Methodology and Results of Lipid Residues Extracted from Naomikong Point and Sand Point Pottery

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Introduction

Five potsherds each from the Sand Point (20BG14) and Naomikong Point sites (20CH2) were submitted for analysis. Exterior surfaces were ground off to remove any contaminants; samples were crushed and absorbed lipid residues were extracted with organic solvents. The lipid extract was analyzed using gas chromatography (GC), high temperature–GC (HT-GC), and high temperature–gas chromatography with mass spectrometry (HT-GC/MS). Residue identifications were based on fatty acid decomposition patterns of experimental residues, lipid distribution patterns, and the presence of biomarkers.

The Identification of Archaeological Lipid Residues

The identification criteria used herein was developed on the basis of changes observed in the fatty acid compositions of fresh foods and the effects of thermal and oxidative degradation. The fatty acid compositions of more than 130 uncooked native food plants and animals from western Canada were determined using gas chromatography (Malainey 1997; Malainey et al. 1999a). When subjected to cluster and principal component analyses, the resultant groupings generally corresponded to divisions that exist in nature. Clear differences in the fatty acid composition of large mammal fat, large herbivore meat, fish, plant roots, greens, and berries/seeds/nuts were detected, but the fatty acid composition of meat from medium-sized mammals resembles that of berries/seeds/nuts (Malainey 1997; Malainey et al. 1999a). The effects of cooking, short-term decomposition at room temperature, and long-term decomposition with oven storage were determined. Observed changes in fatty acid composition of the experimental cooking residues enabled the development of a method for identifying the archaeological residues using the relative percentages of the ten fatty acids (C12:0, C14:0, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1w9, C18:1w11, C18:2) that regularly appeared in archaeological vessel residues (Malainey 1997; Malainey et al. 1999b) (Table 1). Work has continued to understand the decomposition patterns of various foods and food combinations prepared using different cooking techniques (Malainey, Malisza, Przybylski, and Monks 2001; Malainey, Przybylski, and Monks . 2000a, 2000b, 2000c; Quigg et al. 2001). The results of these decomposition studies enabled refinement of the identification criteria (Malainey 2007).

Archaeological scientists working in the United Kingdom have had tremendous success using high temperature–gas chromatography (HT-GC) and gas chromatography with mass spectrometry (HT-GC/MS) to identify biomarkers. High temperature–gas chromatography is used to separate and assess a wide range of lipid components, including fatty acids, long-chain alcohols and hydrocarbons, sterols, waxes, terpenoids, and

triacylglycerols (Evershed, Heron, and Goad 1990; Evershed et al. 2001). The molecular structure of separated components is elucidated by mass spectrometry (Evershed 2000).

Table 1. Criteria for the Identification of Archaeological Residues Based on the Decomposition Patterns of Experimental Cooking Residues Prepared in Pottery Vessels.

Identification	**Medium Chain**	**C18:0**	**C18:1 isomers**
Large herbivore	≤15%	≥27.5%	≤15%
Large herbivore with plant OR bone marrow	low	≥25%	$15\% \leq X \leq 25\%$
Plant with large herbivore	≥15%	≥25%	no data
Beaver	low	low	≥25%
Fish or corn	low	≤25%	$15\% \leq X \leq 27.5\%$
Fish or corn with plant	≥15%	≤25%	$15\% \leq X \leq 27.5\%$
Plant (except corn)	≥10%	≤27.5%	≤15%

Triacylglycerols, diacylglycerols, and sterols can be used to distinguish animal-derived residues, which contain cholesterol and significant levels of both triacylglycerols, from plant-derived residues, indicated by plant sterols, such as β-sitosterol, stigmasterol, and campesterol, and only traces of triacylglycerols (Dudd and Evershed 1998; Evershed 1993; Evershed, Mottram, Dudd, Charters, Stott, Lawrence, Gibson, Conner, Blinkhorn, and Reeves. 1997). Waxes, which are long-chain fatty acids and long-chain alcohols that form protective coatings on skin, fur, feathers, leaves, and fruit, resist decay and can also serve as biomarkers (Charters et al. 1997; Evershed et al. 1991; Evershed, Vaugh, Dudd, and Soles 1997; Heron et al. 1994). Reber et al. (2004) suggested *n*-dotriacontanol could serve as an effective biomarker for maize in vessel residues from sites located in midwestern and eastern North America.

