An application of high performance liquid chromatography to analysis of lipids in archaeological samples

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Abstract Five samples from three different types of 1500-year-old Mediterranean amphorae, as well as from a contemporary oil lamp found in the same deposit, were analyzed for the presence of lipid residues. Each sample of finely ground amphorae powder weighed 1–2 g. The abundance of interfering secondary products makes thin-layer chromatography (TLC) an essential step of the procedure. The fractionation of the extract into its various lipid components by means of TLC was followed by quantitative recovery of the triglyceride (TG) and free fatty acid (FFA) fractions from the plates and by the measurement of their components by high performance liquid chromatography (HPLC) after esterification. The minimum detectable level is 1 ng. The amphorae samples revealed a more abundant FFA fraction than a TG fraction, which is the reverse of what we know about the composition of fresh oil. Despite the considerable age of the amphorae and their preservation under non-ideal conditions, the lipid residues have retained certain identifiable characteristics that enable one to make valid suggestions as to the type of commodity originally transported in the amphorae. The results of these experiments yield important information that enables the biochemist to observe an aging process irreproducible in the laboratory and provides the archaeologist with previously unavailable information about trade 15 centuries ago.—**Passi, S., M. C. Rothschild-Boros, P. Fasella, M. Nazzaro-Porro, and D. Whitehouse.** An application of high performance liquid chromatography to analysis of lipids in archaeological samples. *J. Lipid Res.* 1981. **22:** 778–784.

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The purpose of the study of lipid residues absorbed and retained in the (ceramic) fabric of ancient pottery is to offer a methodology that can corroborate the circumstantial evidence and "pottery sense" presently used by archaeologists to ascertain ceramic function (1). Knowing what a vessel contained is extremely important for the reconstruction of past activity patterns, but this is often impossible without written records. However, locked within the ceramic fabric of ancient wares is evidence which, when extracted with reliable, reproducible methods, can be invaluable to the study of the past.

Following this approach, an original procedure for the extraction and determination of lipids has been applied to material from an archaeological deposit in the Schola Praeconum (2) in Rome. The results offer a clear example of how the study of lipid residues can be of assistance to the archaeologist. Numismatic and ceramic evidence (3) indicate that the deposit was created during the years 425–450 A.D. perhaps between 430–440 A.D. The deposit contained a large number of amphora fragments which, on the basis of petrological and typological studies, can be shown to have originated in several different parts of the Mediterranean basin. Most of the amphorae arrived in Rome as containers for imported foodstuff: mainly olive oil, sesame oil, and wine, but also such products as *garum* (fish sauce). Archaeologists have wondered whether particular types of amphorae were commodity-specific. However, to date they have lacked a technique to test this hypothesis.

In the 5th century A.D., Rome was in decline. Large groups of barbarians invaded the European provinces and the Visigoths under Alaric ransomed and sacked Rome itself in 410A.D. Our assessment of the extent of Rome's decline depends partly on archaeological evidence and in this context the material from the Schola Praeconum is of primary importance. Specifically it is important to know what was in the

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; TG, triglycerides; FFA, free fatty acids; UV, ultraviolet; LC, liquid chromatography.

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amphorae, for this knowledge would throw light on international trade and, therefore, on the economic history of Rome between 425 and 450 A.D. In addition, such information would test the hypothesis that individual amphora shapes were commodity-specific.

Three types of amphorae, each from a different region, were analyzed: *a*) one fragment of a "Micaceous jar" (the color of which varies from 2.5 YR 5/6 to 2.5 YR 4/6 on the Munsell chart), imported from Anatolia (4); *b*) two fragments of "Late Roman type 4" amphora (5 YR 6/6 to 7.5 YR 6/6) from the Gaza region (5); and *c*) two fragments of white-slipped "Africano Grande" amphora (body: 10 YR 5/8; slip 10 YR 8/3) from North Africa (6). In addition, a fifth century oil lamp, found in the same deposit, and a modern terracotta flower pot were examined for comparison.

