Chapter 28
Organic residues

Appendix 28.01. Laboratory protocols for organic residues in pottery

Sample extraction

Pottery powder

Acidified methanol extraction

Lipids were extracted using one-step acid catalysed lipid extraction and methylation with acid-methanol following previously established extraction protocols (Craig et al. 2013). Prior to the extraction, 10μl of internal standard (C34:0, n-tetratriacontane) was added to pottery powder or surface deposit. Methanol (4ml) was added to each sample in clean glass tubes and ultrasonicated for 15min. These were then acidified with concentrated sulphuric acid (H2SO4 – 800μl), vortexed and heated in closed vials at 70°C for 4 hours. Following centrifugation, the acidified supernatants were transferred to clean and sterilized glass vials. The solvent soluble portion was then extracted with 3x 2ml hexane and filtered with potassium carbonate to neutralize the sulphuric acid. Extracts were then dried under a gentle stream of N2. An internal standard (10μl C36:0, n-hexatriacontane) was added to each sample before further analysis by GC-MS and GC-C-IRMS. All acidified extracts were treated with activated spongy copper as they presented a high abundance of sulphur, most likely derived from the surrounding matrix. The analytical protocol was oriented to the identification of the main molecular components, such as saturated and unsaturated fatty acids, branched fatty acids, dicarboxylic fatty acids, alkanes, isoprenoid fatty acids, ω-(o-alkylphenyl)alkanoic acids and phytanic) and the isotopic composition of palmitic (C16:0) and stearic (C18:0) fatty acids.

Conventional solvent extraction

Solvent extraction was also carried out on all pottery samples (Appendix 28.02). As in the acid extraction protocol, 10μl of an internal standard ($C_{34:0}$, *n*-tetratriacontane) was added to pottery powder (~1g) prior to extraction. Each sample was extracted by ultrasonication with 3 aliquots of dichloromethane:methanol (2:1 vol/vol; 5ml). The solvent extract was separated from the powder and, where necessary, treated with activated spongy copper to remove elemental sulphur. The solvent was removed by evaporation to dryness under N_2 (40°C) to obtain a total lipid extract (TLE). Samples were resuspended in hexane (50μl) and 100μl drops of N, O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethyl-chlorosilane were added. Samples were flushed with N_2 before being sealed and isothermally heated at 70°C for 60min. Samples were evaporated to dryness under N_2 (40°C). Finally, the samples were dissolved in 100μl of *n*-hexane with the addition of 10μg of internal standard ($C_{36:0}$, *n*-hexatriacontane). After resuspension in hexane, the obtained extract was analysed directly by high temperature gas chromatography-mass spectrometry (HTGC-MS). These samples were analysed by high temperature gas chromatography mass spectrometry with the aim to identify acylglycerols, waxes and alkylresorcinols.

Bulk isotope analysis of surface deposits EA-IRMS - surface deposits only

$\delta^{13}C$ and $\delta^{15}N$ measurements were made on 60 of the bulk residue samples (Appendix 28.03). No pre-treatment of the samples - for example, to remove humic and fulvic acids - was undertaken prior to analysis. Several authors have noted that this has little effect on the $\delta^{13}C$ (for discussion, see Morton & Schwarcz 2004; but that it is likely to dissolve and remove components of the original foodstuff, Segeberg et al. 1991). Approximately 1–2mg of homogenised material was weighed into 4x 3.2mm tin capsules on a Mettler Toledo 6 d.p. balance in duplicate.

Instrumentation

Lipids extracted from pottery powder Gas Chromatography Mass Spectrometry

GC-MS (gas chromatography-mass spectrometry) analysis was performed with Agilent 7690A Series gas chromatography and Agilent 5975C Inert XL mass-selective detector with a quadrupole mass analyser with Triple-Axis Detector (Agilent Technologies, Cheadle, Cheshire, UK) were used. The splitless injector and interface were maintained at 300°C and 340°C respectively. Helium was the carrier gas at constant inlet pressure. The GC column was inserted directly into the ion source of the mass spectrometer. The ionisation energy was 70eV and spectra were obtained by scanning between m/z 50 and 800. All samples were analysed using a DB5-ms (5%-phenyl)-methylpolysiloxane column (30m × 0.32mm × 0.25µm; J&W Scientific, Folsom, CA, USA) with the temperature program of 2min at 50°C, 10°C per min to 325°C and 15min at 325°C. The results obtained with this method were used for quantification of the lipid concentration and for identification of the main molecular components. The quantification and identification of compounds was conducted with the Agilent ChemStation software according to their mass spectrum, their retention time and with reference to the NIST 2008 library of mass spectra. For quantification proposes, peak integration was carried out using Agilent MSD ChemStation. Automated integration was selected to eliminate inconsistencies; ensuring that the error introduced at this stage was constant in all samples and can therefore be considered insignificant. The lipid content in each sample, omitting the contamination attributed to plasticiser, was quantified using the following formula:

