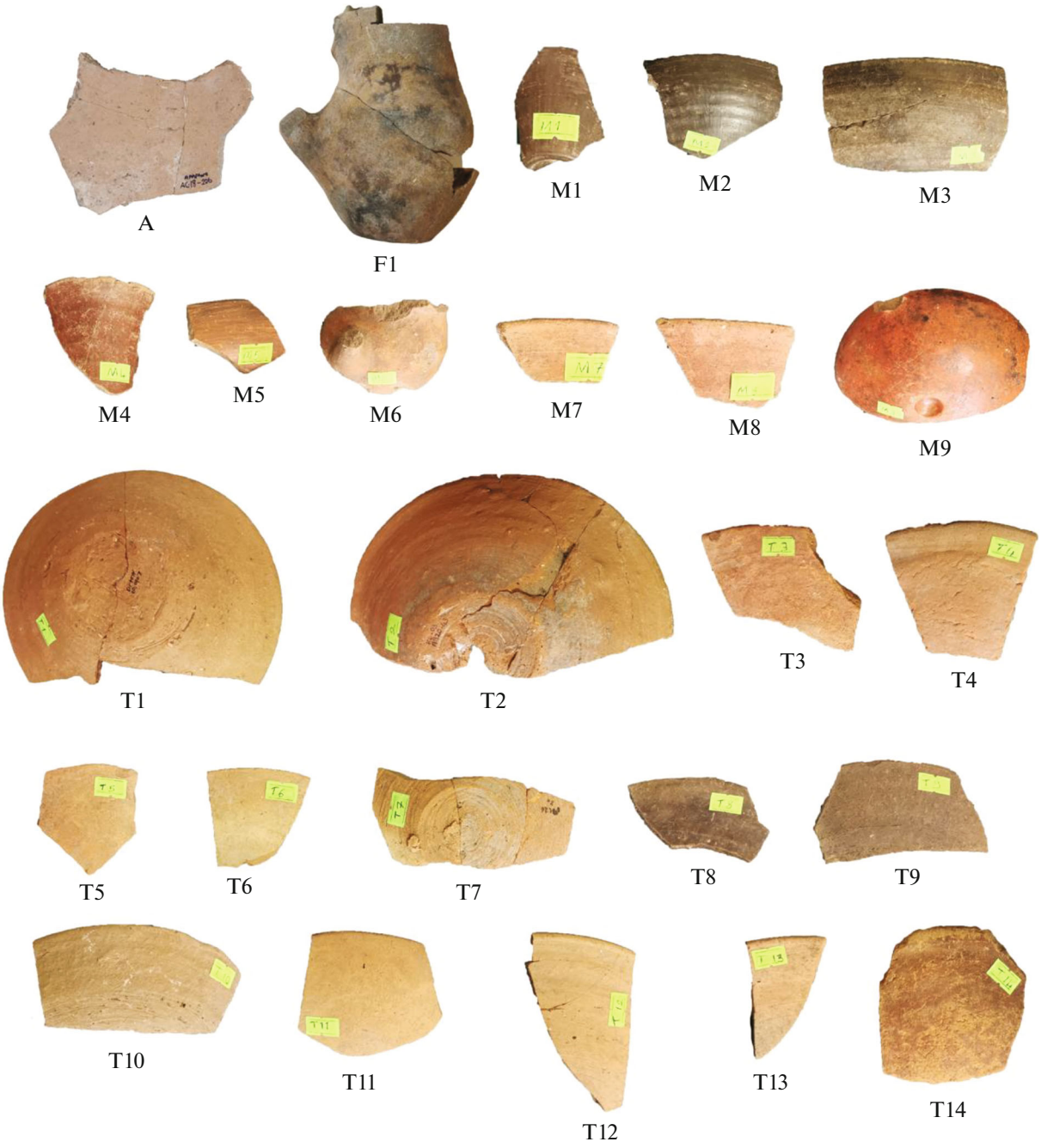

Fig. 1. The images of the archaeological ceramics subjected to the lipid residue determination.

