Fate and preservation of lipids in the soils of archaeological and experimental burials

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Abstract

Archaeological grave soils from two mass burials and a selection of individual burials have been examined as part of a larger study combining soil micromorphology and chemical analysis to recover signatures relating to burials from around the skeletal remains. A strategy involving sampling from up to 17 anatomical positions was employed in the collection of soil samples for chemical analysis. Analysis was performed using combinations of elemental analysis, gas chromatography and gas chromatography mass spectrometry, liquid chromatography mass spectrometry and pyrolysis gas chromatography. Soils from a series of experimental burials of piglets were similarly examined. The analyses reveal notable effects of the burial environment on the preservation of organic signatures from the remains. Anoxia and hydrology emerge as key controls on preservation and the nature of the burial matrix can exert strong influences both on the preservation and on the pathways by which organic residues are transformed. Clear distinction was apparent in a number of graves between the organic signatures from the soil background organic content and those originating from the remains. Distributions of *n*-alkanes, *n*-alkanols and long chain fatty acids attributed to plant matter were differentiated from triacylglycerols, diacylglycerols, monoacylglycerols, fatty acids and *n*-alkanals some of which reveal distributions similar to those of human adipose tissue, reflecting preservation of signatures of the human remains in the soils. Distributions of short chain components, *n*-alkanals and fatty acids, attest to the reduction of adipose-derived fatty acids and their release from triacylglycerols by hydrolysis.

The grave soils also contained a range of 'exotics' including signatures relating to personal effects, dye from clothing and treatments applied to the coffin. Steroidal components from the gut regions also provide broad indications of the dominant nature of last meals, with potential indications of differences relating to social standing. The studies demonstrate the wealth of information contained in organic signatures of burial soils and recommendations for sampling are made for later studies.

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Author's declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented previously for an award at this, or any other, University. All sources are acknowledged as References.

1 Introduction

1.1 Organic matter associated with human remains

Archaeological investigations into burials are of great importance due to the insight they provide on our ancestors cultures, burial practices and morbidity (Brothwell, 1981). These insights, , are typically obtained from inorganic remains and highly resistive materials such as skeletal remains and additional grave goods of a non-organic nature. Organic material in graves is susceptible to degradation with limited chance of survival, yet in extreme cases it can be preserved. Organic matter related to the skeletal structure (collagen and bound proteins) is commonly preserved and lipids distinct from the natural soil background signature can survive in waterlogged, arid and frozen environments (Evershed, 2008). The detection of sterols in soils have shown the presence of faecal material and revealed bulk features about the diets of humans (Bethell et al., 1994a). Further analyses have also been carried out for mummies and bog bodies providing information on signatures directly related to the human remains (see below).

1.1.1 Mummies

Mummies provide a unique insight into organic signatures associated with human remains due to the exceptional level of preservation caused by the mummification process. Mummies can be formed in exceptional burial environments such as ice sheets that provide an arid environment (Barfield, 1994) or by burial practices and conditions such as those of the mummies of Egypt. The highly preserved nature of the remains has led to a large number of investigations. Egyptian mummies have been analysed extensively to understand the practice of mummification and to determine how the remains were preserved. Wrappings and embalming agents have been analysed and compounds including fatty acids of both animal and plant origins, terpenoids of conifer resins and wax esters of bees wax have been identified (Buckley and Evershed, 2001). Terpenoids and specific compounds of bees wax, for example wax esters, are resistant to degradation and their use in embalming has been shown to be significant in the preservation of human remains (Buckley et al., 2004).

Organic signatures from samples of mummified remains show the presence of fatty acids ($C_{14:0}$ to $C_{18:0}$) with dominance of $C_{16:0}/C_{18:0}$ as well as C_{15} and C_{17} *iso* branched chain fatty acids of bacterial origin (Buckley et al., 1999, Gülacar et al., 1990). The low concentrations of branched chain fatty acids in the samples were suggested to relate directly to the remains and not external degraders. Fatty acid compositions of naturally preserved mummies show that varying levels of degradation occurred before being completely arrested/slowed. Profiles either match that expected for adipose tissue (Makristathis et al., 2002) or are dominantly saturated with low abundances of $C_{18:2}$ indicating degradation of adipose tissue. Oxidation products of fatty acids have been observed in

a variety of mummies, with both C_{18} and C_{16} hydroxy fatty acids being indicative of the alteration of the unsaturated $C_{16:1}$ and $C_{18:1}$ components of adipose tissue. Interestingly, ice mummies were the most affected by oxidation (Gülacar et al., 1990). Triacylglycerol preservation has also been investigated and found to vary among mummy samples, with some containing high concentrations (Mayer et al., 1997). This variation in the extent of preservation reflects different extents of hydrolysis during degradation, presumably prior to mummification. Sterols identified in mummies have also been used to reveal features of the diet of the individual, a 4000 year old Nubian mummy showed the presence of cholesterol, 5α-cholestanol and 5β-cholestanol (Figure 1:1) as well as plant sterols in the gut region. Comparison of the ratios of the sterols allowed for the determination of a vegetable based diet, an interpretation in which the sterol origin was supported by distributions of bile acids that derived from human faecal matter being detected in the remains (Gülacar et al., 1990, Lin et al., 1978, Lin and Connor, 2001). Mummies produced by a specific burial practice showed no sterols indicative of faecal material due to the removal of organs on mummification.

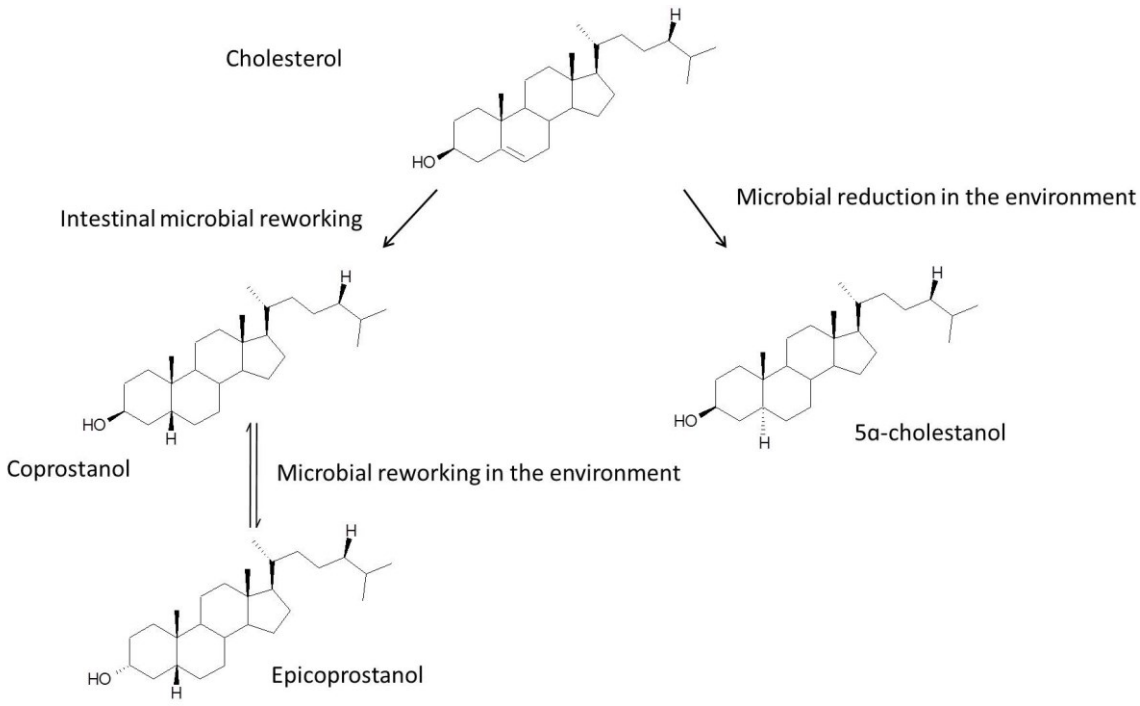


Figure 1:1. Cholesterol and microbial degradation products from both intestinal and environmental degradation. Adapted from Bull et al. 2002.

1.1.2 Burials

The burial of remains in graves is not as well studied as are mummified remains. Analysis of remains in graves, and more importantly organic remains, is highly targeted. Thus, the abdominal cavity and pelvic areas of remains were investigated to examine the stomach contents of the

individuals (Reinhard et al., 1992), such analysis relies on the processes of degradation and movement of organic signatures from the stomach and gut resulting in accumulation in the pelvic basin and sacrum. Analyses of these samples are typically macro and microscopic with pollen, botanical and faunal remains used to determine foodstuffs. This analysis, however, requires large quantities of sample to gain significant evidence and even when samples can be analysed detection of remains is low (Berg, 2002, Reinhard et al., 1992). As well as the macro and microscopic contents of soils from the gut region particular attention and analysis has been given to adipocere, a hard waxy substance found in graves either around the remains or situated on the skeletal structure (Fiedler et al., 2009, Fiedler and Graw, 2003). Adipocere forms after hydrolysis of triacylglycerols to free fatty acids, after which reaction with metal ions from the soils leads to salt formation producing a hard and highly resistant substance. Adipocere consists of saturated fatty acids ($C_{14:0}$, $C_{16:0}$ and $C_{18:0}$), along with 10-hydroxy fatty acids for samples from waterlogged graves (Forbes et al., 2002) suggesting a possible association between oxidation and waterlogging.

1.1.3 Bog bodies

Bog bodies are unique cases of preserved human remains, exhibiting preservation of skin and muscle tissue (Evershed and Connolly, 1988). The peat bogs in which bog bodies have been found are environments that lead to very good preservation owing to cool temperature, acidic conditions and their anaerobic nature which is caused by waterlogging and plant matter (Berstan et al., 2004, Evershed and Connolly, 1988). The reasons for interment in such environments, are debated, some investigators suggest that the burials are intentional, as part of a ritual, and others suggest them to be accidental (Stead et al., 1986). Analysis of bog bodies shows the presence of fatty acids ranging from $C_{12:0}$-$C_{20:0}$ similar to that expected from human tissue. Notably, triacylglycerols are heavily degraded by hydrolysis (Evershed and Connolly, 1988, Evershed and Connolly, 1994). The presence of 10-hydroxy fatty acids in the samples was also taken to suggest that oxidative degradation occurred before degradation was arrested by the anaerobic conditions. Cholesterol and both ketone and stanol degradation products were detected, in muscle and skin tissue. Distributions were dominated by cholesterol with lower levels of stanols and ketones, 5α-cholestanol the environmental reduction product of cholesterol, the most common degradation product (Evershed and Connolly, 1994). The detection of 5β-cholestanol, a degradation product of cholesterol that is formed within the gut of mammals, led to the suggestion that gut microflora aided in the degradation of the remains (Evershed and Connolly, 1994). The likelihood that this process occurred is questionable due to the decrease in temperature on death and the conditions within a peat bog, being likely to significantly alter the

conditions required for those microflora to survive. The more likely explanation for the presence of 5β-cholestanol in samples is the spread of faecal material following degradation.

1.2 Experimental burials

Experimental burials have been used to extend knowledge on both forensic and archaeological burials. The use of human remains for experimental studies poses ethical problems and requires extensive protocols to be followed. Pigs, which are hairless and have similar skeletal and muscle tissues to humans have been shown to also have similar fatty acid and triacylglycerol distributions to that of humans (Notter et al., 2009). Pigs are typically used as analogues for humans due to the close nature and composition as that of humans. Due to the different reasons for conducting experimental burials and range of burial conditions that are relevant, a wide range of environments have been used in such studies.

1.2.1 Laboratory based experiments

Laboratory investigations have been carried out in order to understand the processes involved in the formation of adipocere for forensic (clandestine burials) (Forbes et al., 2005b, Forbes et al., 2005c, Forbes et al., 2005a) and commercial purposes (grave rental in European countries) (Notter et al., 2009, Frund and Schoenen, 2009). Various laboratory experiments have been carried out: Notter (2009) analysed adipocere formation in water over a period of 6 months, detecting fatty acid distributions containing $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ as well as $C_{18:0}$ 10-hydroxy fatty acid, formed from hydration of the unsaturated fatty acids. Similar analysis by Frund (2009) was carried out to understand the formation and degradation of adipocere in different soil matrices over a period of 215 days. Analyses showed that well-drained, oxic, environments did not lead to formation of adipocere, and that the loss of adipocere was most apparent in cases where adipocere formation had already occurred. Studies to aid forensic investigations were carried out by Forbes et al. (2005), examining the extent to which the formation of adipocere relates to the nature of the burial soil, environment and method of burial. All experiments, which were carried out in sealed containers and maintained at a constant temperature, showed the main constituents of adipocere to be saturated fatty acids. In addition, low abundances of unsaturated, oxo, hydroxy and calcium salts of fatty acids were present. Little variation in this distribution was seen between different soil types maintained under the same environmental conditions. Acidic soils and soils containing lime showed different levels of degradation to the other soil types examined (Forbes et al., 2005b). Skin and tissue were found to be present with little signs of degradation. Studies of the effect of burial method showed that coffins were affected by fungal growth and exhibited a lack of adipocere formation, believed to be caused by the more extensive

decomposition due to the aerobic nature of a coffin (Forbes et al., 2005c). Fatty acid signatures from these experiments showed closer distributions to adipose tissue than adipocere suggesting a coffined environment hampers adipocere formation.

1.2.2 Shallow and surface burials

Shallow and surface burials are typically carried out at sites in which clandestine burials have been found, in order to aid the interpretation of the post mortem interval. Surface burials are investigated to determine if signatures from remains can be found in the underlying soil (Benninger et al., 2008, Cassar et al., 2011). Elemental analysis below remains has shown a significant input of nitrogen, which was found to increase over time as degradation progressed (Benninger et al., 2008). Investigations by Cassar et al. (2011) showed the presence of adipocere at a depth of 70 - 80 cm below the carcass after a period of 50 - 60 days, suggesting a correlation between penetration depth and time and allowing for an estimate of how long it had rested on the surface. The finding of signatures at depth below the carcass is explained by the leaching of the sparingly soluble fatty acid metal salts.

1.2.3 Burials at depth

Relatively few investigations of human burials have been conducted. Experimental burials have tended to focus on the stages of decay and the timescales over which these stages occur (Payne et al., 1968). Retardation of decay has also been evaluated, notably the effects of applying lime and quicklime to the remains. Thus, these analyses focused more on the taphonomy of the sample than on chemical signatures. Wilson et.al (2007) carried out a study with three pig carcasses buried in three different locations (pasture, moorland and woodland). Analysis of the degradation over a period of 24 months showed the graves from the woodland and moorland to contain some preserved soft tissue, whereas the remains buried in the pasture were fully skeletonised. Investigation of the remains was carried out more thoroughly than in earlier studies, with temperature, pH and lipid signatures being analysed in addition to visual traits of degradation (Wilson et al., 2007). These investigations showed that the degradation of the corpse altered temperature in the graves with seasonal changes having marked influence on the extent of degradation. Lipid residue analysis was limited, limited examination of fatty acids showed slight alteration to their distributions, in line with degradation experiments carried out by Forbes (2005).

1.3 Body decay

Decomposition of remains starts almost instantaneously after death and follows a specific pattern: the early stages of decomposition are followed by putrefaction, liquefaction and

disintegration, leading ultimately to skeletonisation. Putrefaction is the bacterial breakdown of soft tissue, releasing protein, carbohydrates and lipids from the tissue. This step is followed by liquefaction where organs and body tissues become a pool of organic compounds released by the breakdown of proteins and other complex biomolecules. This, in turn, leads to disintegration and finally, due to the increased mobility of fluids and access of microorganisms, to full skeletonisation (Dent et al., 2004, Carter, 2005, Carter et al., 2007).

The early stages of decomposition and liquefaction of the remains release around 20 % protein, 10 % fats (lipids) and 1 % carbohydrates of the human body. Following their release these compounds undergo further breakdown (Campobasso et al., 2001, Statheropoulos et al., 2007). Proteins are broken down by enzymes into a variety of compounds (peptones, polypeptides and amino acids) which are further degraded by microorganisms to CO_2, H_2S, CH_4 and NH_3 (Dent et al., 2004). Carbohydrates are readily converted to CO_2 and water with the simple sugars being utilised by fungi and bacteria as food sources.

Dent *et. al*. (2008) suggests fats, of which 90 - 99 % is triacylglycerol, undergo hydrolytic degradation mediated by fungi and bacteria to eventually yield free fatty acids (Dent et al., 2004). The fatty acids, in particular the unsaturated components can undergo further alteration *via* hydrogenation or oxidation (Eglinton and Logan, 1991), resulting in changes in the distributions from dominantly unsaturated components to dominantly saturated fatty acids components (Figure 1:2). Triacylglycerols can also undergo auto-oxidation at positions of unsaturation on the acyl moieties as seen in the putrefaction of meat products (Byrdwell and Neff, 2001). This auto-oxidation leads to the formation of epoxides, which on subsequent cleavage can lead to the formation of short chain triacylglycerols (Figure 1:2) (Byrdwell and Neff, 2001, Giuffrida et al., 2004). The shortened triacylglycerols can undergo further degradation *via* hydrolysis.

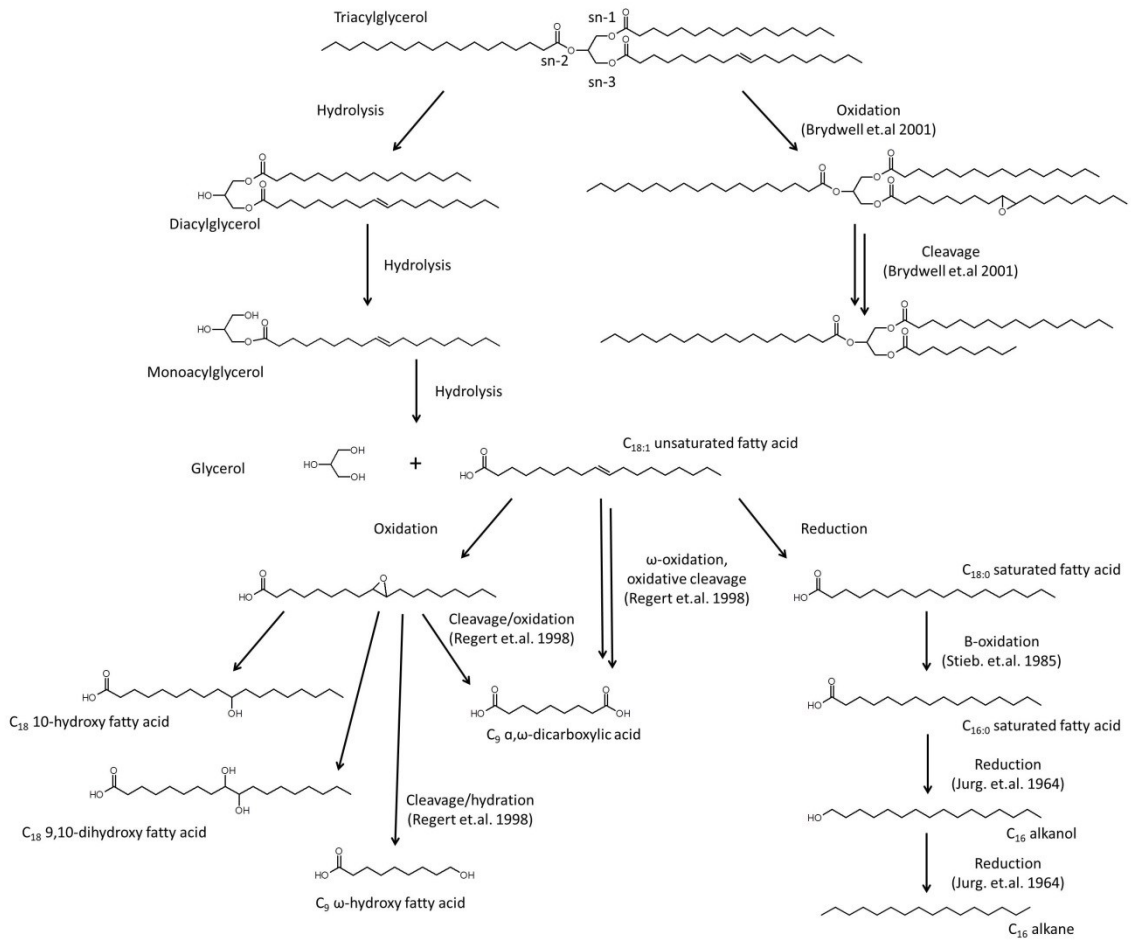


Figure 1:2. Triacylglycerol pathways of degradation *via* biotic and abiotic process, showing the main pathway of degradation, the hydrolysis of triacylglycerols too free fatty acids and the alteration pathways available for unsaturated fatty acids.

1.4 Fate of organic signatures in soils

Soils naturally contain organic matter and hence have individual signatures that relate to their specific location. The distributions and abundances of these biological molecules (hence forth signatures) in soils and from transformation of organic matter have been extensively studied; hence a substantial body of information is available. Details are also discussed in in the individual chapters to which they are relevant. Around 99 % of soil organic matter is estimated to be derived from plant matter (Carter et al., 2007). It is constantly susceptible to alteration under the influences of environmental conditions (physical and chemical) and human activity (Jarde et al., 2009, Bull et al., 1998).

In soils organic signatures related to plant matter show a variety of signatures which include *n*-alkanes ranging from C_{23}-C_{33} and having an odd-over-even predominance and *n*-alkanols ranging from C_{18}-C_{30} with even-over-odd predominance that reflect inputs from higher plant waxes (Eglinton and Hamilton, 1967, Naafs et al., 2004, Kuhn et al., 2010). The distributions of *n*-alkanes

from higher plants typically show dominant signals at C_{29} or C_{31} (Eglinton and Hamilton, 1967, Jambu et al., 1991, Jansen et al., 2008) with both C_{29} and C_{31} shown to be dominant in the soils of woodland and grassland areas(Bull et al., 2000a, Bull et al., 2000b). Potentially, the distributions differ with both location and the nature of plant input to the soil (Eglinton and Hamilton, 1967, van Bergen et al., 1997b). Fatty acids distributions (C_{16}-C_{32}) in soils can also reflect inputs, from plant matter, (Moucawi et al., 1981, Amblès et al., 1994) and have been suggested to be sources of *n*-alkanes in soils (Figure 1:2) (Jurg and Eisma, 1964). Long chain fatty acid profiles are dominated by $C_{26:0}$ or $C_{28:0}$, fatty acids typically related to plants (Eglinton and Hamilton, 1967, Bull et al., 2000a, Bull et al., 2000b). Short chain fatty acids sourced from plant matter that are also present in soils typically have distributions dominated by $C_{16:0}$ and $C_{18:2}$ (Figure 1:3)(Zelles, 1997)

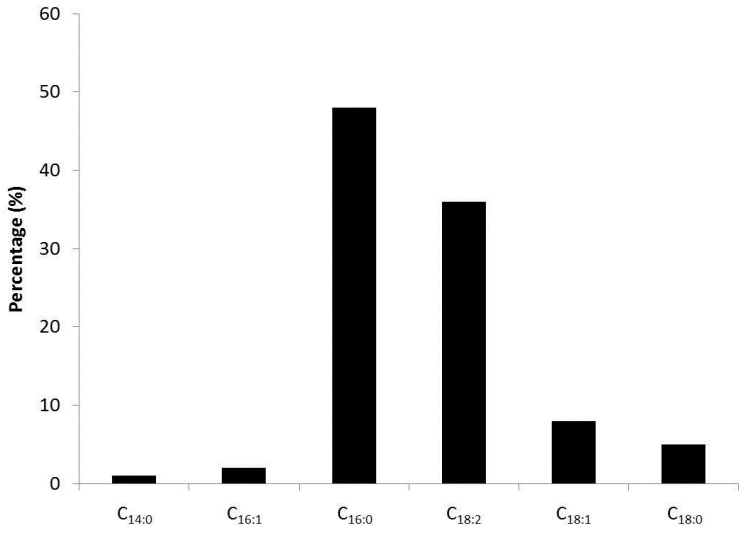


Figure 1:3. Distribution of fatty acids presents in plant material showing a dominance of $C_{16:0}$ and $C_{18:2}$ (Zelles, 1997).

The fatty acids, *n*-alkanes and *n*-alkanols in soils are present through release both on degradation of plant material and from transformation of larger plant derived organic molecules (plant waxes). Short chain *n*-alkanes (C_{19}-C_{21}) in soils are possibly formed *via* the reduction of fatty acids, other potential sources including soil bacteria (Ladygina et al., 2006). ω-Hydroxy fatty acids ranging from C_{22} to C_{28} occur in some soils and are another product of microbial transformation of plant matter they are mainly introduced into the soil environment by the degradation of suberin, a plant root and bark biopolymer (Bull et al., 2000a).

A series of sterols common to plants (β-sitosterol, stigmasterol and campesterol) along with triterpenoid and diterpenoids from plant resins have been found to be present in soils in forests and pasture land (Otto and Simpson, 2005, van Bergen et al., 1997b, Naafs et al., 2004). Stimastan-3-one, stigmasto-3,5dien-7one and sistosterone all oxidative degradation products of

both β-sitosterol and stigmasterol can also be found in soils alongside ergosterol an indicator of fungal activity (Otto and Simpson, 2005). The large variety of organic molecules present in soils, with sources including plants, bacteria and fungi (Eglinton and Hamilton, 1967, Zelles, 1997, Ruess et al., 2002), suggests that organic signatures from these sources will be present in grave soils.

Organic molecules introduced into soils by human activity such as triacylglycerols (which are also naturally present in plant matter) also undergo significant alteration on degradation (Figure 1:2). Hydrolysis of the moieties attached to the glycerol backbone leads to the formation of diacylglycerols and monoacylglycerols and finally to the release of fatty acids into the soil environment (Hita et al., 1995, Notter et al., 2008). These fatty acids released on hydrolysis can then undergo a series of changes (Figure 1:2) with process leading to an environment in which the saturated fatty acids dominate. The simplest of these changes is the transformation of unsaturated fatty acids to saturated fatty acids, a pathway that is most likely mediated by microbes(Fiedler and Graw, 2003, Forbes et al., 2003, Forbes et al., 2005a, Notter et al., 2008). It requires the hydrogenation of the double bond to form the saturated counterpart, which is much more recalcitrant in the soil environment. Oxidation of unsaturated fatty acids leads to additional degradation products and a wider range of possible pathways of alteration of the signatures in the soil environment.

Oxidation of the unsaturated bond on a fatty acid backbone is a process that occurs *via* the formation of an epoxide. The epoxide can undergo a series of alterations, the formation of hydroxyl groups occurring extensively in waterlogged environments (Forbes et al., 2005b). Alternatively, ring opening of the epoxide and subsequent cleavage and oxidation leads to the formation of a series of shorter chain and more polar products such as ω-hydroxy fatty acids and α,ω-dicarboxylic acids (Regert et al., 1998). These smaller and more polar molecules are more susceptible to water movement owing to their greater water solubility. Hence, percolation of water through the soil can affect the soil lipid profile with both short chain and polar molecules being most readily lost.

Fatty acids can undergo further degradation *via* β-oxidation, a microbially induced process leading to cleavage at the β carbon and shortening the fatty acid chain by two carbons, altering the distributions of fatty acids in soil (Hita et al., 1995). This process ultimately progresses until the fatty acid is shortened to acetate, after which uptake by microbes is possible (Hita et al., 1995, Stieb and Schink, 1985, Fiedler and Graw, 2003). Loss of fatty acids, especially the shorter chain lengths, occurs in soils due both to their uptake by soil organisms and via water percolation. Reduction of fatty acids has been suggested to lead to the formation of both *n*-alkanols and *n*-

alkanes in soils (Figure 1:2). Laboratory experiments using *Clostridium butyricum,* a naturally occurring soil bacterium, have shown that fatty acids can be reduce to *n*-alkanols under anaerobic conditions (Naccarat.Wf et al., 1972, Day and Goldfine, 1978), with subsequent reduction of these molecules being suggested to account for the formation of *n*-alkanes.

1.5 Aims and objectives

A lack of knowledge on, and investigation into, organic remains in archaeological burials is evident, despite investigation of human remains being well established. The InterArChive project aimed to combine chemical and archaeological analysis to fill this gap and enhance the information available from archaeological burials. The project aimed to investigate archaeological remains to determine the extent of the information that can be gained from analysis of graves. Experimental burials were included to understand, more completely, the process leading to the organic signatures in archaeological burial soils.

The work presented here considered both archaeological and experimental burials to determine the fate of organic molecules and in particular lipid signatures. The overall aim of the work was to understand the factors that influence the preservation and the degradation of lipid signatures in grave soils. Specific objectives of this investigation were:

I. To determine if signatures from body decay survive in burial soils and can be distinguished from the background soil signature.

II. To identify possible signatures relating to organic materials that may not have physically survived.

III. To understand factors of the burial environment that effect degradation and preservation.

IV. To determine the role of coffins in influencing preservation and degradation of the buried remains.

V. Construct degradation pathways and timelines.

The chapters that follow are organised to consider the study of archaeological graves first, with both mass graves and then single body inhumations investigated to establish their potential to answer the questions stated in the objectives. This is then followed by chapters presenting and

discussing the information gleaned from experimental graves, which, due to the duration of their burial periods prior to excavation, were analysed after the archaeological graves. The order of investigation and reporting has allowed for the nature and range of signatures preserved in archaeological graves to be assessed and suggestions for their origins to be proposed. This is followed by the more direct analysis and comparison of data from experimental graves to formulate clearer understanding of the fate of buried organic matter and the origins of the materials preserved in the archaeological graves. Full experimental details can be found at the end of the thesis in Chapter 7.

2 Organic signatures in the soils and remains of mass burials

2.1 Introduction

The work presented here focuses on organic residues in mass burials and the environmental factors affecting the decomposition and preservation of lipid signatures. Previous analyses of grave soils showed that they can yield lipid profiles that have direct links to the remains/input from interment (Green, 2013, Bull et al., 2009). Mass graves/burials defined as graves in which a large number of corpses are buried due to some form of large scale disaster/atrocity (i.e. famine or war) provide unique environments for analysis due to the large amount of organic material interred. The organic rich nature of mass graves tends towards the generation of anoxia and can be suggested to provide the perfect environment for preservation and for probing the nature and distribution of lipids and exotic compounds that may not survive in an individual burial. The aim of the work described in this chapter was to consider the effect on organic signatures of the unique environments that exist in mass graves, with the fate and preservation of lipids from the being investigated together with the potential archaeological information that may be revealed. In addition, the noted difference between the grave conditions and preservation of organic materials between the two graves from Fromelles posed the question as to whether or not the organic signatures would reflect those differences.

2.2 Sampling and sampling strategies

2.2.1 Sampling, Fromelles, World War 1 mass grave

The village of Fromelles, situated 15 miles west of Lille in northern France, was the site of one of the worst losses experienced by Australian troops during the First World War. The battle that took place over the 19th and 20th of July 1916, involving the 61st British and 5th Australian infantry divisions, was a planned diversionary tactic to prevent German support being sent to the main battle on the Somme, to the South of Fromelles. Allied forces saw combined casualties and fatalities of around 7,000 troops with over 5,000 being sustained by the 5th Australian infantry alone. The unsuccessful attack on the German lines, in particular the salient named the 'Sugarloaf' (due to its shape), left hundreds of dead and dying soldiers behind enemy lines and scattered throughout no man's land.

Such men of the 60th as actually reached the enemy's trenches have been killed or captured. The two companies of the 58th mown down when close to enemy's trench and very few came back.

Message from Major C.A. Denehy of the 58th Battalion, 5th Australian Division to Major-General the Hon James Whiteside McCay. (Cobb, 2010)

After rigorous work carried out by troops in the weeks following the battle and the subsequent amnesty in 1918 most of the missing troops were found. Despite this, 1,600 troops remained unaccounted for. In 2009 work started on the excavation of eight mass graves (thought to have been missed by the post-war burial campaigns) at Pheasant Wood near the village of Fromelles. Earlier investigations by GUARD (Pollard, 2008) had confirmed the presence of mass graves. Of the eight pits ordered and dug by the German forces for the burial of the allied dead within their lines, six were excavated, with five of them producing the skeletal remains of 250 soldiers (Pollard, 2008, Loe, 2014).

On excavation of the pits, the remains found were partially skeletonised with two layers of bodies having been placed in the grave. Waterlogging was a feature of the burials due to the clay-rich nature of the soil (Cobb, 2010, Pollard, 2008, Loe, 2014). Occurrences of a white crystalline material, thought to be quicklime (Pollard, 2008) were noted in the burial pits and suggested to have been used as a disinfectant and aid in degradation (Schotsmans et al., 2012, Forbes et al., 2005b).

Samples from two mass graves (Graves 3 and 4, Figure 2:1 and Table 2.1) were collected from the bottom layer; due to the nature and sensitivity of the mass graves, not all sample positions targeted by the InterArChive low resolution sampling protocol (Usai et al., 2013) could be accessed. Controls were collected for the site but lost during transportation. Soils were sampled adjacent to the remains of three individuals in each grave. One sample was collected from each of the individuals in Grave 3 (Figure 2:2); from the pelvis (2) of SK1673 adjacent to the skull (1) of SK1683 and SK1688. In addition a red sock (A1) was sampled from SK1750 in Grave 3. During preparation of the samples for analysis, roots were found in the soils from SK1683 and SK1688.

A greater number and variety of samples were collected from the soils adjacent to the remains of individuals in Grave 4 (Figure 2:2). SK1523B was sampled at the skull (1), pelvis (2a) and within the pelvic area (2b). Samples taken from the skull (1) and the pelvic area (2b) were found on inspection to contain hair, textile fragments and individual fibres, respectively. Hair and textile remains were separated from the soil as subsamples. Samples were taken at the skull (1), pelvis (2) and foot (3/4) for SK1525B and here also, fibres and cloth were found in each of the samples. SK1525B was located at the North-West edge of Grave 4 (Figure 2:1) and was overlain by the lower parts of the remains of four other individuals. The final burial from Grave 4, SK1527B was sampled at the skull (1) and the pelvis (2). Both samples contained visible remains of organic material with the skull (1) position containing hair and cloth and the pelvis (2) containing fibres and what was believed to be a leathery material. An additional sample, a black sock (A1) and the

surrounding soil, was sampled from SK1527B. The high levels of textiles associated with the remains may be due to the presence of uniforms.

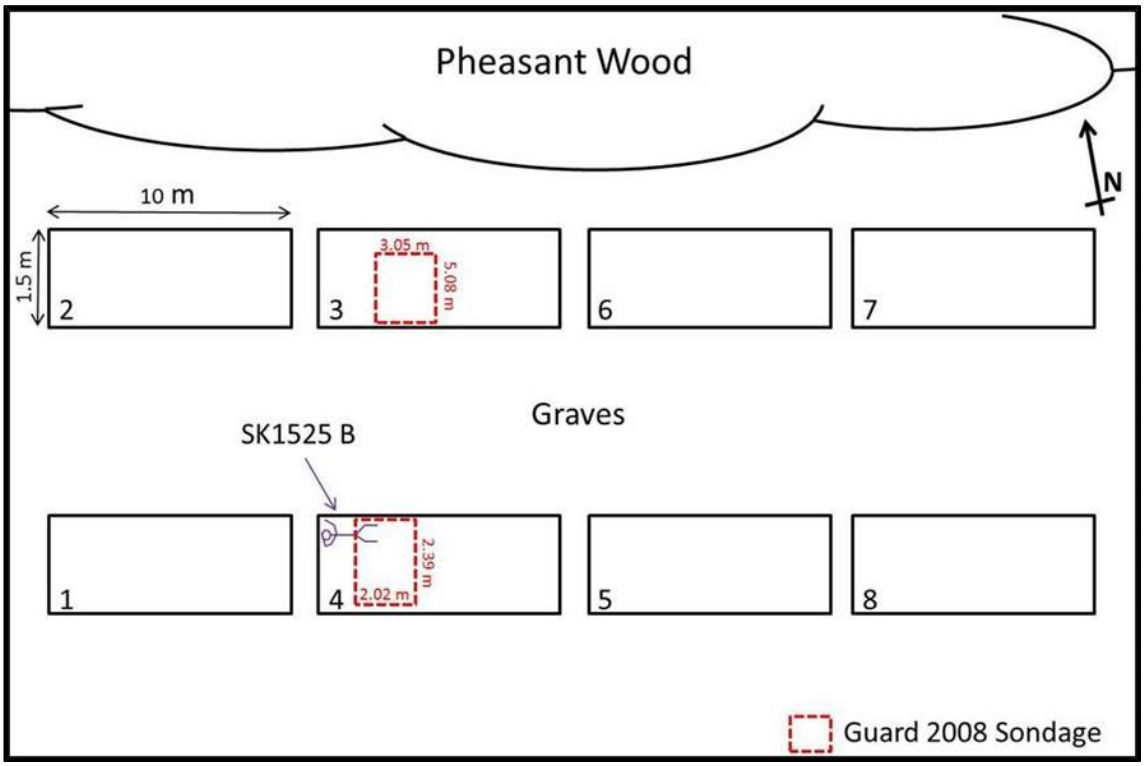


Figure 2:1. Diagrammatic representation of the layout of all eight pits situated at Pheasant Wood, Fromelles. Skeleton position for SK1525B and GUARD 2008 sondages are shown for Graves 3 and Grave 4.

The conditions of the remains noted during the original Guard test dig in 2008 (Pollard, 2008) reflected more extensive degradation in Grave 3 than in Grave 4. This was attributed, at least in part, to the presence of roots from the nearby wood. Excavation of Grave 4 at this time showed good levels of preservation of the remains and constant waterlogging at depths even after periods of dry weather. It is notable that the excavation of the sondage exposed the lower half of SK1525B (Figure 2:1), with a black sock and booted foot being unearthed.

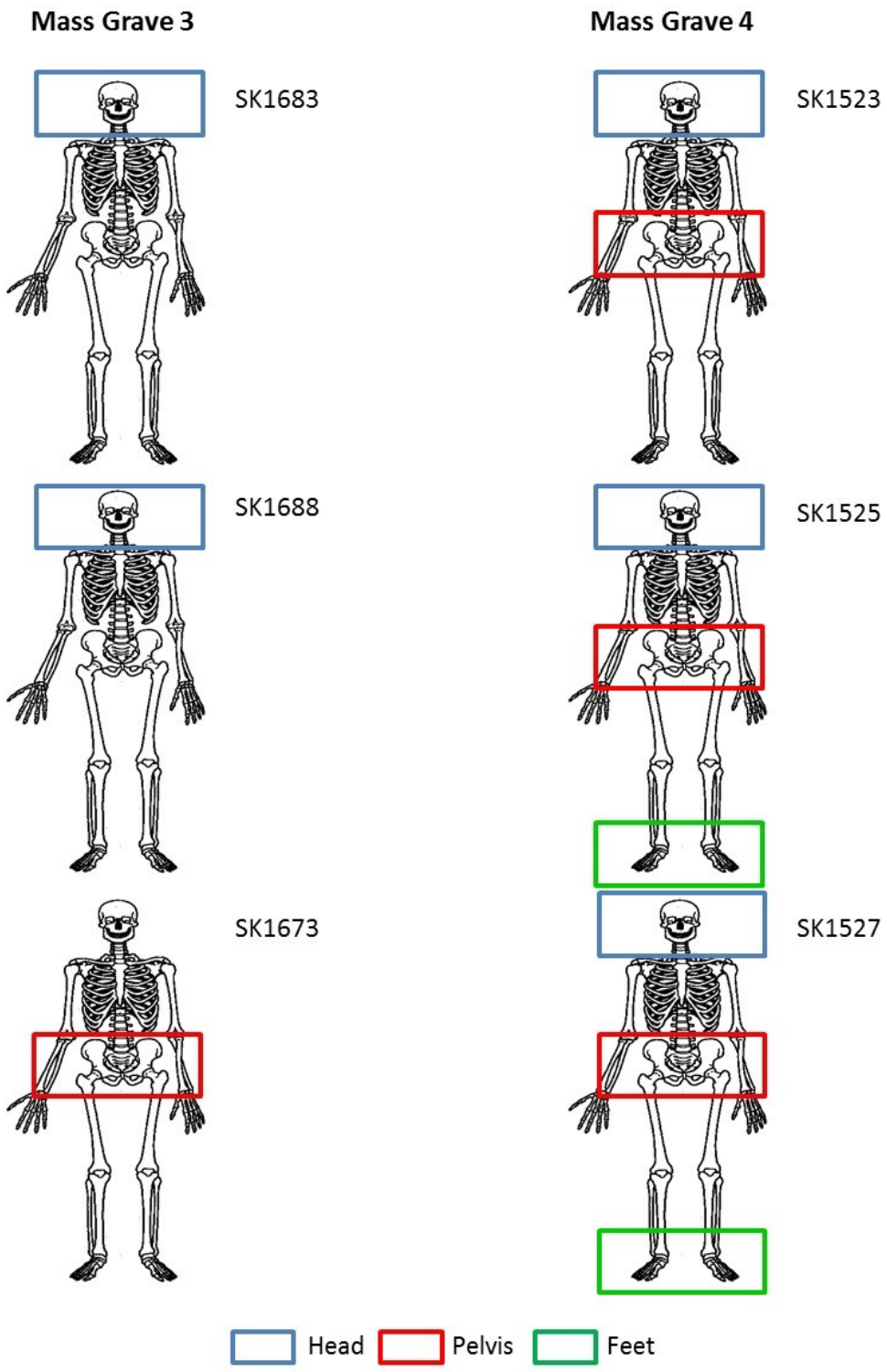


Figure 2:2. Sampled regions for skeletal remains from the two mass graves excavated at Pheasant Wood, Fromelles, France.

Table 2.1. Sample information (location, type and sample number) for mass graves at Fromelles, France.

Grave	Skeletal number	Location	InterArChive sample number	Sample type
3	SK1673	Pelvis	2	Soil
	SK1683	Skull	1	Soil
	SK1688	Skull	1	Soil
4	SK1523B	Skull	1	Soil
	SK1523B	Pelvis	2a	Soil
	SK1523B	Pelvis	2b	Soil
	SK1523B	Skull	A1	Hair
	SK1523B	Pelvis	A2	Textile
	SK1525B	Skull	1	Soil
	SK1525B	Pelvis	2	Soil
	SK1525B	Feet	3/4	Soil
	SK1525B	Skull	A1	Textile
	SK1525B	Skull	A2	Fibres
	SK1525B	Skull	A3	Hair
	SK1525B	Pelvis	A4	Textile
	SK1525B	Pelvis	A5	Fibres
	SK1525B	Feet	A6	Fibres
	SK1527B	Skull	1	Soil
	SK1527B	Pelvis	2	Soil
	SK1527B	Feet	A1	Black sock
	SK1527B	Feet	A2	Soil associated with sock
	SK1527B	Skull	A3	Textile
	SK1527B	Skull	A4	Hair
	SK1527B	Pelvis	A5	Textile
	SK1527B	Pelvis	A6	Fibres
3	SK1750	Feet	A1	Red sock
3	Quicklime?	-	A1	Soil
3	White nodule	-	A2	Soil

2.2.2 Sampling and site description, Mechelen, late 19th century mass grave

The cemetery of St. Rombouts Cathedral situated in Mechelen, Belgium, is believed to have been in use since the 1200's. Excavation of the archaeology at the site was undertaken due to plans for the construction of an underground car park. The grave described here was a mass grave, containing roughly 40 individuals buried in a rectangular pit (2.28 m x 3.16 m) that cut through earlier burials. Analysis of finds and intact textiles dated the grave to the late 18th century. Church records detail the burial of 41 rebels in 1798, their deaths being linked to the rebellion ('Boerenkrijg' or peasants war) against French occupation in various parts of Flanders and

Brabant. Mechelen, one of the scenes of resistance on the 23rd October 1798 (after the rebellion had failed), saw the execution by firing squad of 41 rebel peasants or brigands at the base of the St. Rombouts Cathedral Tower (Depuydt, 2013).

The site exhibited three main layers within the soil. The bottom layer (natural soil), at a depth of 1-2 m below street level, consisted of yellow tertiary sand and anthropogenic layers of slightly loamy sand. Two distinct layers were evident in the latter, the deepest a light brownish layer and the uppermost a dark brown featureless layer. Overall the soil cover in the area is one of a sandy ridge in a wet and sandy area to the north of the river Dijle (Depuydt, 2013). The body positions within the mass grave suggest that little care was taken on placing the bodies in the graves. The textiles present are from remains of clothing, not shrouds (Depuydt, 2013) and their identification (Depuydt, 2013) revealed most of the material to be woollen in nature, some felted, and with some silk fragments present.

Four samples were taken across the grave at positions roughly aligned with the central position of the skeletons in the grave (Figure 2:3). A1 and A4 were taken at either end of the grave with A2 and A3 situated in the centre. Samples A1, A2 and A4 were taken near to pelvises and A3 near to a knee. In addition a series of textiles were sampled (A5-8) and, on processing of A3, a further textile sample was found (A9). A site control (C1) was taken for comparison from a planted area located centrally to the site.