[(Area (Sample) /Area (internal standard)) * Mass (internal standard)]/Mass (pottery powder)

Pottery samples were re-analysed with a DB23, 60m column in single ion monitoring mode (SIM) for better detection of specific ions related to aquatic biomarkers (Hansel et al. 2004; Evershed et al. 2008) with a temperature program of 2min at 50°C, 10°C min to 100°C, 4°C per min to 140°C, 5°C per min to 160°C, 20°C per min to 250°C and 10min at 250°C. The results obtained with this method were used for exploring the presence/absence specific ions characteristic of TMTD, phytanic acid, pristanic and *ω*-(*o*-alkylphenyl)alkanoic acids which can be used to infer the presence of lipids derived from aquatic products. Using this approach, the ratio of the two natural diastereomers of phytanic acid, 3S,7R,11R,15-phytanic acid (SRR) and 3R,7R,11R,15-phytanic acid (RRR) were also determined as this allows aquatic and ruminant sources to be crudely discriminated (Lucquin et al. 2016). The identification of the compounds was conducted with the Mass Hunter for Quantitative Analysis (for GCMS) and ChemStation.

Pottery samples were re-analysed with a DB5-5ms (5%-phenyl)-methylpolysiloxane column (30m × 0.250mm × 0.25µm; J&W Scientific, Folsom, CA, USA), 30m column in single ion monitoring mode (SIM) for better detection of specific ions related to alkylresorcinols (Colonese et al. 2017; Hammann & Cramp 2018) with a

temperature program of 2min at 50°C, 10°C per min to 325°C. Single ion monitoring method explored m/z 268, 492, 520, 548, 576, 604 to identify the alkylresorcinols associated with cereal processing in the pots.

High temperature Gas Chromatography Mass Spectrometry

HTGC-MS was performed in TLE extracts using a 7890A Series chromatograph attached to a 5975C Inert XL mass-selective detector with a quadrupole mass analyser (Agilent Technologies, Cheadle, UK). The carrier gas used was helium, and the inlet/column head-pressure was constant. The ionisation energy of the mass spectrometer was 70eV and spectra were obtained by scanning between m/z 50 and 800. A splitless injector was used to inject the sample (1μL). General screening of TLE was performed using a DB-5 ms (5%-phenyl)-methylpolysiloxane column (30m × 0.250mm × 0.25μm; J&W Scientific, Folsom, CA, USA). Program temperature was set at 50°C for 2min, then raised by 10°C per min to 325°C, where it was held for 15min.

Gas chromatography-combustion-isotope ratio mass spectrometry

Carbon stable isotope ratios were determined on two fatty acid methyl esters, methyl palmitate ($C_{16:0}$) and methyl stearate ($C_{18:0}$), in each extract using a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) linked to a Trace 1310 gas chromatograph (Thermo Fisher) with a ConFlo IV interface (Cu/Ni combustion reactor held at 1000°C; Thermo Fisher). All samples were diluted with hexane and subsequently 1μl of each sample was injected into a DB5 fused-silica column (60m × 0.25mm id × 0.25μm film thickness). The temperature was set for 0.5min at 50°C, and increased by 25°C per min to 175°C, then 8°C per min to 325°C where it was held for 20min. The carrier gas was ultra-high-purity grade helium at a flow rate of 2ml per min. The eluted products were combusted to CO_2 and ionised in the source of the mass spectrometer by electron ionisation. The ion intensities of m/z 44, 45, and 46 were monitored in order to automatically compute the $^{13}C/^{12}C$ ratio of each peak in the extracts. Computations were performed with Isodat 3.0 Gas Isotope Ratio MS Software (version 3.0; Thermo Fisher) and were based on comparisons with a standard reference gas (CO_2) of known isotopic composition that was repeatedly measured. The results from the analysis are reported in ‰ relative to an international standard (V-PDB). Replicate measurements of each sample and a mixture of fatty acid methyl esters (FAMEs) with $\delta^{13}C$ values traceable to international standards were used to determine the instrument precision (<0.3‰) and accuracy (<0.5‰).