A density migration study was also done in order to establish the absence of contamination from the soil. This was done by dividing the sherds into three parts: the interior surface (in contact with the commodity), the core, and the exterior surface. This analysis was applied to one of the Africano Grande sherds, the two Gaza-Rilled sherds, and the modern sherd.

MATERIALS AND METHODS

Criteria for selecting the amphora sherds

The amphora sherds were selected using the following criteria. *1*) They had to come from near the base in order to ensure optimal contact with the contents. *2*) Two North African sherds were chosen because their clay appeared dissimilar. *3*) All the sherds had been cleaned upon excavation with a brush, using water only.

Preparation of the samples

In the case of migration studies, the thickness of the amphora walls was measured (Africano Grande, 90–120 mm; Gaza-Rilled, 70–100 mm) and the sherds were marked for division into three parts. The separation of the three sections was done with dental tools. Each surface was then ground into a fine powder with a mortar and pestle, yielding 1–2 g of material. The modern sample contained a homogeneous mixture of just the interior surface and the core, also yielding 1–2 g.

Extraction of lipids

Samples of finely ground amphorae (1–2 g) were extracted in a Soxhlet apparatus with chloroform–methanol 2:1 (v/v) for 3 hr. The extracts were filtered through a sintered glass, and dried over anhydrous Na_2SO_4. The extracted solutes were recovered after evaporation of the solvent under reduced pressure in a rotary evaporator at below 40°C.

Fractionation of lipid extracts by thin-layer chromatography

The above extracts were fractionated into their various lipid components by TLC on standard thin-layer plates (Stratocrom SI AP, Carlo Erba) coated with a 0.25-mm thick layer of silica gel and activated by heating at 120°C for 20 min. The plates were developed in two successive solvent systems (7): *a*) benzene–petroleum ether (bp 30–50°C) 3:1 (v/v); *b*) petroleum ether (30–50°C)–diethyl ether–acetic acid 70:30:1.5 (v/v/v). In some experiments the lipids on the developed chromatograms were detected by charring at 150°C with 60% (v/v) H_2SO_4.

In other developed chromatograms, only the extreme right-hand column containing standards was stained with bromocresol green (in order to measure the R_f of the reference compounds), while the other parts of the plate, containing the different lipid fractions extracted from pottery samples, were not stained so that they could be used for the following analysis by liquid chromatography.

The areas of silica gel corresponding to the R_f of oleic acid and triolein standards and containing free fatty acids and triglycerides, respectively, were scraped off and extracted with perioxide-free diethyl ether.

Analysis of fatty acids of lipid fractions by liquid chromatography

Recently HPLC has been employed in the separation of fatty acid mixtures. The preparation of UV-absorbing phenacyl derivatives (8–10) was essential to obtain the sensitivity required for samples in the nanogram range. In this study we report the HPLC separation of the *p*-bromophenacyl esters of fatty acids.

Derivatization procedure

The procedure of Fitzpatrick (11) for analysis of prostaglandins was used. The triglyceride fraction eluted from the TLC plates was saponified by treatment with 2 N KOH in methanol and successive acidification to pH 2–3 with 2 N HCl. Free fatty acids were thus obtained.

Free fatty acids samples (standards, eluates of the FFA fraction of TLC, and saponified eluates of the TG fraction of TLC) were dissolved in 0.5 ml of CH_3CN. One mg of *p*-bromophenacyl bromide dissolved in CH_3CN and 2 μl of the catalyst N,N-diisopro-

pylethylamine were added and the reaction volume was adjusted to 1 ml. The mixture was heated to 50–60°C for 10–15 min. Under these conditions complete esterification of free fatty acids to the corresponding, strongly chromophoric, *p*-bromophenacyl esters is achieved. A 100-μl aliquot of the solution was then directly injected into the liquid chromatograph (1084 B Liquid Chromatograph, Hewlett-Packard) provided with either a single wave length (254 nm) UV detector or a scanning spectrophotometer with a wavelength range of 190–540 nm. The liquid chromatograph includes an integrator that gives peak areas and characteristic times for each peak in the chromatogram.

