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Mixed results of seven methods for organic residue analysis applied to one vessel with the residue of a known foodstuff

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Abstract

Several methods of archaeological organic residue analysis were applied to a single unglazed and unseasoned ceramic vessel that had absorbed residues of heated camel milk. Sections of the wall of this vessel were sent to eleven archaeological laboratories. Seven reported their results before the identity of the residue was revealed, during the 70th Annual Meeting of the Society for American Archaeology. Methods included stable carbon and nitrogen isotope ratio analysis, protein analysis and lipid analysis. These laboratory techniques provide a biochemical analysis of the residue in a ceramic matrix, the archaeological interpretation of which can be rather difficult. The exact source of the residue was not identified by any laboratory, but it is evident that residue analysis can provide valuable information, especially when combined with additional archaeological and historical data. We therefore support a close cooperation of those working in this field to develop it to its full potential.

Keywords: Carbon isotopes; Camel milk; *Camelus dromedarius*; Ceramic analysis; Diet reconstruction; Electrophoresis; Fatty acids; Gas chromatography; Lipids; Mass spectrometry; Nitrogen isotopes; Proteins; Residue analysis

1. Introduction

On 31 March 2005 we organized a symposium entitled 'Theory and Practice of Archaeological Residue Analysis' [56]. With assistance of Dr. Jelmer Eerkens (University of California, Davis) and Dr. Ran Boytner (University of California, Los Angeles), this symposium took place in Salt Lake City, during the 70th Annual Meeting of the Society for American Archaeology, as sessions 21 and 46. The proceedings of these will be published in the British Archaeological Reports, International Series. The participants were asked to prepare presentations on their own research, and to report on their analysis of a residue of a foodstuff recently cooked in a new ceramic vessel. Eleven agreed to partake in the latter and were sent a segment of the wall of a vessel in which camel milk had been cooked. The accompanying letter and model report sheet did not provide information on what was prepared in the vessel, nor did it present a list of possibilities. This made the analysis more challenging than is typical of archaeological settings as the provenance, shape and date of a pot usually offer important clues to its former use and contents. Seven participants (almost

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2/3) in this blind 'Round robin' filed a report before the meeting where the source of the residue was revealed. These reports will be discussed here, preceded by some data on camel milk and followed by a discussion on the significance of our findings for the practice of archaeological residue analysis.

2. Creation of the residue

In December 2003 about 200 ml of fresh camel milk, obtained at the camel market in Daraw (Egypt), was placed in a new unglazed earthenware bowl, purchased in Luxor. This was topped off with Baraka mineral water (total dissolved solids 430 mg/l), wrapped in aluminum foil and allowed to sit at room temperature for 24 h. The next day the assemblage was put in a gas oven, heated to approximately 200 °C for one hour, left to cool for four hours, again cooked for an hour and left at room temperature for another 24 h. The vessel was then emptied, rinsed with cold water and air-dried for 10 days. Finally, the vessel was rinsed with cold water to remove the fungus, air-dried for 24 h, stored in a sealed plastic bag and transported to Los Angeles, California. In May 2004 the vessel was machine-cut in 12 pieces, one being the base, after which the 11 wall fragments were distributed among the participants of our blind 'Round robin'.

3. Camels and camel milk

Millions in Asia and Africa rely on camels (the one-humped *Camelus dromedarius* and the two-humped *Camelus bactrianus*) for transportation, milk, meat and leather, especially in areas that are too hot and dry for other large mammals such as cattle and horses [6,18,32,47,49]. A healthy lactating camel produces 5–15 kg milk daily for a period of 9–15 months [4,17,35]. The milk has a relatively high pH (6.5–6.7) and high concentrations of vitamin C and niacin [16,20,46]. The absolute and relative composition of the milk depends on the fodder and the stage of lactation [55], like in other mammals, but also on the state of hydration of the animal. In camels the latter can vary greatly as part of their adaptation to life in an arid environment [53]. Among other things, lack of drinking water will cause an increase in the mineral content of the milk and a decrease of fat, lactose and protein [27,28,51,52]. The fatty acid composition of the fats in camel milk is also rather variable (Fig. 1). In fresh milk the fats are suspended in micelles (globules), about 300 μm in diameter, in which they appear to be bound to proteins [21,43,50].

The fatty acids found in the ceramic matrix of a vessel that was used to contain or process camel milk must have been those present in the milk or the products of oxidation, or other reactions, of these compounds. Their absolute and relative abundance will depend on many factors, including the affinity of the ceramic matrix for each of the various molecules, the stability of those molecules over time and the efficiency of the extraction and detection techniques employed. Given what is known about the fatty acid signatures of fresh camel milk and organic residues typically recovered in archaeological contexts, the saturated fatty acids C16:0 (palmitic or hexadecanoic acid), C18:0 (stearic or octadecanoic acid) and C14:0 (myristic or tetradecanoic acid) were anticipated in the comparatively fresh residue of our blind 'Round robin'. Fair amounts of the mono-unsaturated C18:1 (oleic or octadecenoic acid) and C16:1 (palmitoleic or hexadecenoic acid), and their oxidation products (like dicarboxylic acids), were also expected (Fig. 1). These fatty acids are common in fats and oils of vegetable and animal origin and their presence alone is unlikely to allow