Terpenoid compounds, or terpenes, are long-chain alkenes that occur in the tars and pitches of higher plants. The use of GC and GC/MS to detect the diterpenoid dehydroabietic acid from conifer products in archaeological residues extends over 25 years (Heron and Pollard 1988; Shackley 1982). Lupeol, α-amyrin, and β-amyrin, and their derivatives, indicate the presence of plant materials (Regert 2007). Eerkens (2002) used the predominance of the diterpenoid Δ–8(9)-isopimaric acid in a vessel residue from the western Great Basin to argue that it contained piñon resins.

The presence of lipid biomarkers and distributions of triacylglycerols (TAGs) were determined through HT-GC and HT-GC/MS. The data obtained is useful for distinguishing plant residues, animal residues, and plant-animal combinations. The sterol cholesterol is associated with animal products; β-sitosterol, stigmasterol, and campesterol are associated with plant products. The presence and abundance of TAGs vary with the material of origin. When present, number of TAGs tends to decrease with increasing number of carbon atoms in plant residues (Malainey et al. 2010, Malainey et al. 2014, Malainey et al. in press). The peak arising from C48 TAGs is largest and peak size (and area) progressively decreases, with the C54 TAG peak being the smallest. A line drawn to connect the tops of the C48, C50, C52, and C54 TAG peaks slopes down to the right. This pattern is due to the preponderance of triacylglycerols with fatty acids having carbon chains ranging between 12 and 16 in length; C46 TAG peaks may also be detected. In animal residues, number of

TAGs tends to increase with carbon number, with the C52 or C54 TAG peaks being the largest (Malainey et al. 2010, Malainey et al. 2014, Malainey et al. in press). A line drawn to connect the tops of the C48, C50, C52, and C54 TAG peaks either resembles a hill or the line slopes up to the right. A parabola-like pattern, such as the shape of a "normal distribution," can also occur in the residues of oil seeds that contain high levels of C18:1 isomers (Malainey et al. 2010, Malainey et al. 2014, Malainey et al. in press). This pattern is due to the abundance of triacylglycerols composed of fatty acids with chain lengths mostly of 16 or 18 carbons.

Methodology

Possible contaminants were removed by grinding off exterior surfaces of each sample with a Dremel® tool fitted with a silicon carbide bit. Immediately thereafter, the sample was crushed with a hammer mortar and pestle and the powder transferred to an Erlenmeyer flask. Lipids were extracted using a variation of the method developed by Folch et al. (1957). The powdered sample was mixed with a 2:1 mixture, by volume, of chloroform and methanol (2 × 25 mL) using ultrasonication (2 × 10 min). Solids were removed by filtering the solvent mixture into a separatory funnel. The lipid/solvent filtrate was washed with 13.3 mL of ultrapure water. Once separation into two phases was complete, the lower chloroform-lipid phase was transferred to a round-bottomed flask and the chloroform removed by rotary evaporation. Any remaining water was removed by evaporation with 2-propanol (1.5 mL); 1.5 mL of chloroform-methanol (2:1, v/v) was used to transfer the dry total lipid extract to a screw-top glass vial with a Teflon®-lined cap. The resulting total lipid extract was flushed with nitrogen and stored in a –20°C freezer.

Preparation of FAMES

Either 100 μL (12JS 6 and 7) or 400 μL (12JS 8-10) aliquots of the total lipid extract solution was placed in a screw-top test tube and dried in a heating block under nitrogen. Fatty acid methyl esters (FAMES) were prepared by treating the dry lipid with 3 mL of .5 N anhydrous hydrochloric acid in methanol (68°C; 60 min). Fatty acids that occur in the sample as di- or triglycerides are detached from the glycerol molecule and converted to methyl esters. After cooling to room temperature, 2.0 mL of ultrapure water was added. FAMES were recovered with petroleum ether (2 × 1.5 mL) and transferred to a vial. The solvent was removed by heat under a gentle stream of nitrogen; the FAMES were dissolved in 75 μL of *iso*-octane then transferred to a GC vial with a conical glass insert.