Extraction of the lipid residues by the current method. Acid-catalyzed direct extraction-methylation method which consists of a single step and is suitable for low levels of lipid residues in unglazed ceramics from Southeast Europe and the Middle East, including Anatolia, was used [29, 35–38, 47]. The lipid residues were extracted and methylated, following the protocol as described in Papakosta et al. [38] and Correa-Ascencio et al. [48] with some modifications. According to the method, 1 g of the sample containing 50 μL of *n*-tetratriacontane (1000 mg/L) added as an internal standard was heated with 6 mL of a mixture of MeOH and 98% H_2SO_4 (5 : 1, v/v) at 70°C for 4 h and then cooled. The lipids were extracted with *n*-hexane (3 × 2 mL) and separated off after centrifugation (2500 rpm, 3 × 5 min). In many studies, silylation is also performed to decrease the polarity of the analyte, increase its stability, and improve chromatographic separation [49]. To interpret the effect of silylation processing within the study, the hexane phase col-

Table 1. Information of the archaeological artifacts for which lipid residue determination was performed within the scope of the study

No.	Code	Inventory no.	Ware group	Production method	Form type
1	A	–	–	–	Amphora fragment
2	F1	AC/AD 19–184	Plain	Wheelmade	Tankard fragment
3	M1	AC 26–74	Import (metallic ware)	Wheelmade	Beaker fragment
4	M2	AD/AB 19–302	Gray	Wheelmade	Bowl fragment
5	M3	Z 19–412	Gray	Wheelmade	Bowl fragment
6	M4	AA 29–44	Red coated	Wheelmade	Depas fragment
7	M5	AA 20–121	Brown wash	Wheelmade	Bowl fragment
8	M6	Z 19–263	Brown wash	Wheelmade	Jug fragment
9	M7	AC/AD 18/19–189	Red wash	Wheelmade	Plate fragment
10	M8	X-1	Red wash	Wheelmade	Depas fragment
11	M9	AA 22–102	Red slipped	Handmade	Bowl fragment
12	T1	AC 26–113	Plain	Wheelmade	Plate fragment
13	T2	AA 20–43	Plain	Wheelmade	Plate fragment
14	T3	AA 20–234	Orange	Handmade	Plate fragment
15	T4	AD 19–251	Orange	Handmade	Plate fragment
16	T5	AC 26–96	Orange	Wheelmade	Plate fragment
17	T6	AC 26–72	Orange	Wheelmade	Plate fragment
18	T7	AC 26–61	Orange	Wheelmade	Plate fragment
19	T8	AC 26–?	Gray	Wheelmade	Plate fragment
20	T9	AC 26–124	Gray	Wheelmade	Plate fragment
21	T10	AC 26–116	Gray	Wheelmade	Plate fragment
22	T11	AC 26–124	Import	Wheelmade	Plate fragment
23	T12	X-24	Import	Wheelmade	Plate fragment
24	T13	AC 28/25–10	Import	Wheelmade	Plate fragment
25	T14	AC 26–161	Orange	Handmade	Plate fragment

lected was evaporated to dryness with N_2 gas at room temperature. On the remaining lipid residue, 100 μL of bis(trimethylsilyl)trifluoroacetamide (**BSTFA**) containing 1% trimethylchlorosilane was added and heated at 70°C for 1 h. Silylated lipid residues were dissolved in 100 μL of hexane and passed through a 0.45 μm polytetrafluoroethylene syringe filter and taken into capped glass vials containing inserts. The sample prepared for analysis was subjected to GC-MS analysis.

Gas chromatographic-mass spectrometric analysis. An Agilent (CA, USA) 7890N-5975C GC system consisting of a mass spectrometric detector and a thermostated column oven was employed for analysis. The operating conditions of the GC-MS system equipped with an Agilent (CA, USA) HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm, (5%-phenyl)-methylpolysiloxane) were as follows: the temperature program of the column oven was held at 50°C for 1 min, then increased at 5°C/min from 50 to 300°C, and held at 300°C for 9 min. The temperature of the injection block was 300°C; the carrier gas was helium; the flow rate was 0.7 mL/min. The sample injection volume was 1.0 μL in splitless mode; the electron ionization mode, 70 eV. The temperatures of the source and quadrupole in the MS block were 230 and 150°C, respectively. The MS was set to the scan mode between m/z 40 and 650 with a scan rate of 1.24 per second. The first 10 min of each of the analyses were defined as the solvent delay time. A blank was performed for each sample using the same extraction protocols, taking a great care to check for contamination introduced in the laboratory and to avoid misinterpretations. Chromatographic peaks belonging to the sample and the similarities to the blank were carefully eliminated. Analyses were performed in triplicates, and results were given with standard deviations (**SDs**). The m/z ratios of the chromatographically separated compounds were automatically compared by the MS library data, and the relevant compounds were identified. Compounds identified with a 90% or greater match with the MS library were considered. While the compounds determined with 80–89% match were carefully evaluated, matches below 80%

were not considered. The percentage of the compounds detected in the lipid residues obtained as a result of the relevant extraction was calculated automatically by the device software.