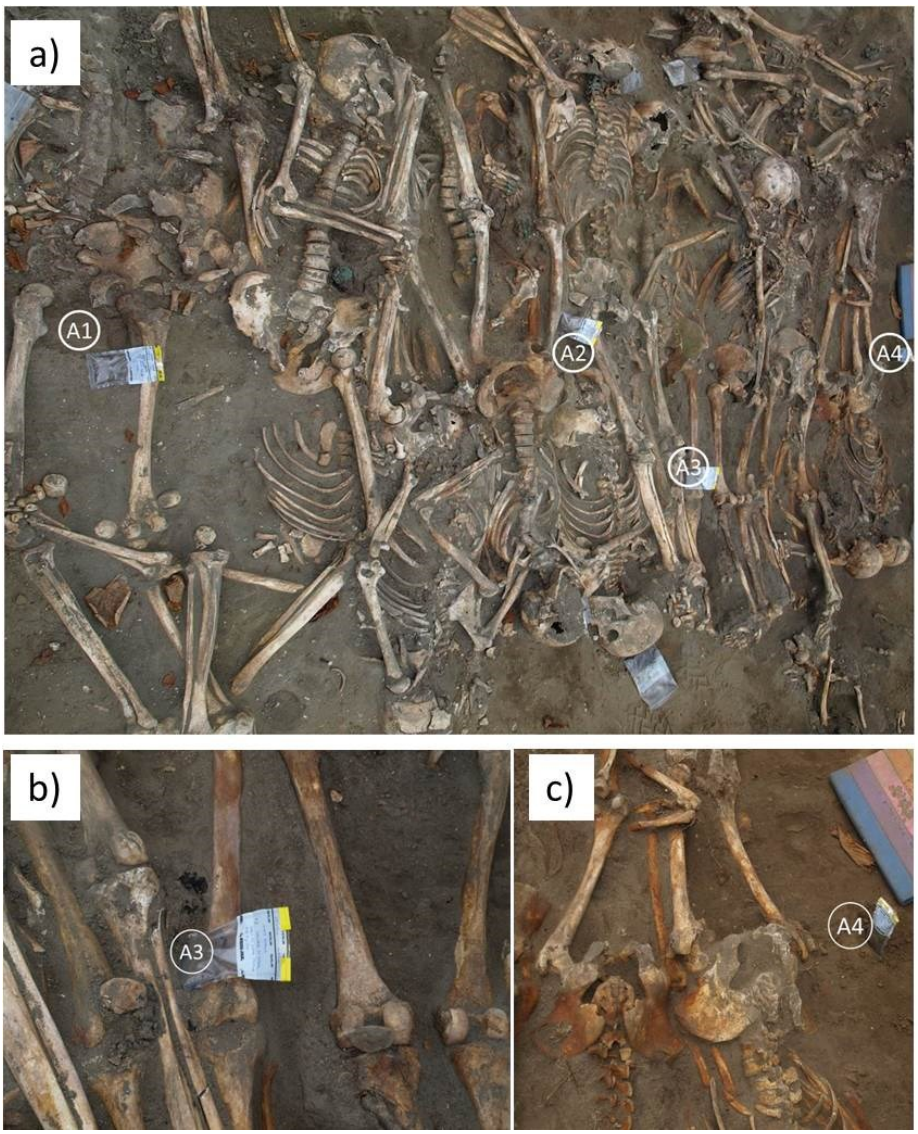


Figure 2:3. Sampling positions in the mass grave MESW G1651 situated in the graveyard of St. Rombouts Cathedral, Mechelen, Belgium. a) Sampling positions across the grave. b) Detail of sample A3 with small amount of black textile remains. c) Detail of sample A4 situated at the edge of the grave fill.

2.3 Results and discussion

2.3.1 Fromelles mass grave

2.3.1.1 Elemental analysis of soil samples

Elemental analysis (CHNS-O) and total organic carbon (TOC) (Table 2.2) was carried out on all soil samples and sub sampled materials. The recognition during excavation of a white chalky material, differing to the natural geology of the site, was interpreted to suggest that a liming agent was added to the grave during burial (Pollard, 2008, Loe, 2014). Micromorphological observation and SEM analysis showed the presence of microscopic accumulations of gypsum crystals in the grave soils (Lang, 2014). The high sulfur contents of some of the soils suggest the addition of material to the grave on burial as the sulfur content of soils is usually 0.00 – 0.05 %. Two samples

representing accumulations of the white chalky material, thought to be the remains of quicklime, were analysed for their elemental composition (Grave 3 A1 and A2; Table 2.2). The absence of sulfur in both samples rules out gypsum ($CaSO_4.2H_2O$) and the ratio of inorganic carbon (2.91 % A1, 4.69 % A2) to oxygen for the A1 and the white nodule (A2) were 1:4 and 1:3, respectively. This ratio suggests the samples are limestone ($CaCO_3$) or impure limestone in the case of the white nodule (A2). The presence of limestone in the graves does not rule out the possibility that the original material added was quicklime: over time reaction with water and CO_2, can effect reversion of quicklime back to limestone (Schotsmans et al., 2012). The presence of the gypsum crystals ($CaSO_4.2H_2O$) could be explained by the conversion of quicklime (CaO) to gypsum via hydration ($Ca(OH)_2$) and subsequent reaction with H_2S/H_2SO_4 produced from the degradation of the organic remains (Hamilton, 1983).

Elemental analysis of the soils from Grave 3 shows varying degrees of total organic carbon (TOC) (Table 2.2). Values range between 0.26 % and 1.03 %, with >1.00 % indicating good preservation of organic matter. The TOC values for Grave 4 samples range from 0.39 % to 4.14 % and are generally higher than those seen for Grave 3. Pelvic samples from Grave 4 exhibit TOC values greater than those of the corresponding skull and feet samples. The higher levels associated with the pelvic area may reflect either greater levels of organic matter input due to organic matter from the gut or an increased level of preservation due to the development of more favourable conditions for preservation. The variations in organic matter content reflected by the TOC values in Grave 4 suggest differing degrees of preservation. Due to the nature of the grave, the factors effecting preservation are likely to include the positions of the remains within the graves, the proximity to other remains and the presence and nature of materials such as clothing.

Table 2.2. Sample masses and elemental abundances of carbon, nitrogen, sulfur and total organic carbon (TOC) in the loose soil samples and sub-samples from two mass graves at Fromelles.

Samples	Sample mass / mg		Elemental abundance / %					
	CHNS	TOC	Nitrogen	Carbon	Hydrogen	Sulfur	Oxygen	TOC
Grave 3								
SK1673 pelvis (2)	13.91	19.66	0.07	0.46	1.11	0.49	-	0.26
SK1683 skull (1)	14.40	12.60	0.18	2.23	1.09	0.00	-	1.03
SK1688 skull (1)	13.36	11.30	0.08	0.85	1.23	0.04	-	0.42
SK1750 red sock (A1)	13.61	-	5.49	18.85	4.85	1.45	12.80	-
Grave 4								
SK1523B skull (1)	16.26	16.87	0.28	0.57	1.07	0.30	-	0.39
SK1523B pelvis (2a)	14.04	11.32	0.37	1.02	0.99	0.46	-	0.55
SK1523B pelvis (2b)	12.71	11.97	0.44	1.30	1.11	0.39	-	0.89
SK1523B skull (hair, A1)	3.31	-	6.64	20.57	3.23	1.69	13.90	-
SK1523B pelvis (textile, A2)	9.17	-	2.56	12.97	1.91	0.46	11.26	-
SK1525B skull (1)	15.71	14.33	0.86	1.92	1.04	0.33	-	1.46
SK1525B pelvis (2)	13.05	13.94	1.79	6.71	1.30	0.17	-	4.14
SK1525B feet (3/4)	14.77	13.88	0.91	2.74	1.21	0.33	-	1.65
SK1525B skull (textile, A1)	9.97	-	6.95	20.20	3.37	1.55	11.16	-
SK1525B skull (fibres, A2)	4.42	-	2.62	17.71	2.58	0.00	14.97	-
SK1525B skull (hair, A3)	4.62	-	3.70	9.45	1.80	0.56	13.28	-
SK1525B pelvis (textile, A4)	5.51	-	9.39	30.37	5.05	1.43	18.74	-
SK1525B pelvis (fibres, A5)	2.10	-	15.25	38.78	5.20	0.00	21.16	-
SK1525B feet (fibres, A6)	1.80	-	6.85	29.91	4.59	0.61	21.57	-
SK1527B skull (1)	13.46	10.66	0.38	0.87	0.86	1.66	-	0.62
SK1527B pelvis (2)	13.26	10.71	0.94	3.32	1.49	2.05	-	2.14
SK1527B sock soil (A2)	14.07	13.63	0.32	1.62	1.24	3.21	-	0.79
SK1527B black sock (A1)	5.95	-	0.43	4.07	1.35	0.78	14.42	-
SK1527B skull (textile, A3)	13.28	-	0.76	3.80	0.76	1.62	20.82	-
SK1527B skull (hair, A4)	6.19	-	5.12	13.46	2.36	2.88	16.81	-
SK1527B pelvis (textile, A5)	3.25	-	19.08	55.85	11.60	4.62	15.12	-
SK1527B pelvis (fibres, A6)	10.98	-	4.34	16.13	2.46	2.17	17.70	-
Quicklime? (A1)	10.95	10.09	0.16	11.15	0.68	0.00	11.91	8.24
White nodule (A2)	15.48	14.14	0.01	11.28	0.07	0.00	12.74	6.59

Grave 4 samples exhibit appreciable nitrogen and sulfur content (Table 2.2). Apart from the soil samples from Grave 3 showing lower N/C ratios than those from Grave 4, discreet groupings are not evident among the data, with significant and non-systematic variations in N/C and S/C between samples from the same anatomical positions (Figure 2:4). The high N/C values maybe due to large quantities of protein degradation products from the body that have remained in close proximity to the remains, as a consequence of the low permeability of the clay rich soil, or to the presence of protein-based textiles in the samples. The finding of fibres during sample processing suggests that, at least in part, the high TOC, sulfur and nitrogen may result from the preservation of textile.

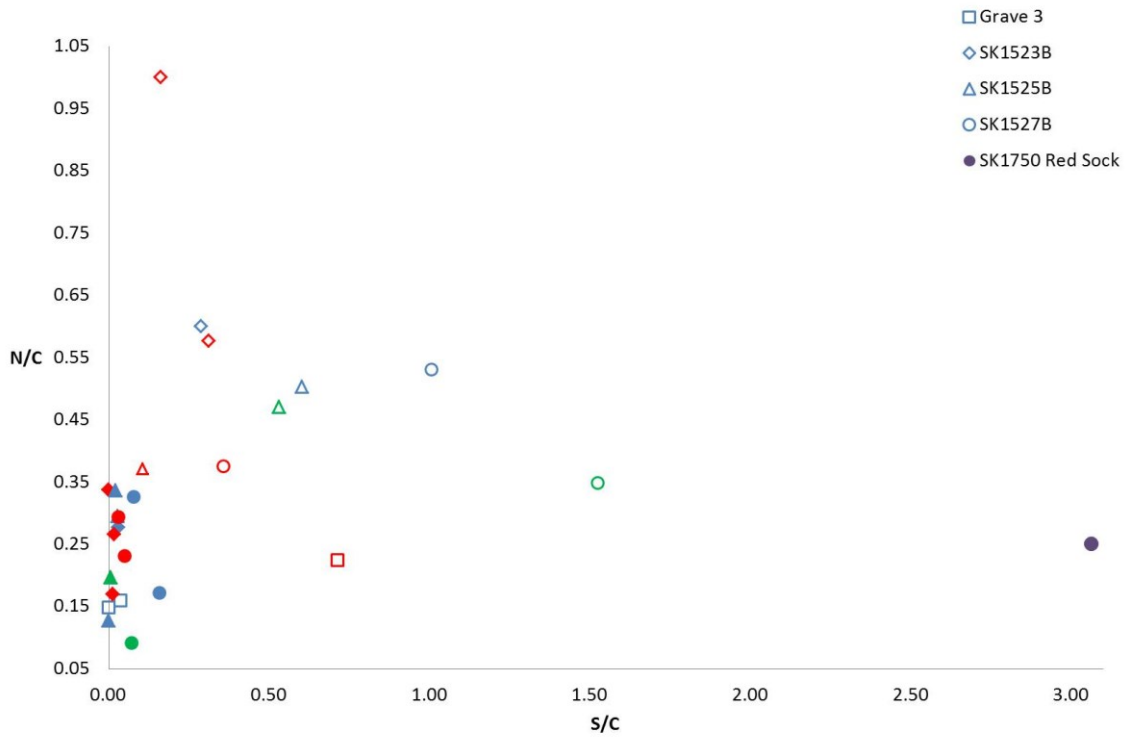


Figure 2:4. Scatter plot showing the atomic ratios of sulfur to carbon (S/C) versus nitrogen to carbon (N/C) for soil (open symbols) and textile samples (closed symbols) from Grave 3 and Grave 4. Positions are denoted using the colour coding from Figure 2:2.

2.3.1.2 Molecular profiles of soil extracts

Solvent extraction yielded differing quantities of extractable organic extract from each of the samples (Table 2.3) with the highest yields from individual remains coming from the pelvic areas, consistent with the high TOC levels for these positions. Gas chromatographic (GC) analysis of the hydrocarbon fractions revealed no significant differences between the distributions of the blank extract and those of the samples adjacent to the skeletal remains. The *n*-alkanes were in very low abundance with carbon chain lengths ranging from C_{21} to C_{35}. The low abundances of

hydrocarbons and similarities between samples and the blank sample suggest that these signals represent background. No aromatic hydrocarbons were detected.

Table 2.3. Masses of soil extracted and total solvent extractable organic matter yields of samples from Fromelles mass graves.

Samples	Dry mass of extracted soil / g	Mass of total extract / mg	Yield / mg g^{-1}
Grave 3			
SK1673 Pelvis (2)	1.98	4.46	2.25
SK1683 Skull (1)	1.93	2.28	1.18
SK1688 Skull (1)	3.98	1.93	0.48
Grave 4			
SK1523B Skull (1)	3.61	5.70	1.58
SK1523B Pelvis (2a)	3.89	15.54	3.99
SK1523B Pelvic Area (2b)	4.16	24.68	5.93
SK1525B Skull (1)	3.21	12.33	3.84
SK1525B Pelvis (2)	2.63	41.14	15.64
SK1525B Feet (3/4)	3.31	23.39	7.07
SK1527B Skull (1)	3.42	17.37	5.08
SK1527B Pelvis (2)	3.14	39.44	12.56
SK1527B Sock soil (A2)	4.06	5.79	1.43

A series of even carbon numbered *n*-alkanols with chain lengths between C_{16} and C_{30} were commonly detected in the medium polar fractions. The distributions for samples from Grave 3 show varying maxima: SK1673 at C_{28}, SK1683 at C_{26} and SK1688 at C_{24} (Figure 2:5). The differences in the maxima suggest different sources of *n*-alkanols at these three positions. The distributions of *n*-alkanols in the samples from Grave 4 also differ. SK1523B and SK1527B are dominated by C_{26} (Figure 2:5), typical of higher plant sources (Freeman and Collarusso, 2001). SK1525B shows C_{26} to be dominant for only the pelvis position with C_{24} representing the maximum for both the head and feet positions. The low molecular weight *n*-alkanols (C_{16}-C_{20}), present in most samples and of unquantifiable amounts, are possibly products of microbial degradation/reworking of fatty acids (Riendeau and Meighen, 1985).

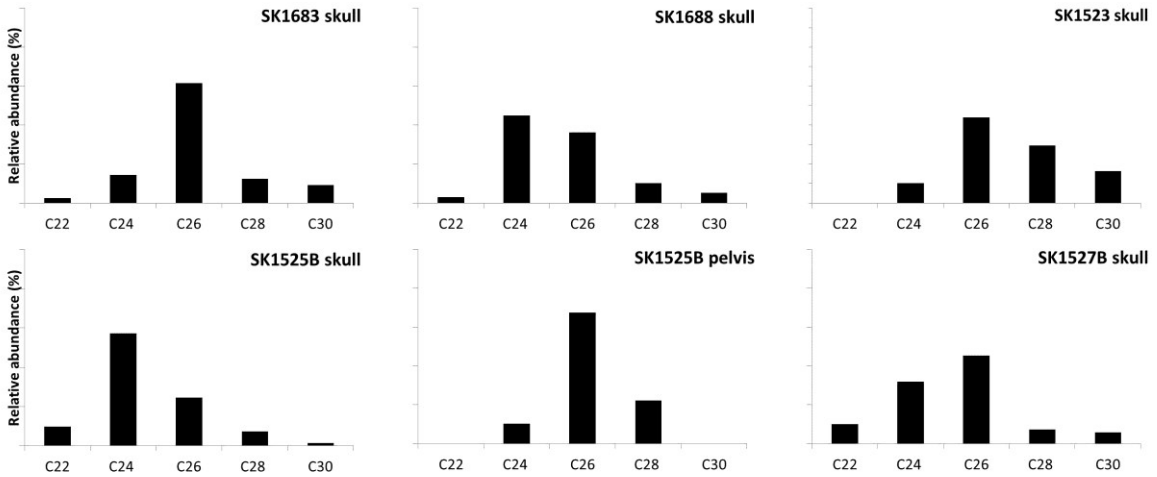


Figure 2:5. Relative abundances of C_{22} to C_{30} *n*-alkanols in samples from Grave 3 and Grave 4 at Fromelles illustrating the variations in the distributions within and between the graves.

The total extracts and the high polar fractions are both dominated by fatty acids, with components in the range C_{14}-C_{21} being more abundant than components in the range C_{22}-C_{30} (Figure 2:6). The soils around the skeletal remains from Grave 4 show similar distributions of fatty acids at each of the three anatomical positions sampled (head, pelvis, and feet). The highest abundance levels occurred in the pelvic samples except for the foot position for sample SK1525B which showed higher abundances than the pelvis sample for this inhumation. The pelvic samples from SK1525B and SK1527B had the highest TOC values whereas the foot sample from SK1525B exhibited a lower TOC value despite, revealing an apparently anomalously high degree of preservation of fatty acids (Table 2.2). The fatty acid distributions in the soils adjacent to the skeletal remains were dominated by $C_{16:0}$, with $C_{18:0}$ being the second most abundant component and with appreciable levels of $C_{14:0}$ being present. Notably, the SK1525B foot position yielded approximately equal abundances of $C_{14:0}$ and $C_{18:0}$, consistent with a lipid profile derived from adipose tissue. The proportions of $C_{14:0}$ fatty acids in bacteria (0.8 %), fungi (0.4 %) and plants (0.2 - 0.3 %) are substantially lower than for all of the burial soils (4.4 - 15.2 %), with the value expected for adipose tissue (≈ 8.0 %) being intermediate in the range (sources for fatty acid compositions: (Forbes et al., 2002, Forbes et al., 2005b, Zelles, 1997, Ruess et al., 2002)).

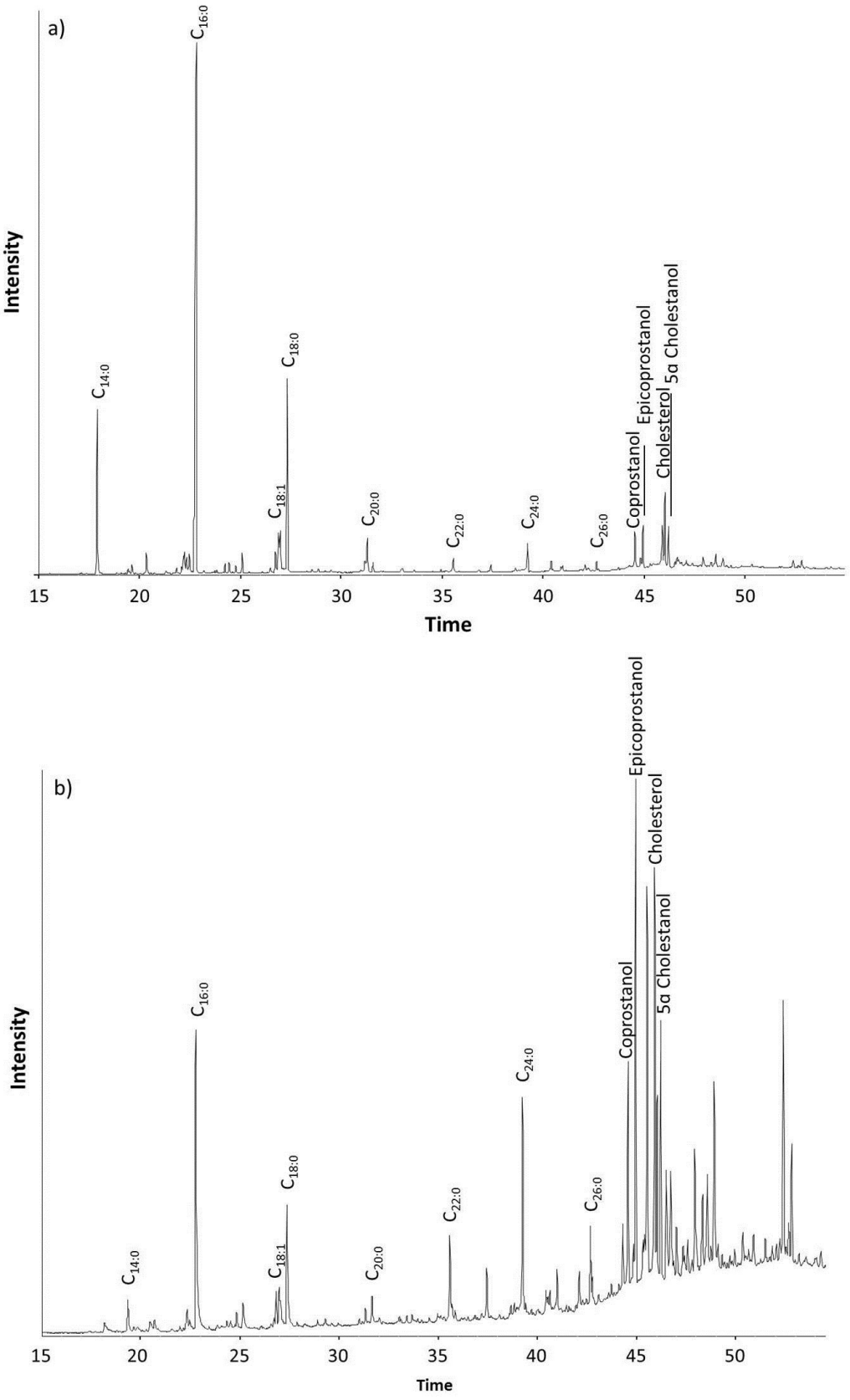


Figure 2:6. Partial gas chromatograms of the total extracts for selected samples from Grave 3 and Grave 4 at Fromelles: a) SK1527 B Feet and b) SK1688 Skull.

The samples from Grave 4 contained higher absolute abundances of the $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ saturated fatty acids than those from Grave 3 and $C_{14:0}$ was absent from the latter. Ratios of $C_{14:0}$:$C_{16:0}$:$C_{18:0}$ fatty acids show that the foot sample for SK1525B, closely matches the expected ratio for human skin tissue (1:4.2:1.5; (Boughton and Wheatley, 1959)), whereas the rest of the samples from Grave 4 show a closer relationship to the ratios expected for degraded human adipose tissue (1:5.5:1.2; (Fiedler et al., 2009)) and fresh adipose tissue (1:7.7:1.2; (Hodson et al., 2008) Table 2.4). Though the soil fatty acid distributions were dominated by $C_{16:0}$ with $C_{18:0}$ occurring in lower abundance, the unsaturated fatty acids, $C_{16:1}$ and $C_{18:1}$, were also identified as low abundance level compounds in Grave 4. Their presence could be attributed either to mammalian triacylglycerols, in which they are significant components (Regert, 2011), or to plant sources in which they occur in various abundance levels and proportions (Kimpe et al., 2004). The levels and proportions are most consistent with origins from adipose tissue. Thus, the unsaturated components in fresh adipose tissue are dominated by $C_{18:1}$, with $C_{18:2}$ also being significant (Notter et al., 2009, Hodson et al., 2008). The ratios of $C_{16:1}$ to $C_{18:1}$ in the soils are similar to those expected for degraded adipose tissue (Forbes et al., 2005b), and the absence of $C_{18:2}$ is also consistent with such an origin. Thus, the low levels of $C_{16:1}$, $C_{18:1}$ relative to $C_{16:0}$ and $C_{18:0}$, and an absence of $C_{18:2}$, suggest significant alteration/degradation in the grave environment with the abundance levels of the unsaturated compounds being preferentially reduced. Water movement and the different solubilities of the fatty acids may provide a possible explanation for the altered distribution. To enable comparison, the fatty acid abundances were normalised to $C_{18:0}$ (Figure 2:7) the component with the highest octanol: water partition coefficient (K_{ow}) and hence the least likely to be affected by water movement. The differences in relative abundances of the fatty acids to those of adipose tissue can be accounted for either by reduction to the saturated counterpart or loss caused by the movement of water through soil. The loss of $C_{14:0}$ (K_{ow} = 6.11 - 6.41) to groundwater is more favourable than for slightly longer fatty acids is, $C_{16:1}$ being the only fatty acid having lower K_{ow}. Thus the apparent loss of both $C_{14:0}$ and $C_{16:1}$ from the grave can be accounted for at least in part. By contrast, $C_{16:0}$ has a higher K_{ow} (7.17 – 7.38), making its removal less likely. The amounts relative abundances compared with the other fatty acids, in particular $C_{18:1}$ (K_{ow} = 7.45 – 7.64) and $C_{18:2}$ (K_{ow} = 6.82 – 7.05) are also consistent with differences in solubility. Losses of $C_{18:2}$ could be accounted for either by reduction or by water movement. The loss of $C_{18:1}$ suggests an additional mechanism for the loss affecting the distribution. Lalman and Bageley (2001), through the study of anaerobic microorganisms (including methanogens) showed the preferential formation of $C_{16:0}$ from the alteration and degradation of $C_{18:1}$. Their studies showed the conversion of $C_{18:1}$ directly to $C_{16:0}$/$C_{16:1}$ without formation/reduction to $C_{18:0}$.

The hypothesis proposed by Lalman and Bagley (2001) is supported by further studies carried out that showed microbial alteration of $C_{18:2}$, as not only low levels of $C_{18:1}$ were found but $C_{16:1}$ also. The formation of $C_{16:1}$ was suggested to occur by β-oxidation with the reduction of the double bond occurring in situ/after β-oxidation (Lalman and Bagley, 2000, Lalman and Bagley, 2001). The methods of degradation proposed by Lalman and Bagley (2001) are supported by the free energies involved in the alteration of the molecules, with routes involving the direct β-oxidation of unsaturated components being, the most favourable (Lalman and Bagley, 2001). Comparison of the distributional differences between fresh adipose tissue and the degraded remains reflects an increase in $C_{16:0}$ that corresponds directly to the decrease in $C_{18:1}$ (Figure 2:8). This change, along with a similar distributional change in $C_{16:1}$ to $C_{14:0}$, supports the suggestion that β-oxidation alter the distributions with alteration of $C_{18:1}$ leading directly to the elevated levels of $C_{16:0}$ in the grave (Pereira et al., 2005, Novak and Carlson, 1970, Lalman and Bagley, 2001).

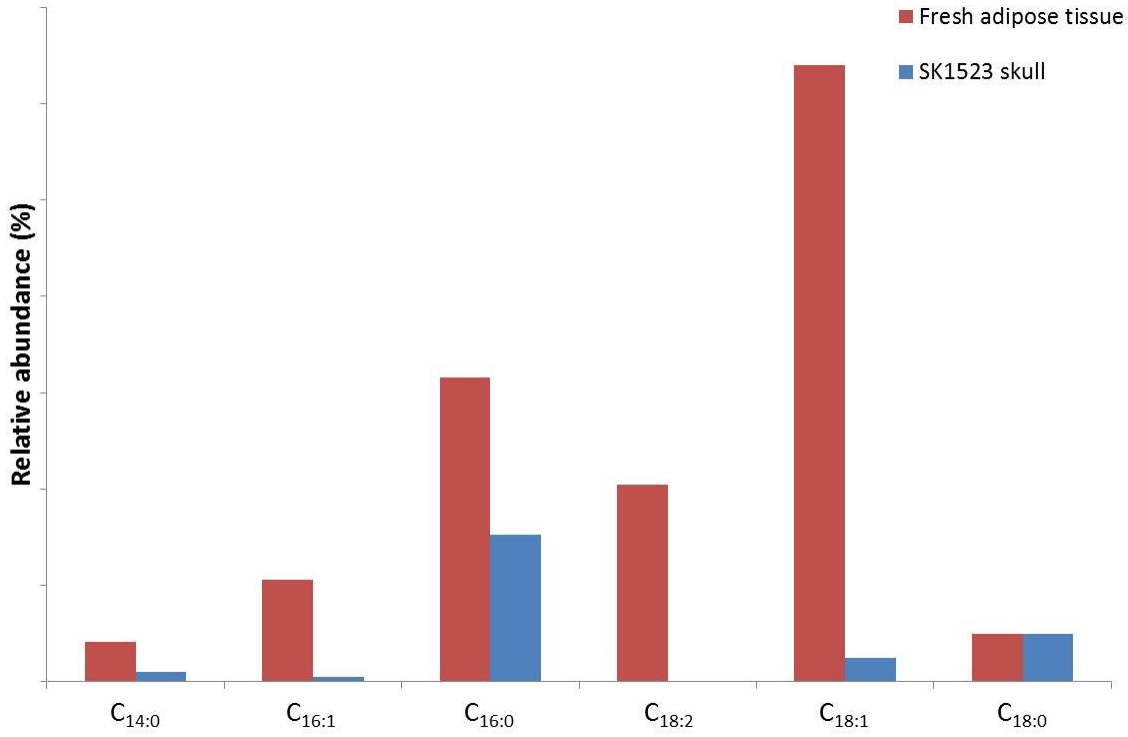


Figure 2:7. Relative abundances normalised to $C_{18:0}$, of selected fatty acids for fresh adipose tissue and SK1523 skull, showing the shift in abundances seen on degradation.

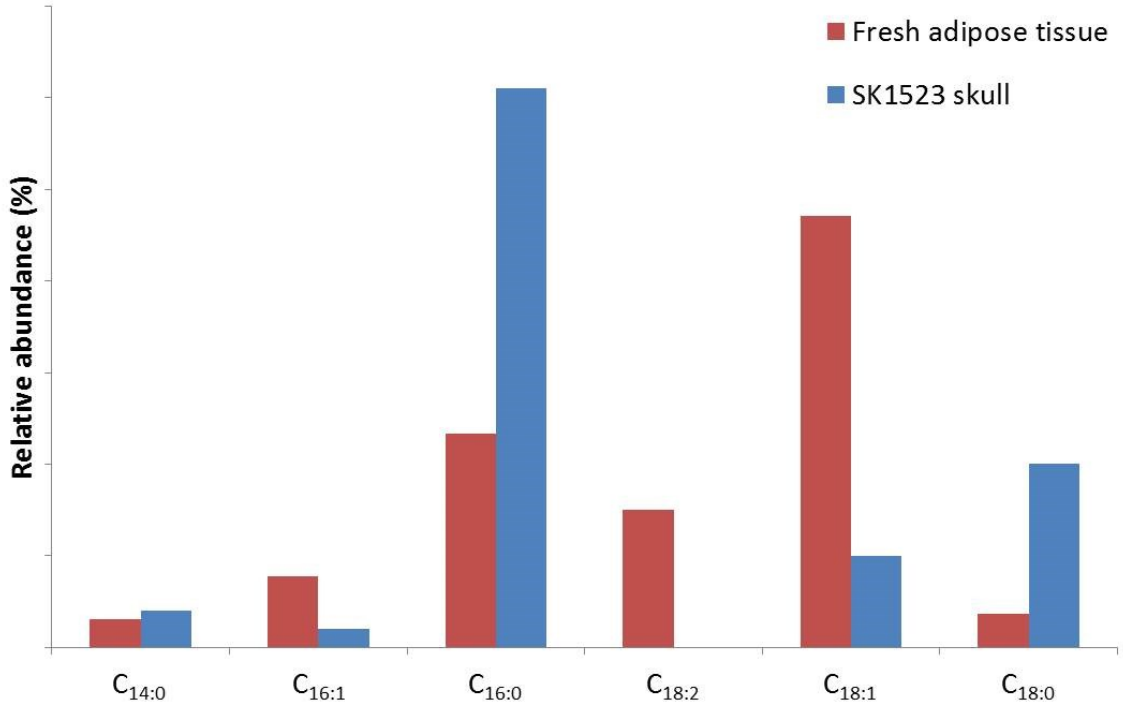


Figure 2:8. Relative abundances of selected fatty acids for fresh adipose tissue and SK1523 skull, showing the shift in abundances seen on degradation.

The difference between the fatty acid distribution of the soil collected adjacent to the foot of SK1525B (greater abundance and $C_{14:0}$:$C_{16:0}$:$C_{18:0}$ ratio) and the rest of Grave 4, especially other SK1525B profiles, may be due to the previous GUARD dig in 2008 exposing the remains. The unearthing of the remains would have affected the movement of water in the grave and may have introduced lipids bound to fine clay material washed in from the other soils to this location. In addition, the open grave environment would have led to increased levels of oxygen, possibly enhancing microbial activity and degradation (Carter et al., 2007).

The abundances of unsaturated relative to saturated fatty acids are similar to those reported from waterlogged environments (Forbes et. al., 2002). Several of the soil samples contained C_{18} 10-hydroxy fatty acid, a product of microbial hydration of the double bond of $C_{18:1}$ that has been observed previously in burial soils (Forbes et al., 2002). The distributions of C_{14}-C_{18} fatty acids and C_{18} 10-hydroxy fatty acids are also similar to those reported for the degradation products of adipose tissue in a wet environment (Forbes et al., 2002) (Table 2.4).

Table 2.4. Percentage contents of fatty acids in Fromelles samples and modern experimental samples taken from Forbes et.al. (2002)†.

Sample	$C_{14:0}$ / %	$C_{16:0}$ / %	$C_{18:0}$ / %	$C_{16:1}$ / %	$C_{18:1}$ / %	$C_{18:0}$ 10-OH / %	$C_{14:0}$ / $C_{16:0}$	$C_{16:0}$ / $C_{18:0}$	C_{14}:C_{16}:C_{18}
Grave 3									
SK1673 Head	0	64	20	10	6	1	0	3.20	0:3.2:1
SK1683 Pelvis	0	60	20	7	13	0	0	3.00	0:3:2
SK1688 Pelvis	0	62	21	5	12	0	0	2.90	0:2.9:1
Grave 4									
SK1523 Head	4	61	20	2	10	3	0.07	3.05	1:15.3:5
SK1523 Pelvis	8	60	20	0	9	3	0.13	3.00	1:7.5:2.5
SK1523 Pelvis	9	55	24	1	7	4	0.16	2.33	1:6.2:2.7
SK1525 Head	8	59	22	1	9	1	0.14	2.68	1:7.4:2.8
SK1525 Pelvis	10	53	24	0	11	2	0.19	2.21	1:5.3:2.4
SK1525 Foot	15	59	15	2	8	1	0.25	3.93	1:3.9:1
SK1527 Head	7	58	21	1	10	3	0.12	2.76	1:8.3:3
SK1527 Pelvis	8	53	26	1	9	3	0.15	2.08	1:6.8:3.3
SK1527 Feet	12	61	15	3	8	1	0.20	4.07	1:5.1:1.3
Forbes Wet Environment									
13 years	9	71	9	1	4	7	0.13	7.89	1:7.9:1
22 years	4	84	7	1	1	4	0.05	12.00	1:21:1.8
26 years	14	71	6	2	6	1	0.20	11.83	2.3:11.8:1

† Data obtained from original paper equate to values greater than 100 %

The presence in the soil extracts of *iso* and *anteiso* C_{15} and C_{17} branched chain fatty acids, uncommon components of organisms other than bacteria, is indicative of bacterial input/reworking in and around the burial (Zelles, 1999). The presence of these particular branched fatty acids infers gram positive bacteria or gram negative sulfate reducing bacteria to be

the most likely organisms (Zelles, 1999). The presence of substantial concentrations of sulfur in the grave soils suggests the latter to be the most likely group. A series of ω-hydroxy fatty acids ranging from C_{22} to C_{26} in the soils are also likely products of microbial activity formed during degradation of plant material: the C_{22}–C_{28} ω-hydroxy fatty acids are the major components of suberin, a plant biopolymer found in the roots and outer surfaces of woody plants (Bull et al., 2000a).

Long chain (>C_{22}) fatty acids occurred in higher abundances in Grave 4 than in Grave 3, though in much lower abundance levels than the short chain components. This difference in relative abundance was less pronounced in Grave 3. Two components, $C_{24:0}$ and $C_{28:0}$, dominated the profiles of >C_{22} fatty acids. The $C_{24:0}$ acid was the most abundant of these components in the soils from the pelvic areas of SK1523B and SK1527B, possible sources being human tissue (skin) and plant matter (Poulos, 1995, Bull et al., 2000b, Buckley et al., 1999). The $C_{28:0}$ component, dominant around the foot position for SK1525B and SK1527B and skulls of SK1523B and SK1525B, is indicative of plant matter (Eglinton and Hamilton, 1967). The differing abundances show better preservation of both short and long chain fatty acids in Grave 4 than in Grave 3. The greater prevalence of the long chain fatty acids in Grave 3 suggests that the short chain fatty acids derived from the degradation of human remains were more extensively degraded than in Grave 4 and the background soil signatures.

The presence of fatty acids in the soils prompted their examination for triacylglycerols (TAGs). Low abundance level TAG distributions, typical of the degraded adipose tissues of mummies, were determined (Ruizgutierrez et al., 1992, Mayer et al., 1997) together with short chain TAGs. Although definitive identification of the short chain TAGs was not possible due to the large number of possibilities their presence suggests mid-chain cleavage of the fatty acids (Giuffrida et al., 2004).

Two plant based sterols β-sitosterol and stigmastanol (Bull et al., 2000b), were identified together with cholesterol and the degradation products cholest-3-one, 5β-cholestanol (coprostanol) and 5α-cholestanol in all of the samples except SK1673, which only contained coprostanol and epicoprostanol (Table 2.5). The presence of coprostanol, which forms only in the mammalian gut (outside the body, degradation proceeds to 5α-cholestanol (Bethell et al., 1994b)), suggests either the presence of faecal matter and therefore the opportunity to determine stomach contents, or that gut bacteria were active in the degradation of the human remains (Dent et al., 2004, Fiedler and Graw, 2003). Notably, however, coprostanol signatures in the soil were not localised exclusively to the pelvic areas. Coprostanol / (5α-cholestanol + coprostanol) ratios greater than

0.7 provide an indication of faecal contamination (Grimalt et al., 1990). The presence of epicoprostanol, a product of anaerobic microbial reworking of faecal matter (Bull et al., 2001), in appreciable amounts in the soils suggested that a modification to the calculation was required (c.f. Bull et.al., 2001) as the original equation was created for modern sewage. The modified equation, allowing for the reworking of faecal material (coprostanol + epicoprostanol) / (5α-cholestanol + coprostanol + epicoprostanol), gave larger values than the original equation, confirming that microbial reworking of coprostanol had occurred and that the grave environment was anaerobic. The inference that the environment was anoxic is further supported by the presence of stanols, which form *via* microbial reworking under anoxic conditions.

The recognition of steroidal components, in particular coprostanol, in the soils prompted further analysis for the presence of bile acids. Acidic cyclic fractions from SK1523B, SK1527B (head and pelvis) and SK1525B (pelvis) were analysed. The head positions contained low levels of lithocholic acid whereas the pelvis samples contained large quantities of lithocholic acid, a bile acid marker formed from bile acids *via* alteration by intestinal bacteria (Bull et al., 2002). Pelvis samples also contained one of the major bile acids of humans, chenodeoxycholic acid (Bull et al., 2002). In addition, low levels of hyocholic acid, a bile acid common to mammals (Bull et al., 2002), were present in the SK1527B pelvis sample. As the reduction of cholesterol to coprostanol is performed by gut bacteria (outside this environment, degradation proceeds to 5β-cholestanol) (Bethell et al., 1994b) and the bile acid signatures are not localised to the pelvic area it can be inferred that the signals for coprostanol most likely come from the movement of gut derived organic matter within the grave. Hence, given that the burial comprises a mass grave and evidence of faecal material occurs throughout the grave, the data requires careful interpretation if they are to be used to infer the gut contents of individuals.

Table 2.5. Steroidal and bile acid components from Fromelles soil samples, along with the retention times and mass fragments. Molecular ion shown in bold.

Component	Fragment ions / *m/z* (alcohols = TMS derivative, acids = methyl esters)
Cholesterol	**458**, 443, 368, 353, 329, 247, 129, 107, 75
Coprostanol	**460**, 455, 403, 370, 355, 257, 107, 75
Epicoprostanol	**460**, 445, 403, 370, 355, 257, 215, 107, 75
5α-cholestanol	**460**, 445, 403, 370, 355, 305, 230, 215, 107, 75
Cholest-3-one	**386**, 316, 231, 217, 81, 55
Lithocholic acid	**462,** 390, 372, 357, 341, 318, 257, 230, 215, 95, 81, 57
Hyocholic acid	**638,** 422**,** 408, 390, 372, 354, 339, 280, 229, 211, 107, 95, 81, 67, 55
Chenodeoxycholic acid	**550**, 478, 406, 392, 388, 370, 355, 339, 273, 255, 213, 145, 107, 93, 81, 55
B-sitosterol	**486**, 471, 396, 381, 357, 275, 129, 73, 57
Stigmastanol	**488**, 473, 431, 398, 383, 305, 215, 147, 107, 75, 57

The ratio of plant sterols to cholesterol and its derivatives can provide an indication of the gut contents at the time of death (Table 2.6). Compared with values from modern stool samples collected from subjects on high and low cholesterol diets (Lin and Connor, 2001), those from Fromelles are closest to those of modern Americans on a high cholesterol diet, indicating a significant intake of animal products. This finding should be treated with caution as the potential for the spread of faecal material and the nature of the grave significantly differ from the coprolites analysed by Lin (2001). The higher ratio at the pelvic regions and the small difference to the other positions suggests that the diet/last meal is consistent with that suggested with little alteration caused by surrounding bodies.

Table 2.6. Ratio of cholesterol and its degradation products to total plant sterols for the samples from Fromelles and modern samples from both high and low cholesterol diets (HC and LC, respectively; data for modern samples from Lin and Connor 2001).

Sample	Plant sterol : cholesterol
SK1673 Pelvis	0.27
SK1683 Head	0.11
SK1688 Head	0.03
SK1523 Head	0.13
SK1523 Pelvis	0.11
SK1523 Pelvic area	0.18
SK1525 Head	0.24
SK1525 Pelvis	0.25
SK1525 Foot	0.26
SK1527 Head	0.24
SK1527 Pelvis	0.22
SK1527 Feet	0.26
Tarahumra Indians LC diet (1983)	0.98 ± 0.19
Tarahumra Indians HC diet (1983)	0.48 ± 0.15
Americans LC diet (1983)	0.50 ± 0.20
Americans HC diet (1983)	0.20 ± 0.09

2.3.1.3 Elemental and molecular profiles of isolated materials

All of the textiles and fibres recovered from the graves were subjected to elemental analysis, to determine element abundance (Table 2.2). The sample of a red sock (A1) from SK1750 shows high carbon content, as expected from organic fibres, and an N/C ratio of 0.25, consistent with the material being proteinaceous. This, along with the value obtained for the O/C ratio (O/C ratio for wool = 0.33 - 0.38) and the elemental sulfur content, (1.45 %; Table 2.2), suggests that the

material is most likely wool-based (wool 3-4 %; (Zahn et al., 1997)). Fibres isolated from the soil samples from SK1523B, SK1525B and SK1527B all showed N/C ratios consistent with that expected for materials of a proteinaceous nature (Figure 2:4). Higher levels of sulfur and nitrogen were determined for the textiles than the corresponding soils (Table 2.2), consistent with the elevated concentrations of N and C in the soils being due to decomposition of textile. Hair samples obtained proximal to the skulls of individuals from Grave 4 show higher levels of both nitrogen and sulfur than any of the soil samples. The N/C ratios range from 0.28 - 0.34, corresponding to what would be expected in human hair (modern human hair measured N/C ratio 0.26 - 0.33, values in paper expressed as C/N (O'Connell et al., 2001, O'Connell and Hedges, 1999)). The crossplot of S/C versus N/C values reveals a tighter clustering of the textiles, fibres and hair than the soils (Figure 2:4). The compositional similarities confirm similar origins of the textiles and, given the context of the burials, it is likely that they are from woollen uniforms. Further analysis of amino acid compositions of the textiles gave results consistent with woollen textile (Pinder, 2017).

Unlike the soil extracts, the textile extracts contained fatty acids ranging from $C_{16:0}$-$C_{30:0}$, with an absence of $C_{14:0}$ and $C_{16:1}$. The skull and pelvis of SK1525B show similar distributions of the fatty acids for both textiles and corresponding soils. The sock from the foot of SK1525B showed a different fatty acid distribution to that of the surrounding soil and the other textiles, with $C_{18:1}$ more abundant than $C_{18:0}$. Interestingly, short chain fatty acids extracted from the textile were in low abundance compared to the soils around the foot, which had the highest abundances of LMW fatty acids of the entire sample set. The difference in abundance and distribution may suggest that the high fatty acid content of the soil at this position relates to the sock acting as a barrier to the movement of the fine clay particles to which the lipid fraction is absorbed (Clemente et al., 2011). The dominance of $C_{18:1}$ over $C_{18:0}$ in the extract from the SK1525B sock could suggest that the fatty acids associated with the textiles are more indicative of adipose tissue and that the matrix of the textile has enhanced the protection of the original signatures.