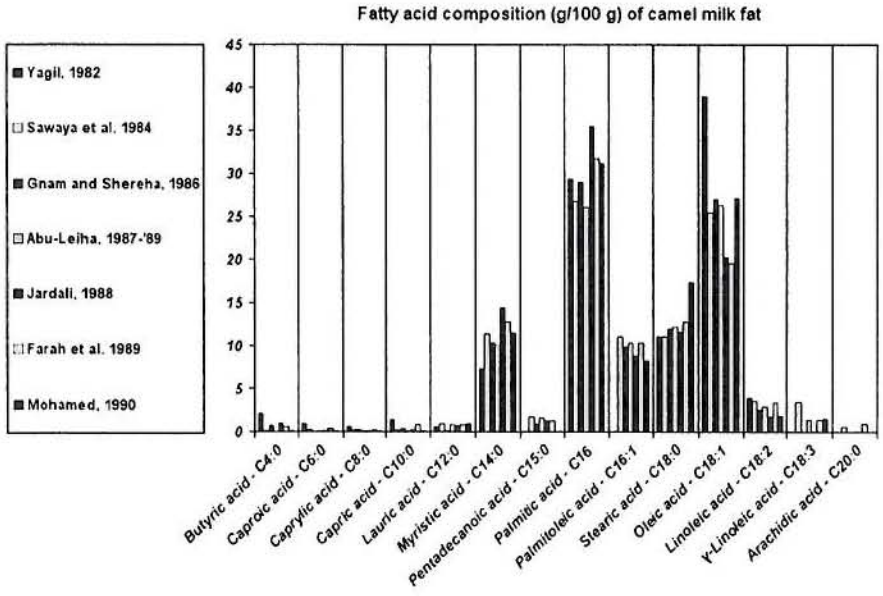

Fig. 1. Comparison of seven fatty acid signatures of fresh camel milk [1,2,21,22,26,33,41,46,50] (after [43]).

identification of an unknown residue such as that of camel milk. Making such an identification does require additional information and further manipulation of the data.

Another analytical technique, which could be used independently or in combination with the identification of specific compounds, entails the determination of the ratios of the stable isotopes of carbon (^{12}C and ^{13}C) and nitrogen (^{14}N and ^{15}N). Families of plants have their own specific preference for one isotope over another and isotope ratios in living matter are therefore dissimilar to those in the environment. In the New World, for instance, maize (*Zea mays*) employs the C_4 pathway for photosynthesis which causes higher $^{13}C/^{12}C$ ratios, while introduced European cereals employ the C_3 pathway, which produces lower $^{13}C/^{12}C$ ratios [44]. This difference in $^{13}C/^{12}C$ ratios is carried up the food chain: herbivores feeding on C_4 plants, and carnivores feeding on such herbivores [5,42], have higher $^{13}C/^{12}C$ ratios than those feeding on C_3 plants. Egyptian camels typically feed on both C_3 (most grasses and trees) and C_4 plants (sorghum, sugar cane). Their milk contains 3–5 g protein (mostly casein) per 100 g [1,2,17,35, 43,46], which should leave small amounts of nitrogen in its residue.

The analysis of proteins such as casein would potentially enable a secure identification of the source of many archaeological residues (Fig. 2), as many proteins are species-specific [24,25]. Ancient proteins have been isolated from archaeological samples with a variety of techniques [3,8,9,19,36,37, 48,54]. That this avenue has not yet been fully explored can be attributed to the facts that proteins are relatively difficult to handle in the laboratory and are expected to have denatured and fractured over time, especially after being heated. Furthermore, not all naturally occurring proteins have yet been fully analyzed, which would be necessary for comparison with archaeological residues [24,25]. Specific antibodies are readily available for only a limited number of proteins, and may not react with severely denatured or damaged proteins, although promising results have been obtained in particular cases [8,9]. Counter or cross-over electrophoresis (CIEP), also employing the principles of antigen-antibody reactions, has been applied on archaeological materials [3,31,36,37,48,54], although some have expressed scepticism as to the reliability of this approach [7,13,15,23]. The rapidly evolving field of proteomics may soon develop new techniques that can also be used for archaeological residue analysis, especially with an increased cooperation between biochemists and archaeologists.

4. Results of our blind 'Round robin': stable isotopes

Laboratory J removed the outside surfaces of the sherd, to avoid possible contamination, after which a total of 12 samples were obtained at evenly spaced intervals from the rim to the base of the vessel segment, six from the interior and six from the exterior. Aliquots of the ceramic powder, weighing an average of 4.9 mg, were manually compressed in tin foil capsules and combusted in a Carlo Erba NA2500