Optimization of the extraction parameters with chemometric modelling techniques. It is of great importance to perform the extraction of the lipid residues found in archaeological artifacts with the highest efficiency that can be achieved with an appropriate extraction method. Because no matter how successful the chromatographic analysis technique is, the residues that cannot be extracted sufficiently will not give meaningful and reproducible results. For this purpose, the extraction time (A), extraction temperature (B), amount of BSTFA added (C), silylation time (D), and silylation temperature (E) parameters available in the literature and belonging to the extraction method were optimized using a response surface methodology (**RSM**). Other parameters were used unchanged. Using an experimental design with 5 factors (A, B, C, D, E) and 5 levels, the study was carried out on the basis of orthogonal central composite design (**OCCD**). All chemometric modeling studies were performed using the Design Expert program. The coded values of the parameters to be optimized are given in Table 2, and the actual level values within the scope of the experimental design are given in Table 3.

Preliminary trials were carried out in the selection of the levels of the parameters (Table 2) to be studied, and the experimental conditions were considered. Since the studied samples are archaeological artifacts and they have a limited amount, the sample amount of 1 g was kept constant. In addition, the amounts of methanol and sulfuric acid to be added were not changed due to the experimental conditions. For the extraction time (A), 0.60 h, i.e., 36 min, was determined to be the minimum required time. The boiling point of methanol, approximately 70°C, was effective in determining the highest level of extraction temperature (B). As a result of the preliminary experiments carried out, the lowest level of BSTFA added (C) was determined as 52 μL, since the amount of BSTFA less than 50 μL could not interact adequately with the lipid residues in which the solvent was evaporated. In addition, it was determined that a period of 12 min for the silylation time (D) was minimum. As a result of another preliminary test carried out, the lowest possible temperature for the silylation temperature (E) was determined as room conditions, so the lowest level value of this parameter was chosen as 26°C.

According to the existing extraction method, the sample with the highest lipid residue concentration was selected, and the extraction processes were carried out according to the experimental conditions contained in each experiment number in Table 3 and analyzed in the GC-MS device according to the conditions. The response value (R) was calculated by dividing the sum of the peak areas of palmitic (C16:0) and stearic acid (C18:0) in both methyl and silyl ester form by the peak area of the internal standard performed for each analysis in Table 3. In this way, the ability of the extraction process to take the fatty acids from the ceramic structure into the solution phase was demonstrated. The reason why palmitic (C16:0) and stearic (C18:0) fatty acids were chosen in the interpretation of the extraction performance is that, as a result of many studies, these fatty acids are the most frequently detected fatty acid types in the lipid residues due to their stable structure, which makes them more resistant to oxidation and degradation [50].

Table 2. The coded values of the parameters to be optimized in the experimental design model (A—extraction time, h; B—extraction temperature, °C; C—amount of BSTFA added, μL; D—silylation time, min; E—silylation temperature, °C)

Parameter	Level				
	−2.37841[a]	−1	0	1	2.37841[a]
A, h	0.60	2	3	4	5.40
B, °C	26	40	50	60	74
C, μL	52	80	100	120	148
D, min	12	40	60	80	108
E, °C	26	40	50	60	74

[a] The level of 2.37841 calculated automatically by the software represents the axis point of OCCD for five independent variables.

RESULTS AND DISCUSSION

Extraction of lipid residues by the current method. Positive results were obtained in approximately 30% of the analyzed ceramic artifacts, and the lipid residues could be detected in 7 of 25 ceramics. The total lipid concentrations (mg/L) of the lipid residues were calculated with the help of the added internal standard and are given in Table 4. They varied from 3.1 (M6) to 6800 μg/g (A). Particularly, ceramic A with a high lipid residue concentration was chosen for the chemometric studies. The percent composition of the biochemical compounds of the artifacts with the lipid residues is given in Table 5.