The other textile samples isolated from the soils of Grave 4 show low abundances of fatty acids, with distributions differing from the corresponding soils only in the absence of the short chain ($C_{14:0}$ and $C_{16:1}$) fatty acids. Given that amino acid analysis (Pinder, 2017) also gave results indicating the textiles to be wool, the ratio of $C_{18:0}/C_{18:1}$ was examined to reveal the possible origin of the wool fibres (Korner et al., 1992). Fibre samples from Grave 4 show similar proportions of $C_{18:0}$ to $C_{18:1}$ fatty acids to those of modern wools from Australia (Table 2.7). The difference in the value for the sock from SK1525B, which was exposed by excavation of the exploratory sondage,

may be due to degradation, absorption of components from the surrounding soil/body or a different original source. The red sock from SK1750 shows a similar proportion of $C_{18:0}$ to $C_{18:1}$ fatty acids to British wool, possibly suggesting a different source for the wool to that of the other textiles. This proxy however may be affected by both absorption of fatty acids from the remains and alteration of the saturated components. The use of both carbon and hydrogen stable isotope analyses could allow for clearer identification of the sources of the wool.

Table 2.7. Ratio of $C_{18:0}/C_{18:1}$ for wools of various origin and textile samples from Grave 4. (reference data from Korner et al. 1992).

Sample	$C_{18:0}/C_{18:1}$
Australia	1.87-2.18
Brazil	3.03
Britain	1.56
Uruguay	2.70
SK1750 Red sock	1.59
SK1523 B Pelvic area textile	-
SK1525 B Skull textile	1.93
SK1525 B Pelvis textile	2.46
SK1525 B Foot sock	0.77
SK1527 B Skull textile	-
SK1527 B Feet sock	1.93

Analysis of the red sock (SK1750) yielded no extractable dyestuff. SEM and amino acid analysis showed that the fibres were heavily degraded, the internal structure having been removed (Pinder, 2017). Further investigation by Pinder suggested the presence of a bacterial degrader, *Bacillus subtilis*, a wool degrader commonly known as 'pink rot' owing to it inducing a pink colouration in wool. Hence, the red colouration may be linked to degradation of the wool and not to the presence of a dye. GC-MS analysis of the extract from SK1750 red sock showed the presence of *iso* and *anteiso* C_{15} and C_{17} fatty acids, known bacterial markers (Zelles, 1999), adding further evidence of microbial degraders and lending support to Pinder's (unpublished) suggestion. As a soil sample was not collected this location, the origin of the *iso* and *anteiso* C_{15} and C_{17} fatty acid signatures cannot be confirmed and it is also possible that they represent components introduced from the adjacent soil.

Extraction and analysis of modern wool samples show that cholesterol is the major steroidal component. In order to assess the steroidal contribution attributable to the textiles steroids extracted from the textiles were normalised to plant based sterols and profiles from the surrounding soils were subtracted (Figure 2:9). Hence, the levels of cholesterol remaining after subtraction may possibly reflect the cholesterol content of the textiles. The high levels remaining imply that a contribution from the soil may also be present. The abundance levels of coprostanol, epicoprostanol and 5α-cholestanol remaining after subtraction may be attributable mainly to absorbed degradation products emanating from the soil, with the protection/pooling of these molecules occurring around the textiles as suggested for fatty acid distributions in SK1525B foot.

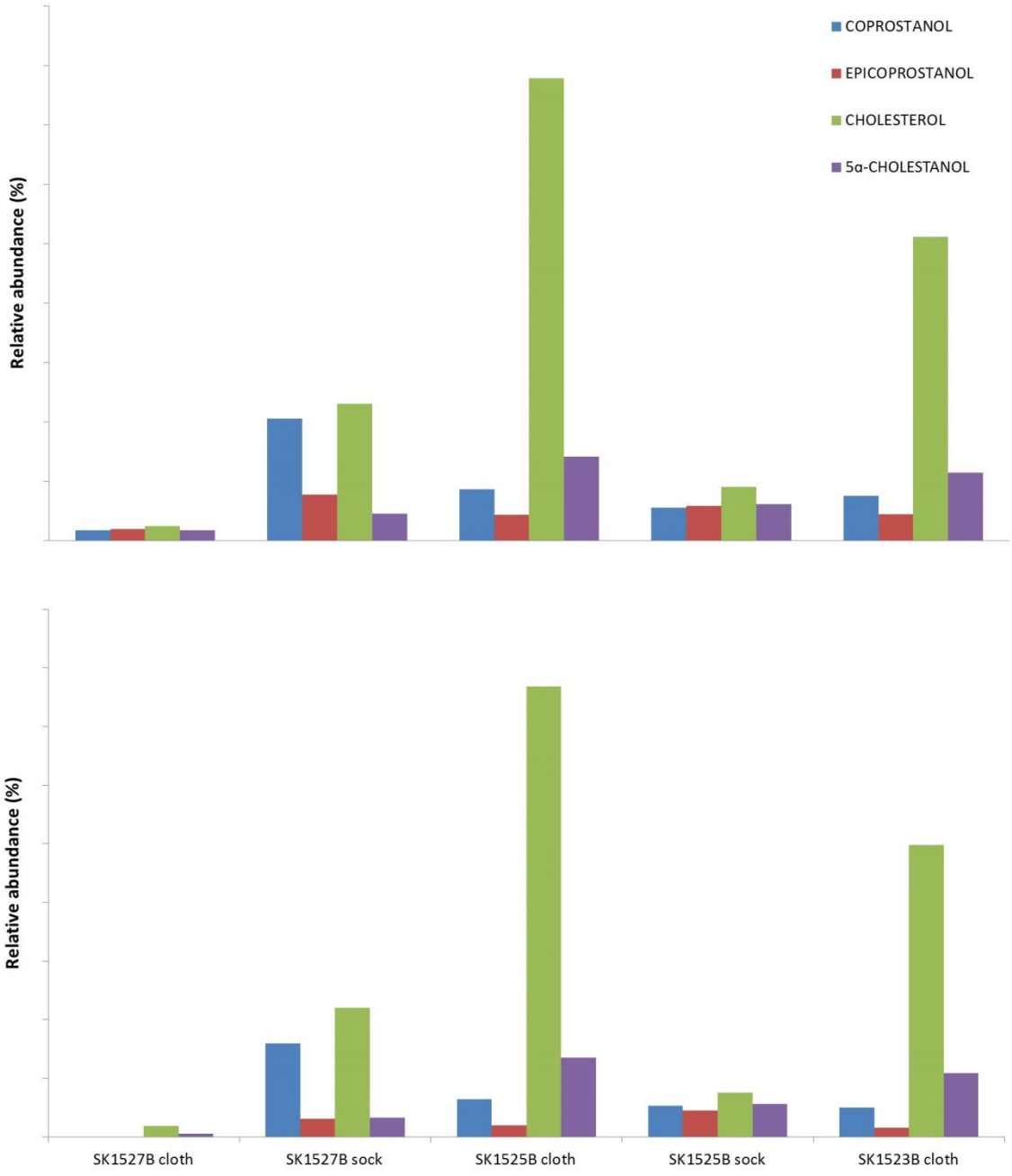
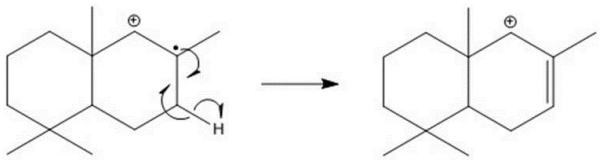


Figure 2:9. Cholesterol and degradation products from textile samples. Top: original abundances determined in the textile samples normalised to stigmastanol. Bottom: cholesterol and degradation products in textiles after the subtraction of the estimated input from the soils.

GC-MS analysis of textiles from the soil adjacent to the skull and pelvis of SK1525B both yielded appreciable levels of two compounds in similar relative abundance to each other. Both compounds gave good NIST library matches to natural product molecules, squalene and ambrein. Neither compound was present in any of the other textile samples from Grave 4, localising its occurrence to the head and pelvis of SK1525B. Squalene occurs in human skin (Evershed, 1993)

and a wide range of sources including marine animals such as sharks and whales (Bakes and Nichols, 1995, Lederer, 1949). Its presence would normally be attributed to contamination of the samples. Notably, however, the presence in the samples of ambrein (Figure 2:11), the main source of which is the sperm whale (Lederer, 1949), and the high abundance levels in the distributions suggest that the most likely source is whale oil. Further analysis of the wax esters and TAGs is required for secure identification.

Identification via MS and analysis of data by NIST gave a close match for ambrein suggesting a strong structural similarity (Figure 2:11). A weak molecular ion was observed at *m/z* 500, consistent with the TMS ether of ambrein (molecular mass 428). The absence of a loss of OH (17 Da) observed in the spectrum of ambrein, the loss from the molecular ion of 90 Da, to give *m/z* 410, and the ion at *m/z* 73 confirm the structure to contain a TMS ether (Diekman and Djerassi, 1967). Thus, the ions at *m/z* 386, 143 and 73 in the component in the soil extracts can be directly related to fragmentation with TMS present on the hydroxyl group. The ions at *m/z* 191 and 192 are consistent with a triterpenoid structure (Figure 2:10) with 191 caused by fragmentation and 192 by rearrangement. The MS is consistent with the TMS derivative of ambrein caused by the routine treatment of extracts with BSTFA prior to analysis.



m/z 192 *m/z* 191

Figure 2:10. Partial fragmentation mechanism showing the formation of the two common fragment ions at *m/z* 192 and 191 in ambrein.

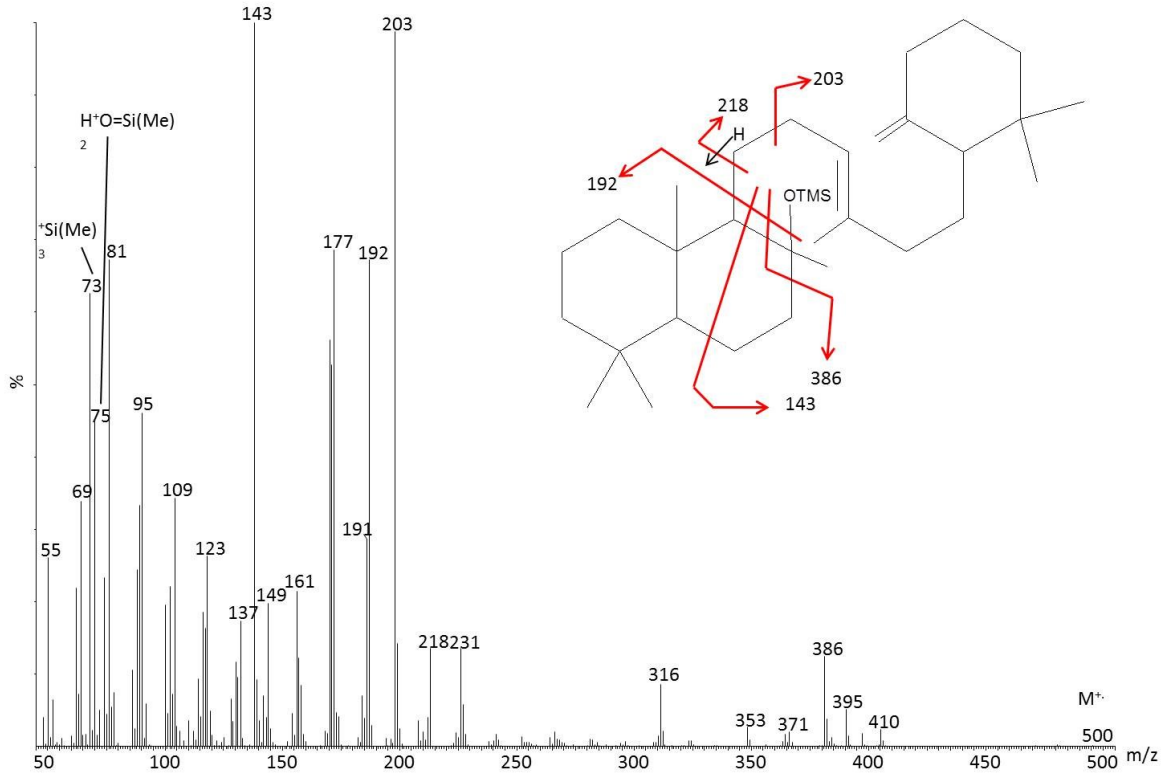


Figure 2:11. Mass spectrum of the trimethylsilyl ether of ambrein in the extract of the soil adjacent to SK1525B skull and pelvis. Arrows detail the generation of key fragments.

The tentative identification of whale oil in the samples promoted investigation of potential reasons for its presence on the battlefield. Research indicated that it was used by soldiers during the First World War to help prevent trench foot (Hughes, 1916), though its use was not very effective. The presence of these signatures in the textiles from the skull and pelvis, and not the fibres adjacent to the foot, may suggest that the oil had been added to the uniform for the purpose of waterproofing. Alternatively its presence may be linked to other sources such as a degraded rifle oil container in the grave. The lack of any signatures in the foot position may be linked to the effects of the exploratory excavation, with oxidation leading to degradation of the unsaturated squalene and ambrein.

2.3.2 Mechelen mass grave

2.3.2.1 Elemental analysis of soil samples

Elemental analysis of the soil samples from Mechelen revealed a range of values for both nitrogen (0.06 - 0.26 %) and total organic carbon content (0.81 – 1.38 %, Table 2.8), with values above 1.00 % TOC representing good preservation. No distinct differences were observed between the site control (C1) and the samples. Sample A3 showed the highest contents of nitrogen and oxygen, textile fragments present in the sample possibly accounting for the higher levels than were evident in the other samples. Another grave, MESW GR422 analysed by Green (2013), contained no nitrogen or sulfur and a low TOC (<0.70 %) with clear differences being apparent between the controls (C2 and C3) and samples. The higher levels of TOC and nitrogen in the soils from GR1651 suggest a higher level of organic matter preservation, suggesting that the particular conditions in the grave may have aided preservation, leading to the retention of nitrogen based compounds (proteins or amino acids).

Table 2.8. Bulk elemental nitrogen, carbon, hydrogen, sulfur and oxygen for grave soils and textile remains from Mechelen grave GR1651 and site control (C1) total organic carbon content for the former. Elemental data are given as weight percent.

Samples	Sample mass / mg		Elemental abundance / %					
	CHNS	TOC	Nitrogen	Carbon	Hydrogen	Sulfur	Oxygen	TOC
Site control (C1)	9.81	13.01	0.06	1.23	0.21	0.00	-	0.92
Soil sample pelvic region (A1)	16.61	15.23	0.18	1.58	0.35	0.00	1.19	1.38
Soil sample pelvic region (A2)	20.71	14.26	0.09	0.98	0.19	0.00	1.06	0.81
Soil sample knee region (A3)	14.08	18.89	0.26	2.66	0.46	0.00	1.51	1.36
Soil sample pelvic region at edge of grave (A4)	26.21	21.47	0.07	1.04	0.22	0.00	1.17	0.67
SK3 brown textile (A5)	0.65	-	6.89	40.55	5.88	2.79	-	-
SK2 leather (A6)	0.68	-	0.00	28.76	3.81	0.31	-	-
SK14 black textile (A7)	0.46	-	0.00	48.31	2.97	0.00	-	-
SK40 felt (A8)	0.49	-	0.00	28.99	4.25	1.50	-	-

2.3.2.2 Molecular profiles of extracted soils

A series of *n*-alkanes were identified in the soils. The distributions ranged from C_{23}-C_{33} with samples from the region of the lower abdomen showing a dominant signal at C_{29} and samples C1 and A4 showing dominant signals at C_{31} (Figure 2:12). These distributions are typical of higher plants (Eglinton and Hamilton, 1967, Jambu et al., 1991, Jansen et al., 2008) with C_{29} often being dominant in woodland areas and grassland (Bull et al., 2000a, Bull et al., 2000b) the differing dominant *n*-alkanes show the possible variation in plant matter. The abundances of the *n*-alkanes

range from 0.05 mg/ mg TE for A1, 0.43 mg/ mg TE for A2, 0.11 mg/ mg TE for A3 to 0.83 mg/ mg TE for A4, with the control giving a value of 2.15 mg/mg TE. The differences in the distributions suggest that the source of *n*-alkanes for samples A1-A3 differ to those of A4 and the site control (C1) suggesting that signatures for A4, like C1, may be directly related to the soil background. The soil samples from the grave and the site control also contained *n*-alkanols ranging from C_{16}-C_{32} with a distinct maxima at C_{26} for all samples apart from A4 (maximum at C_{28}).

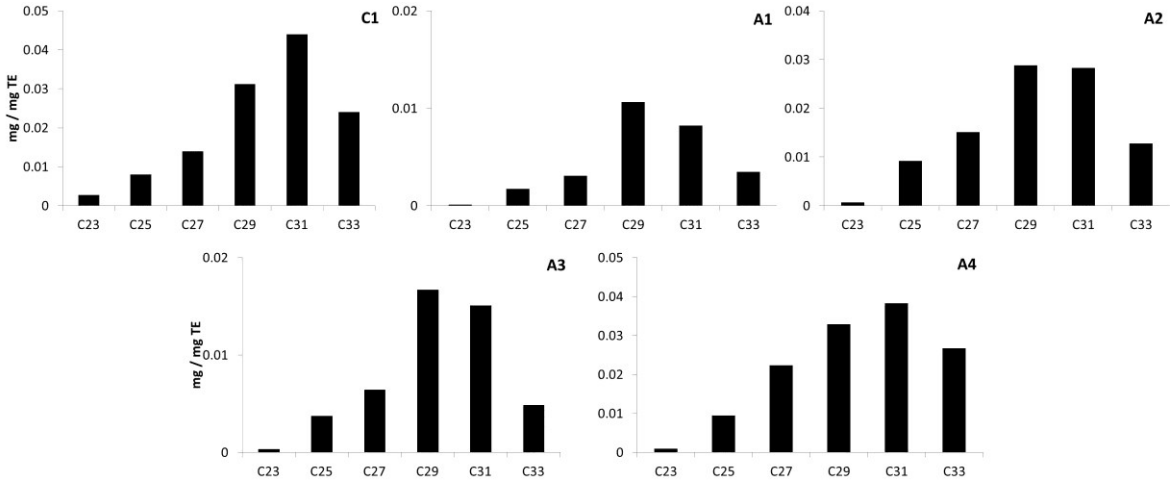


Figure 2:12. Histograms showing the *n*-alkane distributions for samples from GR1651 and site control from Mechelen, illustrating the differences in the distributions.

Fatty acids ranged from C_{14}–C_{30} and included unsaturated components. The abundances ranged from 0.29 - 0.45 mg/ mg TE for samples in the grave and 5.38 mg/ mg TE in the control. Long chain fatty acids (C_{22}-C_{30}) dominated the site control (C1, 4.93 mg/ mg TE) and A4 (0.26 mg/ mg TE) with maxima at C_{28}. The distributions of long chain fatty acids are typical of plants (Eglinton and Hamilton, 1967). By contrast, A1–A3 were dominated by short chain fatty acids (C_{14}–C_{18}, 0.21 - 0.43 mg/ mg TE) with C_{16} being dominant. Unlike Fromelles, the only unsaturated fatty acid present was $C_{18:1}$, perhaps suggesting that stronger reducing conditions occurred than at Fromelles. As was observed in the samples from Fromelles, the distributions contained higher relative abundances of the $C_{16:0}$ fatty acid than occur in fresh adipose tissue (Figure 2:13). This higher proportion of the $C_{16:0}$ fatty acid could suggest an input from other sources such as bacteria or plant matter (Zelles, 1997, Ruess et al., 2002). Alternatively, the high relative abundances of $C_{16:0}$ in Mechelen are accompanied by significantly reduced levels of $C_{18:1}$ suggesting that reduction and β-oxidation may have affected the profiles (Pereira et al., 2005). Comparison of the fatty acid distributions for GR1651 samples with those of samples from grave MESW GR422 from Mechelen (Green, 2013: Figure 2:13) shows similar ratios in the soils. The analysis of GR422 indicated that the distributions of the short chain fatty acids were similar to that expected for adipose tissue degraded in a dry environment (Green, 2013). Given that, apart from the lack of the $C_{16:1}$

unsaturated fatty acid, the distribution of the fatty acids from the GR1651 samples match those of GR422 and are similar to those of degraded adipose in a dry environment, it is likely that the short chain fatty acids are human in origin. The distributions from Fromelles are also similar to those from Mechelen, though the presence of C_{18} 10-OH fatty acid suggests them to be more closely related to degradation in a wet environment.

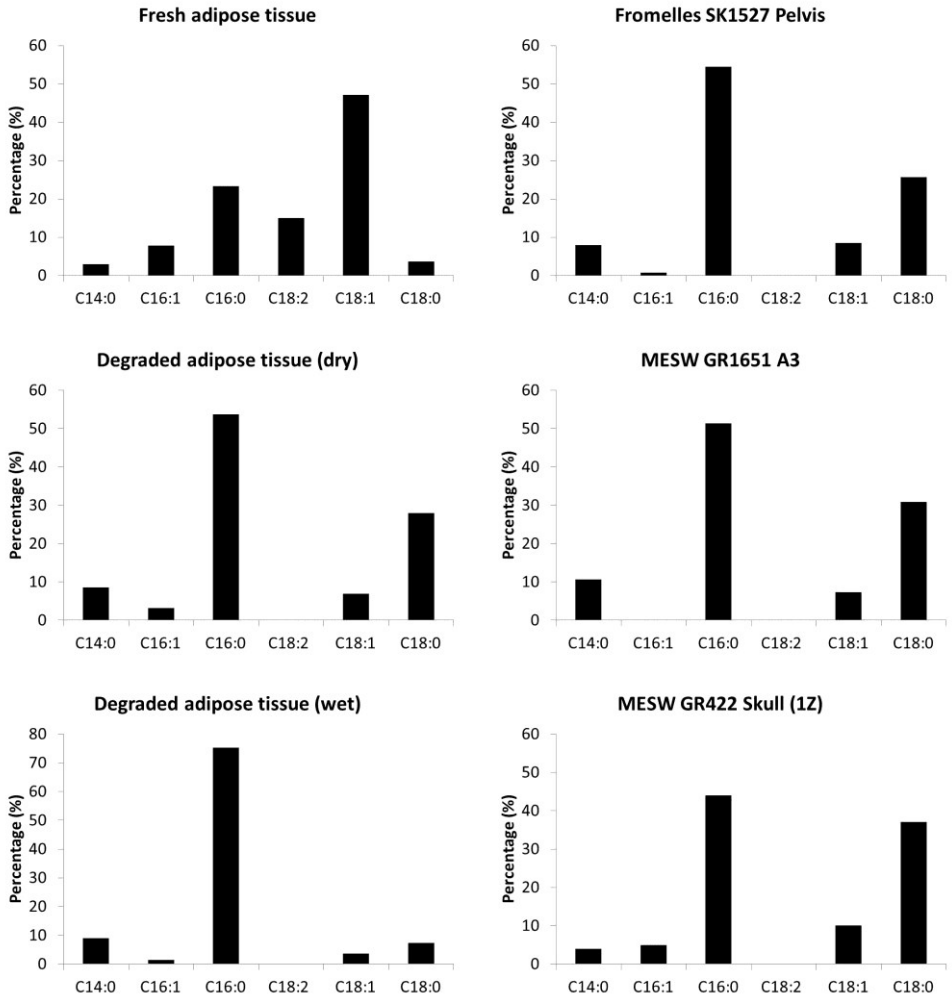


Figure 2:13. Distributions of the short chain fatty acids for a variety of potential sources in soils and selected samples from Fromelles and two graves from Mechelen GR1651 (mass grave) and GR422, (values taken and from (Forbes et al., 2002, Hodson et al., 2008, Green, 2013)).

A series of long chain *n*-alkanones (C_{25}, C_{27}, C_{29} and C_{31}, maximising at C_{27}) were present in A1 - A4 and C1, accompanied by two *n*-alkanals (C_{16} and C_{18}) in samples A1-A3 that were present in higher abundance levels than the methyl ketones. Possible sources of the *n*-alkanones include direct input from plant waxes (Eglinton and Hamilton, 1967) or, as has been suggested by Jansen and Nierop (2009), alteration via the oxidation of *n*-alkanes or reduction of fatty acids (Jansen and Nierop, 2009, Amblès et al., 1993). Direct input from plants has been considered to represent the smallest contribution of methyl ketones to soils, due to the low levels of these compounds in

plant matter (Jansen and Nierop, 2009). The distribution of methyl ketones in the Mechelen grave soils shows no correlation with the distributions of the corresponding *n*-alkanes, suggesting that the latter are not the source of the long chain alkanones. By contrast, the fatty acid distributions correlate much more closely with the methyl ketones distributions, suggesting them to be a possible source. The oxidation and reduction pathways that would be required to form methyl ketones do not appear to be likely, suggesting direct input or alteration on derivatisation to be more likely sources. As well as direct sources from the grave, alteration of *n*-alkanals during derivatisation (methylation) is also a possible source. Notably, short chain *n*-alkanals in samples A1-A3 were accompanied by low abundances of their methylated counterparts, suggesting that methylation occurred with the low abundance longer chain *n*-alkanals being more readily affected. The distributions of the summed *n*-alkanals and methyl ketones (to account for the formation of the latter by methylation, Figure 2:14) show a correlation between the fatty acids, alkanols and alkanals. The presence of alkanals and higher levels of alkanols counterparts of the long chain fatty acids, suggests origins via transformation of fatty acids (reduction through to alkanals and alkanols).

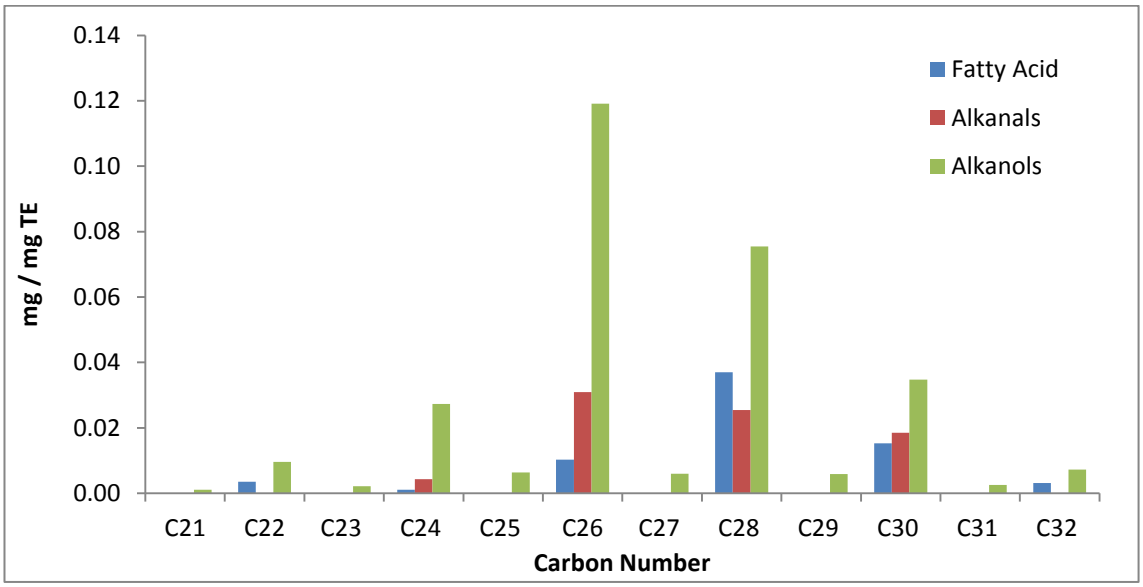


Figure 2:14. Distributions of fatty acids, *n*-alkanals and *n*-alkanols from sample MESW GR1615 A2, showing the relationship between each reduction product.

The presence of C_{16} and C_{18} *n*-alkanals in samples A1-A3 but not in the site control (C1) suggests that they are directly related to the grave environment, either directly sourced or as products of transformation. Mammalian tissue generally contains C_{14}-C_{18} *n*-alkanals (Wittenberg et al., 1956) hence the *n*-alkanals possibly represent free components derived from the body. Alternatively, they could be products of microbial reduction of fatty acids (Day and Goldfine, 1978, Riendeau and Meighen, 1985) formed during the degradation of TAGs. Typically, *n*-alkanal levels in human

remains are very low and are strongly associated with the upper torso and head, organs such as the brain, heart and lungs being the major contributors. The proximity of the samples to the pelvic and knee areas suggests that the *n*-alkanals are more likely to be products of the reduction of fatty acids rather than direct inputs from the original organic matter.

Analysis of samples A1-A4 for the presence of triacylglycerols showed varying amounts of TAGs: A1 contained the highest level (0.24 mg/ mg TE) and A4 the lowest (0.02 mg/ mg TE). Comparison of the TAG distributions of fresh adipose tissue (Ruizgutierrez et al., 1992, Mayer et al., 1997) with the four samples (Figure 2:16, A1-A4) show clear differences, with unsaturated components being considerably lower in the grave soils. The fresh adipose tissue was dominated by POO, PLO and OOO (Mayer et al., 1997) whereas the TAG signatures from Mechelen showed dominance of PPP, PPS and SMP. The clear difference in the degree of saturation is consistent with transformation involving selective loss of unsaturated TAGs. Comparison of the distributions of the saturated fatty acids from the samples with those of fresh adipose tissue (A, Figure 2:15) shows broad similarities, whereas the distributions that would arise from both partial reduction (B, Figure 2:15) and complete reduction (C, Figure 2:15) of unsaturated moieties differ. The similarities with fresh adipose tissue, and differences to the distributions expected from reduction, suggest that the main degradation occurred through hydrolysis/attack, preferentially targeting the unsaturated components of the TAGs. Such specificity is apparent from the levels of $C_{16:0}$ and $C_{18:0}$ differing from that of fresh adipose tissue, with higher $C_{18:0}$ content suggesting additional input (A3).

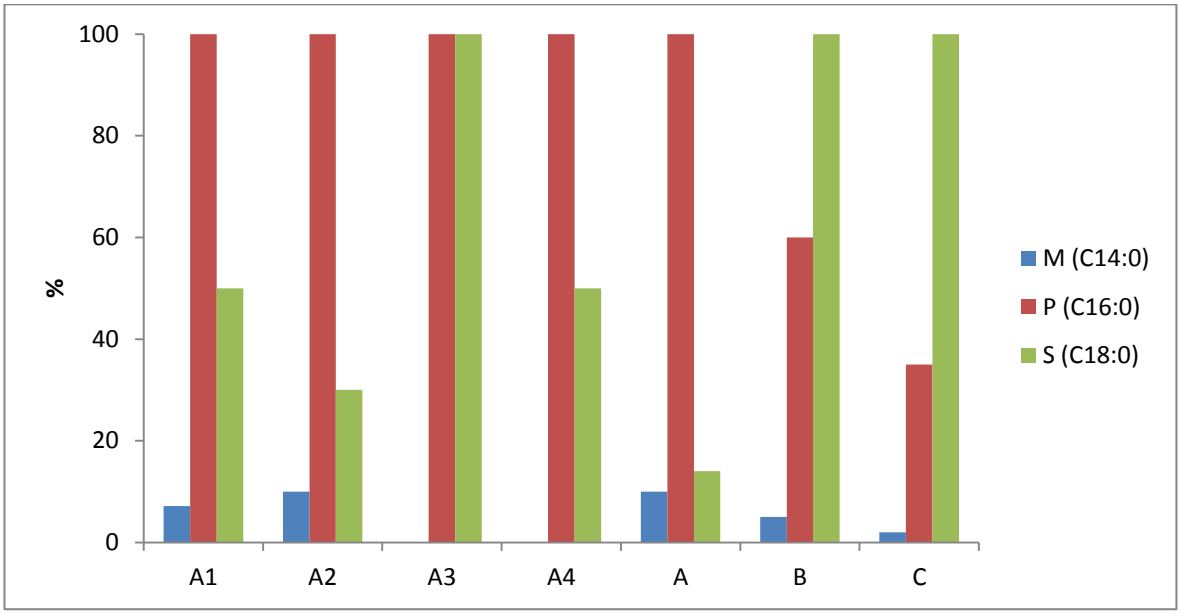


Figure 2:15. Relative abundances of saturated TAG fatty acids normalised to the major component showing comparison of samples from GR1651 to fresh adipose tissue. A = saturated fatty acids for human adipose tissue; B = saturated fatty acids for human adipose tissue with

reduction of all monounsaturated fatty acids to saturated components; C = saturated fatty acids for human adipose tissue with reduction of all unsaturated fatty acids to saturated components.

TAG distributions in extracts from mummies show similar differences in distribution to fresh adipose tissue, with PPP and PPS being the dominant TAGs (Mayer et al., 1997). The ratio of unsaturated to saturated TAGs for fresh adipose tissue is 96:4, compared with 62:38 for A1, 13:87 for A2, 6:94 for A3 and 0:1 for A4. The trend across the grave (from A1-A4) reflects a marked shift from higher levels of unsaturated fatty acid moieties to a dominance of saturated components. Clear evidence of reductive transformation is apparent: the TAG SMP, which was not reported in fresh adipose tissue, is a possible reduction product from TAGs such as MOP or MLP. The presence and high abundance of the TAG PPP suggests either an external source of TAGs (no TAGs of adipose tissue could be reduced to form PPP) or that the extent of degradation has been so high that all other TAGs have been reduced to levels below that of the original PPP content of fresh adipose tissue. The latter scenario is deemed to be the most likely explanation for the distributions. Hence, the remaining TAGs in the distribution from the grave soils represent those most resistant to degradation due to their saturated nature.

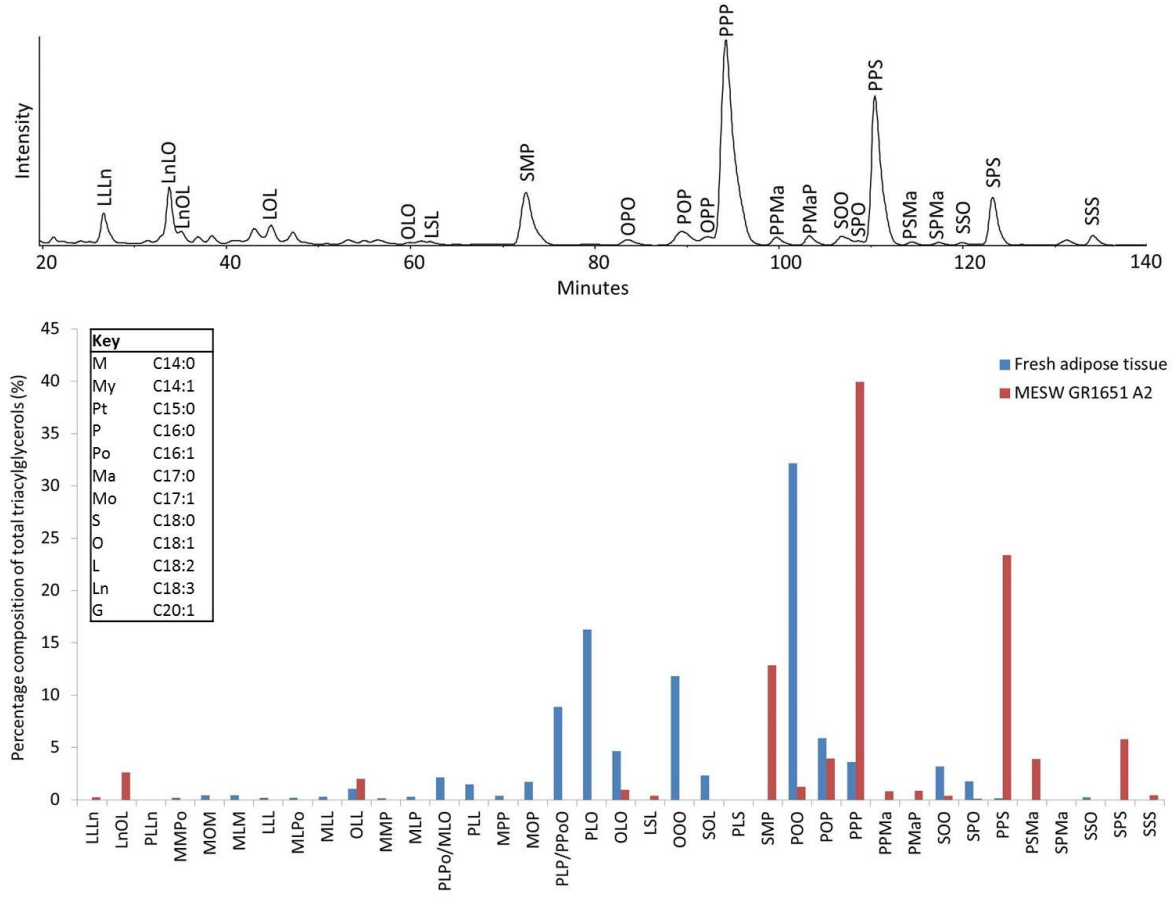


Figure 2:16. Top: Partial LC-MS (*m/z* 300 - 950) of the triacylglycerols from MESW GR1651 A1. Bottom: histogram of fresh adipose tissue TAG proportions (Ruizgutierrez, Montero and Villar 1992) and TAG proportions from MESW GR1651 A2, showing differences in distribution between fresh adipose tissue and the sample A2 with higher abundances of saturated TAGs in the latter.

A mass balance of potential degradation/reduction products of TAGs shows a dominance of *n*-alkanals followed by fatty acids and with TAGs representing minor contributions (Figure 2:17). Both mono- and diacylglycerols, though present, were unquantifiable due to low abundance. Samples A1–A3 show a clear dominance in the reduced products, with A1 having the greatest level of TAGs remaining. Sample A4 was dominated by fatty acids owing to *n*-alkanals being absent and low levels of TAGs.

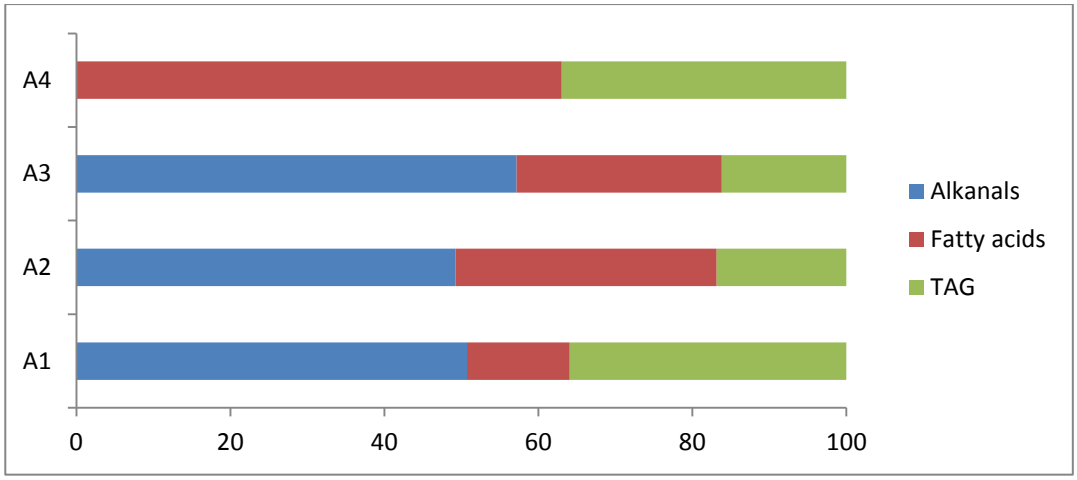


Figure 2:17. Mass balance for samples from GR1651, showing TAGs, fatty acids and *n*-alkanals in the soil samples.

Cholesterol and its degradation products coprostanol, epicoprostanol and 5α-cholestanol were all present in the samples along with the plant sterols β-sitosterol and stigmastanol. Samples A1-A3 all show dominance of the cholesterol degradation products with equal amounts of both environmental (5α-cholestanol) and transformation products formed in the human gut (5β-cholestanol). As with Fromelles, the presence of epicoprostanol indicates reworking of coprostanol in an anaerobic environment. Sample A4 and the C1 control are dominated by the plant based sterols, indicating soil background signatures and lending further support to the association of coprostanol in the grave soils with the human remains. The signatures in the grave soils also match those from GR422 (Green, 2013) suggesting similarities in the organic matter sources and conditions between the two graves. Comparison of plant sterol: cholesterol ratio was carried out, as described previously for Fromelles, to tentatively determine the nature of the gut contents of the inhumed bodies (Table 2.9). Comparison of the ratio with modern values for samples A1–A3 show similarities either to modern low cholesterol diets or to high plant matter, high cholesterol diets (Table 2.6). By contrast, sample A4 showed a value much higher than the other samples, the value corresponding to high plant matter/low cholesterol diets. This suggests either that the individual to whom this sample relates consumed mainly plant material in the days prior to death or, more likely, that the similarities to the C1 control reflect the soil background signature. The similarities in the ratio for A4 and C1, and presence of epicoprostanol, may reflect site background and possibly manuring/faecal contamination of the site. The (coprostanol + epicoprostanol) / (5α-cholestanol + coprostanol + epicoprostanol) ratio for these samples was lower than the 0.7 threshold expected for faecal contamination: C1 gave a value of 0.6 suggesting that manuring may have occurred at this location, accounting for the presence of cholesterol.

Table 2.9. Plant sterol to cholesterol ratios for samples from grave MESW GR1651 (A1-A4) and the site control. See Table 2.6 for comparison.

Sample	Plant sterol : Cholesterol
A1	0.38
A2	0.32
A3	0.41
A4	1.01
C1	0.92

2.3.2.3 Elemental and molecular profiles of isolated materials

Elemental analysis of the textile remains from MESW GR1651 showed that only one sample, A5, contained high levels of nitrogen (6.89 %, Table 2.8) with sulfur being present in a wider number of samples: A5 (2.79 %), A6 (0.31 %) and A8 (1.50 %). The elemental compositions of A5, A6 and A8 suggest a proteinaceous origin with sample A7 suggesting a cellulose origin. The N/C ratio of A5 is consistent with the ratios of woollen textiles from Fromelles. The S/C ratios for A5 and A8 also match those from Fromelles and for modern wools. Molecular analysis of the extracts from samples A5-8 show that A7 (black textile) contains only a few short chain fatty acids. Samples A5, A6 and A8 all contain fatty acids ranging from C_{14}-C_{30}, with plant sterols (β-sitosterol and stigmastanol), cholesterol and its environmental degradation product 5α-cholestanol also present. The presence of high levels of cholesterol in samples A5, A6 further supports an origin from wool textiles.

A notable blue colour of the A3 extract, as well as the presence of textile (A9), suggested that a dyestuff might be present. The UV-vis spectrum of the extracts, revealed an absorption at 610 nm, typical of blue dyestuffs. Blue coloured organic dyes used in the 17th and 18th century had a variety of sources: logwood (*Haematoxylon campechianum L.*), indigo (*Indigofera tinctoria L.*) and woad (*Isastis tincotoria L.*) (Schmidt-Pirzewozna et al., 2004). The chemical species responsible for the colouration in the various sources differ: haematin is the chromophore in logwood (Gulmini et al., 2013), and indigotin is the pigment in woad and indigo (Gulmini et al., 2013, Koren, 2006, Zech-Matterne and Leconte, 2010). HPLC-UV-MS revealed only one signal in the UV-vis (610 nm) chromatogram. APCI mass spectrometry showed a protonated molecule at *m/z* 263 and the expected fragment ions for indigotin (Figure 2:18). Other dye sources (marine animals) of indigotin also contain brominated indigotin (Koren, 2006). The absence of bromo or di-bromo derivatives of indigotin enables these sources to be excluded suggesting that woad or indigo was used as the dyestuff.

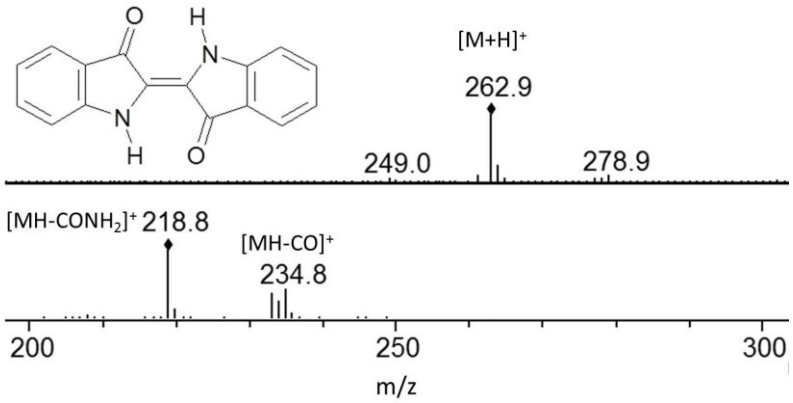


Figure 2:18. Partial APCI mass spectrum (m/z 200 – 300) showing MS^1 and MS^2 of a sample containing indigotin, with fragmentation patterns showing expected fragments for indigotin.

The use of woad or indigo as the dyestuff would suggest that the original textile was of a high quality, as logwood was typically used for cheaper textiles (Gulmini et al., 2013). Extraction of A9 (material isolated from soil sample A3) did not yield the dye, suggesting that the presence of the dye was due to other degraded textiles in the grave soil. On inspection of the soil samples by microscopy, small fragments of textiles could be seen in the sample (Figure 2:19). Pyrolysis-gas chromatography revealed the textile and the microscopic fragments to be cellulose/lignin based (Pinder, 2017). Further investigative work suggests that the textile remaining in A3 is a puesodomorph with complete mineral replacement of the organic structure having occurred (Pinder, 2017). The survival of plant based materials in graves is usually very limited, microorganisms degrade cellulose very rapidly. Enzymatic degradation of cellulose, by cellulase, has been shown to release indigotin from a dyed source (Campos et al., 2000). As the textile in the Mechelen grave soil was heavily degraded and showed no dyestuff on extraction, it is conceivable that enzymatic degradation may have released the dye from the textile matrix, the insoluble nature of indigotin allowing it to be preserved. Retention of the dye may have been enhanced by interaction with clay particles in the soil (Domenech-Carbo et al., 2014).