The separation of the fatty acids, as *p*-bromophenacyl esters, was obtained on a reversed phase column (25 cm × 4 mm I.D.) R P-18, 5 μm (Brownlee Labs., Santa Clara, CA). The chromatographic column was operated at 40°C. Mobile phase: an initial isocratic elution for 5 min (70% CH_3CN in water adjusted to pH 3.10 with H_3PO_4), then a gradient to 100% CH_3CN in 100 min. Flow rate: 1 ml/min; sensitivity: from 4.0×10^{-4} to 256×10^{-4} absorbance units/cm (AV/cm), depending upon the amount of injected substances; chart speed: 0.25 cm/min.

RESULTS AND DISCUSSION

Calibration

Samples (0.5 to 20 μg) of standard carboxylic acids ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$) in 1 ml of reaction mixture were esterified as described under Methods.

One-tenth of the total (for each concentration) was injected into the HPLC column. The outflow from the column was monitored by an UV absorbance detector operating at 254 nm. The absorption maximum for these esters is 255 nm with $\log_{10} \epsilon_M$ values of about 4.5. The detector response was a linear function of the injected quantities for seven *p*-bromophenacyl esters of carboxylic acids in the 0.01 and 2 μg range.

TABLE 1. Recovery of fatty acids from TLC fractions[a]

Lipids	Amount Deposited on TLC Plates	Amount Measured by HPLC after Extraction from TLC Plates and Esterification[b]
	μg	μg
Oleic acid	10	9.55 ± 0.31
Triolein	10 (as oleic acid)	9.28 ± 0.62
Palmitic acid	10	9.70 ± 0.23
Tripalmitin	10 (as palmitic acid)	9.42 ± 0.54

[a] For details see text.
[b] Each result represents the average of five experiments ± SD.

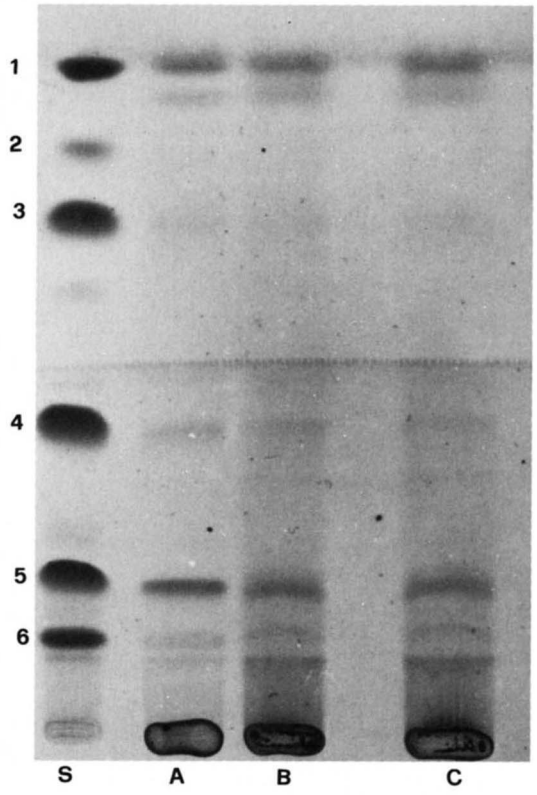

Fig. 1. Thin-layer plate charred with 50% H_2SO_4. S = standards of 1) squalene, 2) cholesteryl oleate, 3) hexadecyl oleate, 4) triolein, 5) oleic acid, 6) cholesterol. A), Gaza-Rilled I; B), Gaza-Rilled II; C), oil lamp.

Recovery of standard samples from TLC plates

It was then ascertained that the fatty acids present in the free fatty acid and triglyceride TLC fractions could be quantitatively recovered from the plates and measured after esterification on HPLC. Various amounts of standard samples were chromatographed on TLC. Standard free fatty acid and triglyceride fractions were extracted from the plates, saponified when necessary, converted to *p*-bromophenacyl esters, and quantitatively measured by HPLC. (**Table 1**).