Preparation of TMS Derivatives

Either a 50 μL (12JS 6 and 7) or 200 μL (12JS 8-10) aliquot of the total lipid extract solution was placed in a screw-top vial and dried under nitrogen. Trimethylsilyl (TMS) derivatives were prepared by treating the lipid with 70 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 percent trimethylchlorosilane, by volume (70ºC; 30 min). The sample was then dried under nitrogen and the TMS derivatives were redissolved in 100 μL of hexane.

Solvents and chemicals were checked for purity by running a solvent blank. Traces of fatty acid contamination were subtracted from sample chromatograms. The relative percentage composition was calculated by dividing the integrated peak area of each fatty acid by the total area of fatty acids present in the sample. It must be understood that the

identifications given do not necessarily mean that those particular foods were actually prepared because different foods of similar fatty acid composition and lipid content would produce similar residues (Table 2). It is possible to say only that the material of origin for the residue was similar in composition to the food(s) indicated. High temperature–gas chromatography and high temperature–gas chromatography with mass spectrometry is used to further clarify the identifications.

Table 2. Known Food Sources for Different Types of Decomposed Residues.

Decomposed Residue Identification	**Plant Foods Known to Produce Similar Residues**	**Animal Foods Known to Produce Similar Residues**
Large herbivore	Tropical seed oils, including sotol seeds	Bison, deer, moose, fall-early winter fatty elk meat, Javelina meat
Large herbivore with plant OR bone marrow		
Low-fat-content plant (plant greens, roots, berries)	Jicama tuber, buffalo gourd, yopan leaves, biscuit root, millet	Cooked camel's milk
Medium- to low-fat-content plant	Prickly pear, Spanish dagger	None
Medium fat content (fish or corn)	Corn, mesquite beans, cholla	Freshwater fish, *Rabdotus* snail, terrapin, late winter fat-depleted elk
Moderate-high fat content (beaver)	Texas ebony	Beaver and probably raccoon or any other fatty medium-sized mammals
High fat content	High-fat nuts and seeds, including acorn and pecan	Rendered animal fat (other than large herbivore), including bear fat
Very high fat content	Very high-fat nuts and seeds, including pine nuts	Freshly rendered animal fat (other than large herbivore)

Gas Chromatography Analysis Parameters

The GC analysis was performed on a Varian 3800 gas chromatograph fitted with an ionization detector connected to a personal computer. Samples were separated using a VF-23 fused silica capillary column (30 m × .25 mm I.D.; Varian, Palo Alto, CA). An autosampler injected a 3 μL sample using a split/splitless injection system. Hydrogen was used as the carrier gas with a column flow of 1.0 mL/min. Column temperature was increased from 80°C to 140°C at a rate of 20°C per minute then increased to 185°C at a rate of 4°C per minute. After a 4.0 minute hold, the temperature was further increased to 250°C at 10°C per minute and held for 2 minutes. Chromatogram peaks were integrated using Varian MS Workstation® software and identified through comparisons with external qualitative standards (NuCheck Prep, Elysian, MN).

High Temperature–Gas Chromatography and Gas Chromatography with Mass Spectrometry

Both HT-GC and HT–GC/MS analyses were performed on a Varian 3800 gas chromatograph fitted with a flame ionization detector and a Varian 4000 mass spectrometer connected to a personal computer. For HT-GC analysis, the sample was injected onto a DB-1HT fused silica capillary column (15 m × .32 mm I.D.; Agilent J&W, Santa Clara, CA) connected to the flame ionization detector, using hydrogen as the carrier gas. The column temperature was held at 50°C for 1 minute then increased to 350°C at a rate of 15°C per minute and held for 26 minutes. For HT-GC/MS analysis, samples were injected onto a DB-5HT fused silica capillary column (30 m × .25 mm I.D.; Agilent J&W, Santa Clara, CA) connected to the ion trap mass spectrometer in an external ionization configuration using helium as the carrier gas. After a 1 minute hold at 50°C, the column temperature was increased to 180°C at a rate of 40°C per minute then ramped up to 230°C at a rate of 5°C per minute and finally increased to 350°C at a rate of 15°C per minute and held for 27.75 minutes. The Varian 4000 mass spectrometer was operated in electron-impact ionization mode scanning from m/z 50 to 700. Chromatogram peaks and MS spectra were processed using Varian MS Workstation® software and identified through comparisons with external qualitative standards (Sigma Aldrich, St. Louis, MO, and NuCheck Prep, Elysian, MN), reference samples, and the National Institute of Standards and Technology (NIST) database.