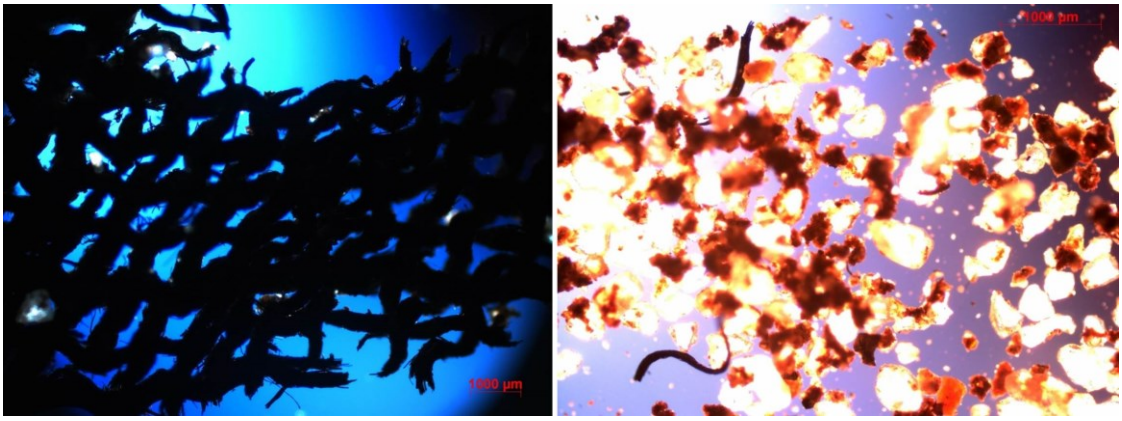


Figure 2:19. Left: microscope image of black textile from sample A3. Right: microscope image of fragments of textile from soil sample A3, the soil sample that yielded a blue extract.

2.4 Conclusions

The nature of mass graves poses a problem with regard to their sampling. The standard InterArChive sampling protocol focuses on the skeletal remains of individual burials, with easily distinguishable sampling positions (Usai et al., 2013). Mass graves, however, are not directly amenable to this approach as the remains are usually intertwined, overlapping or in very close proximity, reducing the sampling positions available. In both of the mass graves discussed in this chapter and in another mass grave site at Ridgeway, Dorset (Pickering et al., 2014) sampling of the graves was carried out in a manner that recovered material from areas that were considered to be of particular interest or to provide a simple representation of all of the remains. Accordingly, due to the nature and quality of the information that can be revealed, the best sampling protocol for mass graves would be to sample from the pelvic regions of the remains (lipid profiles from the remains and indicators of last meal). Further suggestions for sampling positions in mass graves would be the head, feet and any positions that show unexpected features.

Preservation of organic signatures relating to the burials is evident in both Grave 3 and Grave 4 in Fromelles, with Grave 4 showing higher abundances of lipid signatures and higher TOC, consistent with better preservation. This difference in the degree of preservation between graves was noted by the archaeologists during excavation due to the better preservation of organic artefacts (Pollard, 2008). The most likely explanation for the difference between the two graves is the proximity of Grave 3 to Pheasant Wood, impacting on the hydrology and hence oxygenation of the soil. The presence of fatty acid derived *n*-alkanals in Mechelen provides evidence of reducing conditions, with both short chain (C_{16} and C_{18}) and long chain (C_{24}-C_{30}) components being affected. Thus, signatures both from the buried remains and from plant material contained in the soil reveal evidence for a transformation pathway from fatty acids through to *n*-alkanols and possibly alkanes. Short chain *n*-alkanals were also present in the grave of a single individual at Mechelen

(MESW GR422), providing evidence that anaerobic conditions in the grave led to reduction of adipose tissue fatty acids. The presence of short chain *n*-alkanals in the mass grave is also consistent with the existence of a strongly reducing environment.

The dominance of $C_{16:0}$ and $C_{18:0}$ fatty acids in Fromelles and the apparent match of the distributions with that of degraded adipocere (Figure 2:13) indicates these components of the soil organic matter to include substantial contributions derived from the human remains. Evidence of waterlogging from the presence of C_{18} 10-OH fatty acid in the soils concurs with analyses carried out by Forbes 2002 on adipose tissue degraded in a water logged environment, the components being suggested to form by hydration of the double bond in the $C_{18:1}$ fatty acid. The highest levels of fatty acids in Fromelles were typically in the soils around the pelvic areas, coincident with the highest TOC values. Notably, however, the foot position of SK1525B recorded a much higher fatty acid concentration and a distribution distinct from those of the other remains in Grave 4, with higher relative abundance of $C_{14:0}$. The exposure of the foot position during the exploratory excavation may have influenced the lipid signatures at this position. The fatty acids associated with the textile remains at this position show distributions that differ from the soil, contrasting with the other samples from the grave. Two possible scenarios associated with the exploratory excavation of this waterlogged environment are envisaged: (i) alteration of the lipid distribution as a consequence of the changes in the environmental conditions or (ii) movement of water carrying fine clay material with its associated lipid content and impregnating the textile. Textiles in the graves may have served as additional vehicles for the accumulation of fine sediment with associated organic signatures, as seen in SK1750 and the SK1525B sock. The fatty acid distributions from the Mechelen mass grave and the single grave (GR422) show the same range of fatty acids as are present in Fromelles (C_{14}-C_{18}), with dominance of $C_{16:0}$ and $C_{18:0}$ in both cases. By comparison with experiments on the degradation of adipose tissue, this distribution suggests degradation in a dry environment (Figure 2:13). A shift in the dominant fatty acid from that of fresh adipose tissue is evident: fresh adipose tissue is dominated by $C_{18:1}$ whereas the soil distributions have higher levels of $C_{16:0}$. The difference in distribution suggests that degradation of the remains has progressed to an extent in which the level of $C_{16:0}$ is dominant, or more likely that β-oxidation of $C_{18:1}$ to $C_{16:0}$ has occurred via microbial reworking (Lalman and Bagley, 2001). Soil analysis from the mass burial at Ridgeway in Dorset, revealed signatures containing only the $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids, with $C_{18:0}$ dominating. The location of the Ridgeway mass burial, within a shallow pit on a chalk escarpment, precludes waterlogging having been a dominant feature in that particular environment. By contrast, Fromelles was heavily waterlogged and Mechelen is located on a wet sandy ridge. The differences in the distributions between the

graves suggest differences in the pathways by which triacylglycerols are degraded, most likely relating to differences in the nature of the environmental conditions within the burials.

Steroidal components were present in all of the mass graves analysed here and in Ridgeway (Pickering et.al. 2014). The presence of cholesterol together with both the mammalian (coprostanol) and environmental (5α-cholestanol) transformation products reflect contributions from the human remains of alteration products formed both before and after burial. Analysis of the plant and mammalian steroidal components allowed for a tentative assessment of the general composition of the last meals before death. Clear differences were observed between the profiles associated with the individuals buried in each grave: Fromelles remains showed individuals to have had a high cholesterol intake whereas Mechelen showed profiles reflecting both low cholesterol and high cholesterol, high plant content. These differences may shed light on the individuals present, with the high cholesterol contents for the soldiers of Fromelles reflecting army rations (bully beef) and the low cholesterol contents of individuals at Mechelen linking to peasant/prisoner origins. Based on the identification of the textiles and fibres recovered from the graves in Fromelles as woollen textiles and those from the Mechelen mass grave representing both wool textiles and cellulose origins, the lipid profiles provide tantalising indications of differences in geographical origin. The lipid signature associated with the woollen textiles from Fromelles yield information about their possible origins and nature, with ratios of $C_{18:0}/C_{18:1}$ fatty acids, supporting either a British or Australian origin, as would be expected for this particular site. In addition, the textiles from the head and pelvis of SK1525B from Fromelles contained an unusual signature dominated by squalene and ambrein, both of which are present in sperm whale oil. The tentative identification of these two components in the grave suggests that either a waterproofing agent was present on the textile or, perhaps more likely, a degraded canister of rifle oil was present amongst the remains. The lack of squalene and ambrein in the foot region may suggest their removal via oxidative transformation due to the susceptible nature of the unsaturated molecules and the opening of the grave at this position. The soil sample A3 from Mechelen mass grave yielded a blue dyestuff believed to originate from the textile at this location, despite the textiles remains not yielding any dyestuff. The extract from the soil revealed the presence of indigotin originating from either woad or indigo.

Overall, a significant amount of information that would not be possible to ascertain from physical remains has been detected in the graves. This relates both to the transformation pathways of organic remains, having implications for the environmental conditions in the burial environments, and to features specific to the individuals in the graves such as gut contents, colour of clothing

and possible waterproofing /personal effects. Whether or not the preservation of the signatures is due to the nature of the burial environment is not yet fully known and further analysis of single burials is required. What does appear to be evident is that mass graves provide unique environments with high degrees of preservation compared to single inhumations (comparison of graves sampled in Mechelen). Clear effects of the burial environment are reflected in the lipid signatures, with differences between waterlogged and dry environments being apparent as well as clear evidence of reductive degradation and the movement of organic residues associated with the movement of water through the soil. Further analysis of single inhumations and experimental burials will help to clarify these aspects.

3 Organic signatures in the soils and finds from single inhumations

3.1 Introduction

Analysis of the mass graves (Chapter 2) showed that organic signatures from human remains can be recovered from grave soils and that, in part, these reflect the environmental conditions that existed in the grave. Comparison of the mass grave from Mechelen, Belgium, with that of the single inhumation from the same site enabled identification of specific issues that require further investigation. Firstly, the need for in-depth analysis of single inhumations to determine the extent to which the organic signatures differ from those of mass graves was apparent. Secondly, the need to assess the extent to which intra-site variation can influence the preservation and character of the organic signatures was highlighted. Hence, the aim of the work detailed in this chapter was to determine the extent to which single burials reveal signatures from remains and the effects of the environment on preservation of signatures. In order to ensure in-depth analysis, multiple graves from two different sites were examined and the InterArChive sampling protocol (Chapter 7, Figure 7:1) was extended for some graves to consider different depth planes around the skeletal remains. The site of Fewston was chosen for analysis to understand the differences in intra-site variation and preservation, as on excavation greater levels of preservation were noted in-situ and suggested to result from differences in the hydrology within the site. Hence, analysis of graves across this site should lead to identification of different levels of degradation and preservation at the molecular level and allow suggestions as to possible causes.

3.2 Samples and sampling strategies

3.2.1 Medieval graveyard, Haymarket, York

The medieval graveyard (11-13th Century) of All Saints Church, Haymarket (Peasholme Green), North of the river Foss in the centre of York, Yorkshire, UK, was the site of over 500 recorded inhumations. Located next to the historic site of Hungate, the graveyard was located within the flood plain (edge of the Kings Fish pond) of the river Foss, which originally ran closer to the site. The location suggests that the grave may have been susceptible to inundation from groundwater over considerable periods of time.

Grave 83012 was extensively sampled to allow for an in-depth study of organic remains and their distributions around the skeletal remains. Sampling was carried out using the intense sampling strategy of the InterArChive project (Usai et al., 2013) with further samples being taken to provide a profile of the grave (Figure 3:1). Good bone preservation was evident on excavation, except that the feet of the remains were missing due to a service trench cutting through that section of the grave. The control position C3A was not sampled as the position was too close to the skull. Five distinct planes were sampled, upper grave fill (controls C2), lower grave fill (controls C3),

skeletal remains (adjacent to remains (y)), resting plane (samples directly below remains (z)) and below the resting plane (approximately 8 cm below the remains).

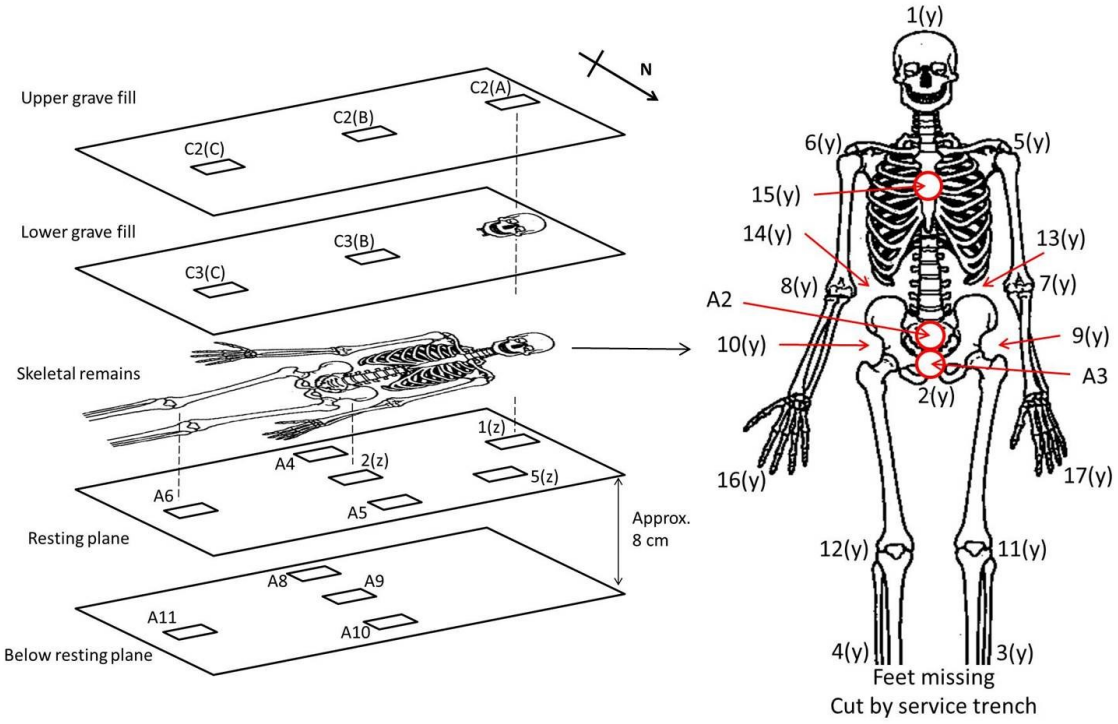


Figure 3:1. Sampling positions for Haymarket 83012 showing all positions sampled in accordance with the InterArChive sampling protocol and reflecting five sampling planes.

3.2.2 18th - 19th century graveyard, Fewston

Part of the Churchyard of St Michael and St Lawrence, situated North of the Swinsty reservoir (last official burial 1896), was excavated due to planned development at the site to allow for the construction of the Washburn Heritage Centre (Figure 3:2). The excavation was intended to identify all burials present, record the archaeological features and effect removal of human remains for reburial. The topography of the graveyard exhibited a steep north-south slope (1 in 6 gradient) as well as a gradual west-east slope (1 in 14 gradient; (Buglass, 2010)). Four sets of remains: SK277; SK331; SK334 and SK408, were selected for examination. Three of the burials, those of SK277, SK331 and SK334 (date unknown, believed to be from an earlier iteration of the graveyard due to ENE-WSW alignment), were situated at the North West corner of the graveyard in a well-drained coarse loamy soil (Figure 3:3). A dated grave (SK310) from this area corresponded to burial *circa.* 1888. A similar age burial, SK408 (*circa.* 1884), was situated at the southern end of the graveyard. At the time of excavation the southern part of the graveyard was heavily waterlogged, the high water levels being evident within the graves (Figure 3:3). Additional graves (SK310, SK319 and SK325) analysed by Pickering are also discussed in order to allow a more

complete examination of intra-site variation (Figure 3:4). Notably, the last of those burials, SK325, was performed after the formal closure of the cemetery and was dated 1921.

Figure 3:2. Site on excavation, Fewston, North Yorkshire.

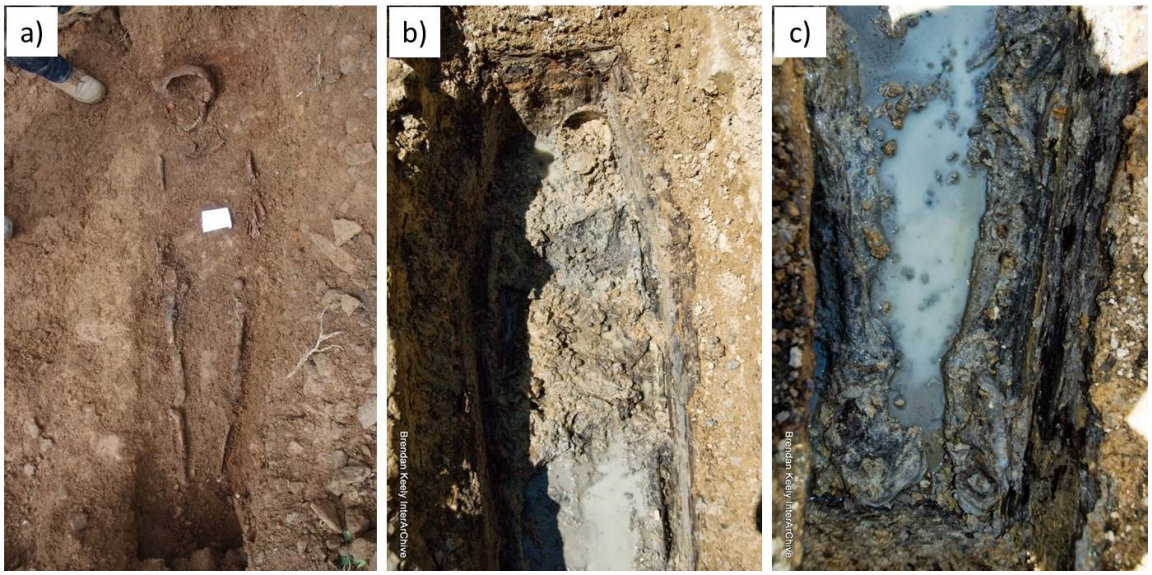


Figure 3:3. Graves sampled from Fewston. a) SK277 situated at northern end of graveyard showing dry nature of grave and bone degradation. b) and c) SK408 situated at the southern end of graveyard showing the waterlogged nature of the grave and remains of the coffin.

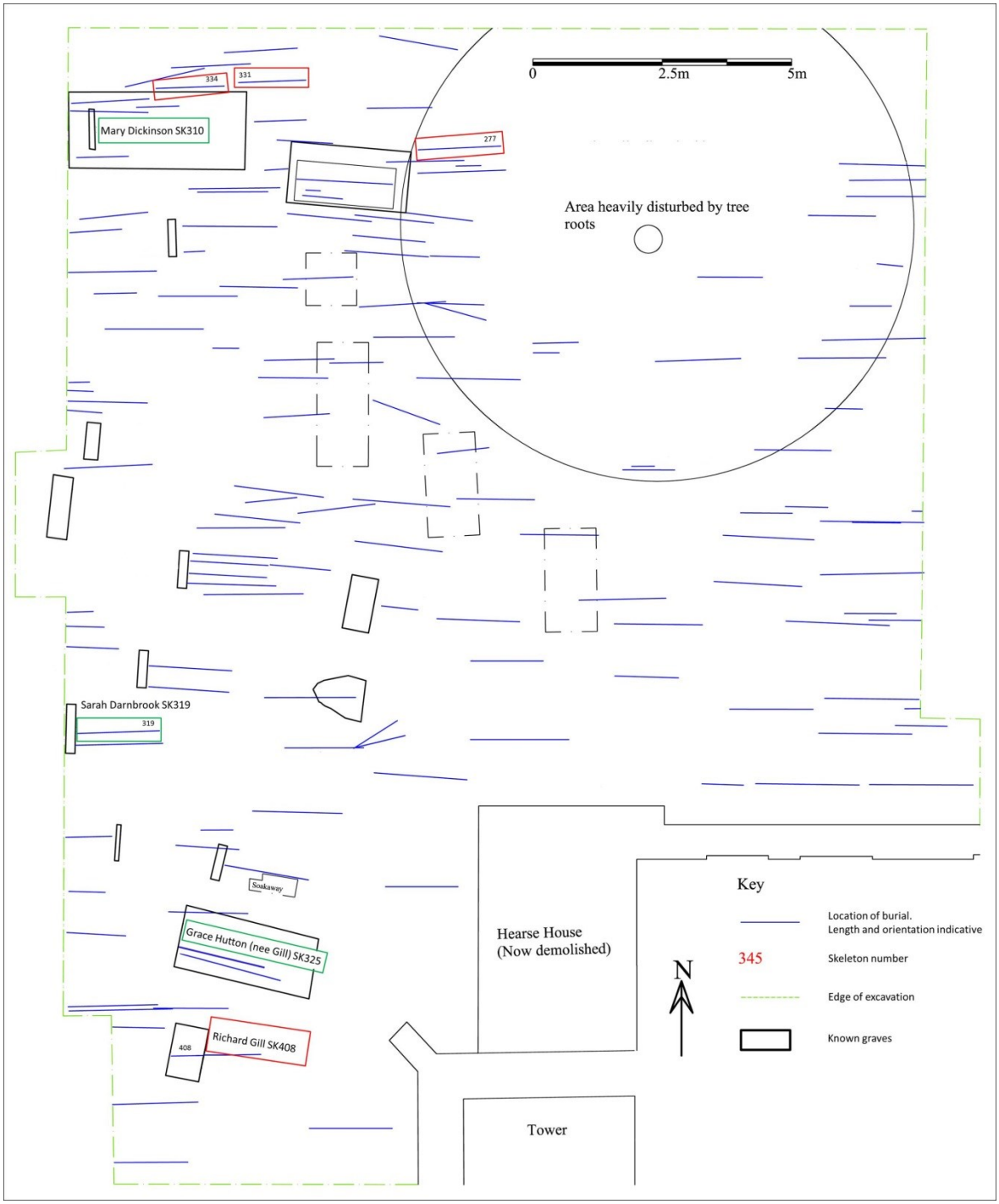


Figure 3:4. Site plan for the Church of St Michael and St Lawrence, Fewston, North Yorkshire. Numbered graves represent sample selection discussed in this chapter, with graves marked in red analysed by the author and green from a previous study (Pickering, unpublished).

The skeletal remains of SK277, SK331 and SK334 were poorly preserved and stains and handles were the only signs of the presence of coffins, though fragments of wood were recovered during the preparation of some of the samples (see below). The grave of SK334 was cut by several later burials. Samples were typically collected from the areas of the skull (1), pelvis (2), hands (16 and 17, collected as one sample) and feet (3/4) (Figure 3:5). Controls and additional samples from notable features were also collected, for example, below the right calf (12) and below the knees (11) of SK331 where a dark colouration of the soil, suggesting elevated levels of organic matter, was noted. During sample processing, SK334 revealed the presence of a fibre in the sample from the pelvis (2). In addition, wood and bone fragments were observed in the soils from the pelvis (2) and feet (3/4). Copper pins, were also found in SK277, SK331 and SK334 during sampling. Graves adjacent to the sampled graves had dates ranging from 1880 - 1890, suggesting the approximate age of the sampled burials to be within this range. Notably, however, the evidence of extensive reuse of the graveyard, along with the alignment of the graves would suggest that these burials may be from an earlier period, no definitive age was obtained (Buglass, 2010).

The remains of SK408 produced textile fragments and a recognisable, though heavily degraded coffin, believed to be made of oak (Buglass, 2010). Soils from the skull (1), pelvis (2) and feet (3/4) were sampled and specific features were also sampled: soil adjacent to fragments of hair, possible remains related to funerary flowers and socks. Further subsamples were collected during processing as substantial amounts of organic plant-like matter was present in the soils adjacent to the right hand side of the body, A2, A3 and A4 (Figure 3:5).

Grave	Sample	Information
SK277	1	Skull
	2	Pelvis
	3/4	Feet
	16	Hands
	A1	Cranial Cavity
SK331	C2	Control 2
	C3	Control 3
	1	Skull
	2	Pelvis
	3/4	Feet
	11	Organic matter below knee
	12	Organic matter below right calf
	16	Hand
	A1	Dark material under left of chin
SK334	C2	Control 2
	C3	Control 3
	1	Skull
	2	Pelvis
	3/4	Feet
	16	Hand
	A1	Resinous material
	A2	Head mout
SK408	1	Bottom of coffin right head
	1A	Hair above
	1B	Hair below
	2	Pelvis
	2A	Pelvic region
	3/4	Feet
	3A	F.B. Feet
	11	Clay above kness
	A1	Edge of right leg
	A2	Plant matter right head
	A3	Plant matter pelvis
	A4	Plant matter edge of right leg
	A5	Socks

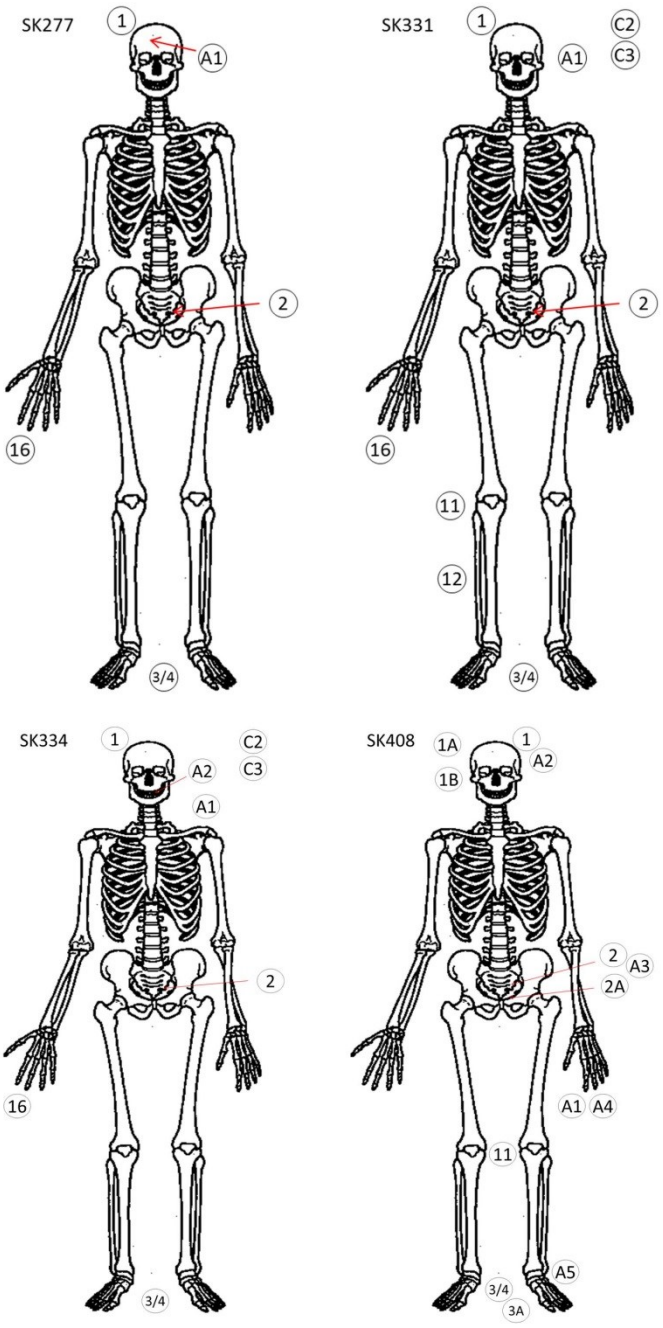


Figure 3:5. Sampling positions for the four graves from the Church of St. Michael and St. Lawrence, Fewston, listing the various subsamples that were obtained during processing of the soil.

3.3 Results and discussion

3.3.1 Haymarket, medieval graveyard

3.3.1.1 Elemental analysis of soil samples

Elemental analysis (CHNS) and total organic carbon (TOC) (Table 3.1) was carried out on all soil samples and error was calculated from the analysis of replicates (n = 3). Sulfur was absent from all samples apart from 2y, with nitrogen levels ranging from 0.07 - 0.22 % (± 0.08, Table 3.1).

Total organic carbon values (0.32 – 1.44 %, ±0.26) varied greatly across the grave (Figure 3:6). The TOC values to the lower right hand side of the remains were generally high (pelvis to feet, 1.05 – 1.44 %) reflecting either greater levels of preservation of organic matter or higher initial organic matter content to the right of the remains (Table 3.1).

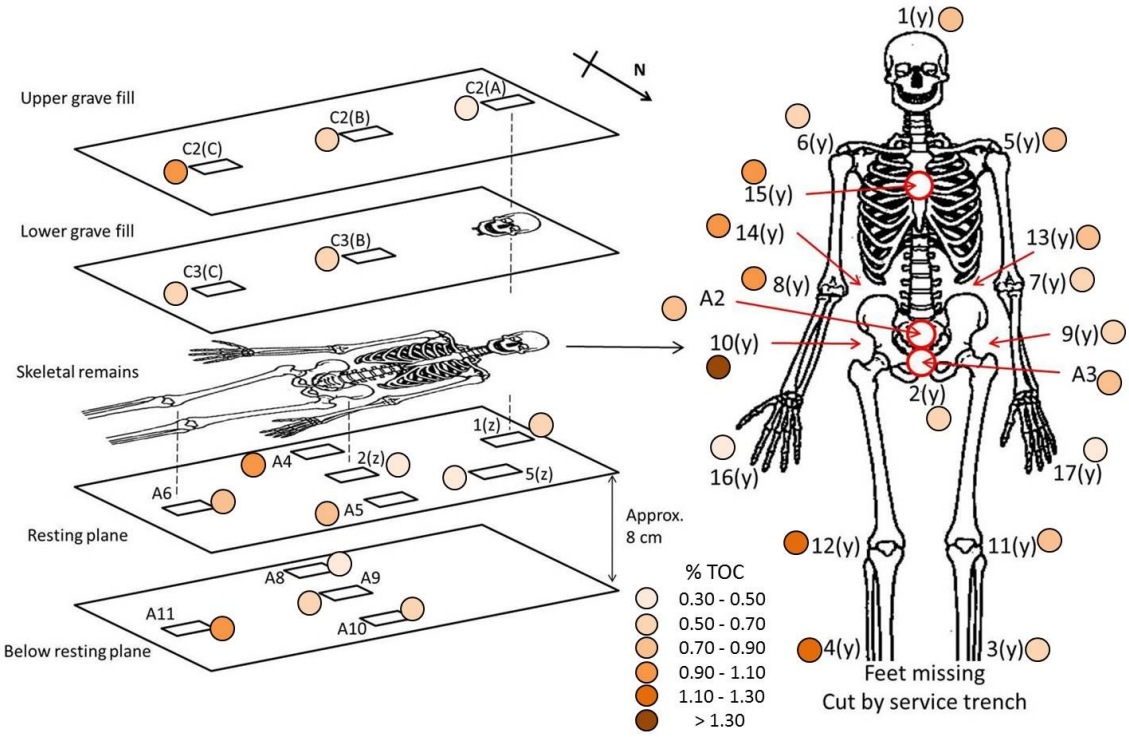


Figure 3:6. Sample positions for Haymarket 83012 showing the total organic carbon contents (TOC) at each position sampled.

Table 3.1. Element abundance (%) of carbon, nitrogen, sulfur and total organic carbon (TOC) in the soil samples from Haymarket, grave 83012. Elemental data given as weight percent.

Sample	Nitrogen (%)	Error (±)	Carbon (%)	Error (±)	Hydrogen (%)	Error (±)	Sulfur (%)	Error (±)	TOC (%)	Error (±)
C2A	0.08	0.07	0.51	0.04	0.32	0.01	0.00	0.00	0.42	0.01
C2B	0.13	0.07	1.07	0.04	0.48	0.02	0.00	0.00	0.62	0.33
C2C	0.13	0.06	1.29	0.02	0.52	0.01	0.00	0.00	1.02	0.19
C3B	0.13	0.06	1.06	0.03	0.53	0.01	0.00	0.00	0.66	0.24
C3C	0.14	0.11	1.02	0.11	0.51	0.03	0.00	0.00	0.68	0.09
1y	0.14	0.06	1.25	0.04	0.51	0.02	0.00	0.00	0.89	0.19
1z	0.13	0.06	1.11	0.05	0.64	0.01	0.00	0.00	0.52	0.36
2y	0.14	0.08	1.28	0.08	0.48	0.03	0.01	0.01	0.66	0.43
2z	0.12	0.05	1.08	0.02	0.66	0.02	0.00	0.00	0.42	0.29
3y	0.16	0.13	1.31	0.05	0.44	0.03	0.00	0.00	0.51	0.42
4y	0.14	0.08	1.46	0.11	0.55	0.03	0.00	0.00	1.11	0.03
5y	0.13	0.06	0.98	0.05	0.50	0.06	0.00	0.00	0.84	0.06
5z	0.11	0.07	0.83	0.03	0.54	0.02	0.00	0.00	0.50	0.31
6y	0.11	0.06	0.85	0.05	0.46	0.02	0.00	0.00	0.58	0.20
7y	0.12	0.07	0.92	0.03	0.49	0.01	0.00	0.00	0.51	0.36
8y	0.15	0.07	1.25	0.05	0.54	0.03	0.00	0.00	1.00	0.17
9y	0.14	0.06	1.23	0.02	0.50	0.01	0.00	0.00	0.63	0.27
10y	0.20	0.10	1.70	0.12	0.60	0.03	0.00	0.00	1.44	0.17
11y	0.22	0.15	1.57	0.22	0.60	0.05	0.00	0.00	0.89	0.60
12y	0.15	0.06	1.46	0.04	0.55	0.01	0.00	0.00	1.22	0.11
13y	0.14	0.06	1.06	0.04	0.53	0.02	0.00	0.00	0.88	0.13
14y	0.16	0.12	1.02	0.01	0.49	0.01	0.00	0.00	0.95	0.07
15y	0.17	0.09	1.49	0.11	0.54	0.02	0.00	0.00	1.10	0.35
16y	0.15	0.07	1.20	0.05	0.56	0.02	0.00	0.00	0.51	0.41
17y	0.12	0.06	0.95	0.03	0.53	0.02	0.00	0.00	0.32	0.40
A2	0.15	0.08	1.26	0.05	0.52	0.04	0.00	0.00	0.87	0.25
A3	0.14	0.07	1.25	0.09	0.54	0.05	0.00	0.00	0.90	0.30
A4	0.15	0.10	1.10	0.03	0.60	0.01	0.00	0.00	1.05	0.06
A5	0.13	0.06	1.16	0.03	0.62	0.03	0.00	0.00	0.72	0.29
A6	0.13	0.07	1.22	0.01	0.63	0.01	0.00	0.00	0.72	0.46
A8	0.14	0.09	1.19	0.01	0.66	0.02	0.00	0.00	0.36	0.24
A9	0.14	0.09	1.11	0.05	0.66	0.03	0.00	0.00	0.69	0.44
A10	0.16	0.11	1.27	0.05	0.69	0.03	0.00	0.00	0.68	0.44
A11	0.17	0.10	1.19	0.04	0.66	0.02	0.00	0.00	0.98	0.03

3.3.1.2 Molecular profiles of soil extracts, Haymarket.

Varying quantities of organic residue were extracted from each of the samples with abundances ranging from 4.60 x 10^{-4} – 5.76 x 10^{-3} mg/ mg TE (Table 3.2). GC analysis of the hydrocarbon fractions reveals *n*-alkanes ranging from C_{19} to C_{33} and displaying a marked odd-over-even predominance (Figure 3:7). All samples apart from 2z, A5 and A10 show C_{31} as the dominant *n*-alkane. The *n*-alkanes from 2z were dominated by C_{29} whereas those from A5 and A10 were dominated by C_{33}. Notably, the A5 samples were collected directly above A10, hence a similar source for the *n*-alkanes at this position seems likely. Within *n*-alkane profiles, dominances of C_{29}, C_{31} and C_{33} are typical of plant sources (Eglinton and Hamilton, 1967, Jansen et al., 2008, Jambu et al., 1991). Whereas the majority of the profiles from the grave are dominated by C_{31}, the dominance of C_{29} at 2z suggests a difference in the input. The short chain *n*-alkanes (C_{19}-C_{21}) may have origins either from gram positive bacteria (Ladygina et al., 2006) or from reduction of fatty acids.

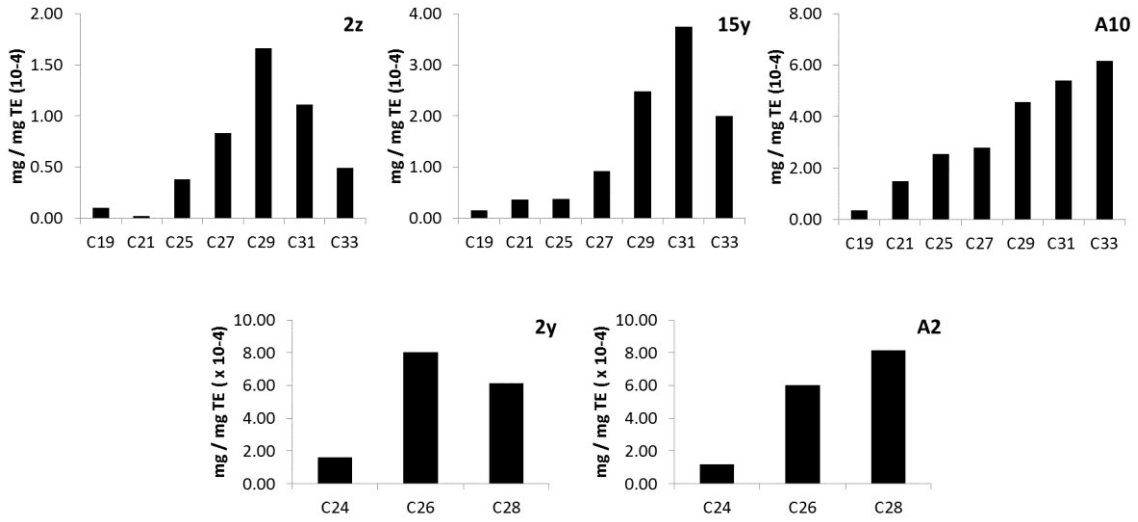


Figure 3:7. Distinctive distributions of *n*-alkanes (top) and *n*-alkanols (bottom) in the soils from Haymarket, grave 83012.

Abundances of *n*-alkanols ranged from 1.31 x 10^{-3} – 6.38 x 10^{-3} mg/ mg TE. The *n*-alkanol distributions from the controls and the grave soils range from C_{22}-C_{28} with the majority of samples showing distinct maxima at C_{26} (Figure 3:7). Eleven of the 33 samples showed maxima at C_{28}. Dominance of C_{28} *n*-alkanol is most significant at both C2B and C3B, the dominance at these positions suggesting the same source. Samples relating to the central location of the remains (7y, 8y and 15y) and in particular the pelvis area (9y, 10y and A2) also show C_{28} to be the dominant *n*-alkanol. Both C_{26} and C_{28} *n*-alkanols are typical of higher plant waxes (Eglinton and Hamilton, 1967, Bull et al., 2000b, Bull et al., 2000a).

Table 3.2. Masses of soil extracted and the total solvent extractable organic matter for soils from Haymarket 83012.

Sample	Average weight of dry soil extracted (g)	Average total lipid extract (mg)
C2A	3.70	0.89
C2B	1.80	0.66
C2C	3.02	2.50
C3B	2.63	0.95
C3C	3.03	1.47
1y	1.95	0.55
1z	2.61	1.15
2y	4.24	1.12
2z	2.16	0.84
3y	2.89	1.21
4y	3.08	3.11
5y	2.78	0.61
5z	2.97	0.69
6y	3.27	0.35
7y	2.47	0.55
8y	2.23	0.62
9y	1.95	0.68
10y	2.79	1.04
11y	3.63	1.35
12y	2.60	0.68
13y	3.26	1.75
14y	2.85	0.73
15y	2.07	0.92
16y	2.47	0.99
17y	3.14	1.66
A2	2.40	0.67
A3	3.46	0.89
A4	2.94	0.80
A5	3.69	2.11
A6	2.91	1.43
A8	2.18	0.63
A9	2.26	0.58
A10	3.06	1.14
A11	2.44	0.41

The extract of 4y was darker coloured then the other extracts. GC-MS analysis showed the presence of fluoranthrene, triphenylene, benzo (k) fluorathrene and benzo pyrene, a series of polycyclic aromatic hydrocarbons (PAH) commonly found in coal tar (Zander, 1995). Micromorphological analysis from this location showed the presence of black staining on a bone fragment present in the sample (Ghislandi, unpublished). The proximity of the service trench to

the location of 4y would suggest that the origin of the material relates to the activity associated with cutting the trench, with leaching/contamination occurring and affecting both the burial soils and the skeletal remains.

The distributions of fatty acids in the soils range from $C_{16:0}$ to $C_{30:0}$ with the only unsaturated fatty acid present being $C_{18:1}$. Overall, long chain fatty acids (6.62×10^{-4} – 1.01×10^{-2} mg/ mg TE) dominate the distributions, with short chain fatty acids (2.00×10^{-5} – 2.54×10^{-3} mg/ mg TE) dominant in only three positions, 1z, 17y and A8. The long chain fatty acid profiles are dominated by $C_{26:0}$, a fatty acid typically related to plant background in soils (Eglinton and Hamilton, 1967, Bull et al., 2000b, Bull et al., 2000a). This finding complements the distributions of the *n*-alkanes and *n*-alkanols, implying related sources. Short chain fatty acid profiles are mainly dominated by $C_{16:0}$ (Figure 3:8) with a few exceptions where $C_{18:0}$ (C2C, C3C, 2z and A2) and $C_{18:1}$ (5y) are dominant. Positions dominated by $C_{18:0}$ fatty acid are spatially close, the controls C2C and C3C being one above the other. The same can be seen in the pelvic areas with A2 and 2z showing marked similarities. Distributions dominated by $C_{16:0}$ show lower levels of $C_{18:0}$ and very low levels of $C_{18:1}$ (Figure 3:8). Comparison of the distributions of short chain fatty acids from Haymarket with fresh and degraded adipose tissue (cf. Chapter 2, Figure 2:13) reveals marked differences: the distribution from 6y is typical of the burial samples (Figure 3:8). By comparison with degraded adipose tissue, the Haymarket soils all lack the $C_{16:1}$ and $C_{14:0}$ fatty acids. The absence of $C_{14:0}$ from all samples may be due to the age of the burial and greater solubility of shorter chain acids relative to those with higher carbon numbers, as reflected by the octanol: water partition coefficient (Bell, 1973). The almost complete absence of unsaturated fatty acids from all of the samples suggests high levels of degradation, with the comparatively high levels of $C_{16:0}$ reflecting the loss or transformation of all other fatty acids, possibly by solubilisation in groundwater, reduction of unsaturated molecules or microbial alteration via β-oxidation and associated reduction, as suggested for the mass graves (Chapter 2). Both controls (C2C and C3C, Figure 3:8) show high levels of $C_{18:0}$ and $C_{18:1}$ relative to $C_{16:0}$. Despite this, the ratio of $C_{18:0}$ to $C_{18:1}$ is consistent with that of 6y. Hence, contributions of $C_{16:0}$ to the controls appears to be lower than in the grave soils or an alternative source of the C_{18} fatty acids contributes to the controls. The fatty acids in 2z and A2 are dominated by $C_{18:0}$ and $C_{18:1}$ with low levels of $C_{16:0}$. The distributions at these positions differ markedly from those in the other samples and from fresh and degraded adipose tissue. The distribution from 5y shows the closest representation for fresh adipose tissue, apart from the absence of $C_{14:0}$, $C_{16:1}$ and $C_{18:2}$ and a slightly elevated level of $C_{18:0}$. The elevated level of $C_{18:0}$ may provide an indication either of the reduction of unsaturated C_{18} fatty acids or of an additional input. Comparison with the proportions of the fatty acids in fresh

adipose tissue shows that the high level of $C_{18:0}$ in controls C2C and C3C match, is consistent origins from reduction of the $C_{18:2}$ and $C_{18:1}$ components of adipose tissue. By contrast, the high abundance of $C_{18:0}$ in sample 2z is more likely to be due to an additional input as the high abundance level does not match the significant decrease in all other fatty acids. In sample 5y, the high levels of $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ can all be attributed to the low levels of $C_{16:1}$ and $C_{18:2}$, indicating reduction of fatty acids in this position. Sample 6y, and therefore the majority of samples in the grave, show low levels of $C_{18:2}$ and $C_{18:1}$ that cannot be explained by the gain in $C_{18:0}$. Accordingly, the levels of $C_{16:0}$ that are not accounted for by reduction of $C_{16:1}$ can be attributed to microbial reworking causing alteration of C_{18} fatty acids to produce $C_{16:0}$. Apart from those differences mentioned above, the rest of the samples have distributions that match those of the controls, suggesting that the signatures most likely represent the soil background and are not directly linked to the interred remains. In part, this may be attributed to the extended use of the graveyard.