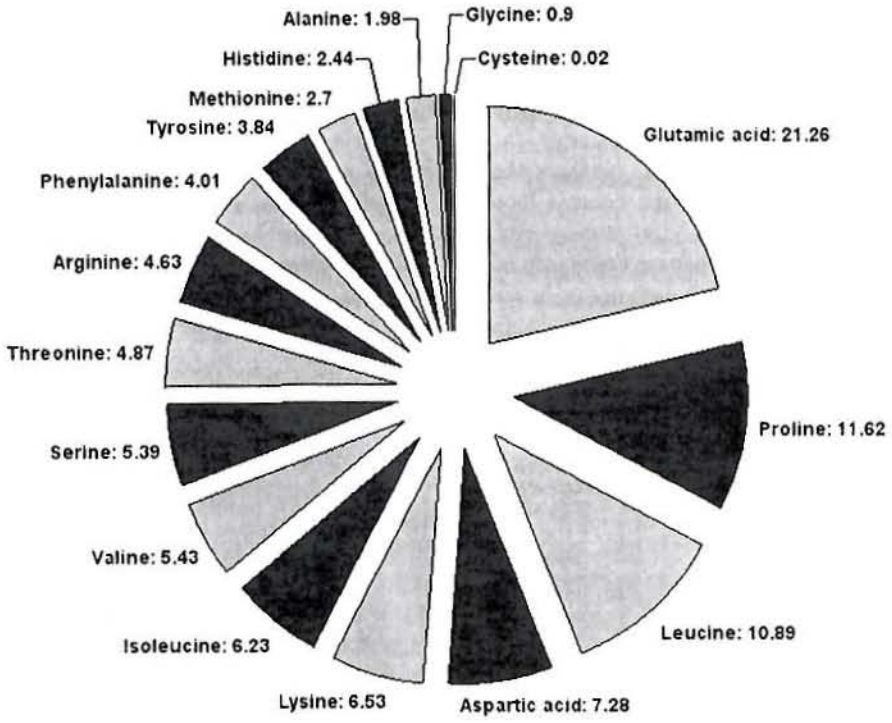

Fig. 2. The amino acid signature of casein in fresh camel milk (after [21]).

elemental analyzer interfaced with a MAT 252 isotope ratio mass spectrometer.

Carbon concentrations averaged 2.69% on the vessel interior and 1.65% on the exterior. Vessel interior $\delta^{13}C$ values were systematically more negative than exterior values. The average $\delta^{13}C$ for the interior was $-22.3‰$; that for the exterior was $-19.2‰$. Interior and exterior $\delta^{13}C$ values varied greatly with the distance from the rim, first increasing by almost 5‰, then decreasing by 4‰ and then systematically increasing toward the base, with the exception of the lowest sample pair (Fig. 3). Interior and exterior $\delta^{13}C$ values co-varied systematically with the distance below the rim (Fig. 4).

Nitrogen concentrations were too low for detection in all exterior samples and in the three interior samples closest to the rim. Nitrogen concentrations in the three lowest interior samples increased systematically from 0.16 to 0.21% with the distance below the rim. Their average $\delta^{15}N$ value was 5.08‰, but these nitrogen samples were too small for accurate isotopic analysis. Therefore, the sample weights were doubled to an average of 10.4 mg to obtain better estimates of nitrogen concentrations and more reliable $\delta^{15}N$ values. Nitrogen concentrations were 0.05 to 0.06% in the three samples closest to the rim and increased systematically from 0.05% to 0.20% between 16 and 41 mm below the rim. Atomic C/N ratios increased slightly and then decreased below the rim. The three lowest samples contained enough nitrogen for reliable isotopic analysis, their $\delta^{15}N$ values averaging 6.00‰.

The principles of archaeological residue analysis and diet reconstruction using stable carbon and nitrogen isotope ratios have been discussed in detail elsewhere [5,42]. The covariance of interior and exterior $\delta^{13}C$ values suggests that the vessel contents were absorbed throughout the thickness of the vessel, with the exception of the exterior sample closest to the base, which did not increase in step with its interior pair. Assuming that part of the carbon on the vessel exterior was absorbed during firing, the difference in $\delta^{13}C$ between the interior and exterior suggests that the vessel's contents had a lower $\delta^{13}C$ value than that of the fuel used to fire the vessel. Had the vessel been 'seasoned', then this pattern could also be due to application of a substance, to either the interior or the exterior of the vessel, with a $\delta^{13}C$ value that differed from that of the fuel. Carbon in the clay and temper may also

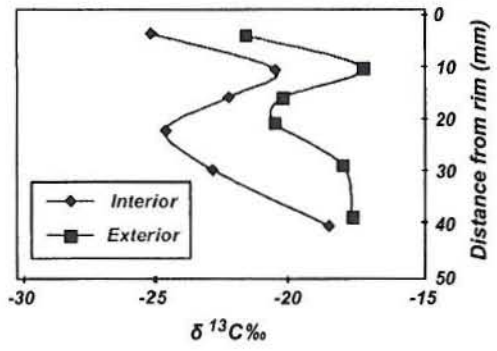

Fig. 3. Variation in interior and exterior $\delta^{13}C$ values with distance below the rim.

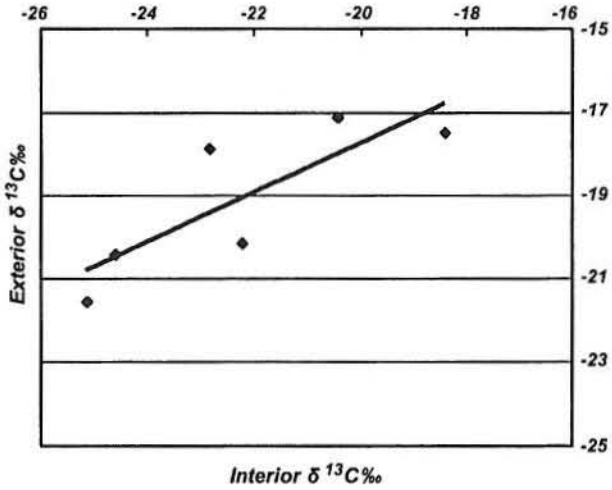

Fig. 4. Covariance of interior and exterior $\delta^{13}C$ values ($Y = 1.138x - 0.497$ $R^2 - 0.679$).

have remained in the matrix after an incompletely oxidizing firing.