The values were also corrected subsequent to analysis to account for the methylation of the carboxyl group. The corrections were based on comparisons with a standard mixture of $C_{16:0}$ and $C_{18:0}$ fatty acids of known isotopic composition processed in each batch as a sample. Instrument precision on repeated measurements was <0.3‰, and the accuracy determined from FAME and *n*-alkane isotope standards was <0.8‰. The accuracy and precision of the instrument was determined on *n*-alkanoic acid ester standards of known isotopic composition (Indiana standard F8-3). The mean ± S.D. values of these were 29.82 ± 0.16‰ and 23.28 ± 0.19‰ for the methyl ester of $C_{16:0}$ (reported mean value vs. VPDB -29.90 ± 0.03‰) and $C_{18:0}$ (reported mean value vs. VPDB -23.24 ± 0.01‰) respectively. Each sample was measured in replicate (mean of S.D. 0.11‰ for $C_{16:0}$ and 0.10‰ for $C_{18:0}$). Values were also corrected subsequent to analysis to account for the methylation of the carboxyl group that occurs during acid extraction. Corrections were based on comparisons with a standard mixture of $C_{16:0}$ and $C_{18:0}$ fatty acids of known isotopic composition processed in each batch under identical conditions. To compare the isotopic values of the main fatty acids (palmitic and stearic), data obtained on ruminant, and non-ruminant adipose fat, dairy and marine derived fatty acids resources throughout Europe have been compiled (Dudd 1999; Spangenberg et al. 2006; Bell et al. 2007; Spiteri 2012; Recio et al. 2013; Cramp

2014a; Cramp 2014b; Carrer et al. 2016) (Table A). The $\delta^{13}C$ values of modern reference fats were corrected for the Suess Effect taking into consideration the date of collection (Hellevang & Aagaard 2015).

Food source	**Proxies**		
Porcine adipose fats	$\delta^{13}C$ $C_{16:0}$	$\delta^{13}C$ $C_{18:0}$	$\Delta^{13}C$ ($C_{18:0}$–$C_{16:0}$)
Mean	-25.2	-24.4	0.8
Standard deviation	0.8	0.9	0.6
N	66	66	-
Ruminant adipose fats	$\delta^{13}C$ $C_{16:0}$	$\delta^{13}C$ $C_{18:0}$	$\Delta^{13}C$ ($C_{18:0}$–$C_{16:0}$)
Mean	-29.1	-30.7	-.,6
Standard deviation	1.3	1.9	1
N	32	32	
Ruminant dairy fats	$\delta^{13}C$ $C_{16:0}$	$\delta^{13}C$ $C_{18:0}$	$\Delta^{13}C$ ($C_{18:0}$–$C_{16:0}$)
Mean	-28.7	-33.6	-4.8
Standard deviation	1.7	2.4	1.4
N	36	36	

Table A. Summary of $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ n-alkanoic acids from modern European authentic reference fats (porcine adipose fats, ruminant adipose fats and ruminant dairy fats)

Bulk isotopic analysis of surface deposits

Stable carbon and nitrogen isotopic values were determined using a Sercon EA-GSL elemental analyser coupled to a 20-22 continuous flow isotope ratio mass spectrometer (Sercon Ltd, Crewe, UK) at the University of York. Stable carbon and nitrogen isotopic compositions were calibrated relative to the VPDB and AIR scales using IAEA-600 and IAEA-N2 and IA-CANE. Measurement uncertainty was monitored using an in-house cold water fish gelatin standard $\delta^{13}C$ -15.32 ± 0.03 ‰, $\delta^{15}N$ + 15.2 ± 0.12 ‰), Precision (u(Rw)) was determined to be <0.35 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ on the basis of repeated measurements of calibration standards and check standards. Sample replicate analysis for both $\delta^{13}C$ and $\delta^{15}N$ of samples was <3‰ and <7‰ respectively.

References

Bell, J.G., T. Preston, R.J. Henderson, F. Strachan, J.E. Bron, K. Cooper & D.J. Morrison, 2007. Discrimination of wild and cultured European sea bass (*Dicentrarchus labrax*) using chemical and isotopic analyses. *Journal of Agricultural and Food Chemistry* 55 (15), 5934–41, doi.org/10.1021/jf0704561.

Carrer, F., A.C. Colonese, A. Lucquin, E.P. Guedes, A. Thompson, K. Walsh, T. Reitmaier & O.E. Craig, 2016. Chemical analysis of pottery demonstrates prehistoric origin for high-altitude Alpine dairying. *PLOS ONE* 11 (4), e0151442, doi.org/10.1371/journal.pone.0151442.