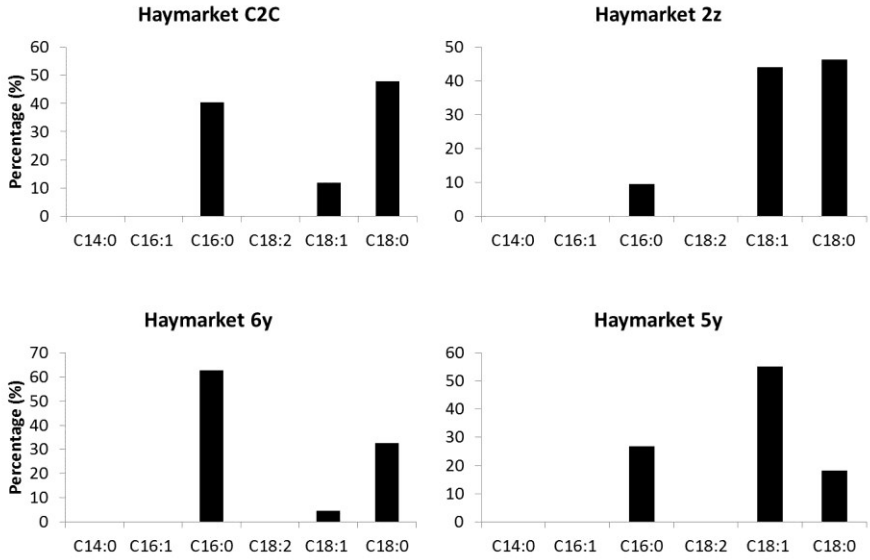


Figure 3:8. Short chain fatty acid distributions from Haymarket, grave 83012.

Signature of bacterial and root activity were also detected in the graves through the presence of $C_{17:0}$ *iso* fatty acid and C_{22} and C_{24} ω-hydroxy fatty acids, respectively. The $C_{17:0}$ *iso* fatty acid is a bacterial fatty acid (Zelles, 1999), and the C_{22} and C_{24} ω-hydroxy fatty acid are found in suberin, a compound that contributes to external structures of woody plants, such as roots (Van Bergen et al., 1998, Bull et al., 2000a). Only three sterols were identified: cholesterol, β-sitosterol and stigmastanol, the latter two being of plant origin (Bull et al., 2000b). All of the sterols are present throughout the grave, with plant based sterols dominating.

3.3.2 Fewston, 18th - 19th century graveyard

3.3.2.1 Elemental analysis of soil samples, Fewston

Results from elemental analysis of grave soils from Fewston are presented in Table 3.3. The sulfur contents were low or non-existent in all but two samples from the same grave (SK408; pelvis (2), S = 1.02 %; edge of the right leg (A1), S = 0.36 %). The measurable levels of sulfur coincide with observations of organic remains during sampling, suggesting that they are due to the preservation of organic matter. The two samples also contain appreciable levels of nitrogen. Notably, however, high nitrogen contents are not universally associated with high sulfur: the samples from SK331 show high nitrogen contents. The TOC contents show a wide range of variation from the lowest for SK277 (0.91 – 1.50 %) to the highest for SK408 (0.29 – 11.14 %). The two positions from SK408 that showed high levels of sulfur and nitrogen also show the highest levels of TOC determined among all of the graves. The high levels for soils of SK408, associated with the pelvis (2) and edge of the right leg (A1), may result from input from an external source: plant matter, maybe remains of a funerary bouquet, noted in the grave during sampling. TOC contents for all of the graves sampled at Fewston range from 1.1 – 23.1 % with the controls exhibiting a much restricted range of 0.5 – 3.2 % (Table 3.4). Overall, the controls show lower levels of TOC than the corresponding samples, indicating preservation of organic matter from the burials. No clear pattern in the TOC contents can be discerned across the site, though the highest average TOC content was for SK325 which was not only waterlogged but situated in a brick lined vault.

Table 3.3. Bulk elemental analysis for carbon, hydrogen, nitrogen, sulfur (CHNS) and total organic carbon contents (TOC) for SK277, SK331, SK334 and SK408. Elemental data given as weight percent.

		Sample weight (mg)		Elemental amount (%)				
Grave	Sample	CHNS	TOC	Nitrogen	Carbon	Hydrogen	Sulfur	TOC
SK277	Skull (1)	11.55	25.69	0.09	0.95	0.57	0.00	0.91
	Pelvis (2)	11.78	13.25	0.13	1.64	0.71	0.00	1.50
	Foot(3/4)	10.61	12.75	0.08	1.11	0.62	0.01	1.25
	Hands (16/17)	14.59	11.97	0.15	1.61	0.70	0.00	1.06
	Cranial Cavity (A1)	11.93	10.42	0.10	0.95	0.58	0.00	0.95
SK331	Control 2 (C2)	21.11	13.63	0.07	0.59	0.44	0.00	0.54
	Control 3 (C3)	19.27	15.96	0.15	1.57	0.67	0.00	1.32
	Skull (1)	11.18	12.91	0.26	4.27	0.90	0.00	3.63
	Pelvis (2)	12.81	10.58	0.08	0.82	0.57	0.00	0.72
	Feet (3/4)	13.40	14.93	0.08	0.92	0.67	0.00	0.80
	Hand (16/17)	16.68	16.53	0.08	1.04	0.63	0.00	1.03
	Organic matter below knee (11/12)	11.53	13.77	0.16	1.67	0.69	0.07	1.53
	Dark material under left of chin (A1)	13.54	11.96	0.36	5.86	1.12	0.01	4.90
SK334	Control 2 (C2)	10.96	13.11	0.10	0.82	0.56	0.00	0.77
	Control 3 (C3)	15.86	19.31	0.05	0.92	0.68	0.00	0.69
	Skull (1)	13.47	13.12	0.18	4.24	1.00	0.00	3.51
	Pelvis (2)	10.66	15.03	0.14	2.85	0.78	0.00	2.56
	Feet (3/4)	10.95	12.33	0.10	2.09	0.75	0.00	1.78
	Hands (16/17)	13.11	15.05	0.16	2.81	0.82	0.00	2.35
	Head Mouth (A2)	20.68	13.75	0.19	2.91	0.80	0.01	2.45
	Fibre from pelvis	0.89	-	8.47	38.35	6.44	1.74	-
SK408	Bottom coffin right head (1)	12.92	12.70	0.43	3.48	1.20	0.09	2.34
	Hair above (1A)	10.09	8.41	0.19	1.46	0.94	0.00	1.35
	Hair below (1B)	10.67	13.92	0.25	1.72	0.95	0.02	1.46
	Pelvis (2)	14.35	15.37	1.64	12.84	2.17	1.02	11.14
	Pelvic region (2A)	20.72	14.38	0.12	0.48	0.82	0.02	0.43
	Feet (3/4)	14.47	17.06	0.12	0.75	0.85	0.00	0.62
	Clay above knees (11/12)	12.16	15.86	0.15	0.43	1.01	0.00	0.29
	Edge of right leg (A1)	16.20	12.99	1.06	7.14	1.51	0.36	6.95
	F. B. feet (3/4 A)	12.00	13.66	0.12	0.58	0.80	0.00	0.48
	Plant matter right head (A2)	3.40	9.36	0.59	26.56	3.64	0.42	13.32
	Plant matter pelvis (A3)	3.24	3.78	2.44	30.14	4.23	1.19	29.91
	Plant matter edge right leg (A4)	8.87	7.64	1.75	19.58	3.02	0.60	17.94
	Long johns (A5)	4.19	6.73	11.91	36.65	5.52	2.17	34.26
	Hair (A6)	1.18	-	14.25	40.51	6.08	2.87	-

Table 3.4. Average total organic carbon contents for samples of grave soils and controls from the Fewston cemetery.

Grave	Controls	Average TOC (%)	Number of samples
SK277	-	1.1	5
SK310	0.6 (C3)	5.5	2
SK319	3.2 (C3)	5.4	20
SK325	-	23.1	19
SK331	0.5 (C2), 1.3 (C3)	2.8	7
SK334	0.8 (C2), 0.7 (C3)	2.5	5
SK408	-	3.0	8

3.3.2.2 Molecular profiles of soil extracts, Fewston

A series of *n*-alkanes ranging from C_{21}-C_{33} were present in the burial fills from SK277 (9.11×10^{-3} – 3.28×10^{-2} mg/ g TOC), SK331 (3.91×10^{-3} – 5.14×10^{-2} mg/ g TOC) and SK334 (1.17×10^{-2} – 3.58×10^{-2} mg/ g TOC), though not in those from SK408 (Figure 3:9). Comparison with the controls reveals similar levels for SK331 (4.24×10^{-2} mg/ g TOC) and much higher concentration levels in the fills of SK334 (8.44×10^{-4} mg/ g TOC). The higher *n*-alkane abundances in the samples from SK334 suggests an input other than from the soil background in this grave, possibly from the placement of a floral tribute. The *n*-alkane profiles from SK277 were all dominated by C_{33}, except for the skull and pelvic positions where *n*-C_{26} dominated. The *n*-alkane profiles for SK331 also show two distinct distributions, being dominated by C_{31} for all of the samples apart from two where the burial matrix was noted to appear distinctive: samples 12 and A1 (Table 3.3). The dominance of the C_{29} *n*-alkane in samples 12 and A1 is consistent with a distinct plant source. By contrast, apart from the control C2 and the pelvis (2), the soils from SK334 all exhibit bimodal distributions with maxima at C_{23} and C_{31}. The C2 control shows a unimodal distribution maximising at C_{31} whereas the pelvis exhibits a bimodal distribution with maxima at C_{24} and C_{33}. Dominance of the C_{23}, C_{24} and C_{26} *n*-alkanes is typical of bacteria or fungal signatures (Han and Calvin, 1969) whereas the longer chain components are typical of plant sources. Assigning an exact source is difficult due to the variations in the dominant *n*-alkanes even within one species (van Bergen et al., 1997a, Rieley et al., 1991). The differences between the profiles from the graves, in particular SK331, SK334 and SK277 may suggest that the signatures reflect the more open nature of the graveyard in the areas around SK331 and SK334 and the close proximity of trees and roots to SK277 (Figure 3:4).

The soils from SK408 did not contain detectable levels of *n*-alkanols, consistent with the lack of *n*-alkanes, whereas graves SK277, SK331 and SK334 contained *n*-alkanols ranging from C_{22} – C_{30} (Figure 3:9). The distributions from SK277 (4.35×10^{-4} – 5.62×10^{-3} mg/ g TOC) are dominated by the C_{26} (skull, cranial cavity and pelvis) and C_{30} (hands and foot) components. Control C3 for SK331 (3.66×10^{-2} mg/ g TOC) is dominated by C_{28} and, as with the *n*-alkanes, shows a similar abundance level to the grave soils. The other samples (5.33×10^{-3} – 1.64×10^{-1} mg/ g TOC) show bimodal distributions dominated by C_{24} and C_{28} for the dark material under the chin (A1) and the organic matter below the right calf (12). All other positions are dominated by C_{24} and C_{30}, apart from the pelvis where maxima were at C_{26} and C_{30}. Both controls from SK334 (9.64×10^{-4} – 4.42×10^{-4} mg/ g TOC) show much lower abundances of *n*-alkanols than the grave soils and, as for the additional sample A2, showed C_{26} to dominate. The feet position was dominated by C_{30} and the remaining samples (8.75×10^{-3} – 2.27 mg/ g TOC) showed maxima at C_{28}. All the dominant *n*-alkanols seen are typical of higher plant signatures (van Bergen et al., 1997a, Bull et al., 2000b). The differences in *n*-alkanol abundances between the controls and burial fills of SK334, together with the difference in *n*-alkane abundances suggests them to have the same source. Overall the differences in *n*-alkanol distributions among the sample positions suggest that a variety of higher plant sources contribute to the profiles in the grave.

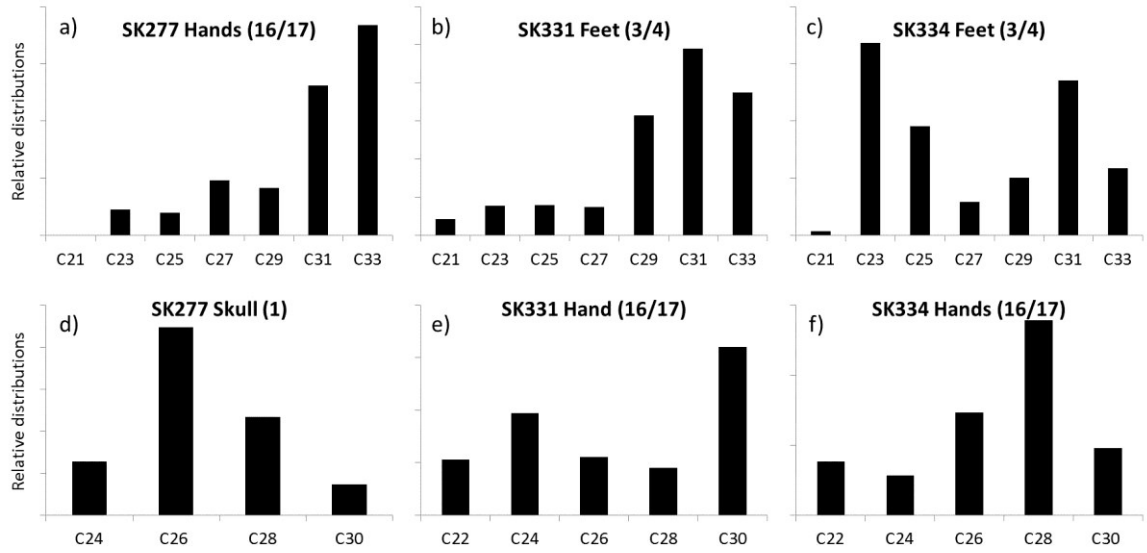


Figure 3:9. Profiles of *n*-alkanes (a to c) and *n*-alkanols (d to f) from the Fewston grave soils.

The fatty acids profiles of the grave soils range from $C_{14:0}$-$C_{30:0}$. In the absence of controls, which could not be obtained for SK277, the distributions were compared with the controls from nearby SK331. The distributions from the controls are dominated by long chain fatty acids typical of plant sources. The soils adjacent to the remains of SK277 show bimodal distributions comprising both short chain fatty acids ($C_{14:0}$-$C_{20:0}$; 2.78×10^{-5} – 5.30×10^{-5} mg/ g TOC) and long chain fatty acids

($C_{22:0}$-$C_{30:0}$; 2.50 x 10^{-5} – 1.05 x 10^{-4} mg/ g TOC)), the abundances of the latter dominating in all samples apart from the pelvis and feet. The short chain fatty acids from the cranial cavity and pelvis were dominated by $C_{18:0}$ with all other samples being dominated by $C_{16:0}$. The distributions of short chain fatty acids match those of degraded adipose tissue (Chapter 2, Figure 2:13) with low levels of $C_{16:0}$ in the samples from the pelvis and cranial cavity, suggesting a lack of microbial alteration.

Long chain fatty acids with maxima at $C_{28:0}$ dominate the controls for SK331. The grave soils show the short chain fatty acids (9.14 x 10^{-6} – 1.08 x 10^{-4} mg/ g TOC) and long chain fatty acids (1.57 x 10^{-5} – 1.39 x 10^{-4} mg/ g TOC) to be of similar abundance. The long chain fatty acids are dominated by $C_{28:0}$ (hand, 16 and feet, 3/4) and $C_{30:0}$ (pelvis, 2 and dark material under left of chin, A1). Short chain fatty acid distributions are dominated by $C_{16:0}$ or $C_{18:0}$ with $C_{18:0}$ being the dominant fatty acid for the controls and positions 2 and 12 (organic matter below right of the calf). The controls for SK334 are also dominated by long chain fatty acids (1.57 x 10^{-6} – 1.88 x 10^{-6} mg/ g TOC vs short chain, 2.20 x 10^{-7} – 3.97 x 10^{-7} mg/ g TOC) with maxima at $C_{28:0}$. The majority of the samples from the grave show short chain fatty acid distributions dominated by $C_{16:0}$ and a dominance of long chain fatty acids (3.51 x 10^{-5} – 2.60 x 10^{-4} mg/ g TOC) with similar profiles to the controls. The skull position is the only one to differ: the distribution is dominated by short chain fatty acids (1.09 x 10^{-5} – 3.47 x 10^{-4} mg/ g TOC) and the long chain fatty acid maximum occurs at $C_{26:0}$. The abundance levels in the controls of both SK331 and SK334 are lower than in the grave soils. This difference may reflect additional inputs associated with the burial, or possibly reflect the coffin and remains acting as barriers to the movement of clay particles with which the fatty acids are associated. As the distributions for the majority of the samples match those of the controls it would suggest the latter scenario to be the most likely.

The grave soils from SK408 show a greater variety of fatty acid distributions, typically with short chain fatty acids (7.61 x 10^{-2} – 5.64 mg/ g TOC) in greater abundance than the long chain fatty acids (8.85 x 10^{-2} – 1.61 mg/ g TOC). Samples of hair collected to the right of the head, above, below and from the bottom of the coffin, show equal abundances of short chain fatty acids and long chain fatty acids. The former are dominated by $C_{16:0}$ and the latter by $C_{26:0}$ (hair above) and $C_{24:0}$. Dominance of the long chain fatty acids occurred only for the pelvic region and clay around the knees, all other positions being dominated by short chain fatty acids.

Graves situated in the northern section of the graveyard have fatty acid distributions typically dominated by long chain fatty acid distributions similar to those of the controls from each specific grave. This, together with the lower levels of fatty acids in the controls than in the burial fills,

suggests that the soils from the graves in the northern section of the graveyard show distributions greatly influenced by the background soil profile with levels above background possibly being caused by the grave and remains, trapping fine clay particles and, hence, associated lipid components. In the southern section of the graveyard, however, SK408 shows the opposite to the northern graves with the burial fills being dominated by short chain fatty acids associated with the degradation of the adipose tissue of the interred remains.

Averaged short chain fatty acid distributions for all graves show SK319 and SK331 to be dominated by $C_{18:0}$ and the rest of the samples by $C_{16:0}$ (Figure 3:10). A few other differences are evident with $C_{14:0}$ being absent in SK310 and SK325 and $C_{18:2}$ only being present in SK310, SK319 and SK325. Uniquely, the profile from SK325 also shows $C_{18:1}$ to be in greater abundance than $C_{18:0}$. The distributions for SK277, SK334 and SK408 are all similar to that expected for degraded adipose tissue (Chapter2, Figure 2:13). Those for SK310 and SK331 are consistent with partially degraded adipose tissue, though $C_{16:0}$ is in lower abundance than might be expected for degraded adipose tissue. The distributions for SK319 and SK325 are consistent with only mildly degraded adipose tissue. The better preservation in these graves is consistent with their location in the region of the graveyard where waterlogging was evident. Comparison of the distributions with those of fresh adipose tissue suggests direct conversion of unsaturated moieties to saturated counterparts through reduction and associated β-oxidation as well as loss through water movement as suggested in Chapter 2. Some samples for SK408 show distributional differences that suggest an external input of fatty acids, the fatty acid profiles match those expected and seen for plant matter in the grave (Section 3.3.2.3).

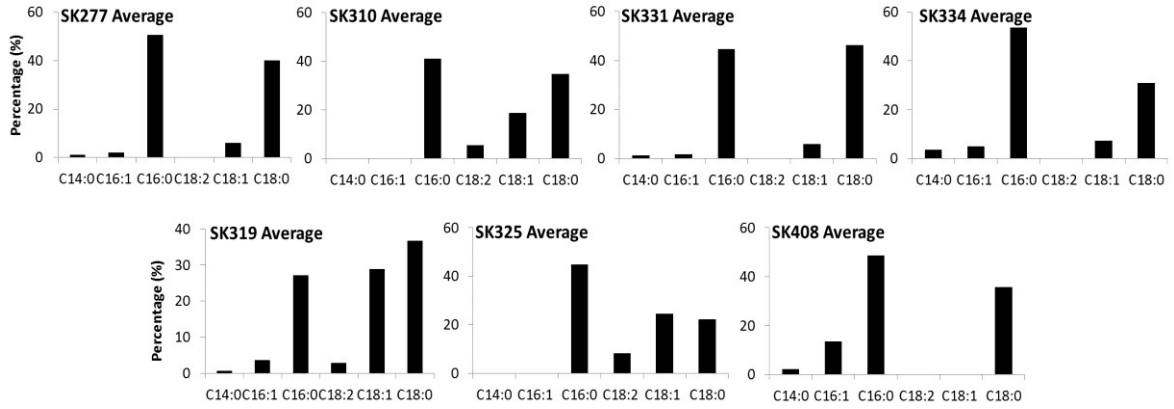


Figure 3:10. Average relative abundances of low molecular weight fatty acids for all graves sampled at the Fewston cemetery (n = 5 – 20)

Branched chain fatty acids were found throughout all sample sets with both C_{15} and C_{17} *iso* and *anteiso* fatty acids indicating bacterial input (Zelles, 1999, Dent et al., 2004). Significant input was

seen by Pickering (unpublished) in and around the body of SK319, with changes noted in the fatty acid abundances for positions that had high abundances of bacterial fatty acid markers, suggesting an association between fatty acid degradation and the presence of high abundances of branched chain fatty acids.

All of the samples from the waterlogged, southern end of the cemetery contained *n*-alkanals (SK319, 7.61 x 10^{-3} – 1.23 x10^{-1} mg/ g TOC, SK325, 7.05 x 10^{-2} – 2.69 x10^{-1} mg/ g TOC and SK408, 0.11 - 3.85 mg / g TOC) whereas none were detected in the graves situated in the northern section of the graveyard, where higher extents of degradation were visually apparent. The C_{16} and C_{18} *n*-alkanals are formed by reduction of fatty acids (Chapter 2), hence their presence is suggestive of oxygen limiting conditions (waterlogging, presence of water reduces oxygen circulation through the soil (Carter, 2005, Swift, 1979, Carter et al., 2007)). The clear divide between the north and south sections of the graveyard may reflect either the waterlogged nature of the southern end of the graveyard or the greater age of the graves situated in the northern section. The presence of *n*-alkanals in the two mass graves discussed in Chapter 2 points more strongly to oxygen limiting conditions being the key factor. The initial aerobic degradation of organic matter utilises the oxygen present in the burial environment, leading to oxygen limited conditions (Dent et al., 2004), conditions favoured in waterlogged environments.

Monoacylglycerols and diacylglycerols were not present in the grave soils for SK277, SK331 and SK334. GC-MS analysis of extracts from SK408 revealed the presence of low levels of monoacylglycerols but absence of diacylglycerols. HPLC-MS analysis revealed low levels of triacylglycerols, which were not present in the other graves, as well as two monoacylglycerols containing the fatty acid moieties $C_{16:0}$ and $C_{18:0}$. The presence of triacylglycerols and monoacylglycerols in SK408 reveals the preservation of organic molecules sourced directly from the degraded adipose tissue of the remains. The fatty acyl moieties of the monoacylglycerols ($C_{16:0}$ and $C_{18:0}$) represent a source for the fatty acids in the samples. Samples analysed by Pickering (unpublished) showed low levels of TAGs and no MAGs or DAGs in SK310 and SK325, yet SK319 showed the presence of MAGs only.

Analysis of the TAGs and their degradation products arising from hydrolysis and reduction (Figure 3:11) shows differences in the distributions over the graveyard. Of the graves in the southern end, SK310, SK325 and SK408 all contained TAGs and SK319 contained low levels only from around the pelvic area, whereas monoacylglycerols occurred at all other positions. Samples from SK319, SK325 and SK408 all contained *n*-alkanals, fatty acid reduction products, and were waterlogged on sampling, consistent with the presence of the anaerobic conditions that are

needed for the formation and preservation of *n*-alkanals. By contrast, SK277, SK331 and SK334 exhibited profiles dominated by $C_{14:0}$-$C_{18:0}$ fatty acids, acylglycerols being absent. Hence, it appears that degradation proceeded to a greater extent than for the graves from the southern end, as would be expected of these possibly older graves and from the visible signs of more extensive degradation. The abundances of the fatty acids at these positions are also significantly lower than those from the southern graves, suggestive of more extensive degradation. Hence, the profiles suggest that, whereas degradation of TAGs was complete in the graves of the northern end of the graveyard, the waterlogged environment in the southern end allowed for better preservation. In particular, the visible remains, presence of TAGs and higher abundances of lipids in the graves of SK325 and SK408 attest to the better conditions for preservation of organic remains.

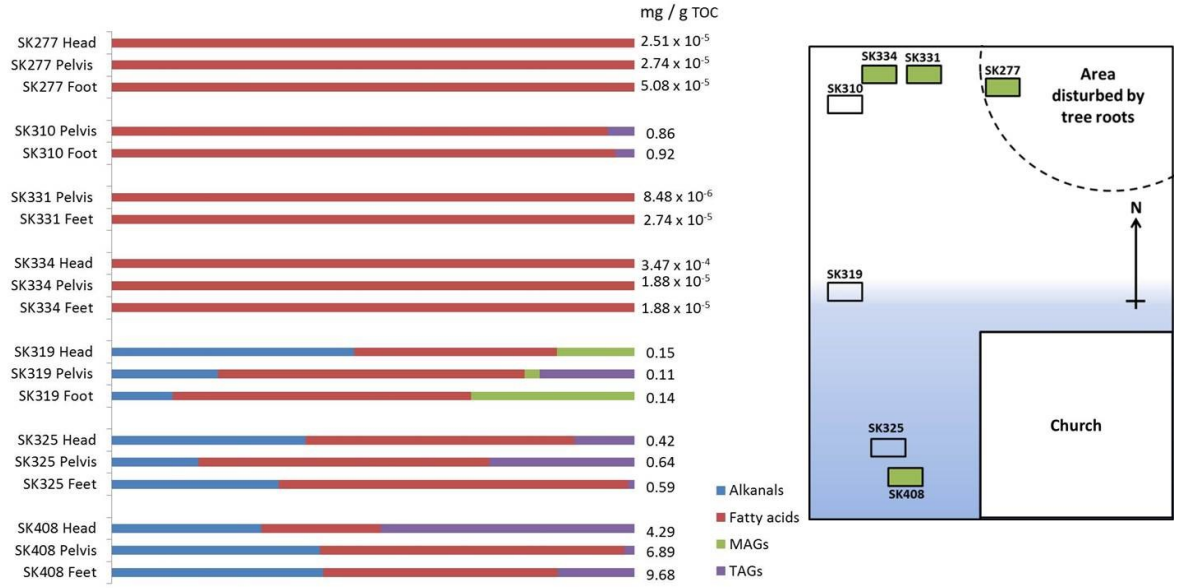


Figure 3:11. Mass balances for triacylglycerols and their degradation products formed by hydrolysis and reduction for all graves from the cemetery at Fewston that were examined during the InterArChive project. Graves shown in green analysed by author, all other graves examined by (Pickering. M).

The total extracts from all graves produced complex gas chromatograms dominated by components eluting in the medium volatility range (Figure 3:13). Further analysis of the total extracts by GC-MS showed the dominant components to typically be abietic acid, didehydroabietic acid, dehydroabietic acid, 7-oxo-dehydroabietic acid and pimaric acid (Figure 3:12). These diterpenoids derive from gymnosperm wood and their presence in the graves, especially SK277, SK331 and SK334 which showed little or no visual signs of coffin wood, confirms the presence of coffins. The terpenoids present suggest the genus *Pinaceae* (Evershed et al., 1985, Colombini et al., 2003). By contrast, the major diterpenoids in the chromatograms for

SK408 were didehydroabietic acid, dehydroabietic acid and 7–oxo-dehydroabietic acid. The absence of abietic acid, in particular, suggests that the coffin wood or treatment may be of a different nature to those for SK277, SK331 and SK334. Alternatively, the differences may relate the extent of degradation (Robinson et al., 1987). The oxidation product of dehydroabietic acid, 7-oxo-dehydroabietic acid was detected in greater relative abundances in the graves of SK277, SK310, SK331 and SK334 than in those at the southern end of the graveyard, indicating more extensive degradation of the resin signature and more oxidising conditions. The evidence for more oxidising conditions in the graves in the northern end of the cemetery is consistent with the absence of *n*-alkanals (discussed above).

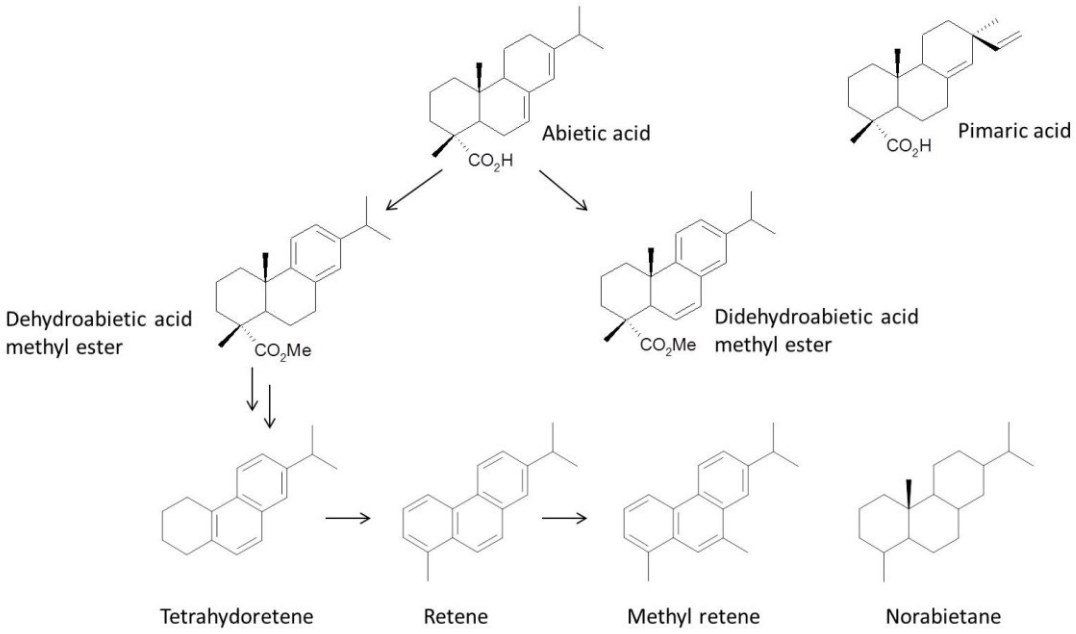


Figure 3:12. Structures of abietic acid and pimaric acid (resin acids), degradation products (dehydroabietic acid and didehydroabietic acid) and thermal degradation products (retene, methyl retene, tetrahydoretene and norabietane) found in samples from Fewston.

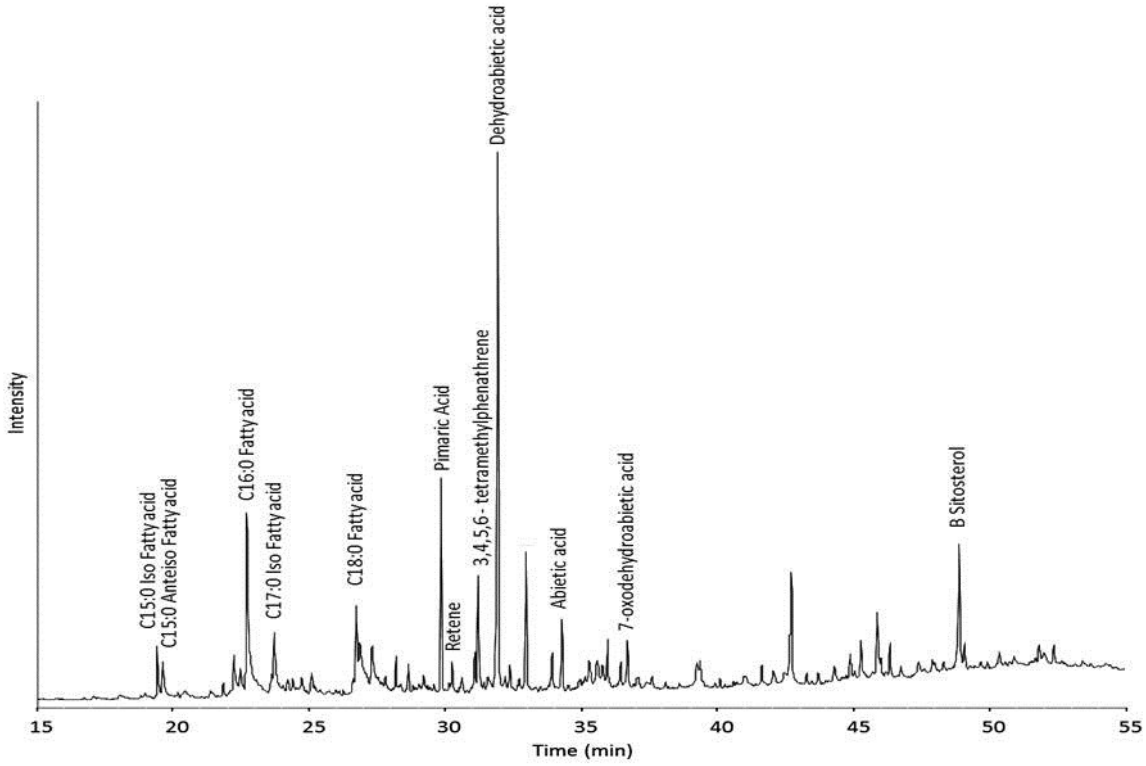


Figure 3:13. Gas chromatogram of the total extract from soil adjacent to the pelvis of SK334. Fewston, North Yorkshire showing the profile to be dominated by fatty acids and terpenoids. All acids were analysed as the methyl esters.

Given the complex nature of the total extract chromatograms, extracts were fractionated. Both high and medium polar fractions were dominated by the terpenoids. Analysis of the aromatic hydrocarbon fraction revealed the presence of methylretene, retene and tetrahydroretene (Figure 3:13). These aromatic transformation products of the diterpenoids abietic and pimaric acid are formed during thermal alteration of conifer resins (Connan and Nissenbaum, 2003, Evershed et al., 1985, Robinson et al., 1987, Colombini et al., 2003, Simoneit et al., 1986). The presence of these compounds, together with several other, minor, thermal alteration products is consistent with the application of a waterproofing or varnishing agent (Evershed et al., 1985, Robinson et al., 1987). The presence of abietic acid, in SK277, SK331 and SK334, a molecule which does not survive thermal treatment (Robinson et al., 1987, Evershed et al., 1985) and hence would not be associated with a thermally treated product, indicates an origin from the coffin itself. The absence of abietic acid in the extract from SK408 most likely reflects the lower extent of degradation than in the graves from the northern end of the cemetery or indicates the wood to be angiosperm; it was suggested by the archaeologists to be oak (Buglass, 2010). Gas chromatography analysis of a small accumulation of resin-like material (A1) recovered during the sampling of SK334 revealed it to comprise mainly terpenoids, predominantly retene and methyl retene (Figure 3:14). These components are expected from a thermally treated resinous material

of *Pinaceae* origin and their presence in the resin and the absence of abietic acid further supports the interpretation of the distributions in the soil extracts.

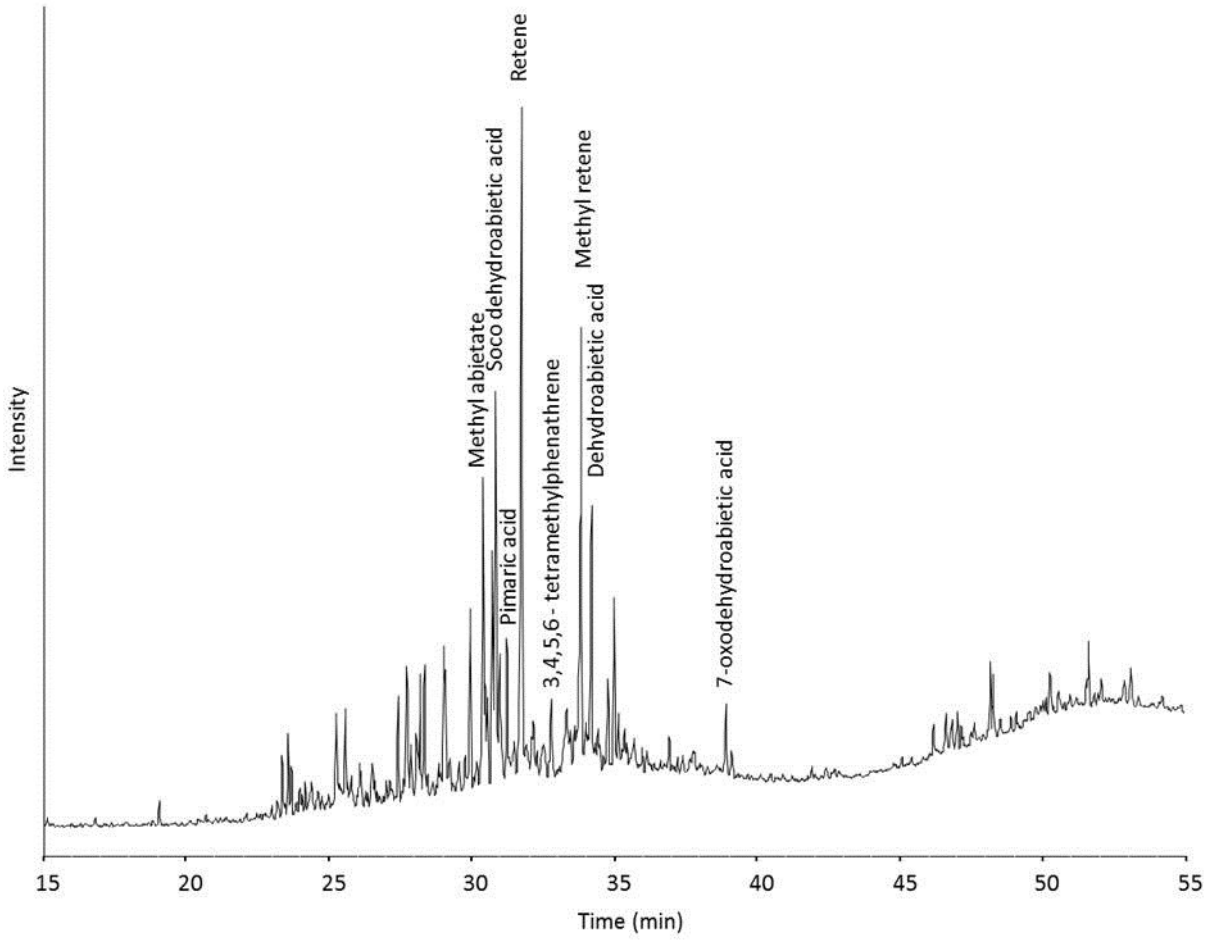


Figure 3:14. Partial gas chromatogram of the total extract of resinous material (A1) from SK334, Fewston, North Yorkshire, showing the presence of thermal degradation products of abietic acid and pimaric acid. All fatty acid components were analysed as the methyl esters unless noted otherwise (SE=silyl ester).

Pickering et al. (unpublished) noted that the presence and abundance of resin acids in the graves, together with the distributions of fatty acids for SK319, suggest that the former may have inhibited microbial degradation. Pickering's work showed that positions around SK319 which contained the highest abundances of resin acids also showed the highest levels of fatty acids and lowest bacterial signatures (branched fatty acids). This is consistent with the similarities, mentioned earlier, in the distributions of fatty acids for SK319 and fresh adipose tissue. Analysis of SK325 and SK408 situated at the southern end of the graveyard, both of which had the high abundances of resin acids, did not show correlation between fatty acid abundance/preservation and resin acid location. This difference may be due to the waterlogged nature of the graves

preventing the degradation of the coffin and limiting the distribution of resin acids throughout the grave, as was apparent for SK319.

Plant and animal sterols were both in low abundance in the grave soils. Cholesterol was only present in SK408, with the other graves only showing sterols of plant origin, β-sitosterol and stigmastanol (Bull et al., 2000b). The presence of the plant sterols in the controls suggests these signals are from the environment. As SK277, SK331 and SK334 contained very limited skeletal remains compared with SK408, the lack of cholesterol, a degradation product also linked to degrading bone (Evershed et al., 1995, Jim et al., 2004), is consistent with the remains being much more degraded. Samples from SK319 showed the presence of both cholesterol and β-sitosterol, the latter being the more abundant of the two.

3.3.2.3 Elemental and molecular profiles of isolated materials

Elemental analysis of the fibre from the pelvis (2) of SK334 reveals that not only are the carbon and hydrogen levels much higher than those of the soil samples but also the nitrogen and sulfur contents (Table 3.3). The ratio of N/C (0.3) shows the fibre to have a composition consistent with proteinaceous material (cf. Chapter 2). Such an origin is supported by the C/O ratio (2.46), the value being consistent with a protein based material such as silk or wool as opposed to cellulose based materials such as linen or cotton. The sulfur content (1.74 %) is significantly higher than that expected for silk 0.0-0.4 %; (Shimura, 1983) indicating the fibre is most likely wool in origin, the latter having typical values in the range 3-4 % (Zahn et al., 1997). The value obtained from the fibre may be lower due either to its partial degradation or to natural variation as the sulfur levels of wool being dependent on the diet of the animal (Reis and Schinckel, 1964). The values for the sock from SK408 are similar to that of the fibre from the pelvis (2) of SK334. The only difference is the higher sulfur content (2.17 %) which, though closer to the expected value for wool, may reflect the lower degree of degradation in this grave than for the SK334 grave. Fibres found in various positions around SK319 also show elemental compositions similar to those recovered from SK408 and SK334. Visual (microscopy) evidence suggests the single fibre from SK334 to be significantly more degraded than the fibres of the sock from SK408 (Pinder, 2017). Further studies of the material using amino acid analysis confirmed both materials to be of wool origin (Pinder, 2017).

Plant matter was found in three of the positions sampled for SK408. The soils of two of these positions were noted to have high nitrogen and sulfur contents. Elemental analysis of the plant matter from these positions (A3 and A4) show levels of sulfur and nitrogen two orders of magnitude higher than those of the soils (cf. Table 3.4). The high levels in the plant matter

suggests that the organic matter in the soil is directly linked, comprising degraded plant matter. TOC contents of the plant materials were also much greater than for the soils, suggesting lower degrees of degradation/dilution with mineral phase. All of the plant matter showed fatty acid distributions similar to that of the soil in which the sub sample was collected, with one noted difference being a higher abundance of $C_{16:0}$. The high level of $C_{16:0}$ can be directly related to plant matter as short chain fatty acid distributions for plants are dominated by $C_{16:0}$. Samples closely related to the plant matter in the grave have marked differences in their distributions from the other soil samples, consistent with inputs of plant fatty acids. The similarity in distributions for both plant matter and soils suggests that the soil signatures are mainly from degraded plant matter.

3.4 Conclusion

Overall, Haymarket samples show no clear differences between the sampled planes. The TOC contents varied over a narrow range (0.32 – 1.44 %), the most noticeable feature being the highest levels representing positions adjacent to the left leg. Micromorphological analysis showed these positions to exhibit dark stains impregnating micro-fissures in the bones. Chemical analysis of the soils showed the presence of polycyclic aromatic hydrocarbons from coal tar. The location and elevated signatures by the left leg can be attributed to a service trench that cut the remains and allowed their contamination.

Elemental analysis of the soils from Fewston showed slight differences between the controls and grave soil, with the highest levels of organic carbon being present in the graves situated at the southern end of the graveyard where better levels of preservation were evident during excavation. Samples from SK408 contained artefacts from the burial practice: plant matter possibly the remnants of a funerary bouquet were recovered and the impact of the plant matter on the elemental composition of the soils was established. Thus, the positions at which plant matter was present showed elevated levels of nitrogen and sulfur in the soils. The organic matter in these soils showed a clear origin from the plant material, the nitrogen and sulfur contents being at least twice that of the grave soils in all other locations. The *n*-alkane profiles in Haymarket reveal low levels in the C2 to C3 controls and slightly higher levels around the remains. Both *n*-alkanes and *n*-alkanols show little variation throughout the grave fills and are typical of plant matter. In Fewston, the *n*-alkanes and *n*-alkanols in graves SK277, SK331 and SK334 also show distributions characteristic of plant material. The similarities in the distributions of the controls and the grave soils suggests these signatures to reflect the background soil organic content.

Fatty acid distributions for Haymarket show clear dominance of plant-derived long chain fatty acids throughout the grave soils and controls, hence the majority of signatures in Haymarket reflect the background soil organic matter. The low levels of short chain fatty acids are heavily degraded remains of adipose TAGs with high levels of reduction/conversion and loss of fatty acids ($C_{14:0}$ fatty acids lost through water movement). The extensive degradation/alteration, reflects either the environmental conditions in the grave or the archaeological age of the burials. The differences in distribution to those from Fewston suggest that age may be a factor influencing the extent of degradation in the signatures. Nevertheless, the presence of signatures from adipose tissue in the older graves from Fewston indicates that age is not the most important factor in the preservation or alteration in the soil organic matter signatures. The better preservation in graves located in areas of waterlogging in Fewston suggests that hydrology is a key factor in preservation. Haymarket may have experienced significant movement of water, leading to alteration in the soil organic matter distributions to a much greater extent than in the graves in the northern section of the graveyard at Fewston.

The lipid abundances in the Fewston graves are significantly lower in the northern graves than in the southern part of the graveyard. The lipids in the soils from the SK277, SK331 and SK334 graves were in low abundances and dominated by long chain fatty acids having distributions indicative of plant organic matter. Short chain fatty acids dominate the profiles from the SK408 grave soils and exhibit variations in the levels of degradation. The best preserved signatures, similar to adipose tissue, are from remains in the southern end of the graveyard. The signatures matching those of degraded adipose tissue and the expected distribution from conversion/reduction of fatty acids. A mass balance of the acylglycerols, fatty acids and *n*-alkanals shows the northern graves (SK277, SK331 and SK334) to be dominated by fatty acids and the southern graves showing MAGs, TAGs and *n*-alkanals. Higher levels of preservation in the southern graves is evidenced by the preservation of TAGs as the dominant species in the grave soils. Signatures derived from the remains were recognised in all graves, with differences in the levels of preservation being observed.