The distinct deflection to the most negative $\delta^{13}C$ values at 21–22 mm below the rim may indicate the 'boil line'. This hypothesis is based on the fact that the $\delta^{13}C$ values of lipids (fats) are approximately 5‰ more negative than those of protein and carbohydrates in foods [11]. If the cooked food was in a liquid form, isotopically lighter fats would have floated to the surface and be absorbed at higher levels than higher density protein and carbohydrates. The high C/N ratios near the rim are consistent with this hypothesis because fats have high carbon contents and lack nitrogen. Variation in concentration of absorbed lipids in this vessel should be evaluated by extraction with organic solvents, gas chromatography and compound-specific isotopic analysis [44]. The increase in nitrogen and decrease in atomic C/N ratios beginning 22 mm below the rim indicates that protein-rich food components were absorbed in lower parts of the vessel (Figs. 5 and 6).

The increase in $\delta^{13}C$ values between the rim and 10 mm below the rim is unlikely to be due to the isotopic composition of the food cooked in the vessel. Perhaps the vessel was fired upside down, with the rim in contact with fuel of C_3 origin. Alternatively, if the pot was seasoned with a ^{13}C-enriched substance, the rim was not treated. Increasing $\delta^{13}C$ values beneath the boil line suggest that a mixture of C_3 and C_4 foodstuffs or proteins of a mixed-feeding animal were cooked in this pot. The $\delta^{15}N$ values are high enough to suggest the presence of meat, milk or blood of an herbivore, perhaps combined with plant foods. The C/N ratios of the three samples closest to the vessel base range from 9 to 27. These are higher than the C/N ratios of pure proteins, but are substantially lower than those of pure carbohydrates and lipids, thus indicating a mix of proteins and carbohydrates. As only one foodstuff was cooked in this vessel it was likely either a protein-rich seed, or a whole milk product. The moderately high $\delta^{13}C$ and $\delta^{15}N$ values exclude ^{13}C-depleted C_3 and ^{13}C-enriched C_4 plant foods.

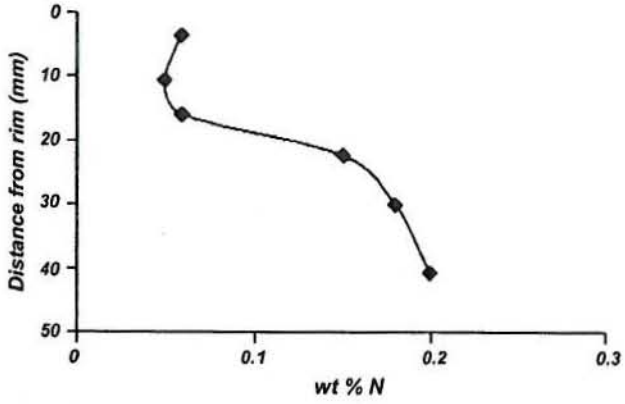

Fig. 5. Change in nitrogen concentrations of the absorbed residue in powdered samples from six positions below the interior of the vessel rim.

The strong co-variance of the interior and exterior $\delta^{13}C$ values of the five samples closest to the rim suggests that the upper residues were readily absorbed in the ceramic matrix. The upper matrix may, however, not have had the same capacity for absorption of higher density proteins and carbohydrates that predominated near the base. The systematic patterns of carbon and nitrogen concentrations and isotopic composition of the interior and exterior of this vessel were entirely unanticipated. However, these patterns are consistent with the preparation of a single foodstuff that had contained lipids and a C/N ratio higher than that of plants but lower than that of pure protein. Variation in carbon and nitrogen isotopic and elemental composition within a vessel used to cook a single substance can be substantial due to systematic differences in the isotopic composition and densities of lipids, proteins and carbohydrates. Control over the sample position within the vessel is therefore essential for the adequate interpretation of carbon and nitrogen elemental and isotopic composition of absorbed organic matter in potsherds. The simple sampling strategy described here, combined with analysis of carbon and nitrogen elemental and isotopic composition, can provide a considerable amount of useful information about the foods prepared in porous ceramic vessels.

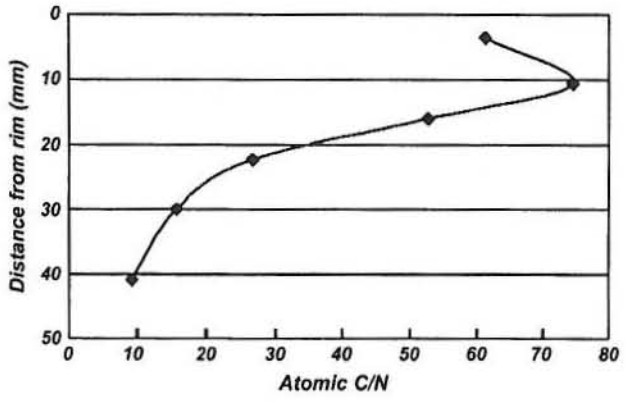

Fig. 6. Change in atomic C/N ratios of the absorbed residue in powdered samples from six positions below the interior of the vessel rim.

5. Results of our blind 'Round robin': proteins

Laboratory A placed the sherd in a plastic dish with 0.5 ml ammonium hydroxide (5%) and sonicated for 5 min. Both dish and contents were then placed in a rotating mixer for 30 min. The resulting solution was transferred into a sterile plastic vial and stored at −20 °C. About 3 μl of this ammonia solution was later transferred into a well punched in an agarose gel in a Helena Laboratories Titan Gel electrophoresis chamber and paired with a second well with 3 μl antiserum. A control positive was prepared in another pair of wells after which an AC current of 105 V was passed through the gel for 45 min. This caused sample and antiserum to migrate, antigens towards the anode and antibodies (antiserum) towards the cathode, and come into contact. If there is protein in the sample that corresponds with the antiserum, an antigen-antibody reaction will occur resulting in the protein precipitating out in a specific pattern. After the run was completed the gel was pressed and dried. The dry gel was immersed in a Coomassie Blue R250 stain for 3 min and then destained in a solution of ethanol, distilled water and acetic acid (5:5:1, v/v) until the background was clear and any positive responses visible. Sterile equipment was used throughout the analysis.