The results in Table 5 show that palmitic (C16:0) and stearic (C18:0) acids were predominant in the samples as expected. Their methyl esters varied from 9.95% (M7) to 56.06% (M6) and 5.86% (M4) to 23.98% (F1) for palmitic (C16:0) and stearic (C18:0) acids, respectively. Except for palmitic (C16:0) and stearic (C18:0) fatty acids and bis-O-trimethylsilyl-palmitinic acid-glycerin-(1)-monoester of artifact A, no other trimethylsilyl ester could be detected. It is thought that this result is due to the esterification of methanol added first in the extraction process with most of the fatty acids and the lack of sufficient fatty acid to react with BSTFA. This reagent could only

Table 3. Experimental design determined in the optimizing of the extraction parameters with orthogonal central composite design (A—extraction time, h; B—extraction temperature, °C; C—amount of BSTFA added, μL; D—silylation time, min; E—silylation temperature, °C)

No.	A level	A, h	B level	B, °C	C level	C, μL	D level	D, min	E level	E, °C	*R*
1	−1	2	−1	40	−1	80	−1	40	−1	40	0.2917
2	1	4	−1	40	−1	80	−1	40	−1	40	0.4584
3	−1	2	1	60	−1	80	−1	40	−1	40	0.6321
4	1	4	1	60	−1	80	−1	40	−1	40	0.9933
5	−1	2	−1	40	1	120	−1	40	−1	40	0.3678
6	1	4	−1	40	1	120	−1	40	−1	40	0.5780
7	−1	2	1	60	1	120	−1	40	−1	40	0.7970
8	1	4	1	60	1	120	−1	40	−1	40	1.2524
9	−1	2	−1	40	−1	80	1	80	−1	40	0.2933
10	1	4	−1	40	−1	80	1	80	−1	40	0.4580
11	−1	2	1	60	−1	80	1	80	−1	40	0.6331
12	1	4	1	60	−1	80	1	80	−1	40	0.9943
13	−1	2	−1	40	1	120	1	80	−1	40	0.3685
14	1	4	−1	40	1	120	1	80	−1	40	0.5787
15	−1	2	1	60	1	120	1	80	−1	40	0.7973
16	1	4	1	60	1	120	1	80	−1	40	1.2528
17	−1	2	−1	40	−1	80	−1	40	1	60	0.2920
18	1	4	−1	40	−1	80	−1	40	1	60	0.4594
19	−1	2	1	60	−1	80	−1	40	1	60	0.6329
20	1	4	1	60	−1	80	−1	40	1	60	0.9940
21	−1	2	−1	40	1	120	−1	40	1	60	0.3674
22	1	4	−1	40	1	120	−1	40	1	60	0.5776
23	−1	2	1	60	1	120	−1	40	1	60	0.7976
24	1	4	1	60	1	120	−1	40	1	60	1.2529
25	−1	2	−1	40	−1	80	1	80	1	60	0.2910
26	1	4	−1	40	−1	80	1	80	1	60	0.4590
27	−1	2	1	60	−1	80	1	80	1	60	0.6331
28	1	4	1	60	−1	80	1	80	1	60	0.9945
29	−1	2	−1	40	1	120	1	80	1	60	0.3688
30	1	4	−1	40	1	120	1	80	1	60	0.5783
31	−1	2	1	60	1	120	1	80	1	60	0.7973
32	1	4	1	60	1	120	1	80	1	60	1.2533
33	−2.37841	0.60	0	50	0	100	0	60	0	50	0.2540
34	2.37841	5.40	0	50	0	100	0	60	0	50	0.6555
35	0	3	−2.37841	26	0	100	0	60	0	50	0.3267
36	0	3	2.37841	74	0	100	0	60	0	50	2.1471
37	0	3	0	50	−2.37841	52	0	60	0	50	0.5166
38	0	3	0	50	2.37841	148	0	60	0	50	0.8319
39	0	3	0	50	0	100	−2.37841	12	0	50	0.4581
40	0	3	0	50	0	100	2.37841	108	0	50	0.4577
41	0	3	0	50	0	100	0	60	−2.37841	26	0.4583
42	0	3	0	50	0	100	0	60	2.37841	74	0.4590
43	0	3	0	50	0	100	0	60	0	50	0.4570

Table 3. (Contd.)

No.	A level	A, h	B level	B, °C	C level	C, μL	D level	D, min	E level	E, °C	*R*
44	0	3	0	50	0	100	0	60	0	50	0.4555
45	0	3	0	50	0	100	0	60	0	50	0.4587
46	0	3	0	50	0	100	0	60	0	50	0.4588
47	0	3	0	50	0	100	0	60	0	50	0.4601
48	0	3	0	50	0	100	0	60	0	50	0.4568
49	0	3	0	50	0	100	0	60	0	50	0.4580
50	0	3	0	50	0	100	0	60	0	50	0.4574

react with fatty acids that remained unesterified with methanol in artifact A which contains a high percentage of fatty acids. According to this result, if only the fatty acids are to be determined, it may not be necessary to use BSTFA as an additional silylation reagent in the acid-catalyzed direct extraction-methylation method. This result is also supported by the chemometric results in the next section.