Results of Archaeological Data Analysis

Descriptions of the Sand Point and Naomikong samples are presented in Table 3; the compositions of extracted lipid residues are presented in Table 4. In Table 5, the term *Area* represents the area under the chromatographic peak of a given fatty acid, as calculated by the Varian MS Workstation® software minus the solvent blank. The term *Rel%* represents the relative percentage of the fatty acid with respect to the total fatty acids in the sample. Hydroxide or peroxide degradation products can interfere with the integration of the C22:0 and C22:1 peaks; these fatty acids were excluded from the analysis.

Table 3. List of Pottery Samples from the Sand Point and Naomikong Point Sites.

Lab No.	Catalog No.	Mass (g)
12JS 1	SP15-956	10.05
12JS 2	SP1-1278	10.008
12JS 3	SP1-1599	11.806
12JS 4	SP12-398	8.891
12JS 5	SP15-367	9.991
12JS 6	76534	8.334
12JS 7	76466	9.927
12JS 8	72875	6.962
12JS 9	76532(2)	11.574
12JS 10	76532(1)	6.938

Table 4. Lipid Compositions of Residues Extracted from Pottery.

Fatty acid	12JS 1		12JS 2		12JS 3		12JS 4		12JS 5	
	Area	Rel%	Area	Rel%	Area	Rel%	Area	Rel%	Area	Rel%
C12:0	114445	2.21	28483	1.16	21742	1.48	14196	2.93	15229	1.39
C14:0	747481	14.42	287671	11.67	80285	5.47	49871	10.31	141463	12.93
C14:1	64522	1.24	24439	.99	12483	.85	4821	1.00	8906	.81
C15:0	36594	.71	64565	2.62	42325	2.88	21264	4.39	40566	3.71
C16:0	3263506	62.95	1572039	63.80	621945	42.34	263855	54.53	702243	64.17
C16:1	49708	.96	38643	1.57	3706	.25	5027	1.04	10072	.92
C17:0	105155	2.03	41894	1.70	63747	4.34	11675	2.41	19856	1.81
C17:1	27741	.54	16813	.68	9967	.68	3512	.73	3764	.34
C18:0	555502	10.72	290572	11.79	527311	35.90	69666	14.40	118567	10.83
C18:1s	76111	1.47	72602	2.95	39430	2.68	23398	4.84	15700	1.43
C18:2	914	.02	129	.01	1261	.09	9	0.00	432	.04
C18:3s	770	.01	0	0.00	2622	.18	1311	.27	0	0.00
C20:0	43673	.84	13707	.56	37190	2.53	7359	1.52	7468	.68
C20:1	0	0.00	963	.04	1107	.08	5664	1.17	565	.05
C24:0	98008	1.89	11587	.47	3675	.25	2249	.46	9566	.87
Total	5184132	100.00	2464109	100.00	1468798	100.00	483879	100.00	1094397	100.00
Peak Ratios of C48, C50, C52 & C54 TAGs	Not Available No p8attern evident		Not Available Distribution similar to Plant & Animal Combination		2.6:1.8:1:1 Plant distribution with animal products present		Not Available Distribution similar to Plant & Animal Combination		2.3:1.6:1:1.7 Plant distribution with animal products present	
Biomarkers	Azelaic acid		Azelaic acid		Cholesterol		Possibly Cholesterol; possibly β-sitosterol		Cholesterol	
Identification	Low-fat-content plant; Plant seeds		Low-fat-content plant; Plant seeds; Some evidence for animal products		Large herbivore and low-fat-content plant, possibly roots		Low-fat-content plant with some animal products		Low-fat-content plant with some animal products	

Table 4 cont'd. Lipid Compositions of Residues Extracted from Pottery.