The antisera used in this study included agave, amaranth, bear, bovine, cactus, cat, cedar, chicken, *Capparidaceae*, *Chenopodiaceae*, *Compositae*, deer, dog, guinea pig, *Graminae*, legume, pine, rabbit, rat and sheep. All the animal antisera are from Cappel, purchased from MP Biomedicals, while the plant antisera were produced by the Department of Biological Sciences at the University of Calgary. All antisera are polyclonal: they recognize epitopes of closely related species. For example, anti-deer serum will react positively to other members of the family *Cervidae* such as elk, moose and caribou. Commercial antisera manufacturers provide product sheets listing cross reactions; the antisera from the University of Calgary are tested against a full suite of other species to ensure that there are no cross reactions.

This method of analysis is known as cross-over (or counter) electrophoresis (CIEP). Prior to the introduction of DNA fingerprinting this technique was commonly used in forensic laboratories to identify residues from crime scenes. Minor adaptations to the original method were made following procedures used by the Centre for Forensic Sciences, Toronto, and the Royal Canadian Mounted Police Serology Laboratory, Ottawa [45]. The solution used to remove residues, ammonium hydroxide, has proven to be the most effective extractant for old and denatured bloodstains without interfering with subsequent testing [12,34].

No trace proteins were detected in the sherd sent for analysis. This may be the result of the degradation of animal proteins that result from cooking. Experiments subsequent to this study have demonstrated that animal proteins, of known origin, heated to 100 °C for 20 min yielded no positive readings using CIEP. This observation has important implications for the application of this particular form of residue analysis on cooking vessels or objects from hearth. Another issue to consider is that many of the taxonomic groups for which

antisera exist, like those used in this study, would not be expected to react to the residues in the 'Round robin' (with the possible exceptions of bovine and sheep).

6. Results of our blind 'Round robin': lipids

Laboratory B ground a small piece of the sherd in a mortar and pestle. The resulting powder was transferred into a sterile test tube with 8 ml acetonitrile (ACN). The mixture was sonicated and centrifuged, after which 5 ml was pipetted into a second test tube. The solvent was evaporated from this test tube by gentle heating under a stream of nitrogen. The dry residue was taken up in 100 μl ACN and treated with 30 μl trimethylsilyl-*N*-methyl-trifluoroacetamide (MSTFA). About 1 μl of the derivitized sample was sandwiched in air and injected, preceded by 1 μl of ACN, into a Varian GC/MS instrument.

Two different pieces of the same sherd were analyzed, by comparing C18:0/C16:0 and C18:1/C16:0 ratios. One sample showed a C18:0/C16:0 ratio of 0.72 and a C18:1/C16:0 ratio of 1.15 (Fig. 7). The second sample showed ratios of 1.03 and 0.32, respectively. Fatty acid amounts in known foodstuffs were obtained from the USDA Nutrient Data Laboratory website and used as a comparative data set. The peaks of all the fatty acids that were tested for (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C20:0) were compared with those in the known foodstuffs. The only peak that differed dramatically was that for C18:1 (oleic acid). This may be due to the oxidation of this unsaturated fatty acid, which may have also caused the difference in ratios between the two pieces tested. The chromatogram containing the higher amount of C18:1 was compared with the data of known foodstuffs. The results were tentatively interpreted as indicative of the residue of some animal product and most likely veal, eggs or goat milk.

Laboratory C received the sherd broken into two vertical strips. The upper portion of one strip was selected as the 'time 0' sherd. The remainders of the pieces were stored in an oven at 75 °C. After six days the lower portion of the first strip was removed from the oven and stored in a freezer at −20 °C. The same was done, after 12 days, with the upper portion of the second strip. Residues were then extracted from the 'time 0' sherd and from those stored in the oven for six and 12 days. Contaminants were removed by grinding off the surfaces after which the sherds were crushed. The resulting powder was mixed with 30 ml of a chloroform and methanol mixture (2:1, v/v) and sonicated (2 × 10 min). Solids were removed by filtering into a separatory funnel. The solvent mixture was washed with 16 ml ultra-pure water and left until it separated into two phases. The lower chloroform-lipid phase was transferred into a flask from which the chloroform was removed by rotary evaporation. Any remaining water was removed by evaporation with 1.5 ml benzene. The dry residue was transferred into a vial with 1.5 ml of the chloroform and methanol mixture and stored. A 200 μl sample of the solution was dried under nitrogen and treated with 6 ml 0.5 N anhydrous hydrochloric acid in methanol. After cooling 4 ml ultra-pure water was added. The fatty acid methyl esters (FAMEs) were recovered with 3 ml petroleum ether and transferred into a vial. The solvent was removed by heat under a gentle stream of nitrogen. The dry residue was transferred into a GC vial with 1 ml iso-octane.