Resin acids dominated the HP fractions and total extracts of all grave soils from Fewston. Graves SK277, SK331 and SK334 showed resin signatures from the coffins, which indicate them to have been of gymnosperm (*pinacea*) origin. A resinous aggregate from the grave of SK334 showed a strong signature for products of thermal degradation of wood resins, indicative of a pine pitch wood treatment. The presence of high relative abundances of 7–oxodehydroabietic acid, an oxidation product of dehydroabietic acid, suggests oxic environments in these graves. Abietic

acid, absent in SK408, suggests the coffin to be angiosperm in origin (coffin believed to be oak in nature, (Buglass, 2010)). Signatures from resins are lower in abundance in the southern end of the graveyard and may be linked to the waterlogged nature of the graves and preservation of the coffin. The waterlogged graves contained *n*-alkanals, reduction products of fatty acids, suggesting the environment to be anoxic and therefore conducive to organic preservation.

The aims of the investigation detailed in this chapter were to determine if single inhumations show signatures related to the buried remains. Such signatures were identified and provide evidence for the environmental conditions within the grave and site with both oxic and anoxic environments being identified. Notably, the single inhumations examined did not show the same level of lipid preservation as occurred in the mass graves, the signatures being dominated by background soil signals. Multiple graves across a site also showed that marked variation between graves can be evident within the same site.

4 Experimental burials in defined mineral compositions

4.1 Introduction

The study of experimental burials has proved to be of considerable value to the forensic and archaeological communities in aiding interpretation of real-life situations. The use of pig cadavers as analogues for human bodies limits ethical considerations, the decomposition of pig carcases having been shown to exhibit close parallels to those of humans (Dent et al., 2004, Notter et al., 2009). Thus, forensic investigations on pig carcases have aided the determination of the time elapsed since deposition, development of approaches to confirm the presence/absence of a body and determining if a clandestine burial has occurred (Carter et al., 2007, Benninger et al., 2008, Cassar et al., 2011). These approaches include evaluation of the incorporation of organic components from the body into surface soils and shallow graves during short-term burials (Carter et al., 2007, Benninger et al., 2008, Cassar et al., 2011). The absence of studies considering the incorporation and preservation of organic residues in the context of longer terms of interment and greater depth of burial prompted these investigations into lipid biomarkers in conventional depth graves having distinctly different burial matrices. As part of the InterArChive project (Usai et al., 2014), experimental burials, coffined and non-coffined were buried in five locations to aid the interpretation of archaeological graves. The locations were chosen to encompass variations in soil type and environment and, in four cases, to utilise sites that had previously yielded archaeological finds.

The work presented here concerns two burials alongside a control piglet (not buried), in which the matrix used to fill the grave was highly controlled, giving a clear starting point to allow identification of lipids from the remains. The overall aim was to study the effects of the environment on the organic remains in the burial in order to gather information that could further understanding of the significance of the findings in archaeological graves.

4.2 Sampling and sampling strategies

Piglets were buried at Hovingham (HO12), Yorkshire, UK for a period of 3 years. The piglets were buried in wooden coffins; the coffins were filled with the burial matrix (to ensure that the matrix interacted with the degrading remains as the coffins were not expected to degrade in the time span of the experiment). For ethical reasons all piglets used in the experiments died of natural causes (stillborn or suckling death). Each burial was augmented by a selection of grave goods placed in two separate fabric bags, the first placed at the snout and the second in the abdomen (Table 4.1). The grave goods were chosen to include materials that have been found in archaeological sites as well as a range of foodstuffs. The filled coffins were placed in a grave of a depth of 0.5 m that had been shored with plywood and partly filled with the appropriate matrix,

sand or limestone. The graves were backfilled with more of the matrix, covered with mesh to prevent excavation by animals. The coffins used were purchased and believed to each be of a single wood type. Further investigation found them to be made from a variety of wood materials (Pinder, unpublished).

Table 4.1. Additional materials added to grave in the location of the stomach and snout.

Fabric bag containing foods in stomach area:	Fabric bag close to snout containing foods:
Rolled oats 25 g	Florets of cloves 60 g
Desiccated coconut 25 g	Amber leaf tobacco 5 g
Dried clementine peel 5 g	Beeswax 5 g
Dried apple peel ~2 g	
Ground ginger 1 teaspoon	
Ground cinnamon 1 levelled teaspoon	
Red kidney beans 25 g	

The graves were excavated in September 2012, approximately three years after burial. At that time the top surfaces of the graves were covered in leaf litter. Samples of loose sediment for chemical analysis were collected in the closest representation to the InterArChive sampling protocol (Usai et al., 2013), translated to the pig skeletal structure (Chapter 7, Figure 7:1). A control piglet (piglet C; Table 4.2) was also obtained in order to determine the lipid residue profiles in undegraded porcine tissue. The sampling for the piglet burials from Hovingham, piglet S (sand, Figure 4:1) and piglet L (limestone, Figure 4:2) included several controls of the grave fill as well as samples collected additional to the defined anatomical locations (Figure 4:3). Thus, C0 was the original burial matrix added to the graves (piglet S, sand and piglet L, limestone), a C1 control was collected from the surrounding soil of the site, C2 and C3 at differing depths in the grave fill and C4 within the coffin and just below the lid. The coffins in both burials were intact (Figure 4:1 and Figure 4:2) and showed some fungal activity. Inside the top of the limestone grave flies, pupal cases and tunnels were present, providing clear evidence of movement of organisms through the limestone (Figure 4:2). On excavation leaf litter was noted on the surface of the grave fill, though, unfortunately, this was not sampled.

Table 4.2. Positions sampled from the control piglet

Sample position	Composition
Stomach	Organ and internal contents
Ham	Subcutaneous fat
Shoulder	Subcutaneous fat
Gut	Intestines and contents
Belly	Subcutaneous fat
Loin	Subcutaneous fat

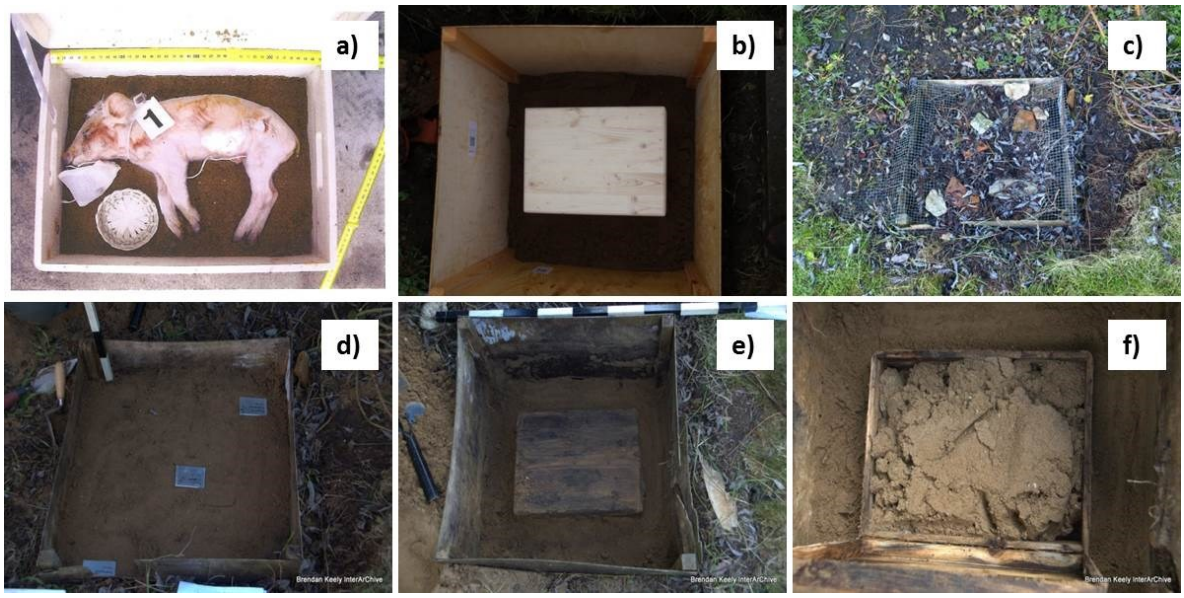


Figure 4:1. Piglet S prior to burial at Hovingham in a sand matrix, top left, located in the grave(middle) and at various stages in the excavation showing leaves above the grave fill, positioning of tins from micromorphology, condition of the coffin and the fill within the coffin. a-b) coffin and grave layout before infilling and burial of remains. c) grave site before excavation showing overlying leaf litter and open nature of the soil matrix. d) Start of excavation and the collection of first controls (C2). e-f) state of coffin lid on excavation and the inside on opening of the coffin.

Figure 4:2. Piglet L situated at Hovingham in a limestone matrix all pictures from excavation with clear signs of leaf litter above the grave, condition of coffin and the remains of flies and casters in the top of the coffin fill along with small burrows through the fill. a-b) the grave site before excavation showing overlying leaf litter and the surface of soil matrix on leaf litter clearence. c) state of coffin lid on excavation with the presence of white rot. d-e) inside of the top of coffin on excavation and the presence of small holes and fly pupae.

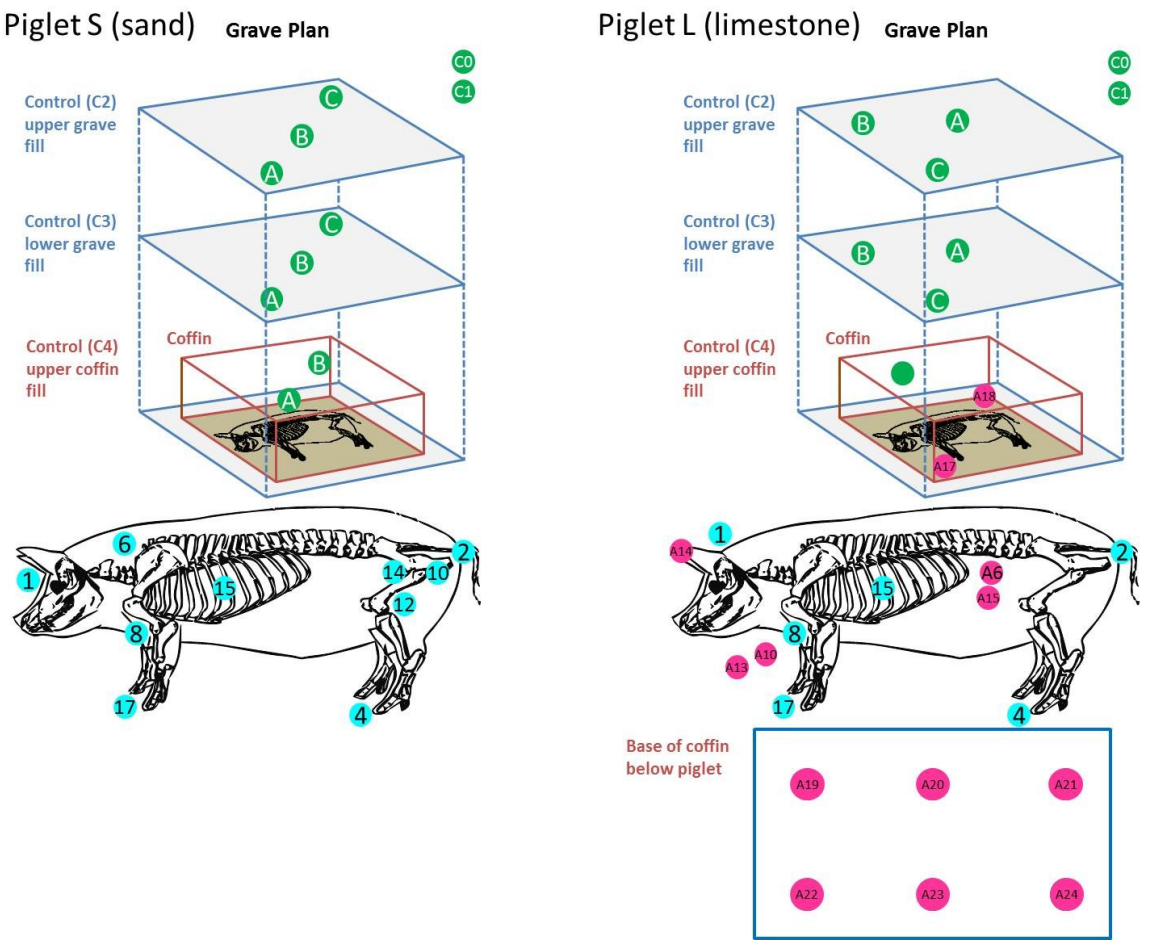


Figure 4:3. Positions sampled from piglets buried at the Hovingham site. Piglet S buried in a sand matrix and piglet L in limestone matrix. Positions were sampled from the top of the grave fill to the base of the coffin. Controls are in green, skeletal positions in blue and additional samples in pink.

4.3 Results and discussion

4.3.1 Elemental analysis of soil samples

Bulk elemental analysis (CHNS) of the control soils (C2-C4) and the original burial matrices (C0) showed low levels of nitrogen and an absence of sulfur (Table 4.3 and Table 4.4). The total organic carbon (TOC) contents for the controls match that of the original burial matrix (C0) with a clear difference from the natural soil of the site (C1) (Table 4.3 and Table 4.4). The highest TOC value was for C1 (site control), consistent with it being a loamy soil having regular input from degrading plant matter. TOC values for the upper coffin fill (C4) for both piglets are similar to the rest of the fill controls (C0–C3). The TOC contents of the soils in the coffins were higher than for the controls, suggesting contributions of organic carbon from the carcass and/or grave goods. No sulfur was present for piglet S and only sample A15 from piglet L, which was taken from in the gut bag, contained sulfur. Nitrogen levels varied across both graves with piglet S ranging from 0.06 - 0.85 % and piglet L showing a much greater range (0.00 - 3.66 %) with the highest positions

relating to fly pupal cases (A5), the wicker basket (A12) and foodstuffs from the gut bag (A15). Positions located adjacent/near to the grave goods also show the highest levels of TOC, values greater than 1.00 % indicating good preservation and incomplete degradation of some of the added materials. The TOC contents of the fills from positions located around the abdomen of piglet S (Figure 4:4) were higher than for other samples, as might be expected due to the high levels of organic matter associated with the body and grave goods. Sample position 8 gave the highest TOC value external to the body, probably reflecting its proximity to the organic matter in the fabric bag situated at the snout. The control positions C2A and C3A for piglet L also gave elevated TOC contents tentatively suggested to reflect input of organic matter from the top of the fill. The TOC contents for positions around the centre of the body of piglet L were higher than those in the soils adjacent to the remains (Figure 4:5). Exceptionally, the front foot position (16/17) gave a high value which may be associated with the organic matter in the fabric bag at the snout position and the wicker basket in the grave. The positions directly related to the contents of the fabric bags gave high TOC values, similar to the positions surrounding the bags. The sample position A13 taken from beneath the fabric bag in the gut region gave a low TOC value compared with the contents of the bag. This suggests that the majority of the organic matter remained localised to that specific position. Samples taken from the base of the coffin for piglet L also show low TOC values similar to that of the background (C0) material, suggesting little input from the piglet and grave goods. The carbonate nature of the matrix for piglet L may have affected the TOC levels as although extensive acid treatment as carried out, complete removal of carbonate material does not appear to have been achieved.

Table 4.3. Elemental analysis (CHNS) and total organic carbon (TOC) results for Hovingham piglet S, sand matrix.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 - sand matrix	0.08	0.52	0.12	0.16	0.12
C2A	0.00	0.56	0.11	0.00	0.11
C2B	0.02	0.72	0.15	0.00	0.24
C2C	0.01	0.51	0.12	0.00	0.14
C3A	0.00	0.46	0.12	0.00	0.12
C3B	0.01	0.52	0.12	0.00	0.12
C3C	0.02	0.70	0.14	0.00	0.11
C4A	0.03	0.59	0.13	0.00	0.19
C4B	0.02	0.39	0.13	0.00	0.10
1y - skull	0.11	0.90	0.18	0.00	0.61
1z - skull	0.20	1.47	0.24	0.00	0.78
2y - pelvis	0.06	0.62	0.16	0.00	0.31
4y - back feet	0.06	0.58	0.16	0.00	0.29
6z - shoulder	0.13	0.93	0.22	0.00	0.74
8z - elbow	0.85	6.84	1.00	0.00	2.83
10z - pelvis	0.08	0.67	0.16	0.00	0.32
12z - knee	0.20	1.32	0.28	0.00	1.02
14z - pelvis	0.28	1.95	0.34	0.00	1.93
15z - abdomen	0.36	2.27	0.38	0.00	1.90
16y - front feet	0.20	0.92	0.23	0.00	0.13

Values given as mass percentage.

Table 4.4. Elemental analysis (CHNS) and total organic carbon (TOC) results for Hovingham piglet L, limestone matrix.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 - limestone matrix	0.01	10.84	0.05	0.00	3.78
C1 - site control	0.54	6.01	1.03	0.00	4.98
C2A	0.25	12.83	0.24	0.00	2.59
C2B	0.06	10.33	0.15	0.00	0.51
C2C	0.08	10.47	0.18	0.00	0.62
C3A	0.29	14.53	0.28	0.00	4.31
C3B	0.07	10.66	0.17	0.00	0.65
C3C	0.00	7.89	0.07	0.00	0.30
C4 - control coffin fill	0.03	11.12	0.06	0.00	0.20
1y - skull	0.00	11.15	0.10	0.00	3.21
1z - skull	0.00	8.90	0.10	0.00	4.91
2z - pelvis	0.33	12.19	0.33	0.00	7.85
3/4z - back feet	0.37	12.33	0.26	0.00	4.48
15z - abdomen	0.31	11.85	0.30	0.00	6.08
16/17y - front feet	0.05	14.18	0.11	0.00	8.06
A5 - insect larval casings	3.66	22.75	2.26	0.00	14.91
A6 - crust on roof of cavity	0.01	12.67	0.06	0.00	6.74
A10 - sample of beeswax	0.01	79.65	13.94	0.00	80.65
A12 A - base of wicker basket	0.80	16.87	1.05	0.00	3.40
A12 B - base of wicker basket	2.79	35.63	4.08	0.00	33.41
A13 - below fabric bag at snout	0.16	12.06	0.19	0.00	0.81
A14 1 - adipocere skull	0.20	11.44	0.22	0.00	0.64
A14 2 - adipocere skull	0.33	11.81	0.31	0.00	5.27
A15 - contents of gut bag	1.87	20.76	1.91	0.05	6.49
A17 - North east corner of coffin	0.00	11.48	0.06	0.00	0.16
A18 - South west corner of coffin	0.02	11.53	0.07	0.00	0.17
A19 - soil in contact with base of coffin	0.10	11.98	0.16	0.00	0.47
A20 - soil in contact with base of coffin	0.05	11.63	0.09	0.00	0.30
A21 - soil in contact with base of coffin	0.10	11.72	0.11	0.00	0.35
A22 - soil in contact with base of coffin	0.06	11.51	0.08	0.00	0.31
A23 - soil in contact with base of coffin	0.03	11.61	0.07	0.00	0.34
A24 - soil in contact with base of coffin	0.04	11.58	0.09	0.00	0.23

Values given as mass percentage.

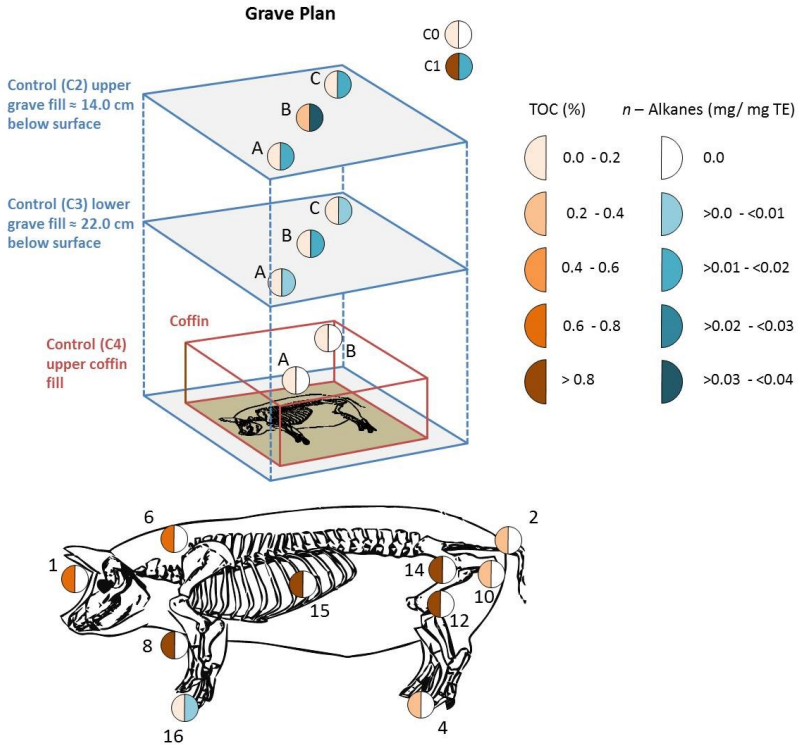


Figure 4:4. Positions sampled for piglet S sand matrix, indicating total organic carbon contents and *n*-alkane abundances in controls and around the carcass.

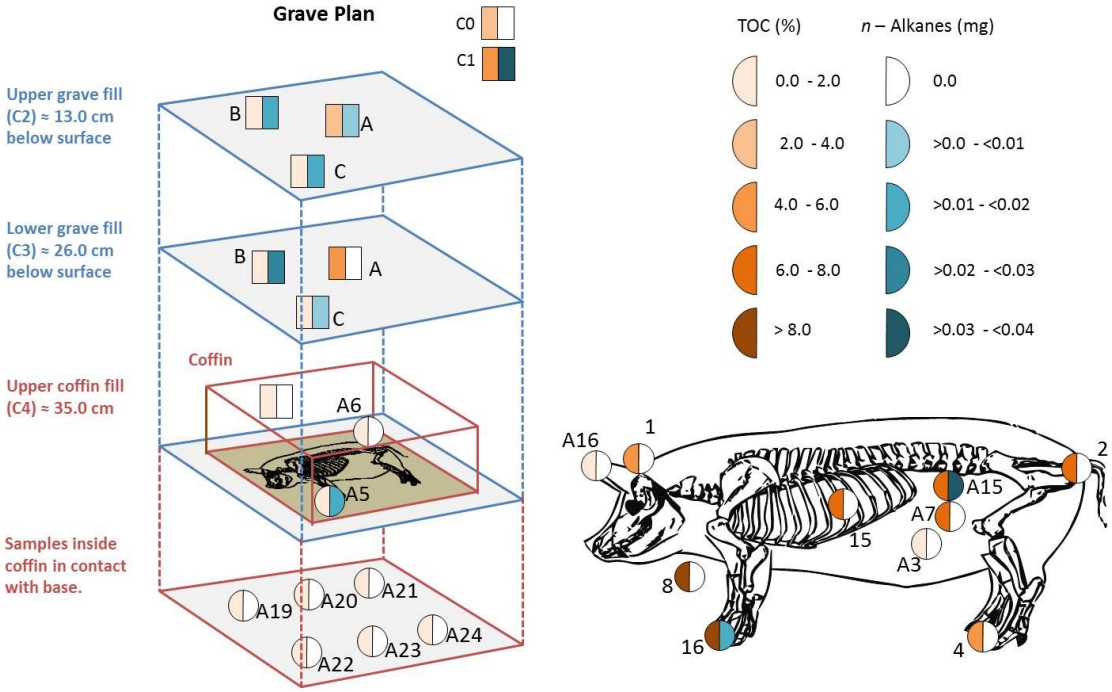


Figure 4:5. Positions sampled for piglet L, limestone matrix, indicating total organic carbon contents and *n*-alkane abundances in controls (squares, C0-C4) and around and below the carcass (circles).

A pseudo van Krevelen diagram of H/C(TOC) vs N/C(TOC) for piglet S (Figure 4:6) shows a clear difference between the controls and samples related to the remains, with a distinct difference to the original matrix material (C0) showing additional input. The majority of samples from positions close to the remains cluster in three groups, samples from the head region, abdomen and rear/pelvic area. These clusters reflect the close proximity of the samples due to the size of the remains and samples taken, with clusters formed by samples taken near or even overlapping each other. The sample 16y is a clear outlier suggesting a major input of organic material at this position. As this sample was taken near to the remains of the wicker basket and fabric bag at the snout the noted difference may be due to residues of wicker and plant matter in the sample. The groupings are strongly influenced by the TOC contents, a plot of H/C vs N/C for piglet L shows a large spread in the data with no clear clusters. This spread and lack of groupings may be due to the nature of the matrix and the difficulties of removing all inorganic carbon providing unreliable results.

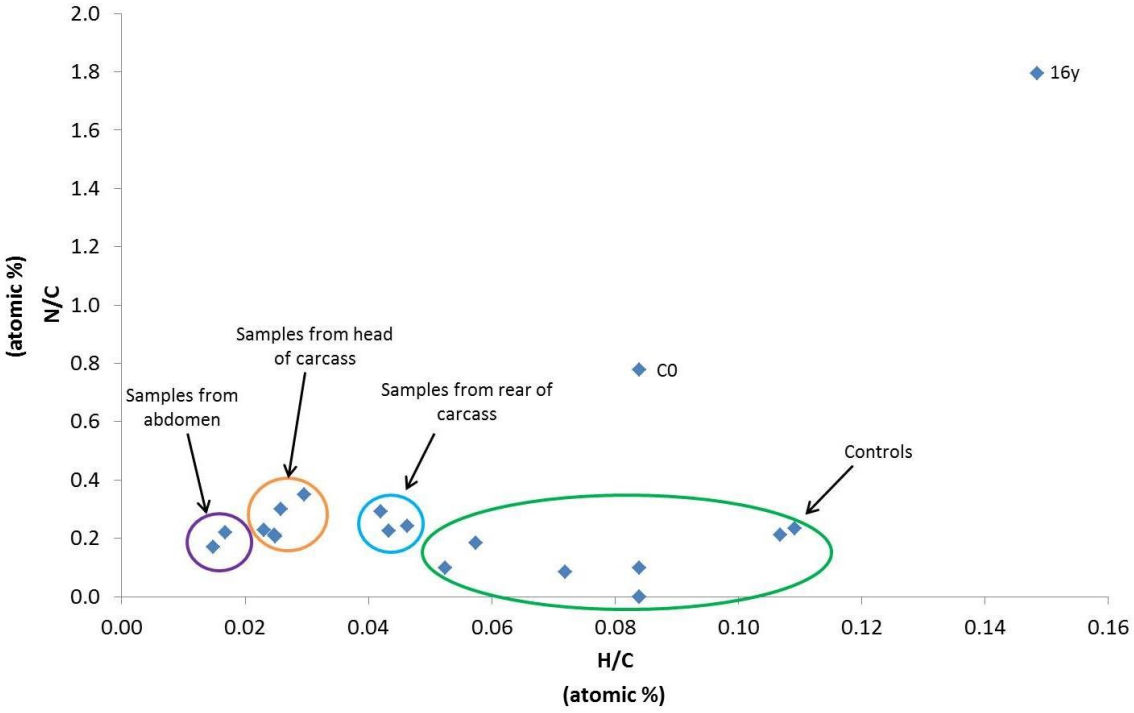


Figure 4:6. Pseudo van Krevelen diagram for H/C vs N/C of piglet S, showing the similarities of samples in the groupings of controls, samples from rear of carcass, abdomen and head with clear distinction from C0. Ellipses show sample groupings.

4.3.2 Molecular profiles of extracted soil

The C0 controls were essentially devoid of lipids, with only $C_{16:0}$ and $C_{18:1}$ fatty acids present in minor amounts (piglet S; 2.99 x 10^{-3} mg/ mg TE and piglet L; 1.47 x 10^{-3} mg/ mg TE). Hence, the presence of lipids in the burial samples from the graves can be attributed directly to inputs from the surrounding environment and the organic remains placed in the grave. Controls for both graves contained *n-a*lkanes, *n*-alkanols and fatty acids typical of plant matter. The *n*-alkanes ranged from C_{21}-C_{33}, with C_{29} dominant, a distribution typical of plant leaf waxes (Bull et al., 2000b, Baker, 1974, Eglinton and Hamilton, 1967). The abundances of *n*-alkanes in the C1, C2 and C3 controls (piglet S; 1.46 x 10^{-3} – 2.11 x 10^{-2} mg/ mg TE and piglet L; 4.59 x 10^{-4} – 1.63 x 10^{-2} mg/ mg TE) in the grave fills above the coffin contrasts with the absence in samples from C0 control (Figure 4:4 and Figure 4:5). The soils also contained *n-a*lkanols ranging from C_{22}-C_{28} with distributions typical of plant leaf waxes (Bull et al., 2000b, Baker, 1974, Eglinton and Hamilton, 1967). In all sampled from piglet L the C_{26} component was dominant (6.67 x 10^{-4} – 4.02 x 10^{-2} mg/ mg TE) and C_{24}, C_{26} and C_{28} components were dominant for piglet S (6.01 x 10^{-4} - 1.46 mg/ mg TE). Fatty acid distributions for the control samples from both piglets differ to that of the samples from the coffin and from both C1 and C0. The C1 control from the surrounding site shows a distribution typical of plants (Bull et al., 2000a), with a unimodal distribution ranging from C_{22}-C_{30} and maximising at the C_{26} fatty acid. Controls C2 and C3 for piglets S and L show distributions containing similar abundance levels of both long chain fatty acids (> C_{22}) with C_{26} dominant (piglet S: 1.34x10^{-3} - 1.19x10^{-2} mg/ mg TE; piglet L: 1.19 x 10^{-4} – 1.01 x 10^{-2} mg/ mg TE) and short chain fatty acids (C_{14}-C_{18}) (piglet S: 5.55 x 10^{-3} - 2.29 x 10^{-1} mg/ mg TE; piglet L: 1.17 x 10^{-3} – 1.71 x 10^{-2} mg/ mg TE). The short chain fatty acids, may have origins in fungal, bacterial or faunal activity (Haack et al., 1994, Ruess et al., 2002, Schoenen and Schoenen, 2013), with the long chain fatty acids being indicative of plant matter contributions (Eglinton and Hamilton, 1967, van Bergen et al., 1997a). These distributions differ greatly to that of samples from in the coffin, where *n*-alkanes and *n*-alkanols were not present within the majority of samples and long chain fatty acids were present in much lower abundances (piglet S: 1.29 x 10^{-4} - 2.41 x 10^{-3} mg/ mg TE; piglet L: 1.28 x 10^{-3} – 8.19 x 10^{-3} mg/ mg TE). The profiles show distinct differences to those of the grave controls. Piglet S sample 16y and piglet L samples 16y, A5 and A16 all showed the presence of *n*-alkanes and, for all samples apart from 16y for piglet L, *n*-alkanols. All of these positions in the graves can be associated with the food stuffs added in the snout and gut regions and the wicker basket placed near the front feet (16y). Hence, these plant materials imparted distinctive signatures to the burial matrices. Interestingly, the beeswax appeared both visually and chemically to be completely intact after 3 years, suggesting that it made little or no contribution

of organic compounds to the burial matrix. As the *n*-alkanes and *n*-alkanols are present in the controls (except C4 and C0) yet in low abundances or not in the samples directly related to the remains their presence most likely reflect leaf waxes incorporated into the overlying soil through decomposition of vegetation and bioturbation. The similar *n*-alkane distribution in both graves suggests similarities in the origins with leaf litter noted on the overlying soil at the time of sampling being the likely source (Figure 4:1 and Figure 4:2). The distinct decrease in abundance within the coffins suggests that it acted as a physical barrier, preventing the ingress of *n*-alkanes and *n*-alkanols. Thus, it is evident that movement of organics, in this case from leaf matter from the surface, occurred through the grave fill, until it was arrested when it reached the coffin and further movement was prevented. Furthermore, evidence of this movement and the localised nature of the *n*-alkanes and *n*-alkanols in the coffin allows for interpretation of the presence of components adjacent to other areas around the body to be directly related to the degradation and alteration of the carcass.

Signatures of adipose tissue (triacylglycerols (TAGs), diacylglycerols (DAGs), monoacylglycerols (MAGs) and fatty acids (FAs)) occurred in the soil extracts from around the skeletal remains. TAGs were found in samples from both piglets S and L (Figure 4:9). Samples analysed (piglet S, 1z, 2y, 8z, 14z, piglet L, 1z, 2z and 15z) give a comprehensive picture across the remains with greater amounts of TAGs present in piglet L ($1.91 \times 10^{-2} – 9.50 \times 10^{-2}$ mg/ mg TE) than piglet S ($6.62 \times 10^{-4} – 2.30 \times 10^{-2}$ mg/ mg TE). The TAGs in samples from the control piglet show high abundances and complexity (Figure 4:9). The chromatograms from piglets S and L both differ greatly, with large numbers of triacylglycerols being absent and hence the distributions notably different. Although the retention times between sample runs varied, identification of peaks was carried out via tandem MS. Thus, the identification of the triacylglycerol at the retention time of 105 minutes (Figure 4:7) was based on the assignment of ion in MS^1 for both the $[M+NH_4]^+$ *m/z* = 878 and $[M+H]^+$ *m/z* = 861, suggesting a TAG present of *m/z* = 860. Peaks present in MS^2 suggest diacylglycerol ions for $[SO]^+$ *m/z*= 605 (33 %), $[SP]^+$ *m/z* = 579 (79 %) and $[PO]^+$ *m/z* = 577 (30 %). The most intense DAG ion arises through loss of the acyl group from the *sn*-2 position of the glycerol backbone this being *m/z* 579 in Figure 4:7 indicates the TAG to contain S and P in the *sn*-1 and *sn*-3 positons (Hasan, 2010). As the other two DAG ions present contain PO and SO, the TAG molecule can identified as SOP, which has a mass of 860.

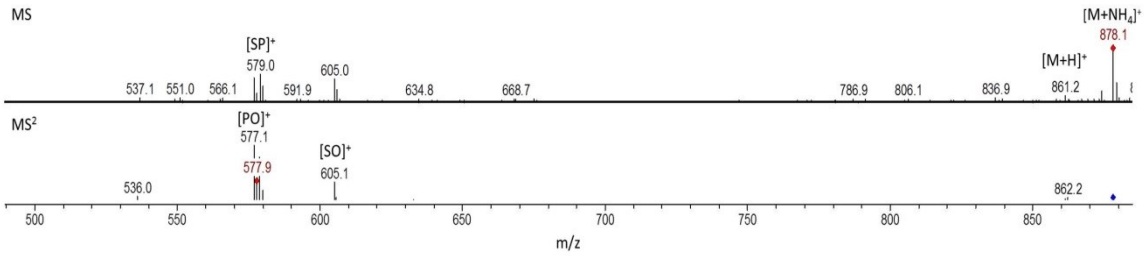


Figure 4:7. Tandem mass spectrum for the triacylglycerol SOP at the retention time of 105 minutes for piglet S.

Similarly, the peak at a retention time of 100 minutes for piglet L (Figure 4:8) gave $[M+NH_4]^+$ at *m/z* 838 and $[M+H]^+$ at *m/z* 821, with the $[M+H]^+$ being extensively fragmented. DAG ions in the MS^2 are $[SP]^+$ *m/z*= 579 (28 %), $[PMa]^+$ *m/z* = 565 (100 %), $[PP]^+$ *m/z* = 551 (45 %) and $[MMa]^+$ *m/z* = 537 (37 %), suggesting the presence of more than one TAG combination. The main DAG ion being $[PMa]^+$ suggests a TAG with fatty acid moieties in the *sn*-1 and *sn*-3 positions to be P and Ma. As no DAG ions are present for $[SMa]^+$ or $[PM]^+$ the possibility of the TAG containing S and M, as suggested by the presence of $[SP]^+$ and $[MMa]^+$ DAG ions, is unlikely. Hence, the most likely candidate for the TAG is PPMa, which incorporates both $[PMa]^+$ the highest intensity DAG ion present and $[PP]^+$. The TAG structure suggested gives the expected molecular mass of 860.

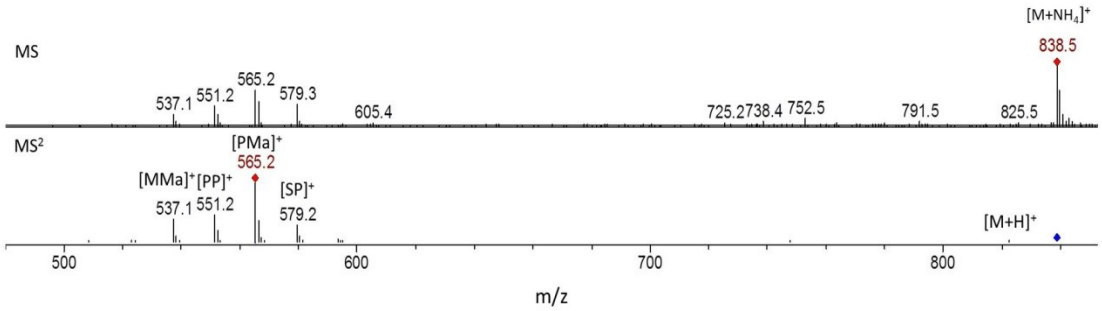


Figure 4:8. Tandem mass spectrum for the triacylglycerol PPMa at a retention time of 100 minutes for piglet L.

Notably, piglet S (Figure 4:9) shows the greatest extent of TAG degradation, with only a few of the expected TAGs remaining. This significant change in the TAGs has occurred in all positions for which TAG analysis was performed. Piglet L, however, had a higher abundance of TAGs and more of the original TAGs remaining. Certain TAGs decreased in abundance, their removal leading to the increased levels of the saturated $C_{16:0}$ and $C_{18:0}$ fatty acids. The distributions of TAGs and their derivatives show degradation of TAGs in all sampling positions for piglet S.

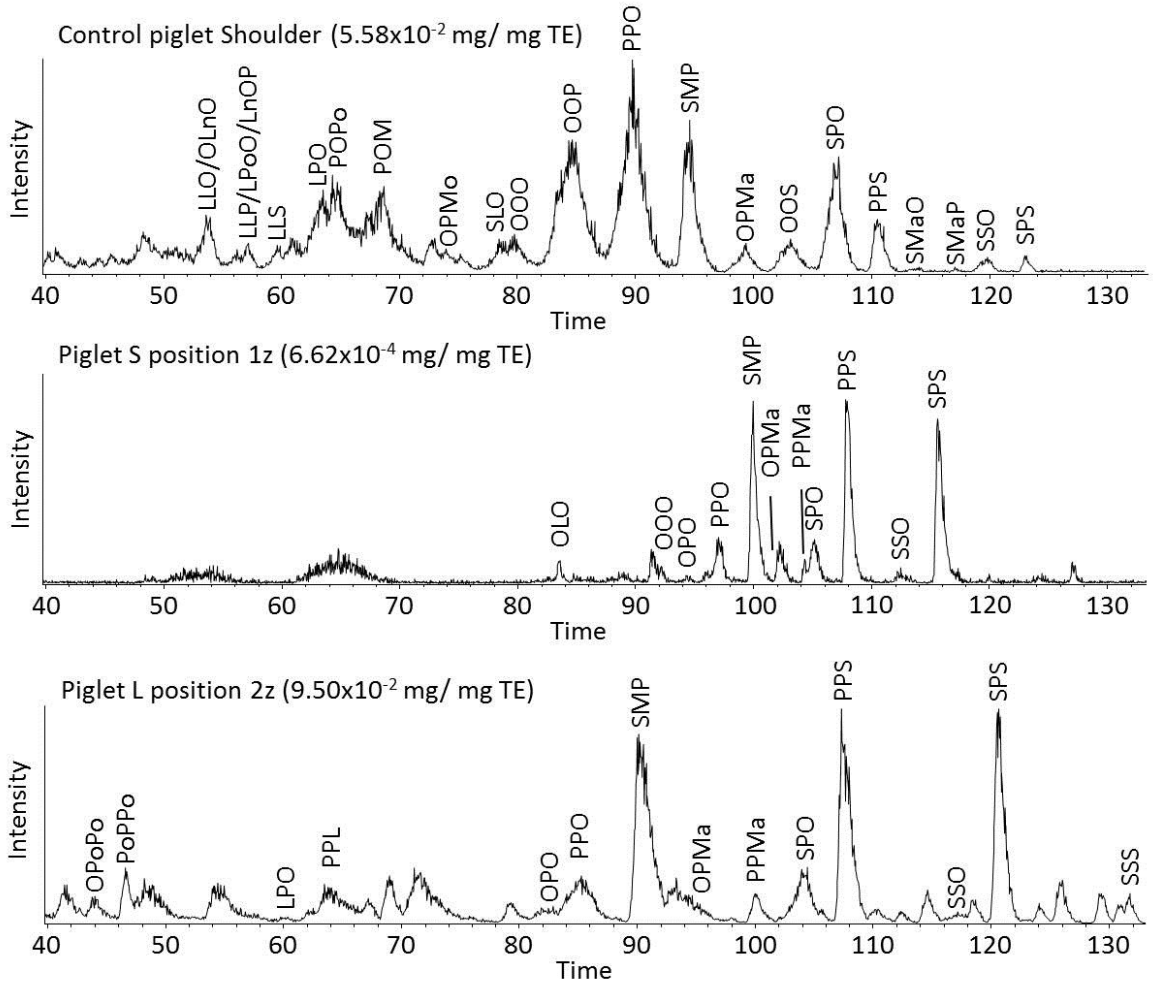


Figure 4:9. Partial LC-MS chromatogram for the shoulder position of the control piglet, position 1z piglet S and piglet L position 2z showing distributions for porcine adipose tissue and the altered abundances seen in samples from the graves.

The abundances of unsaturated relative to saturated TAGs for piglet S (Figure 4:10) are slightly lower than for the distribution for piglet C, the slightly higher levels of saturated TAGs possibly suggest that reduction occurred. Piglet L yielded a greater abundance of TAGs than piglet S but with the ratio of unsaturated/saturated TAGs opposite to that in piglet C and piglet S. The lower ratio indicates preferential degradation of the unsaturated TAGs. The clear difference between piglet S and piglet L indicates different degradation pathways operating in these two distinct burial matrices.

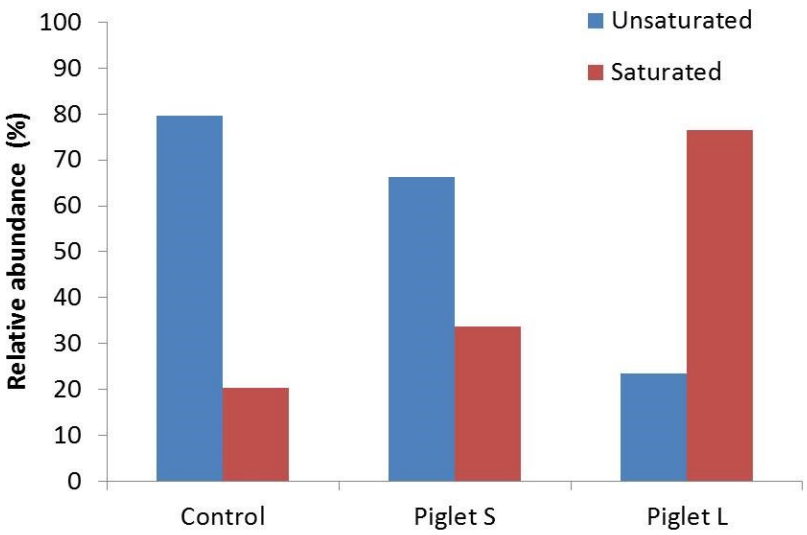


Figure 4:10. Comparison of unsaturated to saturated TAGs for the control, piglet S and piglet L. Showing the levels of alteration in TAG distribution from the control to piglet S and piglet L in the samples affected by the grave environment.

Analysis of the alteration of the TAG distributions to that expected, with partial and complete reduction of TAGs (Figure 4:11), shows the distribution of saturated fatty acids to differ slightly from that of porcine adipose tissue with piglet S showing a slight increase in the level of TAGs containing C_{18} moieties and piglet L showing a greater increase in C_{18} TAGs and an absence of TAGs containing $C_{14:0}$ moieties. The small change seen in piglet S suggests that very little reduction has occurred with hydrolysis of the TAGs being the main degradation pathway. The similar change in piglet L would suggest that hydrolysis of TAGs instead of reduction is the main contributor to TAG degradation. The difference between the levels of unsaturated and saturated components suggests that the mechanism of hydrolysis in both graves differs with selective hydrolysis of the unsaturated TAGs dominating for piglet L and non-selective hydrolysis for piglet S. For both piglets LC-MS analysis revealed the presence of short chain TAGs and possible oxidation products, similar to that seen in Fromelles (Chapter 2). Due to the large number of structural possibilities, clear identifications were not possible at this time.