In terms of monounsaturated fatty acid structure, oleic acid (C18:1) was detected in M1 and M4, while palmitoleic (C16:1) acid was observed in M1, M4, and T1. Linoleic (C18:2) acid was detected in only M4. To assess the potential origin of these fatty acids, their ratios can be useful, but they need to be compared to and integrated with the presence/abundance of other compounds. For instance, if stearic acid (C18:0) is lower than palmitic acid (C16:0), and pentadecanoic acid (C15:0), margaric acid (C17:0), and oleic acid (C18:1) are also present, then the investigated residue may be a fatty compound obtained from ruminant animals which may be fat or dairy products [44]. In the analyzed ceramics, M1 fits this fatty acid profile (Table 5). Also, ruminant carcass fats are characterized by a higher amount of stearic (C18:0) compared to palmitic (C16:0) fatty acid [44], and only A conforms to this pattern. Also, the presence of palmitinic acid-glycerin-(1)-monoester in A is considered a product of triglyceride hydrolysis. Although it is thought that this result may indicate that the glyceride structure in the A sample has started to degrade relatively recently, this molecule will not allow to comment on the lipid type studied. Since the archaeological artifacts studied have been under the ground for many years, the glyceride-type structures in their content have a very low chance of surviving due to hydrolytic degradation.

The most common and most stable oxidation products of unsaturated fatty acids are dicarboxylic acids which survive particularly well in geographies with dry climates [51] such as the Near East. Dicarboxylic acids that ranged from C5 to C12 with azelaic acid as the main compound [13, 40, 44] have for instance been determined in ceramic samples from a 4000-year-old Nubian burial [52]. Considering Table 5, azelaic acid was determined in M1 and T1, while adipic and pimelic acids were observed in only T1. Especially azelaic acid is a hydrolysis product of unsaturated fatty acids with a double bond at the ninth carbon position in the original fatty acids [53] such as oleic acid (C18:1). Roffet-Salque et al. [50] suggest that dicarboxylic acid compounds are more likely to be found in plant oils. The presence of dicarboxylic acids and oleic acid (C18:1) strengthens the possibility that plant oil or derivative mixtures might have been major components of the samples M1 and T1. Under anaerobic conditions, oleic (C18:1) acid tends to transform into azelaic (C9:0) and palmitic (C16:0) acids through oxidative decay [51, 54]. M1 contains oleic (C18:1) acid along with azelaic acid (C9:0) and a large amount of palmitic acid (C16:0) (Table 5), suggesting that the lipid residue in these samples may be of plant-origin oil. When all the data in Table 5 are evaluated, it can be said that although the lipid residue in A may indicate an animal source, M1 and T1 mostly point to a plant source. Although a composition could not be

Table 4. Total lipid concentrations of artifacts with lipid residue detected

Ceramic	Data	Total lipid concentration, μg/g (lipid/ceramic powder)[a]
A	Mean	6800.21
	SD	0.56
F1	Mean	160.81
	SD	0.83
M1	Mean	219.65
	SD	33.72
M4	Mean	301.36
	SD	9.38
M6	Mean	3.11
	SD	1.68
M7	Mean	42.75
	SD	3.02
T1	Mean	345.66
	SD	2.33

[a] SD—standard deviation from triplicate analysis.

Table 5. Percentage by weight of the biochemical composition of the artifacts found to contain lipid residues

Compound	A		F1		M1		M4		M6		M7		T1	
	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD
Pelargonic (nonanoic) acid methyl ester	2.83				0.24	0.00	0.12	0.00			1.11	0.08	4.16	0.16
Adipic (hexanedioic) acid methyl ester													1.11	0.04
Capric (decanoic) acid methyl ester					0.65	0.01	0.28	0.01					5.21	0.20
Heptanedioic (pimelic) acid methyl ester													0.41	0.01
Tetradecane											1.57	1.50		
Undecanoic acid methyl ester					0.23	0.01	0.46	0.01					1.49	0.02
Suberic (octanedioic) acid methyl ester					0.34	0.02								
Lauric (dodecanoic) acid methyl ester			1.40	0.53	1.95	0.03							6.65	0.18
Azelaic (nonanedioic) acid methyl ester					0.42	0.01							3.07	0.10
Hexadecane					0.52	0.02	0.36	0.01			1.72	0.66		
Tridecanoic acid methyl ester					0.46	0.01							0.71	0.04
Dotriacontane							0.41	0.01						
Heptadecane											2.20	2.70		
Hexadecane, 2,6,10,14-tetramethyl					0.82	0.02					1.04	0.06		
Myristic (tetradecanoic) acid methyl ester			18.18	0.05	9.49	0.31	3.36	0.01	4.99	0.01	2.49	0.59	22.33	1.80
Octadecane											5.48	1.04		
Pentadecanoic acid methyl ester			1.04	0.01	3.93	0.09	1.17	0.03					9.05	0.86
Palmitoleic (9-hexadecenoic) acid methyl ester					5.60	0.10	1.55	0.02					2.54	0.11
Palmitic (hexadecanoic) acid methyl ester	17.70	0.27	29.72	0.42	27.97	0.15	51.35	0.19	56.06	21.31	9.95	1.00	25.44	0.01
Methyl 14-methylhexadecanoate					2.65	0.01								