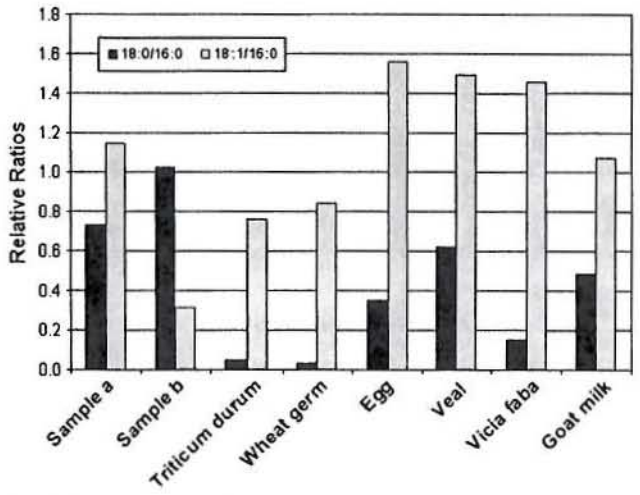

Fig. 7. Graphic representation of the C18:0/C16:0 and C18:1/C16:0 ratios in two samples of the 'Round robin', established in Laboratory B, compared to those in six foodstuffs.

Analysis was performed on a Varian 3800 gas chromatograph fitted with a flame ionization detector. Chromatogram peaks were integrated using Varian Star Chromatography Workstation software and identified through comparisons with several external qualitative standards (NuCheck Prep, Elysian, MN). To identify the residue the relative percentage composition was determined, first with respect to all fatty acids present in the sample, and second with respect to the ten fatty acids utilized in the identification criteria: C12:0; C14:0; C15:0; C16:0; C16:1; C17:0; C18:0; C18:1ω9; C18:1ω11 and C18:2. Medium chain fatty acids represent the sum of C12:0, C14:0 and C15:0; while C18:1 is the sum of all isomers. It must be understood that the identifications given do not necessarily mean that those foods were actually prepared because different foods of similar fatty acid composition and lipid content can produce similar residues.

Significantly more fatty acids were recovered from the upper portions than of the lower portions of the sherd and, consequently, these provide the best information about the residue. The characterization is based on the relative fatty acid composition of the residue extracted from the upper portion of the second strip. Due to the low level of unsaturated fatty acids in the residues, it is possible to establish decomposition trends after only 12 days of oven storage. The sum of medium chain fatty acids exceeds 30%, while the C18:0 and C18:1 isomer levels are low. By applying published criteria it is possible to say that the partially decomposed residue is typical of decomposed plants [39,40]. This category includes low fat-content plants such as roots or tubers, greens as well as certain berries and seeds. North American foods known to produce residues with high levels of C14:0 when boiled are biscuit root and *Chenopodium* seeds.

Laboratory G ground off about 0.1 g of the internal surface of the sherd, mixed the resulting powder with 1 ml of a chloroform and methanol mixture (2:1, v/v), sonicated and centrifuged. The supernatant was decanted and dried under a stream of nitrogen. The dry residue was treated with 30 μl N,O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) and about 1 μl of the derivatized sample was injected into a Hewlett Packard 5972 CG/MS.

Next to a trace of C12:0, the sample appeared to contain a number of free fatty acids: C14:0, C16:0, C18:0 and C18:1 as well as the methyl-esters of C16:0 and C18:0 (Fig. 8). Neither short chain fatty acids nor poly-unsaturated fatty acids were identified. No attempt was made to determine the ratios of the free fatty acids but C16:0 appeared predominant. There were traces of odd carbon number fatty acids, but these were in low abundance. Cholesterol was present in abundance along with cholesterol oxidation products and a trace of squalene. No phytosterols or alkanes were found. No terpenoids and no wax esters were identified, indicating that the source most likely did not contain a resin or a wax. On the basis of these results it can be suggested that the residue is of animal origin, the absence of short chain fatty acids pointing to a non-dairy source like veal or eggs. This identification should be confirmed by saponification of the sample, hydrolysis of the acylglycerols resulting in glycerol and fatty acid salts, followed by an analysis of the result by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS) as discussed by Laboratory J above [44].

Laboratory H ground the sherd in a mortar and pestle. Lipids were extracted in a chloroform and methanol mixture by sonification. This was centrifuged and decanted after which the solvents were evaporated under nitrogen. The dry residue was treated with BSFTA, saponified with sodium methoxide in methanol and methylated with a BF_3-methanol complex. The sample was then analyzed in a GC-17A Shimadzu Chrom-Perfect gas chromatograph, with a 30 m Agilent DB-5 column raised 5 °C/min from 40 °C to 280 °C, after a split injection. No activity was found in the received sherd.

Laboratory K cut the sherd into four pieces, two of which were crushed into a fine powder using a mortar and pestle.

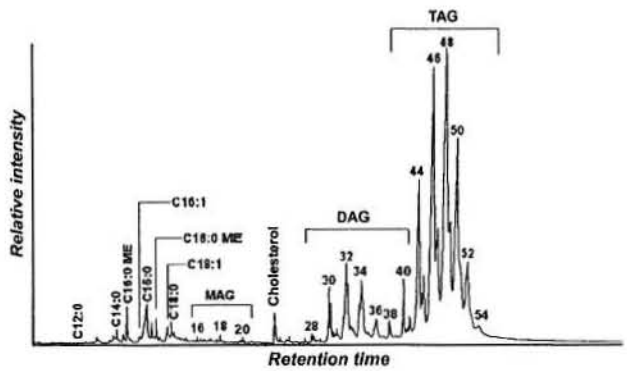

Fig. 8. Chromatogram showing the lipid profile of the 'Round robin' as established by Laboratory G. ME: methyl-ester; MAG: monoacylglycerols; DAG: diacylglycerols; TAG: triacylglycerols (cf. Fig. 9).