Colonese, A.C., J. Hendy, A. Lucquin, C.F. Speller, M.J. Collins, F. Carrer, R. Gubler, M. Khun, R. Fishcher & O.E. Craig, 2017. New criteria for the molecular identification of cereal grains associated with archaeological artefacts. *Scientific Reports* 7 (1), 6633, doi.org/10.1038/s41598-017-06390-x.

Craig, O.E., H. Saul, A. Lucquin, Y. Nishida, K. Tache, L. Clarke, A. Thompson, D.T. Altoft, J. Uchiyama, M. Ajimoto, K. Gibbs, S. Isaksson, C.P. Heron & P. Jordan, 2013. Earliest evidence for the use of pottery. *Nature* 496, 351–54.

Cramp, L.J.E., J. Jones, A. Sheridan, J. Smyth, H. Whelton, J. Mulville, N. Sharples & R.P. Evershed, 2014a. Immediate replacement of fishing with dairying by the earliest farmers of the northeast Atlantic archipelagos. *Proceedings of the Royal Society B* 281, 20132372, doi.org/10.1098/rspb.2013.2372.

Cramp, L.J.E., R.P. Evershed, M. Lavento, P. Halinen, K. Mannermaa, M. Oinonen, J. Kettunen, M. Perola, P. Onkamo & V. Heyd. 2014b. Neolithic dairy farming at the extreme of agriculture in northern Europe. *Proceedings of the Royal Society B* 281, 20140819, doi.org/10.1098/rspb.2014.0819.

Dudd, S.N. & R.P. Evershed, 1998. Direct demonstration of milk as an element of archaeological economies. *Science* 282, 1478–81.

Evershed, R.P., M.S. Copley, L. Dickson & F.A. Hansel, 2008. Experimental evidence for the processing of marine animal products and other commodities containing polyunsaturated fatty acids in pottery vessels. *Archaeometry* 50 (1), 101–13, doi.org/10.1111/j.1475-4754.2007.00368.x.

Hammann, S & L.J.E. Cramp, 2018. Towards the detection of dietary cereal processing through absorbed lipid biomarkers in archaeological pottery. *Journal of Archaeological Science* 93, 74–81, doi.org/10.1016/j.jas.2018.02.017.

Hansel F.A., M.S. Copley, L.A.S. Madureira & R.P. Evershed, 2004. Thermally produced omega-(o-alkylphenyl)alkanoic acids provide evidence for the processing of marine products in archaeological pottery vessels**.** *Tetrahedron Letters* 45 (14), 2999–3002.

Hellevang, H. & P. Aagaard, 2015. Constraints on natural global atmospheric CO_2 fluxes from 1860 to 2010 using a simplified explicit forward model. *Scientific Reports* 5, 17352, doi.org/10.1038/srep17352.

Lucquin, A., A.C. Colonese, T.F.G. Farrell & O.E. Craig, 2016. Utilising phytanic acid diastereomers for the characterisation of archaeological lipid residues in pottery samples. *Tetrahedron Letters* 57 (6), 703–07.

Morton, J.D. & H.P. Schwarcz, 2004. Palaeodietary implications from stable isotopic analysis of residues on prehistoric Ontario ceramics. *Journal of Archaeological Science* 31 (5), 503–17.

Recio, C., Q. Martin & C. Raposo, 2013. GC-C-IRMS analysis of FAMEs as a tool to ascertain the diet of Iberian pigs used for the production of pork products with high added value. *Grasas y Aceites* 64 (2), 181–90, doi.org/10.3989/gya.130712.

Segeberg, A., G. Possnert, B. Arrhenius & K. Liden, 1991. Ceramic chronology in view of 14C datings. *Laborativ Arkeologi* 5, 83–91.

Spangenberg, J.E., S. Jacomet & J. Schibler, 2006. Chemical analyses of organic residues in archaeological pottery from Arbon Bleiche 3, Switzerland – evidence for dairying in the late Neolithic. *Journal of Archaeological Science* 33 (1), 1–13, doi.org/10.1016/j.jas.2005.05.013.

Spiteri, C.D., 2012. Pottery use at the transition to agriculture in the western Mediterranean: evidence from biomolecular and isotopic characterisation of organic residues in Impressed/Cardial Ware vessels. (PhD thesis.) University of York, etheses.whiterose.ac.uk/3391/.