Fatty acid	12JS 6		12JS 7		12JS 8		12JS 9		12JS 10	
	Area	Rel%	Area	Rel%	Area	Rel%	Area	Rel%	Area	Rel%
C12:0	74119	.67	56883	.26	30254	5.08	161728	2.14	12188	.71
C14:0	1112133	10.11	940847	4.33	100431	16.88	339370	4.49	167240	9.72
C14:1	116432	1.06	107188	.49	2349	.39	55449	.73	0	0.00
C15:0	270625	2.46	515230	2.37	22139	3.72	139954	1.85	65632	3.82
C16:0	6858124	62.37	14017750	64.47	285194	47.93	4768229	63.03	1033103	60.05
C16:1	144871	1.32	17994	.08	10189	1.71	20752	.27	31353	1.82
C17:0	222138	2.02	209856	.97	14005	2.35	202579	2.68	49910	2.90
C17:1	79549	.72	28717	.13	0	0.00	42968	.57	6603	.38
C18:0	1666064	15.15	5043127	23.19	53512	8.99	1508274	19.94	247652	14.40
C18:1s	234501	2.13	452223	2.08	49992	8.40	154247	2.04	58717	3.41
C18:2	5014	.05	8049	.04	2848	.48	10905	.14	6826	.40
C18:3s	15462	.14	68470	.31	7677	1.29	16819	.22	2219	.13
C20:0	105982	.96	169007	.78	8225	1.38	89108	1.18	15879	.92
C20:1	5323	.05	0	0.00	4453	.75	10086	.13	2636	.15
C24:0	78251	.71	104283	.48	3809	.64	41632	.55	20399	1.19
C24:1	6669	.06	3766	.02	0	0.00	2564	.03	0	0.00
Total	10995256	100.00	21743389	100.00	595077	100.00	7564664	100.00	1720357	100.00
Peak Ratios of C48, C50, C52 & C54 TAGs	Not available C48 TAG larger than C50 TAG		None detected		7:2:1.7:1 Plant distribution		11.4:5.7:2.2:1 Plant distribution, some animal		Not available C48 TAG larger than C50 TAG	
Biomarkers	Cholesterol		Cholesterol		Cholesterol; β-sitosterol		Cholesterol; β-sitosterol		Cholesterol; β-sitosterol	
Identification	Low-fat-content plant and animal		Animal, some plant products		Low-fat-content plant, traces of animal products		Plant and animal		Low-fat-content plant, some animal products	

Sand Point

The lipid yield from all potsherds was impressive. Typically our lab uses 400 µL of the total lipid extract (TLE) for the preparation of FAMEs and injects 3 µL of the derivatives for routine gas chromatography of archaeological residues. In order to prevent the column from becoming overloaded, we took only 200 µL of the TLE extracted from the Sand Point potsherds and injected 1 µL or less of the FAMEs onto the column (i.e., not more than 1/6 the usual amount).

The fatty acid compositions of residues 12JS 1, 12JS 2, and 12JS 5 are practically identical and that of residue 12JS 4 is quite similar to them. The level of medium-chain fatty acids (sum of C12:0, C14:0, and C15:0) in all four samples ranges between 15.45 percent and 18.02 percent, which is a characteristic of residues produced from low-fat-content plants, such as plant greens, roots, and berries. The level of C18:1 isomers preserved in these samples is very low, at less than 5 percent. The level of C18:0 is also quite low, ranging between 10.72 percent and 11.79 percent in residues 12JS 1, 12JS 2, and 12JS 5; it is only slightly higher in residue 12JS 4, measuring 14.40 percent. The plant sterol β-sitosterol may occur in residue 12JS 4. The biomarker azelaic acid occurs in residues 12JS 1 and 12JS 2; this short-chain dicarboxylic acid is associated with the oxidation of unsaturated fatty acids (Regert et al. 1998). Unsaturated fatty acids are most abundant in seed oils, so it is possible that these residues in part reflect the processing of plant seeds. Evidence for animal products in these residues includes the presence, or possible presence, of the animal sterol cholesterol in residues 12JS 4 and 12JS 5. The ratio of C48, C50, C52, and C54 TAG peak size areas in residue 12JS 5 is 2.3:1.6:1:1.7, which is similar to a plant and animal combination; the C50 TAG is below detectable levels in residues 12JS 2 and 12JS 4, but the distribution of the other peaks is most similar to a plant and animal combination. Given the low levels of both C18:0 and C18:1 isomers, it is probable that only lean animal flesh, perhaps rabbit or another animal that has lean flesh, was processed in these vessels. The faunal assemblage should be consulted in order to determine the most likely animal sources. In summary, these four pottery residues primarily reflect the processing of low-fat-content plants. There is some evidence that plant seeds were processed in the vessels from which residues 12JS 1 and 12JS 2 were extracted. Traces of animal products (other than larger herbivores) were detected in residues 12JS 2, 12JS 4, and 12JS 5; the animal flesh was very lean.