TLC analysis of extracts from the amphorae and reference samples

The detection (before charring) on developed TLC plates of the main fractions of lipids extracted from three ancient amphorae is represented in **Fig. 1**. Unfortunately, a percent quantification of each fraction by photodensitometry would be meaningless because of trailing. This can be attributed either to an abundance of secondary products of oil autoxida-

tion or, possibly, to the presence of unidentified organic compounds extracted by chloroform–methanol.

The abundance of secondary products visible in TLC and the high number of unidentified peaks in HPLC (see below) is not surprising if we assume "fifteen-centuries-old" oil to be the main source of the material and if we consider the chemical instability of oil and the variety of the reactions that can occur in it. Moreover, oxidation phenomena may have been facilitated by the fact that the lipid material extracted from the jar existed mostly as a thin film, i.e., in a physical form that favours oxidation by air.

The variety of fatty material found in the jar indicates that prepurification and fractionation by TLC is indeed an indispensable prerequisite for the quantitative determination of identifiable components by HPLC.

In each sample, the free fatty acid fraction (FFA) was more abundant than the triglyceride (TG) fraction. This is contrary to what we know about the composition of fresh oil, where glycerides, and particularly triglycerides, largely predominate over free fatty acids. This finding could be due to one or more of the following events: *1*) autoxidation of the TG fraction; *2*) hydrolysis, either spontaneous or by lipases from microorganisms that may have grown on the amphorae walls; *3*) inadequate oil processing procedures in ancient times, which led to a greater acidity of the oil; or *4*) preferential penetration and adhesion to the amphorae clay of smaller and more polar FFA.

HPLC analysis of the free fatty acids present in the "FFA" and "saponified TG" fractions obtained from TLC of lipids extracted from amphorae and reference samples.

Figs. 2A and **2B** show liquid chromatographic separation, respectively, of a synthetic mixture of standard fatty acids and of fatty acids present in the saponified TG fraction from the Gaza-Rilled I sample.

The fatty acid composition of the saponified triglyceride (TG) and free fatty acid (FFA) fractions found in the amphorae in toto (i.e., without distinction between the interior, core, and exterior samples) is reported in **Table 2**. Because of the large number of unknown components present in the starting material, it is possible that many of the peaks assigned to known FA on the basis of their chromatographic behavior may contain more than one component. The scarcity of starting material prevented the characterization of each peak by mass spectrometry. However, the absorbance spectrum of each peak was measured in the scanning spectrophotometer connected to the liquid chromatograph; the recorded spectra

TABLE 2. Fatty acid composition (%) of the TG and FFA fractions found in the amphorae in toto

Type of Amphora	$C_{18:0(2OH)}$[a]		$C_{14:0}$		$C_{18:2}$		$C_{16:0}$		$C_{18:1}$		$C_{17:0}$		$C_{18:0}$		$C_{20:0}$		Others[b]	
	TG	FFA	TG	FFA	TG	FFA	TG	FFA	TG	FFA	TG	FFA	TG	FFA	TG	FFA	TG	FFA
1. Gaza-Rilled I	0.70	0.85	0.36	0.64	2.85	1.64	5.82	5.71	15.40	13.94	1.13	tr	8.78	7.22	3.28	2.80	61.67	67.10
2. Gaza-Rilled II	3.06	3.91	0.48	0.76	0.15	tr	5.99	6.10	16.31	14.64	0.46	1.03	5.13	5.89	tr	0.21	68.32	67.36
3. Micaceous jar	1.93	2.76	0.76	0.46	0.26	tr	17.74	20.66	14.25	11.17	0.25	tr	6.08	4.45	tr	tr	58.63	60.40
4. Africano Grande, white slipped (NAz)	4.45	3.82	0.70	0.45	tr	tr	4.88	5.63	13.67	11.93	tr	0.33	4.30	4.89	0.87	0.17	71.03	72.68
5. Africano Grande, white slipped (NA 4)	5.41	6.13	0.83	0.90	tr	tr	4.94	6.86	11.13	7.99	0.28	0.49	3.68	4.16	0.45	0.30	73.18	73.07
6. Oil lamp	13.81	14.76	1.02	0.70	tr	tr	6.42	10.87	13.40	12.45	tr	0.18	5.49	5.33	tr	tr	59.76	55.61
7. Modern terracotta pot																		

[a] $C_{18:0(2OH)}$ = dihydroxystearic acid.
[b] Non-identified secondary products of autoxidation of oils with carboxyl groups or other organic UV-absorbing compounds that migrated on TLC plates with the same R_f of TG and FFA fractions. Generally they were eluted on the reverse phase RP 18 column after the non-reacted excess of esterifying reagents.