Lipids were extracted by sonification from 500 mg of the powder in 2 ml chloroform/methanol (2:1, v/v) after which the solvents were evaporated under a stream of nitrogen. 50 μl tetratriacontane (CH_3-$(CH_2)_{32}$-CH_3) was added as internal standard and the dry residue was then treated with BSTFA with 1% trimethyl-chlorosilane (TMCS). The lipids in another 500 mg were saponified with 0.5 ml 2 N KOH, acidified with HCl and extracted with diethyl-ether. The solvent was then evaporated under a stream of nitrogen and treated with BSTFA with 1% TMCS after the internal standard was added. The derivatized residues were analyzed in a Hewlett Packard 5890 series II gas chromatograph (HTGC) and a Hewlett Packard 5890 gas chromatograph with a 5972 mass selective detector (GC/MS). The HTGC was used with a DB-1HT fused-silica capillary column (15 m × 0.32 mm, 0.10 μm). The HTGC oven temperature was initially set at 50 °C and increased with 10 °C/min to 380 °C, at which temperature it was held for another 5 min. The GC/MS was equipped with a fused-silica capillary column (30 m × 0.25 mm, 0.25 μm). The GC oven temperature was programmed to start at 50 °C, to increase with 10 °C/min to 340 °C and finally to be held at 340 °C for 10 min.

The analysis of the total lipid extract, by HTGC, showed the presence of triacylglycerols (TAGs) with a total carbon number between 44 and 56, the main constituent having 52. After saponification and GC/MS, the main fatty acids appeared to be C12:0, C14:0, C16:1, C16:0, C18:1 and C18:0. The C16:0/C18:0 ratio (P/S ratio) for one part of the sherd was 3.175 by HTGC, and 5.741 by GC/MS. For the other part of the sherd the C16:0/C18:0 ratio was 3.09 by HTGC. The distribution of triacylglycerols indicates a residue of animal origin. A C16:0/C18:0 ratio of around 3 may be explained by the presence of egg. It was therefore concluded that an animal product was the source of the residue.

Analysis of samples taken eight months later, using another HTGC (an Agilent Technologies 6890N) with the same column, showed some interesting differences even though the sherd had been stored in a refrigerator (Fig. 9). All fatty acids except C18:0 had decreased, while the most common triacylglycerol now had a total carbon number of 48 instead of 52. It seems that triacylglycerols containing mainly C18:0 had hydrolyzed to form free C18:0 and monoacylglycerol C18:0. The new C16:0/C18:0 ratio obtained after saponification appeared very low compared to the first ratio, which is concordant with an increase in C18:0. Other ratios, like C16:1/C18:1 and C16:0/C18:1, showed little variation after eight months. It therefore seems likely that C18:0 was released, probably because of the temperature changes to which the sherd was exposed.

7. Discussion

The first conclusion of our blind 'Round robin' must obviously be that none of us could pinpoint the source of the residue. This may seem disappointing but should be hardly surprising as our methods still only allow us to look at a small selection of molecules and not directly at foodstuffs

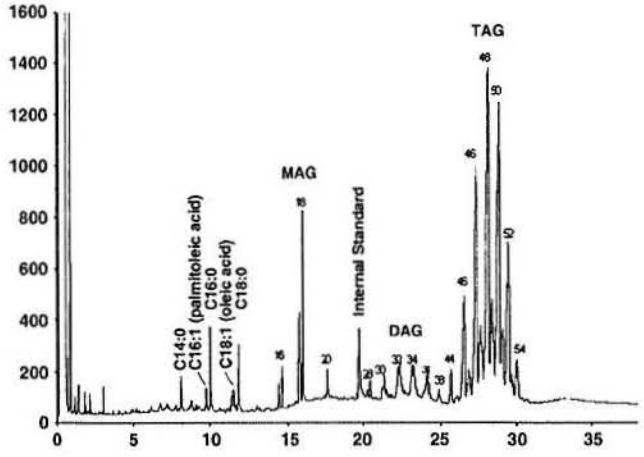

Fig. 9. Chromatogram showing the lipid profile of the 'Round robin' after eight months of refrigerated storage as established by Laboratory K. The internal standard is tetratriacontane; MAG: monoacylglycerols; DAG: diacylglycerols; TAG: triacylglycerols (cf. Fig. 8).

themselves. In the reduction from camel milk to fatty acids too much information is lost and it is impossible to retrieve all of this. The driving motive behind the 'Round robin' was to get an overview of the methods currently available to recover some of this information in order to arrive at archaeologically relevant conclusions. We purposefully include the negative results of two of the laboratories as the trend to only publish successful projects, though fully understandable, has contributed to a biased representation of the state of the field and unrealistic expectations.

The second conclusion is that, with the current technology, it will only be possible in certain cases to assign archaeological residues a source more specific than 'milk of an herbivore' or 'low fat-content plant'. And it will rarely be feasible to infer such determinations directly from the chemical analysis alone. Usually additional archaeological and historical information combined with data from the analysis of the residues of, preferably artificially aged, known foodstuffs is required. A residue of camel milk, for instance, need not be expected in vessels from North America or in pots from Pharaonic Egypt. That residue analysis can contribute to this assemblage of information is clear from the overview of our interpretations in Table 1. This illustrates the necessity of combining the chemical data with archaeological and historical information. Laboratory C, for instance, specialized in the study of North American hunter-gatherers who did not partake in dairying activities. In order to characterize their archaeological residues, using fatty acids, a collection was made of foodstuffs from that region and residues were prepared and subjected to oven storage to simulate the effects of long-term decomposition. As Laboratory C was under renovation from spring 2004 to spring 2005 the preparation of new reference materials, relevant to the possible residue in the circulated sherd, was not possible.