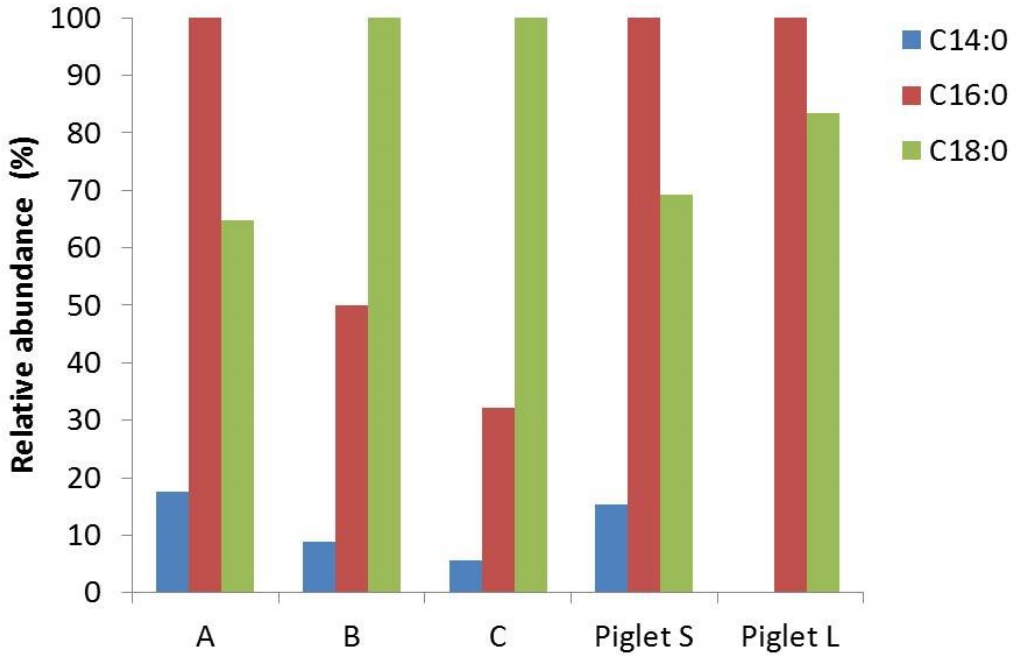


Figure 4:11. Relative abundances of saturated TAG fatty acids normalised to the major component showing comparison of samples from piglet S and piglet L to fresh porcine adipose tissue. A = saturated fatty acids for porcine adipose tissue; B = saturated fatty acids for porcine adipose tissue with reduction of all monounsaturated fatty acids to saturated components; C = saturated fatty acids for porcine adipose tissue with reduction of all unsaturated fatty acids to saturated components.

Diacylglycerols in the burial matrices for piglet S and L are exclusively saturated, reflecting preferential hydrolysis of unsaturated FA's moieties. In both graves the DAG fatty acid moieties were $C_{16:0}C_{16:0}$ and $C_{16:0}C_{18:0}$, with $C_{14:0}C_{14:0}$ only being present for piglet S. The presence of appreciable levels of DAGs in the limestone matrix (Figure 4:12) indicates selective hydrolytic degradation affecting the adipose tissue. The depletion of TAGs in the sand matrix, and presence of low abundances of DAGs, indicates extensive TAG hydrolysis/degradation. Hence, the soil distributions reflect additional contributions from TAG degradation. The MAG abundances for piglet L are most similar to the control piglet which only contained MAGs with $C_{16:0}$ fatty acid moieties. The low levels of MAGs in samples from piglet L reflect the low extent of degradation in the burials with mainly DAGs being produced from the TAG degradation.

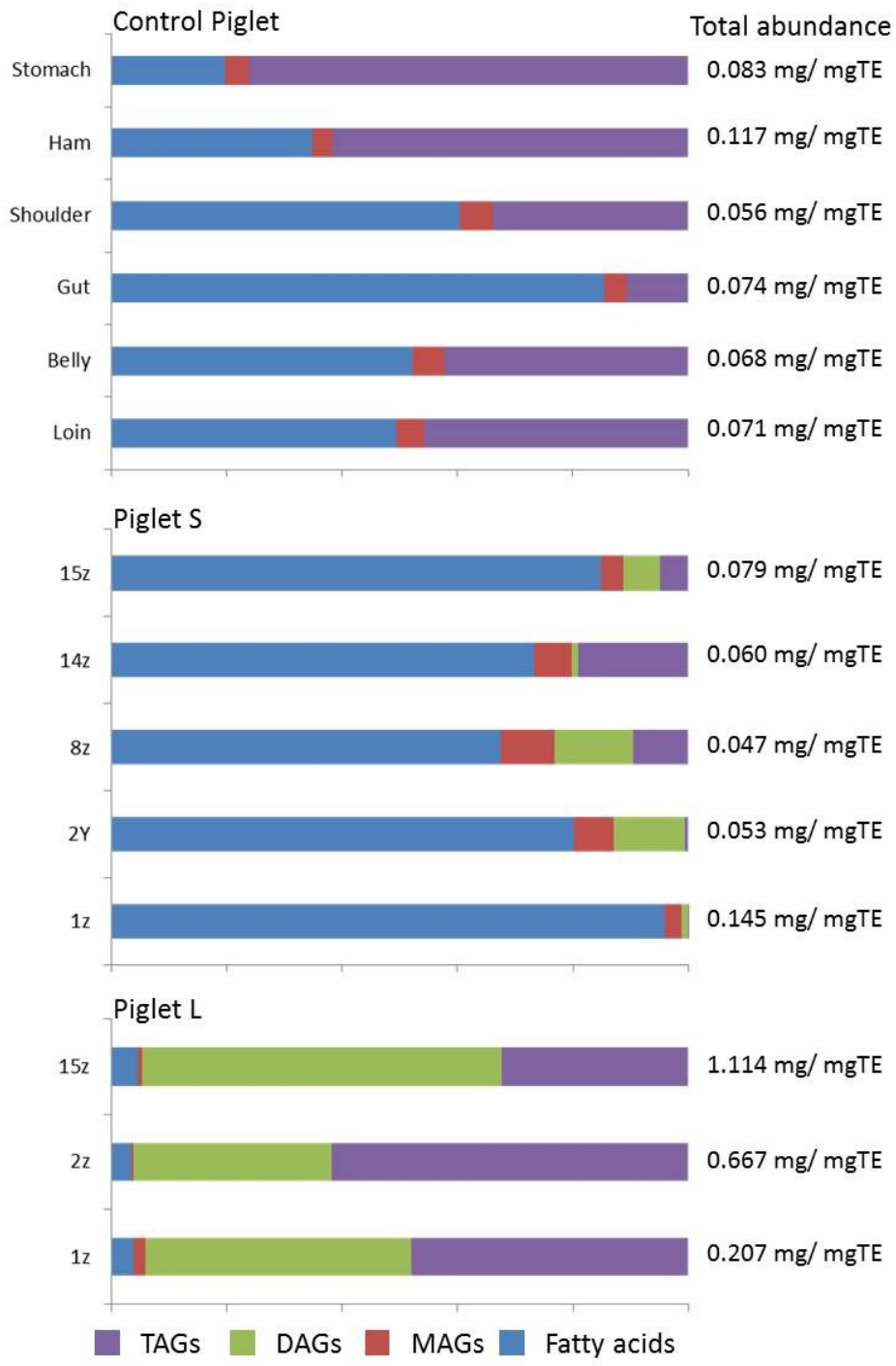


Figure 4:12. Mass balance for the major lipid components of adipose tissue from triacylglycerols (TAGs), through the hydrolysis products, diacylglycerols (DAGs), monoacylglycerols (MAGs) to free fatty acids. Showing the relative distributions in the control, piglet S and piglet L along with the abundance of this particular lipid fraction.

Fatty acids ranged from $C_{14:0}$ to $C_{30:0}$ with saturated, unsaturated and branched chain moieties (C_{15}–C_{17}) being present (Figure 4:13 and Figure 4:14). Samples from around the carcass (Figure 4:13 and Figure 4:14) and coffin fill (piglet L, A17-A24) are dominated by short chain fatty acids ranging from $C_{14:0}$ – $C_{18:0}$ (piglet S: 4.05 x 10^{-3} - 1.07 x 10^{-1} mg/ mg TE; piglet L: 4.87 x 10^{-3} – 9.09 x

10^{-2} mg/ mg TE), with unsaturated $C_{16:1}$, $C_{18:2}$ and $C_{18:1}$ components being present, providing further evidence of selective hydrolysis to produce the DAG species.

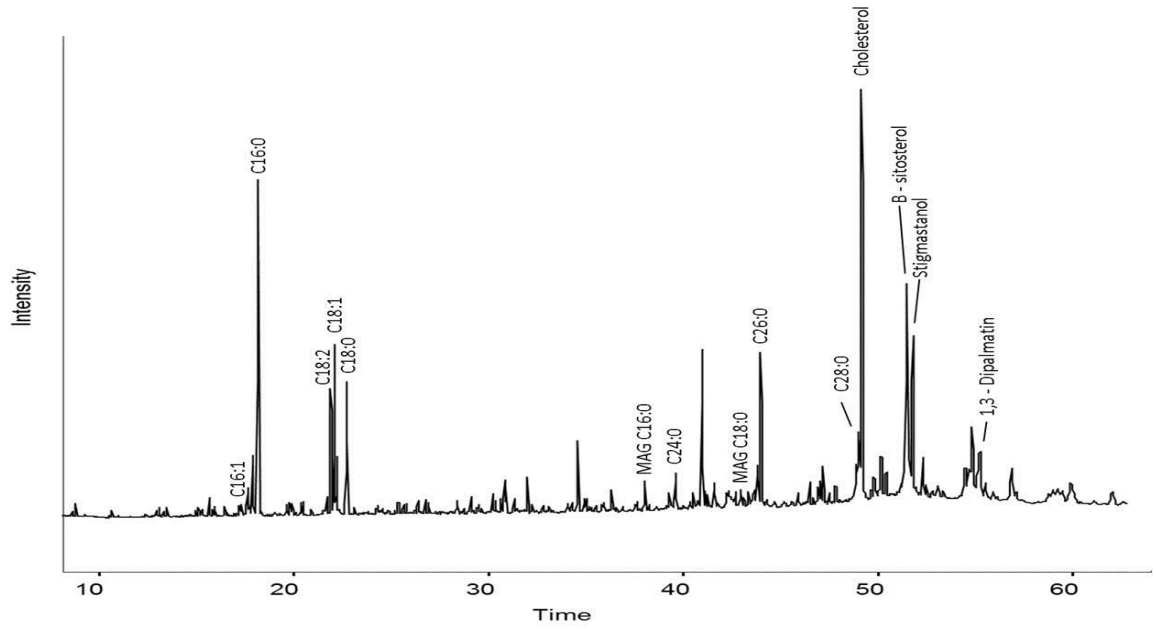


Figure 4:13. Partial gas chromatogram of the total extract for sample 12z, piglet S, sand matrix buried at Hovingham for three years, showing a small selection of the major components present in the samples including fatty acids, mono and diacylglycerols as well as sterols.

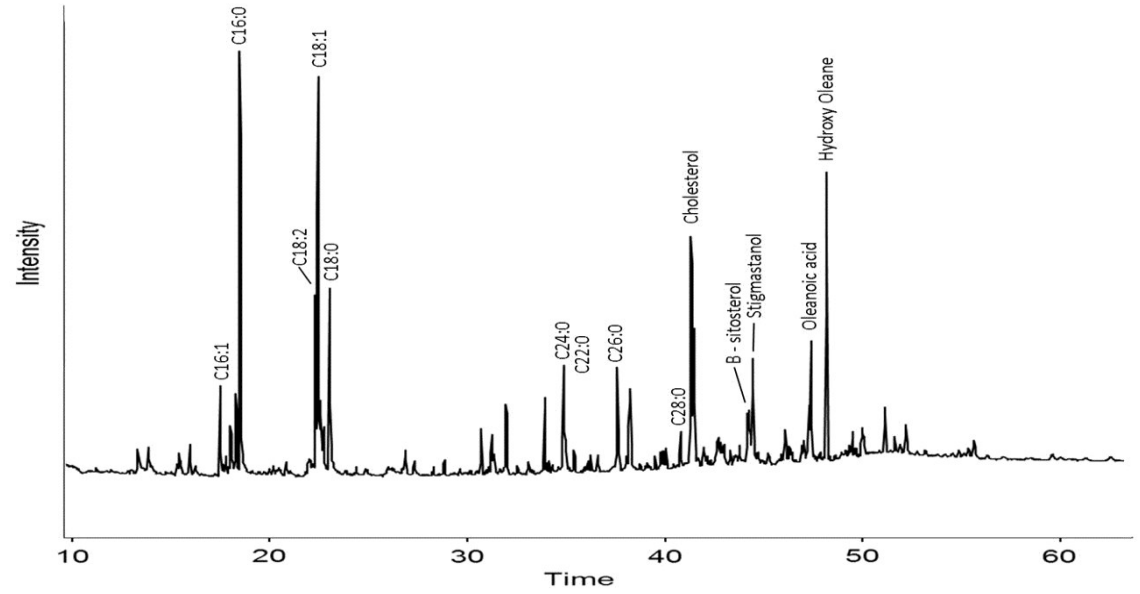


Figure 4:14. Partial gas chromatogram of the total extract for sample A17, piglet L, limestone matrix buried at Hovingham for three years, showing a small selection of the major components in the samples including fatty acids, mono and diacylglycerols as well as sterols.

Fatty acid distributions for the majority of piglet S samples are similar to those of the control piglet (Figure 4:15) and previous work by Hassan (2010) with 2y, 8z and 16y showing the same distributions. Samples 1y, 12z and 14z are similar to that of the control piglet; yet differ due to dominance of $C_{16:0}$ fatty acid. This selective increase in the abundance of $C_{16:0}$ relative to the other fatty acids may be due to one or more of several degradation pathways discussed in previous chapters (water movement, reduction, β–oxidation and associated reduction).

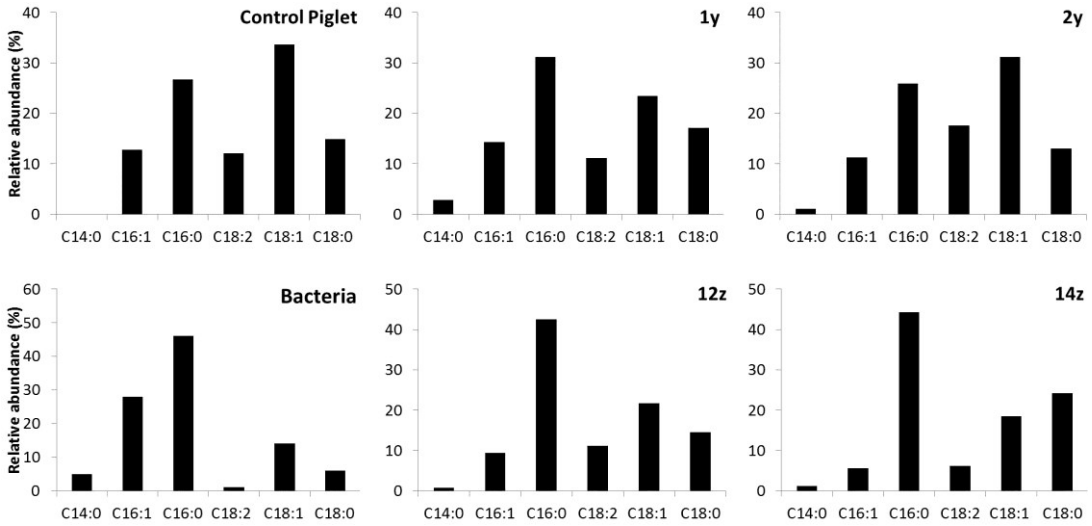


Figure 4:15. Relative distributions of free fatty acids in the control piglet (*n*=6), bacteria and sampled positions from piglet S showing the various distributions seen in the samples. Bacterial signature adapted from (Zelles et al., 1992).

Comparison of the control distribution to that of 1z (Figure 4:16) shows that the changes in distribution with increase in $C_{16:0}$ for 1z, correspond with the decrease in $C_{16:1}$, $C_{18:2}$ and $C_{18:1}$. This change in distribution may be the result of the operation of the β-oxidation and associated reduction pathway suggested in Chapter 2 and Chapter 3. This pathway, suggested to be attributable to microbial processes, is supported by fatty acid distributions typically seen for bacteria: they show greater abundances of $C_{16:0}$ to the other components in the majority of samples.

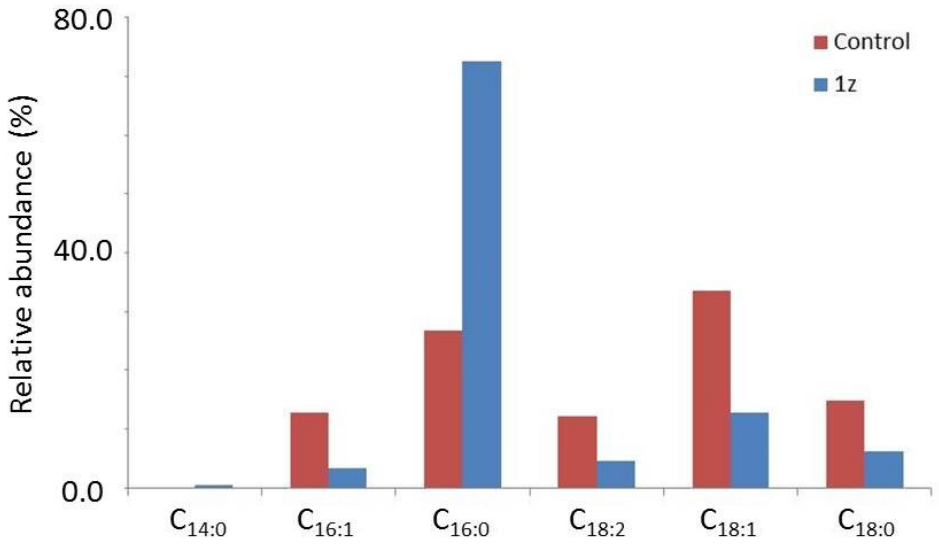


Figure 4:16. Comparison of the distribution of fatty acids for the control piglet (*n*=6) and 1z (head) of piglet S showing the change in distribution on degradation and alteration in the grave.

Comparison of the same data normalised to $C_{18:0}$, in order to test the potential effects of water movement (Figure 4:17), shows that the distribution has altered very little in relation to all fatty acids apart from $C_{16:0}$. The increased proportion of $C_{16:0}$ along with the presence of $C_{14:0}$ could possibly imply a contribution to the fatty acid profile from bacteria (Figure 4:15).

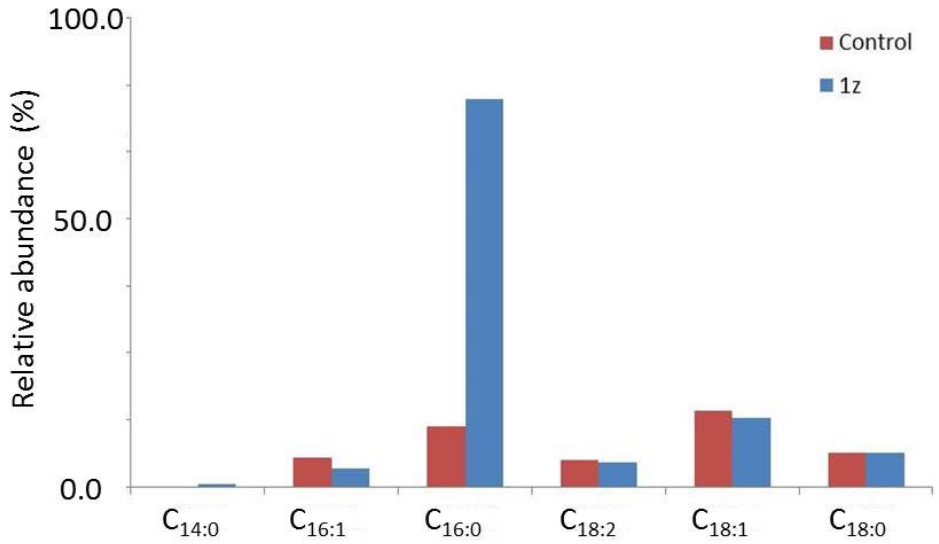


Figure 4:17. Comparison of the distribution of fatty acids for the control piglet (*n*=6) and 1z (head) of piglet S normalised to $C_{18:0}$ showing the change in distribution on degradation and alteration in the grave.

Branched chain $C_{15:0}$ *iso* and *anteiso* fatty acids were also identified in samples from piglet S though they were absent from piglet L samples. These signatures are typically related to bacterial input (Haack et al., 1994) with small amounts attributed to fungal activity (Ruess et al., 2002). The

presence of these signatures in piglet S samples suggests that microbial activity has played a significant part in degradation. Low levels of branched chain $C_{15:0}$ *iso* and *anteiso* FAs in the piglet S matrix reflect signatures derived from heterotrophic bacteria (Parkes and Taylor, 1983).

Piglet L short chain fatty acid distributions (Figure 4:18) differ from that of the control piglet and Hassan (2010). The majority of samples are dominated by $C_{16:0}$ with lower levels of $C_{18:1}$ (Figure 4:18, 1y and 15z). Samples 1z and 2z (skull and pelvis, respectively) were dominated by $C_{16:0}$ and $C_{18:2}$ and A6 by $C_{18:0}$. The $C_{18:0}$ fatty acid and unsaturated $C_{18:1}$ and $C_{18:2}$ distributions are significantly different for A6, with $C_{18:0}$ dominating the distribution, unlike that of other samples, piglet S and the control piglet (Figure 4:15).

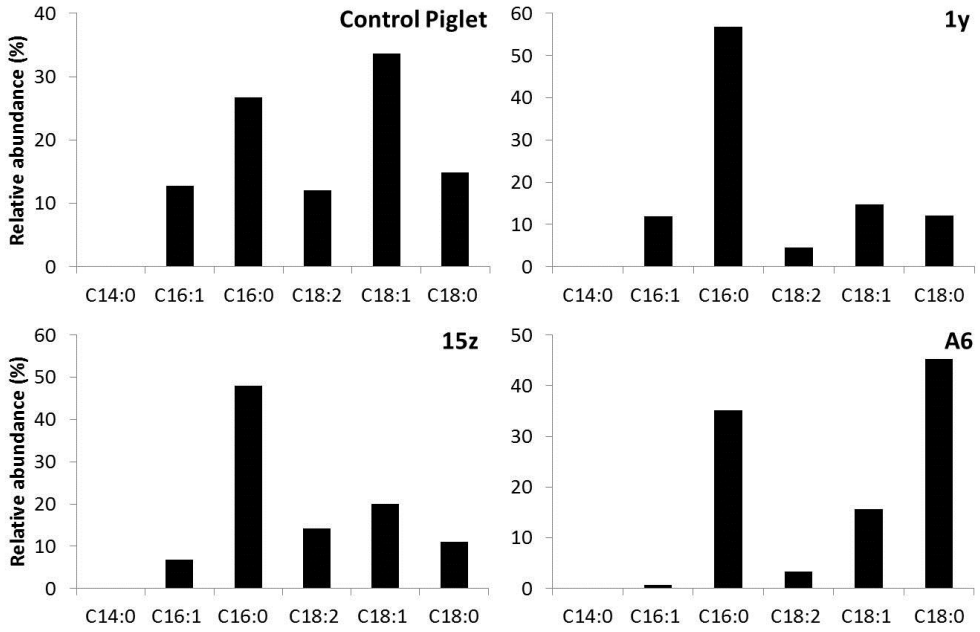


Figure 4:18. Relative abundances of free fatty acids in the control piglet (n = 6) and sampled positions from piglet L showing the distributions among the samples.

Comparison of the control piglet and sample distributions clearly shows reduction in the relative abundances of $C_{16:1}$ and $C_{18:unsat.}$ components. Together with the elevated levels of $C_{16:0}$ (Figure 4:19), via β-oxidation and associated reduction appears to be viable explanation. Normalising the data in Figure 4:19 to $C_{18:0}$ shows little evidence of differences that could be attributed to alteration via water movement through the soil or and reduction (Figure 4:20).

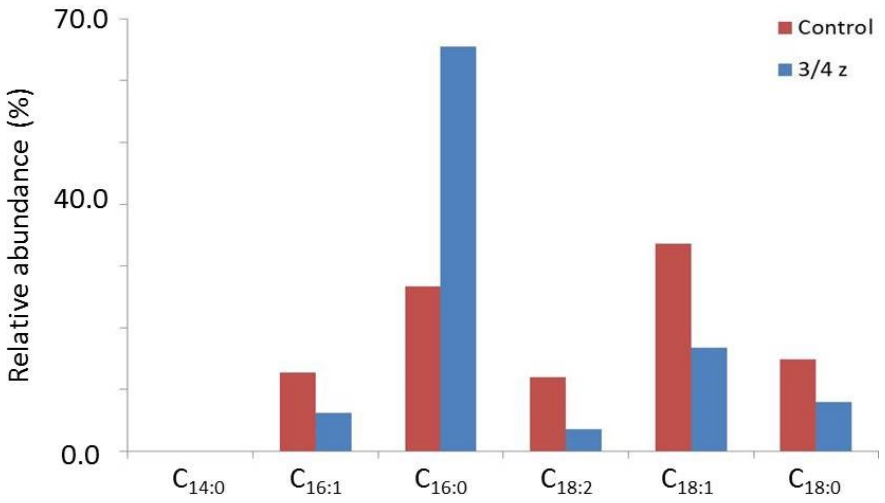


Figure 4:19. Comparison of the distribution of fatty acids for the control piglet (n = 6) and 3/4z (foot) for piglet L showing the change in distribution on degradation and alteration in the grave.

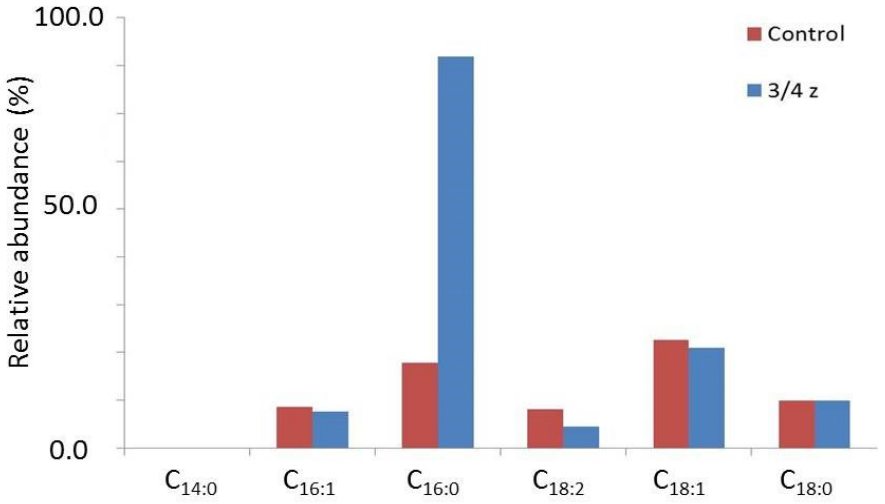


Figure 4:20. Comparison of the distribution of fatty acids for the control piglet (n = 6) and 3/4z (foot) for piglet L normalised to $C_{18:0}$ showing the change in distribution on degradation and alteration in the grave.

Overall, a significant increase in $C_{16:0}$ can be seen for piglet L, suggesting that the distribution has been altered heavily with respect to $C_{16:0}$ (Figure 4:20). Fatty acid distributions at positions A13 and A15 taken from the body cavity are similar to that expected for pig fat, suggesting that hydrolysis of the triacylglycerols occurred and that the signatures were preserved relatively unaltered due to a lack of bacterial activity. A white fatty substance noted on excavation (possibly adipocere, A14) at the head position may account for the alteration of the lipids profiles. The generation of adipocere involves the formation of sodium or potassium salts of fatty acids which are later altered by replacement of the metal ions with magnesium or calcium from the environment (Dent et al., 2004, Forbes et al., 2005b, Forbes et al., 2002). The low abundances of

fatty acids in extracts from around the body and higher abundances in samples situated at the bottom of the coffin (A19-A24), the latter having distributions similar to that expected for pig fat, suggests that fatty acids were transported to the base of the coffin, probably as salts. This movement of fatty acids as salts would have different impacts to movement of native fatty acids in water and may account for the observed distributions of free fatty acids.

In order to test if hydrolysis of triacylglycerols could occur in the soil matrix of piglet L, samples of pure pig fat were saponified using either KOH or limestone as base (60 °C, 24 h). Saponification experiments showed changes in the distributions of fatty acids on heating with limestone (Figure 4:21). While the distributions did not match those for piglet L, they did match those of the control piglet and the majority of piglet S distributions. Further experimentation with an increased reaction time may show that the distributions do eventually match those of piglet L. As the distribution of fatty acids from limestone hydrolysis does not match samples from piglet L it would suggest a different pathway of degradation. The fatty acid distribution from limestone hydrolysis showed a good match to those from samples A13 and A15 lending support to their formation via hydrolysis of triacylglycerols. Similarly, the match to the distributions from piglet S samples further suggests that hydrolysis of triacylglycerols occurred in this grave. The match in the distributions from saponification tests of pig fat using the limestone matrix and the piglet S fatty acids may indicate that degradation was of a chemical nature. Conversely, the lack of a match to the piglet L distributions indicates a different, possibly microbial transformation route, possibly with loss of FA's as salts and water movement altering the distribution.

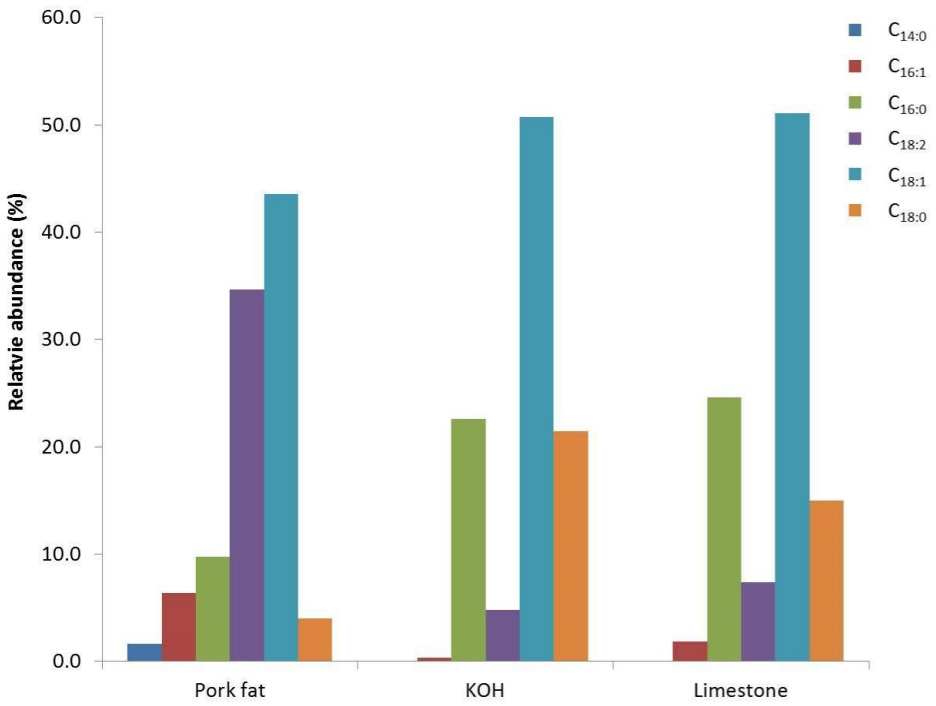


Figure 4:21. Distributions showing the relative abundances seen for pork fat and the saponification experiments using both potassium hydroxide (KOH) and the limestone material used within the grave of piglet L as the base catalyst for hydrolysis.

The mass balances show clear differences between the control piglet, piglet S and piglet L (Figure 4:12). Piglet L shows a dominance of TAGs and DAGs, suggesting either low levels of degradation, the distributions matching that of the control piglet, or greater loss of fatty acids through salt formation than occurs for piglet S. The piglet S distributions match those for the gut region of the control piglet, and suggest extensive degradation; the distributions dominated being dominated by fatty acids and MAGs, by a process similar to digestion, i.e. hydrolysis.

Short chain *n*-alkanals (C_{16} and C_{18}), most likely formed by microbially mediated reduction of free fatty acids, as suggested for in the mass graves (Chapter 2) and waterlogged graves (Chapter 3, c.f. (Riendeau and Meighen, 1985)), were detected in the sand and limestone burials but not in the control soils. Their presence indicates an oxygen limited environment (Pickering et al., unpublished). Due to the signatures in the grave being exclusively from the remains, the presence of these molecules confirms their origin from the body, presumably via direct transformation of fatty acids via reduction.

Sterols indicative of animal, plant and fungal markers were present in the graves, confirming cholesterol, the most abundant sterol in the grave, to originate directly from the interred remains. The microbial alteration product of cholesterol, 5α-cholestanol, was also present in some samples (Bethell et al., 1994a, Bull et al., 2002, Bull et al., 1999b). Plant sterols, stigmasterol, stigmastanol

and β-sitosterol (Bull et al., 2003) were present in the controls from both piglets whereas their presence inside the coffin was limited to the piglet S burial. Given the evidence that the coffins represented isolated environments, the plant sterols can be attributed to the grave goods. Two fungal sterols, ergost-2,22-diene-3-ol and ergost-5-en-3-ol were identified in both sets of controls for the piglets, providing evidence of fungal activity in the soil (Kieber et al., 1955, Appleton et al., 1955). Fungal sterols were only found within the coffin of piglet S. The presence of these sterols in the grave and the high levels of degradation suggest that fungal activity played an active role in the degradation of the grave goods, coffin and/or body.

4.3.3 Molecular profiles of isolated materials

Signatures directly related to the grave goods were limited. Caryophellene, isoeugenol, eugenol and coumarin were identified in the region of the snout bags for both piglets reflecting localised signatures from cloves and tobacco. Beeswax was observed intact during excavation and no molecular signatures relating to its presence were observed in the soils, signifying its high degree of stability. Cinnamon was the only substance from the materials placed in the gut to leave a recognisable signature, cinnamic acid, in the soils from piglet S and piglet L. The lack of signatures from other foodstuffs in the gut region is most likely due to their chemical compositions; they were mainly carbohydrate or protein based. Sample A15 for piglet L was the only position to show the presence of fungal sterols in the grave, suggesting that fungal activity played a role in the degradation of organic molecules present from the cellulose based foodstuffs.

4.4 Conclusion

Elemental analysis showed the total organic carbon contents for both piglet graves to be higher around the remains than for the grave controls and original starting matrix. This suggests that the elevated levels of TOC in burial soils can be attributed to the presence of a body. In addition the presence of sulfur and high levels of nitrogen reflect the additional materials placed in the grave, with elevated levels corresponding directly to the locations where they were placed alongside the carcass.

The analysis of the two graves discussed in this chapter is unique in that the original starting material was controlled and devoid of major lipid signatures. Hence, this environment allows for the identification of the sources of the inputs to the soil either from external sources or from alteration of the signatures produced by the degrading remains. Two observations of particular significance resulted from this level of control in the study design. The first is that *n*-alkanals associated with both piglet burials are shown to originate directly from the remains, presumably via reduction of free fatty acids. Their occurrence must reflect a reducing environment denoting environmental conditions that persisted in grave fill during decomposition. The result confirms that *n*-alkanals in archaeological graves represent decomposition products from the body in a reducing environment. Secondly, *n*-alkanes and *n*-alkanols in the grave fills of piglet S and L are typically only present above the coffin lid with samples underneath the lid showing their presence only in locations of the added grave goods. These signatures can be attributed to plant waxes, their distributions reflecting movement of fine particles through the soil profile. This interpretation is supported by the presence of leaf litter on the overlying exposed soil surface at the time of sampling. The migration of these components from the top of the fill to the coffin lid implies that their transportation through the grave fill was probably mediated by percolation of water through the grave fill. The hydrophobic nature of both compound-classes implies that their movement is linked to the movement of soil particles to which the lipids were associated (Clemente et al., 2011). The absence of *n*-alkanes and *n*-alkanols in the soils from the upper coffin fill indicates that the coffin lid acted as a barrier to movement of soil particles.

Fatty acid distributions of piglet S and piglet L samples shows that the majority from piglet S are similar to that of the control piglet and what would be expected on hydrolysis of porcine TAGs to free fatty acids, suggesting hydrolysis has played a major role in degradation. Piglet L differed greatly from the controls and the majority of samples from piglet S, suggesting either that a different method of degradation/alteration took place, such as β-oxidation and associated reduction (suggested as a possible method for altering the distribution in the archaeological graves), or that hydrolysis of TAGs occurred with subsequent alteration of the distribution via

water movement. Given that the distributions around the remains differed greatly from that of the control piglet, yet samples from the bottom of the coffin showed higher abundances of fatty acids and similar distribution of fatty acids to that of the controls, salt formation appears likely to have played a role in their transport, the presence of small quantities of adipocere providing supporting evidence for salt formation.

Triacylglycerol analysis showed that the distributions for piglet S contained high levels of unsaturated to saturated TAGs with piglet L revealing the reverse. In addition it appears that degradation of the TAGs occurred mostly via hydrolysis, with oxidation and cleavage being minor processes as evidenced by the presence of TAGs with short chains that are unidentifiable at this time. Mass balances show that clear differences can be seen for the distribution of TAGs and degradation products, piglet S is heavily dominated by fatty acids and piglet L by TAGs and DAGs suggesting lower levels of degradation in the latter grave.

Overall, signatures from the overlying vegetation track the transport of fine soil particles and associated organic matter through the soil profile and reveal that the coffin provides a barrier to the movement of fine particles. Lipid signatures generated from the body tissues (e.g. triacylglycerols and fatty acids) show clear differences between different burial environments, reflecting different conditions and pathways of transformation. The highest levels of bacterial signatures occur close to the skeletal remains and short chain *n*-alkanals associated with the skeletal remains are attributed to microbial reduction of fatty acids, indicating the development and persistence of oxygen limited conditions leading to and since the time of their formation. Organic signatures from the buried remains can be preserved in grave soils and distinguished from soil organic matter, providing insight into the burial environment and the potential application in burial archaeology.

5 Experimental burials in natural soils

5.1 Introduction

The study of experimental burials with a defined burial matrix provided valuable information on the alteration and degradation pathways within a soil devoid of any initial lipid signature and fauna or flora. The design of the InterArChive project (Usai et al., 2014), included the setting up experimental burials of piglets at a range of sites including some in natural soils. Three of those sites, being of differing soil matrix and both with a coffined and one non-coffined burial were selected for inclusion in this work. The locations, chosen to encompass variations in soil environment and to utilise sites that had previously yielded archaeological finds, allow for the extension of observations reported in Chapter 4 to a more active soil environment. The intention was to bridge the gap between the experimental burials in a controlled matrix and archaeological burial remains. The three graves selected also allow assessment of the effects of the presence of a coffin both on the nature of the organic signatures and the rate/effect of environment on degradation. The work presented here covers six burials, on which the burial matrix was soil from the burial site. The aim was to study the effects of the environment on lipids in each unique environment, comparing coffined and non-coffined burials, in order to gather information that can further develop ideas presented in Chapter 4.

5.2 Sampling and sampling strategies

Six piglet burials were examined in the work described in this chapter. The burials at three sites of differing soil matrix, all situated in the County of Yorkshire, UK and buried for three years. The graves were prepared as described in Chapter 4 but with soils from the burial site incorporated into the grave and coffin fill. The first set of piglets discussed are from a site at West Heslerton (WH), North Yorkshire, situated near an Anglo-Saxon cemetery that was the subject of and extensive archaeological investigation between 1986 and 1995 (Powlesland, 1986). The soil consisted of a windblown sand of an alkaline nature (Powlesland, 1986). Two piglet burials were sampled from this site, WH1 (coffined) and WH2 (un-coffined). The sampling positions were analogous to those defined for the InterArChive project (Chapter 7) for the soils around the remains and augmented for the grave fills (Figure 5:2). Thus, controls of the grave fill were collected across the horizontal extent (Figure 5:1, f) with additional samples for WH1 taken from the base of the coffin (A20-23). The second set of piglet burials discussed were buried at Folkton (FLK), North Yorkshire, a site consisting of a peaty soil in a location with a high water table. The two piglet burials sampled from this site, FLK1 (coffined) and FLK2 (un-coffined) were also extensively sampled (Figure 5:3). On excavation at Folkton the water level was found to be above the bottom of the grave cut. Hence in addition to sampling from the base of the coffin for FLK1, soil was also collected from below the coffin. Additional samples were also taken from the

location of the grave goods. The final site and piglets discussed in this chapter were from burials at Heslington East (HE), University of York campus, UK. This sites located on a glacial mound consisting of a varied matrix with piglets buried in a sandy loam. Previous excavations at the site yielded graves of varying ages. Samples were taken from HE1 (coffined) and HE2 (un-coffined) as specified in the InterArChive protocol and across the bottom of the coffin as performed for the other experimental coffined burials (Figure 5:4). On excavation water was found to be free flowing through the site.

Figure 5:1. a–b) Folkton coffined piglet (FLK1) before burial and filled coffin. c) Folkton un-coffined piglet (FLK2). d) Coffined piglet at Heslington East (HE1). e) Un-coffined piglet from Hesligton East (HE2). f) Piglets buried at West Heslerton showing both coffined and un-coffined remains and location in grave.

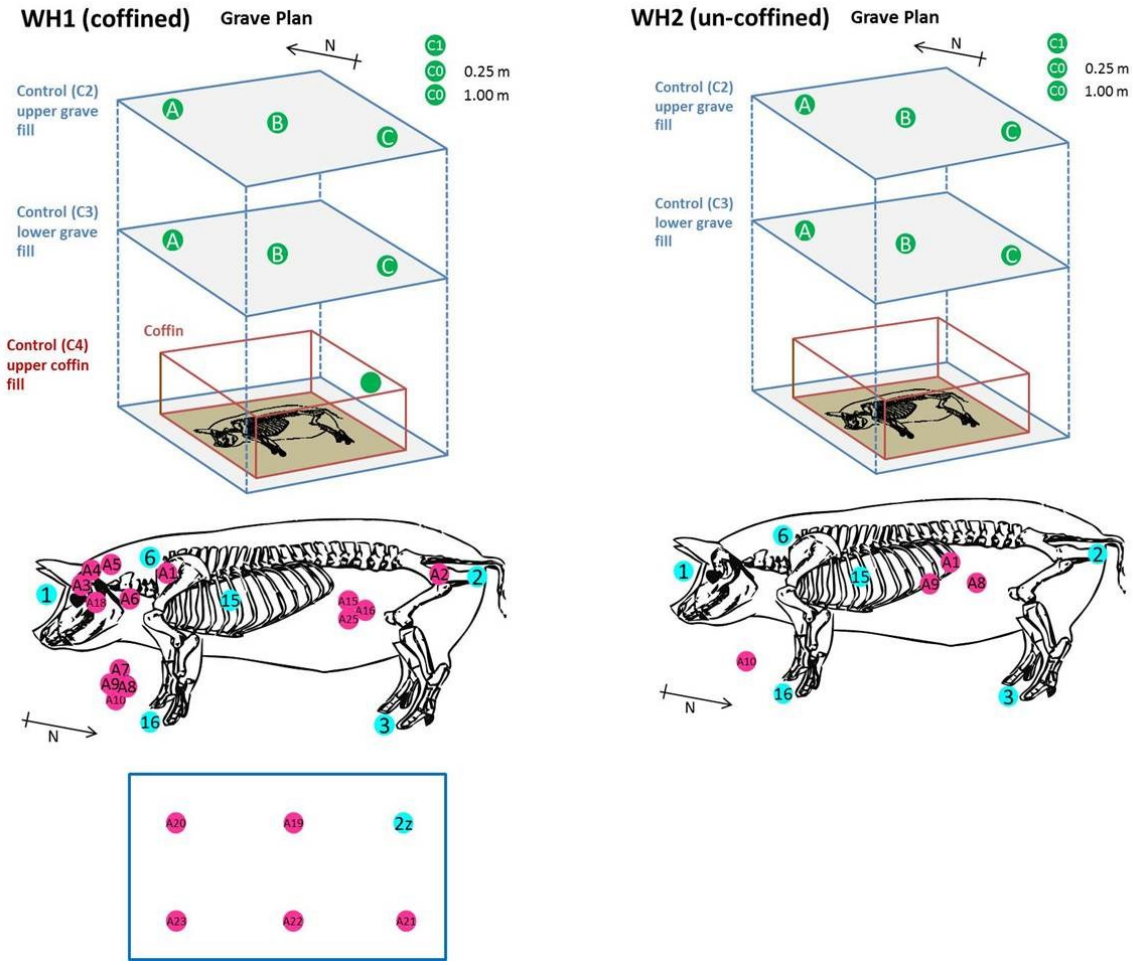


Figure 5:2. Positions of samples collected from piglet burials at West Heslerton, WH1 (coffined) and WH2 (un-coffined). The plane below the diagram of WH1 piglet indicates samples from the base of the coffin. Control samples are shown in green, samples from the remains in blu e and additional samples in pink. x, y, z refer to the location of sampling with respects to the remains, x, above remains, y, adjacent to remains and z, below remains .