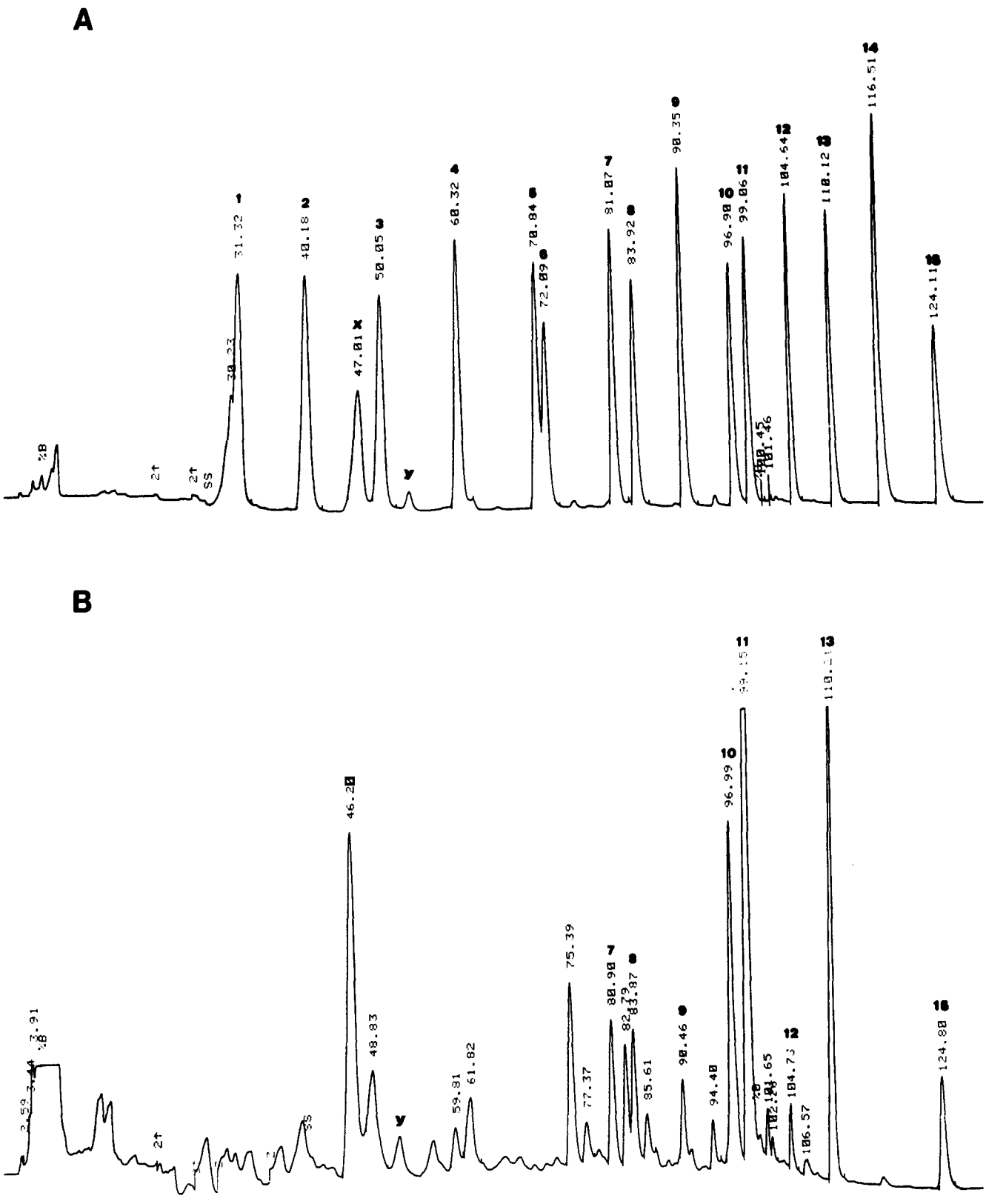

Fig. 2. Separation of a mixture of the *p*-bromophenacyl esters fatty acid from standard FA (A) and of the saponified TG fraction from Gaza-Rilled I sample (B). Conditions are described in Methods. Amount injected: 0.5–0.75 μg for each standard compound. Peak identifications: 1, $C_{9:0}$; 2, $C_{10:0}$; ×, unknown; 3, $C_{11:0}$; y, $C_{18:0\,(2\,OH)}$; 4, $C_{12:0}$; 5, $C_{13:0}$; 6, $C_{18:3}$; 7, $C_{14:0}$; 8, $C_{18:2}$; 9, $C_{15:0}$; 10, $C_{16:0}$; 11, $C_{18:1}$; 12, $C_{17:0}$; 13, $C_{18:0}$; 14, $C_{19:0}$; 15, $C_{20:0}$. Many unidentified peaks were observed; these were not investigated further.

corresponded to those of *p*-bromophenacyl esters that give a characteristic absorbance spectrum with a maximum at 225 nm. The presence of other UV absorbing components was not observed.

The identification in samples 1 to 5 of $C_{14:0}$, $C_{18:2}$, $C_{16:0}$, $C_{18:1}$, $C_{18:0}$, and $C_{20:0}$ fatty acids, which are typical components of vegetable fats, strongly suggests that the amphorae studied had indeed contained vegetable oils. In contrast, the modern sherd produced nonquantifiable traces of $C_{16:0}$ and $C_{18:0}$ on the areas of the TLC plates corresponding to the oleic and triolein standards.

The analysis of samples obtained from different parts of the amphorae, named interior surface, core, and exterior surface, shows that the TG content decreases from the interior through the core to the exterior of the amphorae (see **Table 3**), suggesting that oil slowly migrated through the porous ceramic fabric of the amphorae.

Moreover, in the four cases studied, the unsaturated fatty acids of TG fractions were always higher in the core than in the interior or exterior surfaces (see Table 3), possibly because they were more protected from autoxidation.

Interpretation

Conclusions of the present work should be drawn at two levels, technical and interpretative. At the technical level we should ask ourselves whether the method is reliable and practical; the above results show that it is both. By combining specific extractions, TLC and HPLC, it is possible to obtain lipid material from the amphorae, to fractionate it into identifiable components, and to measure, with a good degree of accuracy and precision, the most significant fatty acids present in both the free acid (FFA) and the triglyceride (TG) fractions. It is worthwhile to note also the reasonable similarity between the results obtained with two samples from two different amphorae of the same type (Africano Grande in Table 2). Moreover, by esterification, before HPLC, of fatty acids with a strongly UV-absorbing derivative it has been possible to reach nanogram sensitivity that allows accurate analysis of very small samples. The present TLC + HPLC method is therefore more suitable for the study of lipids in archaeological material than direct gas–liquid chromatography (12) both in terms of sensitivity (mg instead of g of starting material) and of chemical significance (the interference from the vast number of secondary products is practically abolished). This "technical" conclusion is very important because it provides an indispensable basis for further considerations.

At the level of interpretation, conclusions are much harder to draw. We can approach the problem by successive approximations, asking if a chemical analysis on lipid material expected to be 15 centuries old can provide at least indicative evidence that the material is a vegetable oil.