The composition of residue 12JS 3 differs from the others in that the level of C18:0 is high (35.90 percent). A high level of the fatty acid C18:0 is a characteristic of the decomposed cooking residues of large herbivores, such as deer, bison, and moose; other foods known to produce similar residues include javelina meat and the seed oils of certain tropical plants, such as sotol (see Tables 1 and 2). The level of medium-chain saturated fatty acids (sum of C12:0, C14:0, and C15:0) in this residue is about 10 percent, which indicates that low-fat-content plants also occur. The level of C17:0 is somewhat elevated (4.34 percent), which suggests that certain plant roots, such as that of the cattail, may have contributed to the residue. As with the other residues from the Sand Point site, the level of C18:1 isomers is very low, measuring only 2.68 percent. The animal sterol cholesterol occurs in residue 12JS 3, and the distribution of TAGs suggests at least some animal products were processed in the pot. The ratio of C48 to C50, C52, and C54 TAG peak areas is 2.6: 1.8:1:1; if only plant products were processed, the C50 TAG peak would probably be less than one-half the size of the C48 TAG peak. Unless there is reason to believe javelin or

tropical seed oils were available, the vessel from which residue 12JS 3 was extracted was likely used to process low-fat-content plants, including plant roots, in combination with lean large-herbivore flesh.

Naomikong Point

While plant and animal products seem to occur in all vessel residues, some pots appear to have been used primarily for the preparation of plant materials and others were used more for the preparation of animal products. High levels of medium-chain fatty acids (sum of C12:0, C14:0, and C15:0) in residues 12JS 8 (25.65 percent), 12JS 10 (14.24 percent), and 12JS 6 (13.25 percent) indicate these vessels were used primarily for the preparation of low-fat-content plants. The plant sterol β-sitosterol was detected in residue 12JS 8, and the ratio of C48, C50, C52, and C54 TAGs in this residue is 7:2:1.7:1. The presence of animal products in residue 12JS 8 is indicated by the animal sterol cholesterol. Given the low level of C18:0 (8.99 percent) and TAG distribution, only trace levels of animal products likely occur.

Based on its relative fatty acid composition, plant products likely dominate residue 12JS 10, but the level of C18:0 (likely from animal products) is somewhat higher, measuring 14.40 percent. Only the C48 and C50 TAGs were detected in these residues, and the C48 TAG peak was almost three times higher than the C50 TAG peak, which is suggestive of plant material. Both the plant sterol β-sitosterol and the animal sterol cholesterol occur in residue 12JS 10.

While residue 12JS 6 is similar in fatty acid composition to that of residue 12JS 10, the level of medium-chain fatty acids is slightly lower and the level of C18:0 is slightly higher. Only the C48 and C50 TAGs were detected in residue 12JS 6, but the C48 TAG peak was only slightly larger than the C50 TAG peak, pointing to a low-fat-content plant and animal combination. Only the animal sterol cholesterol was detected in residue 12JS 6.

Its relative fatty acid composition shows the presence of animal products is even stronger in residue 12JS 9, but both plant and animal products occur. The level of C18:0 in residue 12JS 9 is 19.94 percent, and the level of medium-chain fatty acids is 8.47 percent. Both the plant sterol β-sitosterol and the animal sterol cholesterol were detected, and the ratio of C48, C50, C52, and C54 TAGs shows that plant products were an important component of residue 12JS 9.

The level of C18:0 in residue 12JS 7 is 23.19 percent, and the level of medium-chain fatty acids is 6.96 percent. Animal products dominate residue 12JS 7, but the somewhat elevated levels of medium-chain fatty acids suggest the presence of some plant material. Only the animal sterol cholesterol occurs in residue 12JS 7 and no TAGs were detected.

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