Table 5. (Contd.)

Compound	A		F1		M1		M4		M6		M7		T1	
	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD	mean, %	SD
Eicosane							0.45	0.01			1.63	0.45		
Margaric (heptadecanoic) acid methyl ester					1.23	0.13								
Linoleic (9,12-octadecadienoic) acid methyl ester							16.80	1.01						
Oleic (9-octadecenoic) acid methyl ester					19.28	0.98	8.06	0.79						
Palmitic (hexadecanoic) acid trimethylsilyl ester	2.44	0.12												
Stearic (octadecanoic) acid methyl ester	19.91	0.30	23.98	0.42	8.54	0.16	5.86	0.12	17.14	6.66	7.82	0.59	14.66	0.01
Docosane			1.29	0.23	0.31	0.04					1.02	0.13		
Stearic (octadecanoic) acid trimethylsilyl ester	3.11	0.01												
Tricosane			1.03	0.04										
Arachidic (eicosanoic) acid methyl ester					0.53	0.01							0.59	0.00
Tetracosane			1.14	0.01										
Docosanoic (behenic) acid					0.23	0.01								
Heptacosane					0.52	0.02								
Bis-O-trimethylsilyl-palmitinic acid-glycerin-(1)-monoester	5.20													
Tetracosanoic (lignoceric) acid methyl ester					0.59	0.04							0.64	0.01
Others[a]	48.81		22.22		13.50		9.77		21.81		63.97		1.94	

[a] Compounds with less than 80% matches in MS.

determined in the residues of other ceramics (F1, M4, M6, M7), it can be mentioned that the lipid residue they have is of plant origin due to the high ratio of palmitic (C16:0) to stearic (C18:0) acids. It should be considered that the stable isotope assay of these fatty acids (palmitic and stearic) should be performed to make more precise interpretations about the origin of the lipid residues.

The pottery found stratigraphically in the systematic archaeological excavations in Küllüoba and

included in this study belongs to the EBA III period, between 2400 and 2100 BC, when the layers in which they were found and the dating of these layers are evaluated. Since the bowl forms (M2, M3, M5, and M9) in these are produced for daily use and for the consumption of liquid-containing foods, they mostly consist of slipped samples. Different ware groups in bowl forms indicate interregional relations [3, 4].

Plates which are represented with the highest number of samples in the analyzed group (M7, T1–T14) primarily sign the innovations in pottery production technology. These plates which are called Troy A2 plates in the literature started to be produced with the use of potter wheel technology in Anatolia. The form in question has been found in various settlements in Northwestern Anatolia and the Aegean World, especially in Troy, and it constitutes one of the most important pieces of evidence that the potter wheel technology was first used in Anatolia in 2400 BC alongside handmade pottery [55, 56]. In addition, among these samples, there are also imported samples which are foreign to the region, especially in terms of fabric and firing properties. It is understood that the technology in question reached Anatolia from Syria-Mesopotamia and spread to the Aegean World.

The forms known as depas (M4) and tankard (F1) in the study are vessels that point to new drinking habits and were used for the first time at the end of the EBA. The use of these containers also indicates some changes in the social hierarchy [57]. A sample of the transport amphorae (A) which is thought to carry the beverages in these containers was also evaluated in the aforementioned analyses. Amphorae were also used for the first time in this period [58]. Finally, the form known as the beaker (M1) whose examples are mostly known from Syria is considered one of the important pieces of evidence of the trade that took place with distant regions within the samples of Küllüoba.

Chemometrics. As a result of the analyses made in the previous section, it was determined that the artifact with the highest lipid concentration was the A-coded amphora piece. For this reason, artifact A was chosen as the stock sample in the chemometric model studies carried out. Artifact A was sampled, and then the extraction processes were carried out according to the experimental conditions contained in each test number in Table 3.