The considerations reported under Results and Discussion make it seem very probable, though not certain by the criteria of experimental science, that the three types of ancient amphorae and oil lamp, but not the modern flower pot, had indeed contained

TABLE 3. Fatty acid composition (%) of TG fraction from interior surface, core, and exterior surface samples of three amphorae

Type of Amphora	Section	Amount of FA/g Powder[a]	$C_{18:0(2\,OH)}$	$C_{14:0}$	$C_{18:2}$	$C_{16:0}$	$C_{18:1}$	$C_{17:0}$	$C_{18:0}$	$C_{20:0}$	Others
		μg					%				
Gaza Rilled I	Int.	8.80	0.85	0.61	1.99	6.21	13.70	1.45	9.12	3.80	62.22
	Core	5.20	0.51	0.38	3.06	5.87	18.85	0.57	8.74	2.75	59.27
	Ext.	2.10	1.05	0.55	0.81	9.36	11.30	tr	8.80	3.06	64.97
Gaza Rilled II	Int.	6.20	3.55	0.73	tr	4.46	14.73	0.71	6.46	tr	67.26
	Core	4.30	3.01	0.41	0.27	5.82	17.09	tr	5.61	tr	67.69
	Ext.	1.20	3.48	0.49	tr	6.81	15.51	0.37	6.18	tr	67.06
Africano Grande, white slipped (NAz)	Int.	3.70	4.65	0.86	tr	5.25	13.06	tr	4.86	0.91	70.31
	Core	1.75	3.63	0.51	tr	4.51	14.25	tr	4.21	0.84	71.95
	Ext.	tr									
Oil lamp	Int.	15.51	14.13	1.12	tr	8.31	10.71	tr	6.36	tr	59.27
	Core	12.18	12.73	0.95	tr	5.46	18.76	tr	5.20	tr	56.80
	Ext.	6.71	14.46	1.01	tr	9.17	11.48	tr	5.94	tr	57.84

[a] The amounts of fatty acid per g of powder were calculated by the sum of peak areas of identified fatty acids in the chromatograms and reference to the corresponding areas of standard compounds.

$C_{18(2\,OH)}$, dihydroxystearic acid; tr, traces.

a vegetable oil that had partly permeated its walls. The identification of the type of oil is more difficult. Some suggestive indications, however, are provided by a careful examination of the quantitative results. Beginning with the easier case, the composition of the lipids obtained from the oil lamp is slightly different from that of the lipids from the amphorae. In particular, a peak identifiable as 9–10 dihydroxystearic acid is much more abundant in the case of the lamp (Table 2), suggesting that it may have contained rancid oil, which normally contains 9–10, dihydrostearic acid. Incidentally, it is worth noting that rancid oil is still used for lamps by peasants in the Apennine hills east of Rome.

A comparison among the results obtained with the three types of amphorae is also suggestive. The fatty acid patterns of both the FFA and TG fractions from the Africano Grande, the Micaceous jar and the Gaza-Rilled II are similar and compatible with the assumption that they contained olive oil. The patterns from the Gaza-Rilled I sample are quite different: notice, in particular, (Table 2) the relatively high amounts of the polyunsaturated linoleic acid ($C_{18:2}$) and of the arachic acid ($C_{20:0}$). It is known that sesame oil contains a high percentage (35–40%) of linoleic acid and appreciable amounts (0.5–1%) of arachic acid (13, 14), which is not generally found in olive oil. The survival of the polyunsaturated linoleic acid could be explained in sesame oil by the presence, in the latter, of the antioxidant compound, sesamol (14). It can therefore tentatively be assumed, as a working hypothesis, that the amphora Gaza-Rilled I contained sesame rather than olive oil.

The above considerations show both the advantages and the limits of the present approach. The significance of the results obtained from the chemical analysis of the residues of ancient products, and of lipids in particular, will certainly increase with the number of samples analyzed, so that a general rather than a particular view will be obtained. Also, suggestions coming from the interpretation of one set of chemical analysis could be substantiated by other independent sources of information, including other sets of analyses. For instance, in the present case, the suggestion that the amphora Gaza-Rilled I may have contained sesame oil should be followed through by looking for the presence of other species-specific compounds including numerous antigens, present in sesame oil.[5]

Finally, the above line of work is of some interest to the biochemist as well as to the archaeologist because it may provide a way of studying the effects on biochemicals of very protracted aging, such as can hardly be reproduced in the laboratory.

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[5] Further analysis is of particular archaeological importance as no extant written source mentions sesame oil in connection with this particular type of amphora.

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