In our 'Round robin' comparisons between unknown residues and those of known foodstuffs were made in two manners that are different but not mutually exclusive. Some residues preserve 'biomarkers', which are more or less specific for certain classes of foodstuffs. Such molecules can be alkaloids (such as caffeine), steroids (such as cholesterol), terpenoids (a large diverse group of mostly polycyclic compounds synthesized by plants [10,30]), but also fatty acids like C22:1 (erucic or docosenoic acid) and phytanic (3,7,11,15-tetramethyl-hexadecanoic) acid [29]. Respectively, these compounds naturally occur in coffee, animal products, certain families of plants (the terpenoids are well-studied and can be very specific [10,30]), members of the *Brassicaceae* (*Cruciferae*) family (such as mustard, nasturtium or rape seed) or fish and finally, in the products of ruminants or fish [29]. As is evident from the last two examples the same molecule is sometimes considered a biomarker for two apparently unrelated classes. Some compounds can mark modern contamination of the sample. This is obvious in the case of man-made organic molecules like phthalates, in plastics to keep them flexible, but must also be considered for naturally occurring compounds like anthraquinones, commonly used as dye in textiles and paper, or 13-docosenamide (erucamide), coated on many plastic objects to prevent them from sticking together. Proteins are inherently more specific than the relatively simple molecules mentioned above, and would theoretically be better biomarkers, but their identification from an archaeological context is still problematic in many cases. As some residues will not contain or preserve detectable concentrations of any biomarker, protein or otherwise, this approach does not always yield helpful results.

The second way to match unknown with known residues is by comparing the ratios of the abundance of common fatty acids. Results can be sometimes be obtained with a simple C16:0/C18:0 ratio (P/S ratio) and sometimes after the two-dimensional plotting on a double logarithmic scale of C16:1/C18:1 versus (C15:0 + C17:0)/C18:0 [14]. Such ratios tend

Table 1
Summary of the results of our analysis of the residue in the 'Round robin'

Lab.	Technique	Result	Interpretation
A	CIEP (20 antisera)	No proteins detected	Proteins degraded after cooking?
B	GC/MS	C18:0/C16:0 = 0.72–1.03, C18:1/C16:0 = 0.32–1.15	Veal, egg or goat milk
C	GC	Medium chain fatty acids >30%, low C18:0 and C18:1 isomer levels	Decomposed roots, tubers or berries
G	GC/MS	C14:0, C16:0, C18:0, 18:1 and cholesterol	Veal or egg
H	GC	No activity	–
J	IRMS	$\delta^{13}C$ = 19.2 (ext.) −22.3‰ (int.) $\delta^{15}N$ = 5.08–6.00‰	Milk of an herbivore
K	HTGC and GC/MS	triacylglycerols (44–56 C-atoms), C16:0/C18:0 = 3.175–5.741	Animal product

to bring the residues from the same class of foodstuffs together rather well, but often fail to fully separate the different classes. The fact that these ratios may furthermore change over time, as different fatty acids oxidize at different rates, further complicates the application of this method. One way to approach this is be monitoring such changes in known residues in sherds that are stored in an oven to simulate the effects of long-term decomposition [38]. The previously mentioned lipid biomarkers and fatty acids are usually found with the same analytical techniques, which enables the use of both methods on the same dataset. This approach would greatly benefit from a database, preferably accessible through the Internet, in which the raw data on residues from known sources is stored together with detailed information on the methods used to obtain these data.

The determination of food types by stable carbon and nitrogen isotope ratios is hampered by the similarities in isotopic composition of food types within a few broad classes such as C_3 or C_4 plants, or animals feeding on these classes of plants. As with other methods, additional archaeological and historical data on the studied material benefits the analysis. This technique requires specific instruments and combining it with those discussed above demands additional samples, time and funding. One way to cope with this would be cooperation and the sharing of data, combined with the analysis of more known samples to evaluate cross-laboratory variation. Perhaps our most important conclusion is that such is not only necessary but also possible.

Other issues in need of attention by those working in the field include the formation processes related to organic residues. It is always assumed that the encountered residues are related to the food that was once present in the vessel. It is unclear, however, if the residue represents the first food to come into contact with the ceramics, after which the available binding sites are saturated, or the last, if older residues are continually replaced by new ones, or a combination of all food ever to have been inside the vessel, if the molecules that make up the residue compete for the available binding places. If the first is the case it must be taken into account that many unglazed vessels are 'seasoned', treated with oil or milk to make them less porous, before being used. This means that the ceramic matrix can indeed be saturated, but also that the residues will tell us more about the seasoning agent than about the actual use of the vessel. If we assume, on the other hand, that residues are easily replaced, we must account for the fact that vessels may end up on trash dumps or in graves where they can come into contact with organic materials that are not related to their original use. Finally, if there is competition for the available binding places it will be difficult to separate these different sources, especially if vessels have been in use for a long time for a variety of purposes.

Organic residues in potsherds may also originate from sources other than food but nevertheless related to the use of the vessel. Ceramics can have been employed for 'industrial' purposes (such as the preparation of organic dyes or glues) or as censers, smoking pipes or coffins (especially for infants), or to store a multitude of things. On the other end of the spectrum are foodstuffs that never come into contact with ceramic vessels but are eaten raw, roasted over a fire or prepared and consumed in other ways that do not involve ceramics. Archaeological residue analysis, as discussed here, can therefore never be more than complementary in the reconstruction of the diet of an ancient people. For our laboratory techniques to reach their full potential as archaeological tools the study of more material with experimental or ethno-archaeological origins will prove as indispensable as a further cooperation and sharing of data by those working in this field.

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