The extracts obtained as a result of the relevant extractions were analyzed by GC-MS according to the previously described conditions, and the sum of the palmitic (C16:0) and stearic (C18:0) acid peak areas in the form of both methyl and silyl esters was calculated, and the response value (R) was obtained by dividing this sum value by the internal standard peak area obtained in the relevant analysis. After the response values were placed in Table 3, the available data were processed with the software used, and the optimum values of each studied parameter and their effects on the efficiency of the extraction process were determined by creating the perturbation and RSM plots given in Figs. 2 and 3, respectively.

When Table 3 is examined, it is seen that the lowest (0.2540) and highest (2.1471) response values were obtained in analyses 33 and 36, respectively. In these analyses, only the extraction time and temperature parameters showed changes, while the other parameters were the same. It is seen that the extraction time is at the lowest level in analysis 33, where the lowest response value is taken, while the extraction temperature is at the highest level in analysis 36, where the highest response value is taken. These results indicate that the parameters affecting the extraction efficiency most from the parameters examined are the extraction time and temperature.

The perturbation plot showing the codes of the parameters to be optimized within the scope of the study is given in Fig. 2. In this plot, the parameters that are located farthest from the central black point and tend to draw curves represent the parameters that mostly affect the result in the analysis. According to the related plot, it is seen that the extraction temperature corresponding to B is the parameter that has the most effect on the results obtained, that is, the parameter responsible for the changes in the relevant results. Although not as much as B, the extraction time (A) and amount of silylation reagent (C) also had relative effects. It was determined that the silylation temperature and time which were coded with D and E, respectively, had almost no effect (Fig. 2). According to the results in Fig. 2, it can be seen that the most effective parameter in the extraction process of the lipid residues in the archaeological ceramic artifacts is the extraction temperature. The reason why the silylation process is not very effective may be the methanolic sulfuric acid solution added at the beginning of the extraction process. Fatty acids which can be taken into the solution environment from the ceramic structure with the strong catalyst role of sulfuric acid reacted with methanol to give methyl esters and there may not be enough fatty acids left to yield the silyl ester with the BSTFA reagent. When Table 5 is examined, it is seen that most of the currently detected fatty acids are in the form of methyl esters, and only palmitic (C16:0) and stearic (C18:0) fatty acids of artifact A are in the form of silyl esters. Since the silylation temperature and time which are coded with D and E, respectively, have almost no effect, only RSM plots with A, B, and C parameters are drawn and interpreted in Fig. 3.

Figure 3 shows the RSM plots with binary combinations of the parameters A, B, and C. As stated before, since the parameter with the extraction temperature coded with B is the most effective, more changes can be seen in the plots with this parameter. Figure 3a shows the effects of A and B parameters on each other and on the response value. It is seen that the response value increases as both the extraction tem-

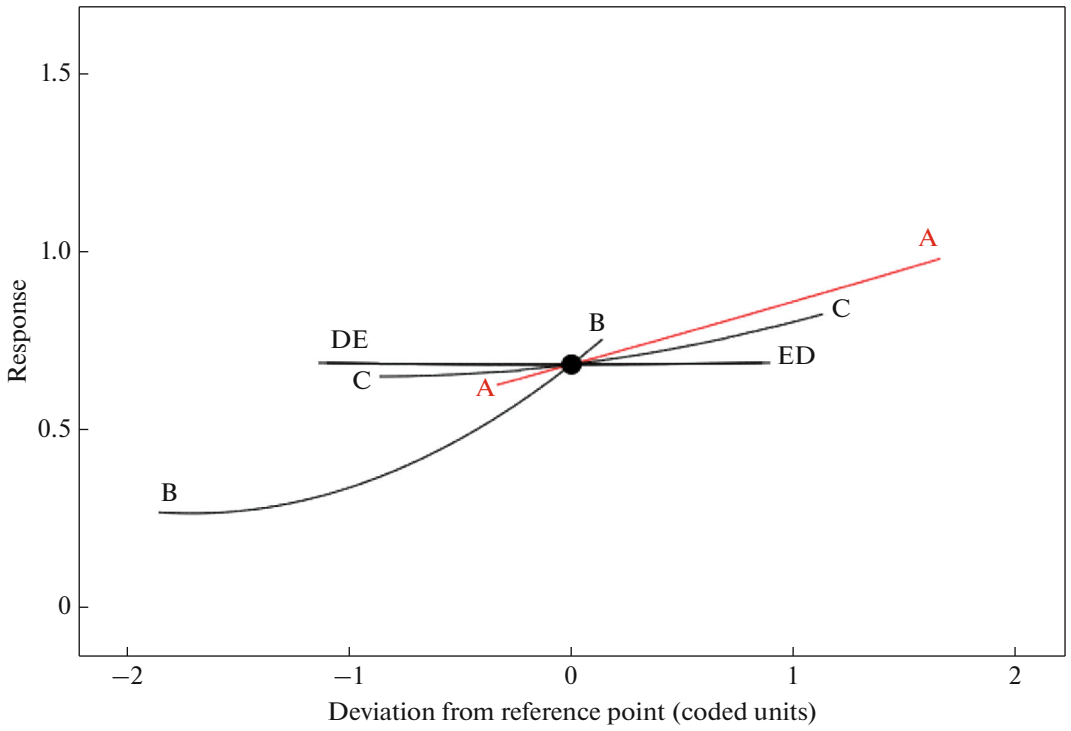

Fig. 2. The perturbation plot showing the codes of the parameters to be optimized within the scope of the study (A— extraction time, h; B—extraction temperature, °C; C—amount of BSTFA added, μL; D—silylation time, min; E—silylation temperature, °C).

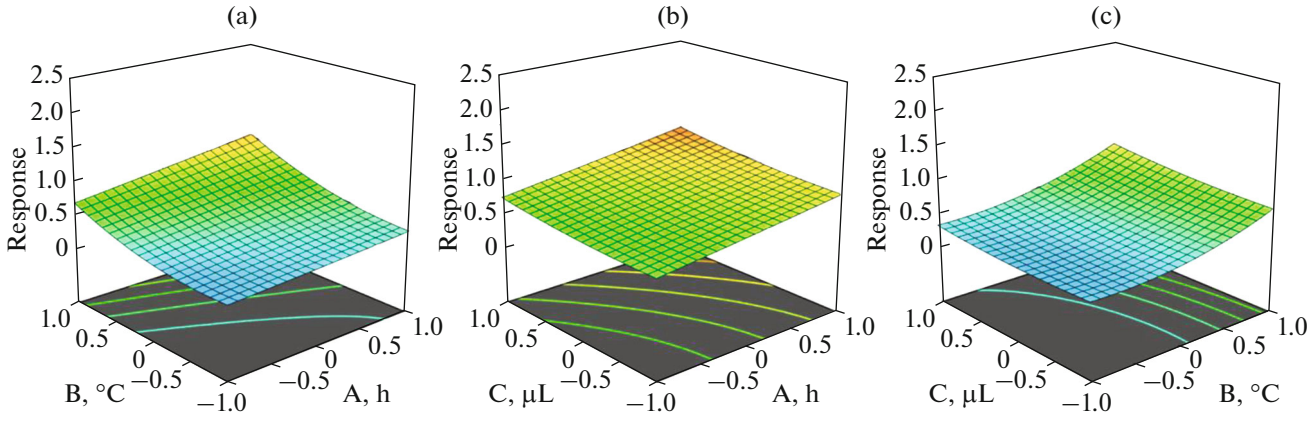

Fig. 3. RSM plots affected by the method parameters for the extraction of the lipid residues: (a) A—extraction time and B—extraction temperature; (b) A—extraction time and C—amount of BSTFA added, μL; (c) B—extraction temperature and C—amount of BSTFA added, μL.

perature and time increase and, accordingly, it is seen that the ratio of extractable fatty acids increases. Figure 3b shows the effects of A and C parameters on each other and on the response value. This plot also shows that as the extraction time and amount of silylation reagent increase, the fatty acid yield increases, but it is not as effective as the extraction temperature. In Fig. 3c, it is seen that there is an effective change due to the extraction temperature.

CONCLUSIONS

Valuable interpretations can be made in terms of archeology by taking the lipid molecules which are highly likely to be found in ceramic artifacts into the solution environment with appropriate extraction methods. In this regard, the extraction of lipid molecules is of great importance. In the present study, the parameters of the acid-catalyzed direct extraction-methylation method which is widely used in the liter-

ature for the extraction of lipids and ORs in archaeological ceramic artifacts were investigated. When the results were evaluated, it was determined that the extraction time and temperature should be high as long as the experimental conditions and the environment allow for the extraction of the lipid residues in the ceramic artifacts, and the silylation process has a limited positive effect on the yield, but the extraction with methanol can be achieved successfully when the silylation process is not performed.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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