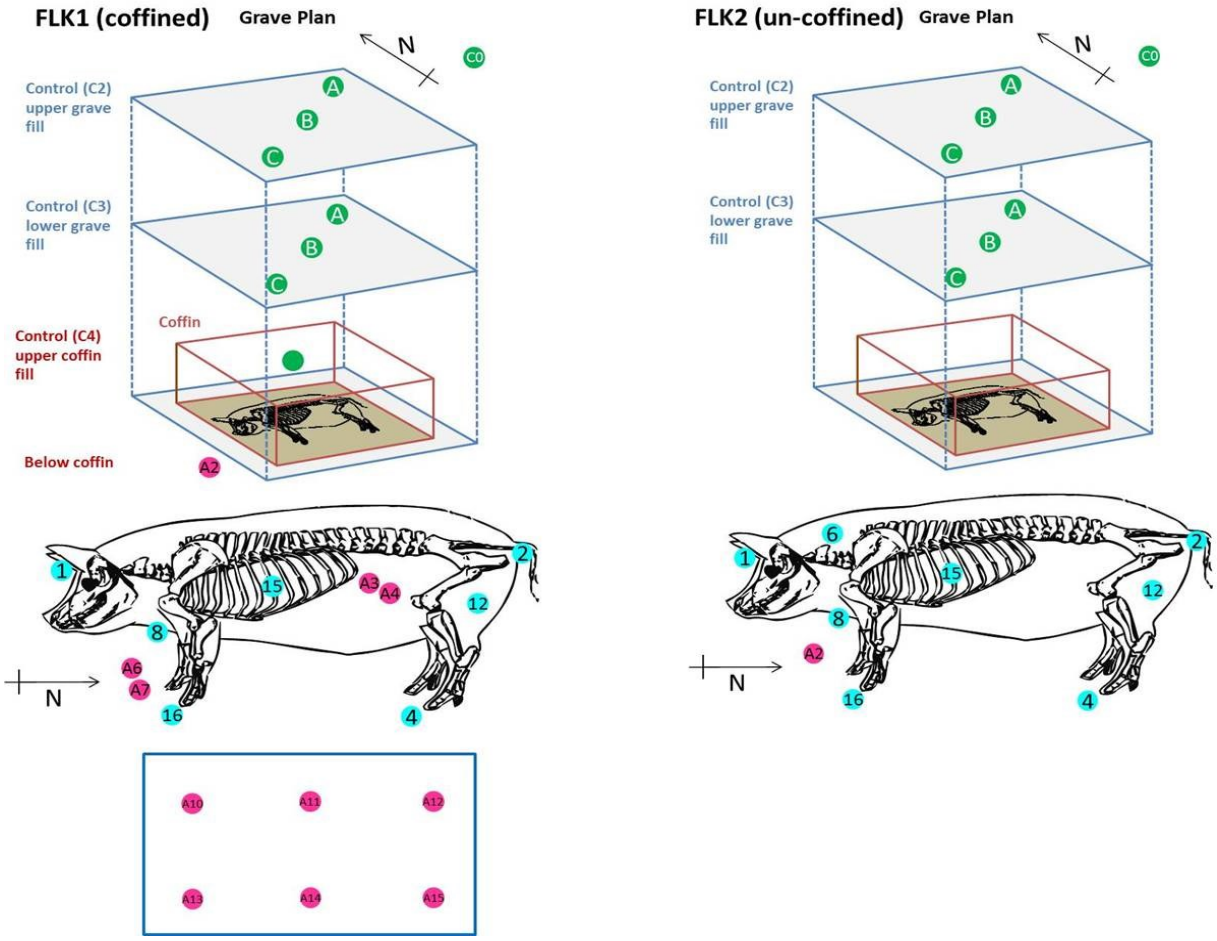


Figure 5:3. Positions of samples collected from Folkton, FLK1 (coffined) and FLK2 (un-coffined) piglet burials. The plane below the diagram of FLK1 piglet indicates samples from the base of the coffin. Control samples are shown in green, samples from the remains in blue and additional samples in pink. x, y, z refer to the location of sampling with respects to the remains, x, above remains, y, adjacent to remains and z, below remains .

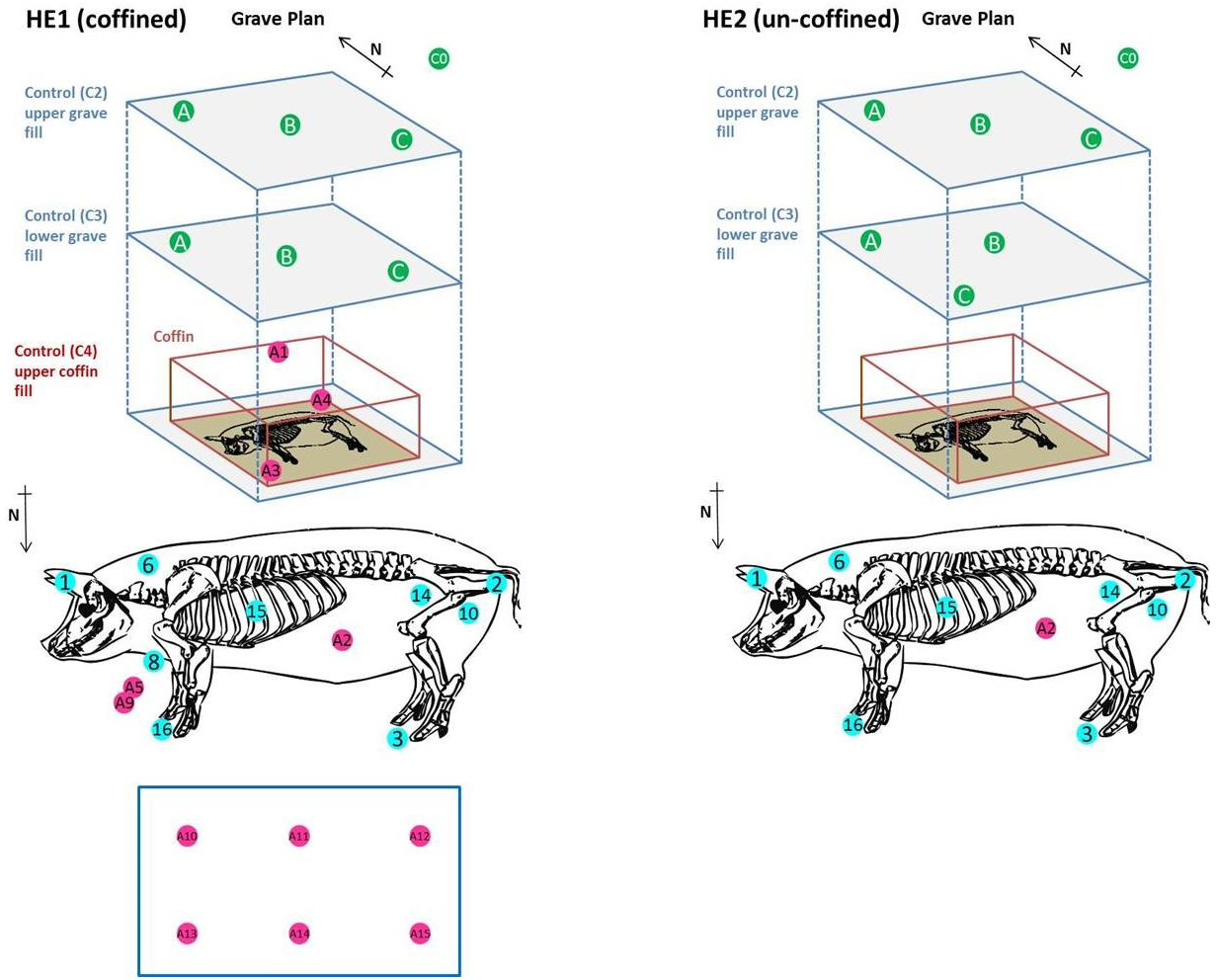


Figure 5:4. Positions of samples collected from Heslington East, HE1 (coffined) and HE2 (un-coffined) piglet burials**.** The plane below the diagram of HE1 piglet indicates samples from the base of the coffin. Control samples are shown in green, samples from the remains in blue an d additional samples in pink. x, y, z refer to the location of sampling with respects to the remains, x, above remains, y, adjacent to remains and z, below remains .

5.3 Results and discussion

5.3.1 Elemental analysis of soil samples

Bulk elemental analysis (CHNS) and total organic carbon (TOC) of control soils and original burial matrices showed low levels of nitrogen and an absence of sulfur for WH piglets (Table 5.1 and Table 5.2). TOC for the two C0 controls at West Heslerton exhibited significant differences at the two depths sampled (0.25 m and 1.00 m). The upper level C0 exhibited similar values to C1 and that at 1.00 m depth was significantly lower. The TOC contents of the C2 and C3 controls increased with depth in the grave fill. WH1 burial samples reflect a substantially higher TOC content than controls C2 and C3, suggesting contributions of organic carbon from the carcass and/or grave goods. Positions located adjacent/near to the grave goods also show elevated levels, indicating incomplete degradation of some of the added materials. WH2 (un-coffined) shows little variation in the TOC among all of the samples. Average TOC in the coffin of WH1 (1.05 ± 0.24 %, *n*=10) and that around the carcass of WH2 (0.69 ± 0.16 %, *n*=12) differ, suggesting a difference in the containment/degradation of organic matter depending on the presence/absence of a coffin. The burials soils adjacent to the two piglets from Folkton (Table 5.3 and Table 5.4, FLK1 and FLK2) show TOC and nitrogen values much higher than those at West Heslerton (WH1 and WH2), this difference can be explained by the differing soil matrix, Folkton being highly organic in nature. Elemental results for FLK1 and FLK2, show FLK2 to be devoid of sulfur and the majority of FLK1 samples not to contain sulfur, though there are a few exceptions (A8, A14 and A24). No correlation between sample position and sulfur content could be discerned; hence the origin of the sulfur remains unclear. As seen for West Heslerton the coffin burial FLK1 shows a clear difference between the TOC of the controls and samples from within the coffin, reflecting input from the remains. In addition, the un-coffined (FLK2) remains show no difference between the controls and burial samples. This similarity between the controls and samples in FLK2 matches that in WH2, further suggesting that a clear difference between preservation/degradation is evident between un-coffined remains and coffined remains. Average TOC for FLK1 (36.74 ± 5.15 %, *n*=23) and that around the carcass of FLK2 (34.46 ± 3.56 %, *n*=12) show similar values, further supporting the similar organic composition from the original soil matrix. Graves from Heslington East show levels of nitrogen similar, to those of West Heslerton and are completely devoid of sulfur. TOC for HE1 (coffined) shows a clear difference between the controls and samples from around the remains in the coffin (Table 5.5 and Table 5.6). HE2, however, shows similar TOC to that of the controls and in some cases lower values. The similarity of the values for the un-coffined burial to the controls and the difference between the coffined burials and controls matches what was observed for the West Heslerton and Folkton sites.

Average TOC for HE1 (0.20 ± 0.08 %, *n*=14) and that around the carcass of HE2 (0.84 ± 0.31 %, *n*=14) show significant organic matter input to the HE2 grave. The low TOC content seen compared to the controls suggest that significant loss of organic material has occurred; this may be due to degradation or loss caused by the hydrology of the site.

Table 5.1. Elemental analysis (CHNS) and total organic carbon (TOC) results for West Heslerton WH1, coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 – initial matrix before burial from depth 25 cm	0.14	1.60	0.35	0.01	1.21
C0 - initial matrix before burial from depth 1 m	0.01	0.14	0.14	0.00	0.10
C1 - site control	0.11	1.13	0.25	0.00	1.78
C2A	0.05	0.60	0.17	0.00	0.65
C2B	0.06	0.70	0.18	0.00	0.51
C2C	0.08	0.93	0.22	0.00	0.33
C3A	0.08	0.87	0.22	0.00	0.91
C3B	0.06	0.69	0.16	0.00	0.77
C3C	0.07	0.75	0.20	0.00	0.83
C4	0.11	1.17	0.25	0.00	1.31
1y - skull	0.08	0.75	0.18	0.00	0.81
1z - skull	0.16	1.38	0.29	0.00	1.25
2y - pelvis	0.13	1.06	0.20	0.00	1.55
2z - pelvis	0.11	1.12	0.24	0.00	1.22
3y - back feet	0.17	1.23	0.24	0.00	1.11
3z - back feet	0.07	0.66	0.16	0.00	1.15
6y - shoulder	0.12	0.92	0.21	0.00	0.78
15z - abdomen	0.13	0.95	0.21	0.00	0.98
16/17y - front feet	0.07	0.64	0.16	0.00	0.83
16/17z - front feet	0.09	0.81	0.20	0.00	0.79
A1 - top of cavity around shoulder	0.07	0.81	0.20	0.00	0.59
A2 - top of cavity from pelvis	0.12	1.07	0.24	0.00	0.38
A3 - concretion on skull	0.12	0.83	0.21	0.00	0.79
A4 - next to skull below A3	0.11	0.92	0.22	0.00	0.80
A5 - dark organic material	0.14	1.06	0.23	0.00	1.10
A6 - hair from around neck	0.14	1.20	0.25	0.01	0.92
A7 - cloves	0.48	6.12	0.77	0.02	4.59
A8 - white flakes from in snout bag	0.00	0.00	0.00	9.73	-
A9 - dark deposit from snout bag	0.23	2.62	0.43	0.00	3.74
A10 - base of snout bag	0.09	1.02	0.21	0.00	1.02
A12 - fur	0.23	1.77	0.33	0.00	1.75
A14 - possible leather	8.47	30.79	4.90	0.11	21.56
A15 - underneath stomach bag	0.15	1.20	0.26	0.00	1.13
A18 - soil related to skull	0.11	0.86	0.20	0.00	1.03
A19 - bottom of coffin	0.09	0.90	0.20	0.00	0.77
A20 - bottom of coffin	0.10	0.96	0.23	0.00	0.94
A21 - bottom of coffin	0.12	1.24	0.27	0.00	1.16
A22 - bottom of coffin	0.09	0.99	0.24	0.00	0.77
A23 - bottom of coffin	0.07	0.71	0.17	0.00	1.00
A24 - bottom of coffin	2.24	21.44	2.84	0.28	31.88

Table 5.2. Elemental analysis (CHNS) and total organic carbon (TOC) results for West Heslerton WH2, un-coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 - initial matrix before burial from depth 25 cm	0.14	1.60	0.35	0.01	1.21
C0 - initial matrix before burial from depth 1 m	0.01	0.14	0.14	0.00	0.10
C1 - site control	0.11	1.13	0.25	0.00	1.78
C2A	0.05	0.60	0.17	0.00	0.65
C2B	0.06	0.70	0.18	0.00	0.51
C2C	0.08	0.93	0.22	0.00	0.33
C3A	0.08	0.87	0.22	0.00	0.91
C3B	0.06	0.69	0.16	0.00	0.77
C3C	0.07	0.75	0.20	0.00	0.83
C4	0.11	1.17	0.25	0.00	1.31
1y - skull	0.04	0.69	0.17	0.00	0.56
1z - skull	0.13	1.43	0.27	0.01	1.05
2y - pelvis	0.09	0.80	0.20	0.00	0.74
3y - back feet	0.05	0.83	0.20	0.00	0.71
6y - shoulder	0.04	0.56	0.16	0.00	0.48
8y - elbow	0.04	0.63	0.15	0.00	0.58
12y - knee	0.06	0.63	0.17	0.00	0.43
15z - abdomen	0.09	0.81	0.21	0.01	0.66
16/17y - front feet	0.08	0.89	0.21	0.00	0.82
16/17z - front feet	0.12	0.96	0.18	0.00	0.82
A1 - crust material over abdomen	0.08	0.78	0.20	0.00	0.69
A9 - beneath the centre of the body	0.11	0.91	0.21	0.00	0.70

Table 5.3. Elemental analysis (CHNS) and total organic carbon (TOC) results for Folkton FLK1, coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 - initial matrix before burial	3.38	36.92	4.71	0.00	33.26
C2A	2.10	33.40	4.16	0.35	26.45
C2B	1.96	33.20	3.97	0.91	25.97
C2C	2.25	36.51	4.41	0.00	34.37
C3A	2.96	33.29	4.38	0.00	22.83
C3B	3.28	38.63	5.57	0.00	28.38
C3C	2.74	34.24	4.88	0.00	24.64
C4	2.40	31.30	5.58	0.00	28.98
1y - skull	3.37	41.47	5.65	0.00	28.86
1z - skull	3.05	40.88	5.07	0.20	39.33
2y - pelvis	2.92	40.90	5.06	0.16	39.12
2z - pelvis	3.39	42.62	5.50	0.00	27.77
4y - back feet	3.27	43.53	5.75	0.00	40.10
4z - back feet	2.93	39.00	4.96	0.16	36.07
6y - shoulder	3.68	42.44	5.81	0.00	38.71
8y - elbow	3.53	46.83	5.93	0.15	43.23
12y - knee	2.64	42.45	5.31	0.13	41.77
15z - abdomen	4.16	45.69	5.95	0.00	31.57
16/17y - front feet	3.45	44.48	5.95	0.00	34.10
16/17z - front feet	2.72	39.35	4.84	0.27	38.15
A2 - below coffin	3.27	43.34	5.35	0.00	34.24
A3 - top of cavity	3.66	51.66	5.32	0.00	30.43
A4 - string	3.81	44.91	5.88	0.23	29.09
A6 - bee's wax	3.93	48.25	5.92	0.12	41.79
A7 - soil snout bag region	2.02	28.80	4.15	0.10	28.11
A10 - bottom of coffin	4.07	48.76	6.42	0.00	35.66
A11 - bottom of coffin	3.79	45.65	5.78	0.00	44.64
A12 - bottom of coffin	3.14	42.72	5.14	0.48	42.80
A13 - bottom of coffin	3.42	41.00	5.30	0.00	38.72
A14 - bottom of coffin	3.47	45.88	5.77	0.00	39.97
A15 - bottom of coffin	2.92	42.30	5.04	0.43	40.82

Table 5.4. Elemental analysis (CHNS) and total organic carbon (TOC) results for Folkton FLK2, un-coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C2A	2.40	36.32	4.12	0.00	31.27
C2B	2.33	33.67	3.97	0.00	33.34
C2C	2.50	32.41	4.25	0.09	29.93
C3A	2.52	40.54	5.06	0.00	38.24
C3B	2.28	39.12	5.01	0.00	38.51
C3C	2.63	33.97	4.11	0.00	28.25
1y - skull	2.41	37.17	4.59	0.00	36.62
1z - skull	3.11	39.91	4.94	0.00	38.42
2y - pelvis	2.34	33.84	2.78	0.00	25.59
2z - pelvis	2.28	40.75	4.97	0.00	38.18
4y - back feet	2.29	34.22	4.36	0.00	31.63
4z - back feet	2.53	40.45	5.02	0.18	35.70
6y - shoulder	2.30	34.72	4.40	0.00	34.80
8y - elbow	2.57	34.84	4.21	0.00	30.75
12y - knee	2.19	35.91	4.60	0.00	34.68
15z - abdomen	2.72	35.24	4.29	0.00	33.53
16/17y - front feet	2.48	35.07	4.22	0.00	38.17
16/17z - front feet	2.74	37.15	4.45	0.00	35.40

Table 5.5. Elemental analysis (CHNS) and total organic carbon (TOC) results for Heslington East HE1, coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C0 - initial matrix before burial	0.06	1.76	0.65	0.00	0.59
C2A	0.07	1.50	0.57	0.00	0.12
C2B	0.12	1.61	0.53	0.00	0.17
C2C	0.10	1.47	0.60	0.00	0.13
C3A	0.11	1.51	0.58	0.00	0.13
C3B	0.14	1.49	0.55	0.00	0.16
C3C	0.15	1.74	0.57	0.00	0.12
1y - skull	0.21	1.87	0.62	0.00	0.24
1z - skull	0.23	1.72	0.55	0.00	0.30
2z - pelvis	0.15	1.64	0.63	0.00	0.21
3y - back feet	0.08	1.41	0.54	0.00	0.21
4y - back feet	0.15	1.45	0.51	0.00	0.14
6y - shoulder	0.15	1.49	0.54	0.00	0.23
8y - elbow	0.22	1.84	0.63	0.00	0.19
10z - pelvis	0.21	1.62	0.60	0.00	0.17
14z - pelvis	0.12	1.63	0.57	0.00	0.10
15z - abdomen	0.20	1.71	0.57	0.00	0.19
16/17y - front feet	0.19	1.50	0.48	0.00	0.23
A1 - above body between front and hind legs	0.11	1.54	0.58	0.00	0.16
A2 - gut bag contents	0.48	3.88	0.86	0.00	0.50
A3 - corner of box south west corner	0.11	1.22	0.55	0.00	0.14
A4 - corner of box north east corner	0.17	1.53	0.56	0.00	0.16
A5 - fabric bag snout contents	0.18	1.71	0.53	0.00	0.23
A9 - below A5	0.20	1.64	0.55	0.00	0.16
A10 - bottom of coffin	0.17	1.44	0.48	0.00	0.21
A11 - bottom of coffin	0.14	1.39	0.50	0.00	0.19
A12 - bottom of coffin	0.19	1.45	0.59	0.00	0.14
A13 - bottom of coffin	0.17	1.46	0.56	0.00	0.22
A14 - bottom of coffin	0.14	1.37	0.52	0.00	0.10
A15 - bottom of coffin	0.17	1.34	0.47	0.00	0.23

Table 5.6. Elemental analysis (CHNS) and total organic carbon (TOC) results for Heslington East HE2, un-coffined. Elemental data given as weight percent.

Sample	Nitrogen / %	Carbon / %	Hydrogen / %	Sulfur / %	TOC / %
C2A	0.38	1.58	0.61	0.00	1.27
C2C	0.45	1.65	0.65	0.00	0.90
C3A	0.50	1.39	0.49	0.00	1.06
C3B	0.49	1.67	0.58	0.00	1.68
C3C	0.46	1.69	0.57	0.00	1.49
1y - skull	0.52	1.60	0.55	0.00	1.02
1z - skull	0.48	1.65	0.56	0.00	1.06
2z - pelvis	0.53	1.88	0.58	0.00	0.73
3y - back feet	0.59	1.64	0.52	0.00	0.72
3z - back feet	0.37	1.75	0.64	0.00	1.03
4y- back feet	0.42	1.68	0.61	0.00	1.01
4z - back feet	0.42	1.58	0.59	0.00	0.77
6y - shoulder	0.44	1.60	0.60	0.00	1.16
10z - pelvis	0.45	1.67	0.55	0.00	0.72
12z - knee	0.54	1.62	0.55	0.00	0.80
14z - pelvis	0.41	1.64	0.57	0.00	0.00
15z - abdomen	0.52	1.75	0.58	0.00	0.48
16/17y - front feet	0.59	1.80	0.61	0.00	1.15
16/17z - front feet	0.41	1.61	0.56	0.00	1.15

5.3.2 Molecular profile of extracted soil

All C0 controls for the piglets contained lipids attributed to the soil organic matter (lipids in samples of fill excavated for the grave cut are dominated by long chain *n*-alkanes, *n*-alkanols and fatty acids from plant waxes). The WH1 *n*-alkane distributions ranged from C_{23} – C_{33} (Figure 5:5, a-b) with WH2 *n*-alkanes having a smaller range from C_{23}–C_{31} (Figure 5:5, c-d), both graves showed *n*-alkane distributions to have maxima at C_{29} or C_{31}. Distributions for the controls of West Heslerton match that seen for sample 1z (Figure 5:5, a) with C0 *n*-alkane abundances (7.44 – 3.66 x 10^{-3} mg/ mg TE) lower than those of the controls from the grave fill (6.69 x 10^{-3} – 1.51 x 10^{-2} mg/ mg TE) indicating accumulation of *n*-alkanes in the grave over the burial period. WH1 samples show abundances (5.35 x 10^{-4} – 9.72 x 10^{-3} mg/ mg TE) typically lower than those controls and C0, with WH2 having abundances similar to those of the controls (7.13 x 10^{-3} – 1.17 x 10^{-2} mg/ mg TE). The *n*-alkane abundances suggest that the presence of the coffin prevented the accumulation of *n*-alkanes that is evident for the WH1 controls and WH2 samples. A likely explanation for the difference in abundance levels relates to the movement of organic matter absorbed on clays, as discussed in Chapter 4. Thus, the disturbance associated with digging and subsequently filling the graves may have led to increased movement of fine particles, transporting *n*-alkanes through the grave fill and affecting the WH2 burial. In the case of WH1 burial the presence of the coffin would have prevented the ingress of fine clay material. Distributions of *n*-alkanols were found to be

detectable only in WH1, ranging from C_{22}-C_{30} with maxima at C_{30} (Figure 5:5, e-f). Profiles shown in Figure 5:5 are typical of distributions throughout the grave fill, including controls, with similar abundances of *n*-alkanols in both controls and samples.

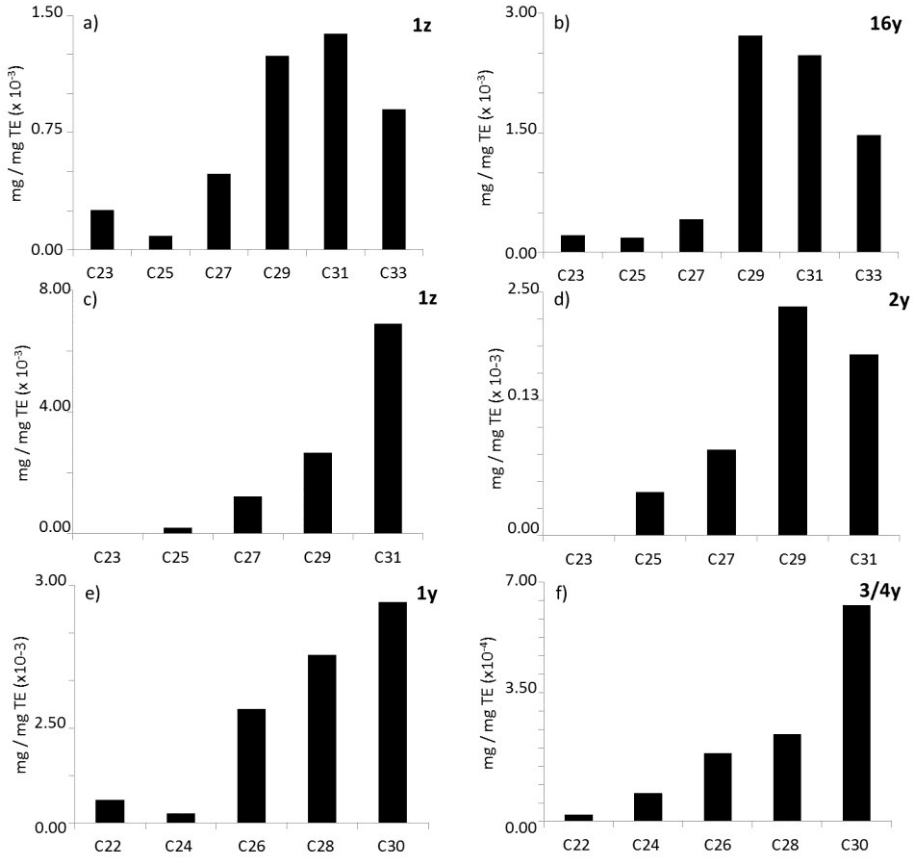


Figure 5:5. a-b) Distributions of *n*-alkanes typical of samples from the coffin burial West Heslerton (WH1). c-d) Distributions of *n*-alkanes typical of samples from the un-coffined burial West Heslerton (WH2). e-f) Distributions of *n*-alkanols of burial samples for WH1.

The *n*-alkanes in the FLK1 soils ranged from C_{23}–C_{33} (Figure 5:6, a-b) and those in the FLK2 soils ranged from C_{23}–C_{31} (Figure 5:6, c-e). The *n*-alkane distributions for FLK1 maximise at C_{31} and those for FLK2 maximise at C_{29} or C_{31}. The distributions in the controls for FLK1 match that from sample C3A (Figure 5:6, a) and other samples in the grave (2z, Figure 5:6, b). Abundances of *n*-alkanes for FLK1 (controls: 4.23×10^{-3} – 1.16×10^{-2} mg/ mg TE, sample: 2.55×10^{-3} – 1.24×10^{-2} mg/ mg TE) and FLK2 (controls: 4.88×10^{-4} – 1.28×10^{-3} mg/ mg TE, sample: 3.63×10^{-4} – 1.05×10^{-3} mg/ mg TE) show similar abundance levels consistent with the high organic content (Table 5.3 and Table 5.4) of the peaty soil.

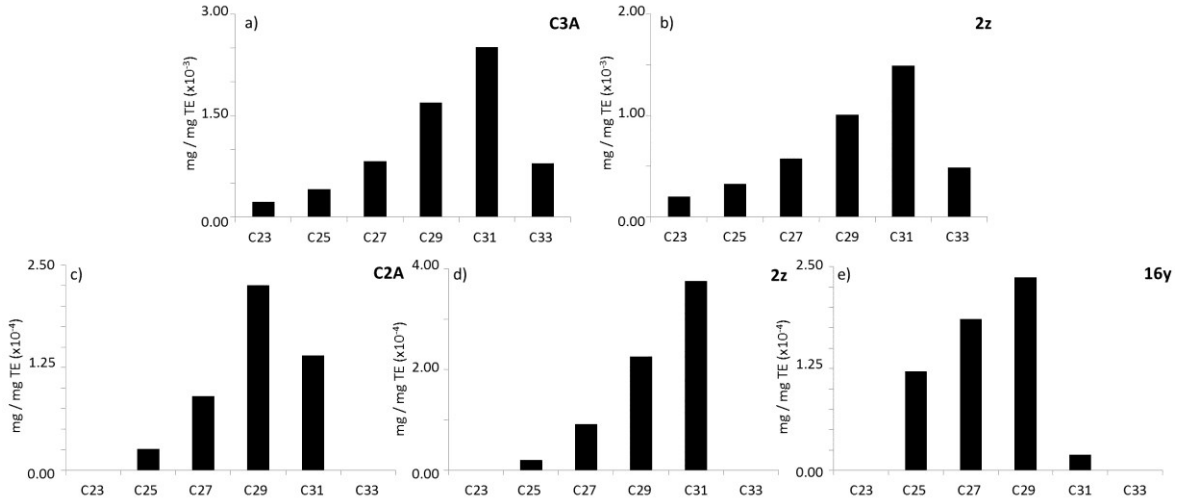


Figure 5:6. a-b) Distributions of *n*-alkanes typical of samples from the coffin burial at Folkton (FLK1). c-e) Distributions of *n*-alkanes typical of samples from the un-coffined burial Folkton (FLK2).

HE1 *n*-alkanes range from C_{25}–C_{33} (Figure 5:7 a-b), with HE2 *n*-alkanes ranging from C_{25}–C_{31} (Figure 5:7, c-d), both graves have distributions with maxima at C_{29} or C_{31}, with control distributions matching those of the samples (HE1, 10z and HE2, 1z, Figure 5:7). The *n*-alkane abundances is HE1 (controls: 2.97×10^{-3} – 8.90×10^{-3} mg/ mg TE, sample: 1.00×10^{-3} – 1.42×10^{-2} mg/ mg TE) and HE2 (controls: 9.31×10^{-4} – 7.92×10^{-3} mg/ mg TE, sample: 1.49×10^{-3} – 7.71×10^{-3} mg/ mg TE) show similar abundances reflecting the background soil contribution to the grave fill. Distributions of *n*-alkanols were only detected in HE1, with a range from C_{26}-C_{30} and maximum at C_{28} (Figure 5:7, e), similar abundance levels were present in the controls and burial samples, as was also noted for the *n*-alkanes.

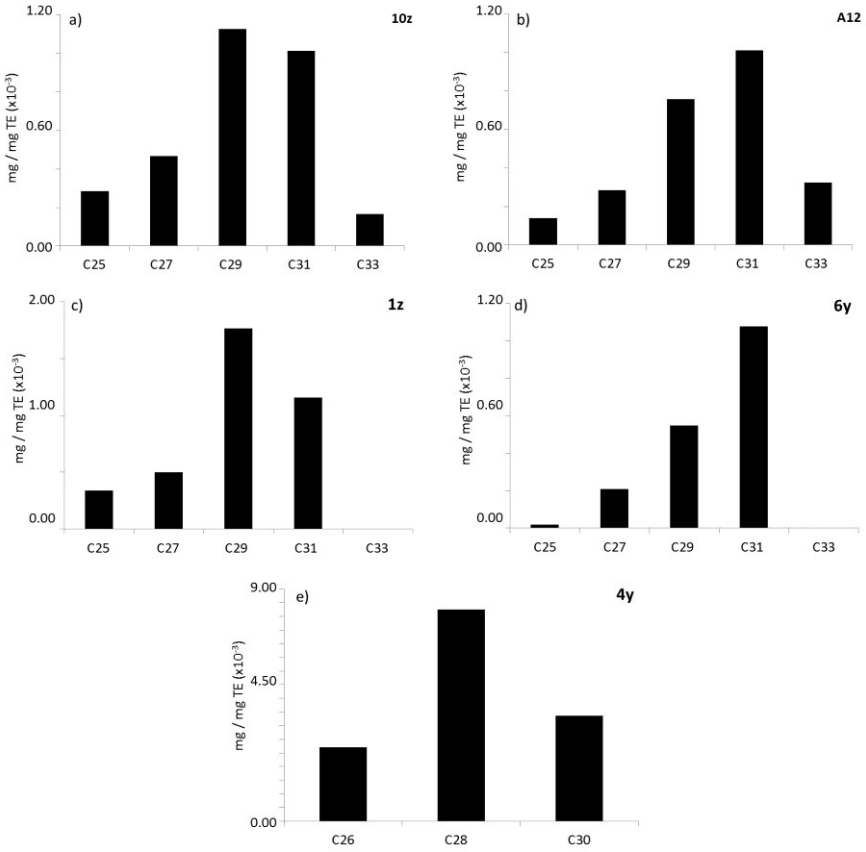


Figure 5:7. a-b) Distributions of *n*-alkanes typical of samples from the coffin burial Heslington East (HE1). c-d) Distributions of *n*-alkanes typical of samples from the un-coffined burial Heslington East (HE2). e) Distributions of *n*-alkanols typical of HE1.

Triacylglycerols were found in graves from all three sites with both FLK2 and HE2 showing either low abundances or no TAGs in the samples. Comparison of the triacylglycerol distributions of coffined and un-coffined graves (Figure 5:8, Figure 5:9, Figure 5:10) shows that the number of TAGs are lower in non-coffined burials than in the control piglet soils (Chapter 4, Figure 4:9) with loss of unsaturated TAGs being more pronounced than in the coffined burials. The TAG distributions, where present, all contained potential degradation products having short acyl chains or being oxidised (c.f Chapter 4).

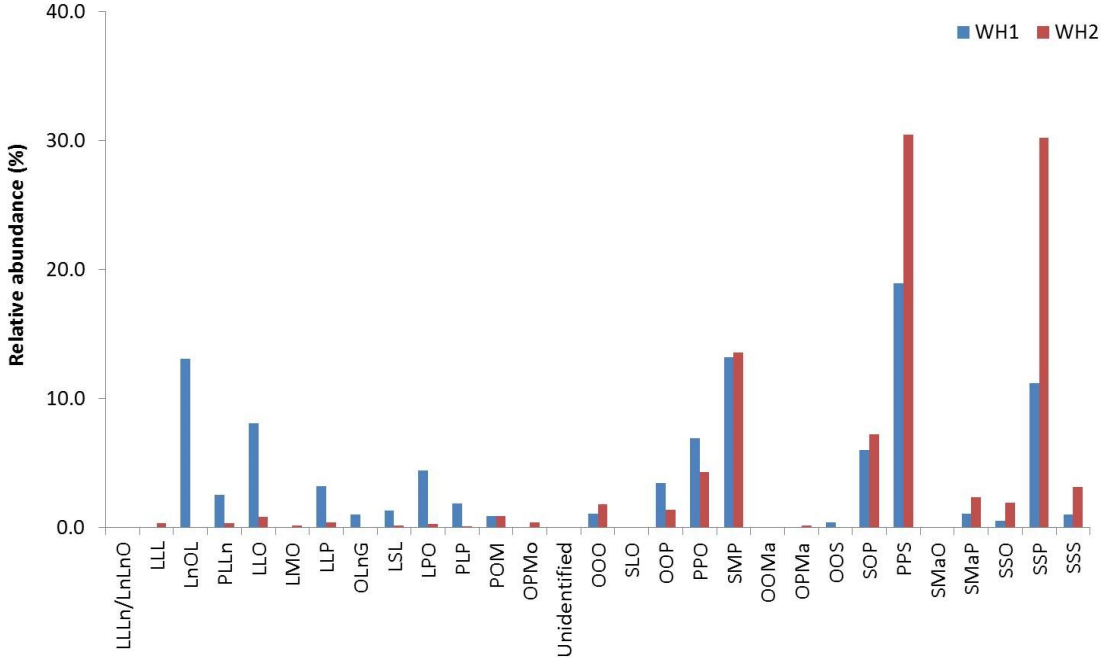


Figure 5:8. TAG distributions for West Heslerton graves, WH1 (coffin) shown in blue and WH2 (un-coffined) shown in red.

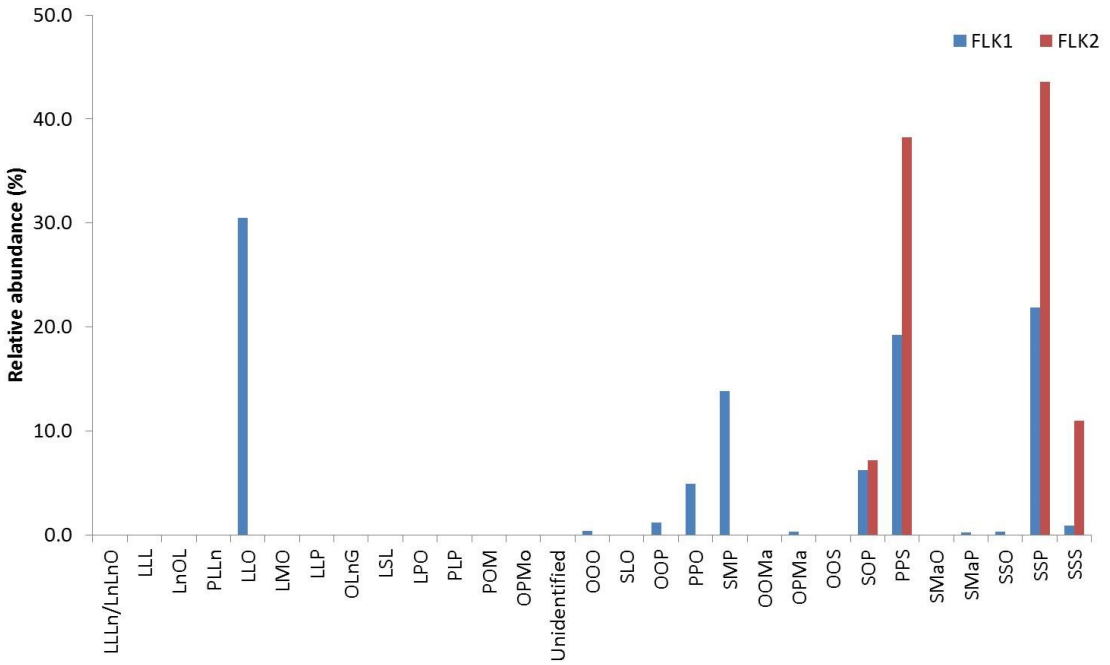


Figure 5:9. TAG distributions for Folkton graves, FLK1 (coffin) shown in blue and FLK2 (un-coffined) shown in red.

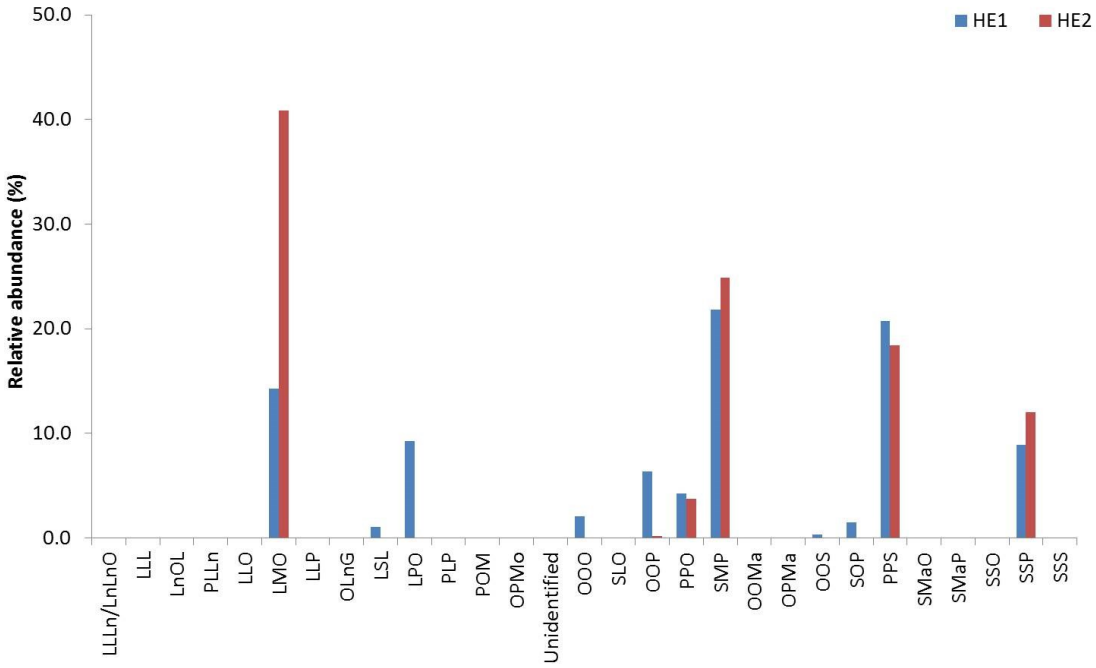


Figure 5:10. TAG distributions for Heslington East graves, HE1 (coffin) shown in blue and HE2 (un-coffined) shown in red.

As well as TAGs the grave soils also showed other potential components derived from adipose tissue, MAGs and DAGs. Both West Heslerton graves showed MAGs containing $C_{14:0}$, $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ acyl moieties and DAGs with $C_{14:0}C_{14:0}$, $C_{16:0}C_{16:0}$, $C_{18:0}C_{18:0}$ acyl groups. FLK1 was the only grave from Folkton that showed the presence of MAGs with only the $C_{16:0}$ fatty acyl moiety being represented. Heslington East, contained MAGs in HE1, the acyl moieties being $C_{16:0}$, $C_{18:1}$, $C_{18:1}$ and $C_{18:0}$, and both MAGs and DAGs in HE2, the former having only $C_{16:1}$ and $C_{16:0}$ moieties and the latter $C_{16:0}C_{16:0}$. The similarity in the acyl moieties of the MAGs and DAGs in West Heslerton and Heslington East suggest a direct relationship between the components and a degradation pathway from TAGs in the soil. An even stronger relationship is apparent between the dominant fatty acids in HE1 and HE2 and the MAG fatty acyl moieties.

C1 and C0 controls for all sites show fatty acid distributions typical of plants, with a unimodal distribution ranging from C_{22}-C_{30} and centred on the C_{26} fatty acid. West Heslerton fatty acids have a broader range, encompassing C_{14}–C_{30}, whereas the distributions for WH1 exhibit a mixture of short chain (5.27×10^{-4} – 3.05×10^{-2} mg/ mg TE) and long chain fatty acids (1.75×10^{-4} – 3.26×10^{-2} mg/ mg TE). The distributions in WH2 were typically dominated by long chain fatty acids (0.10 – 0.54 mg/ mg TE). Long chain fatty acid distributions for both graves have C_{26} predominant, indicative of plant/soil lipids (Eglinton and Hamilton, 1967, Baker, 1974, Bull et al., 2000b, Bull et al., 2000a). The greater dominance of long chain fatty acids in WH2 than in WH1 suggests that the majority of long chain fatty acids originate from the soil, the open nature of the un-coffined

grave allowing for constant influx, different from the coffined grave. Short chain fatty acid distributions for both WH1 (Figure 5:11) and WH2 (Figure 5:12) contain unsaturated fatty acids as well as $C_{14:0}$ a fatty acid not present in the control piglet distribution suggesting its origin to be from formation or liberation within the grave. The WH1 distributions differ somewhat to that expected for porcine adipose tissue, with varying levels of alteration to the fatty acid distributions across the sample set. The majority of samples show distributions that differ from the control, mostly with one component, typically $C_{16:0}$ becoming dominant (most evident for 2y and 15z).

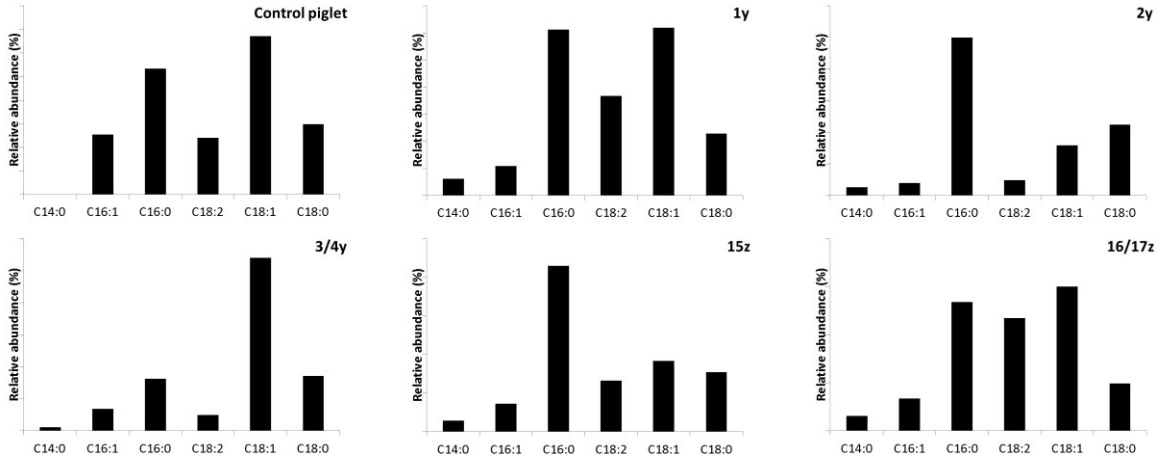


Figure 5:11. Fatty acid distributions for West Heslerton coffined burial (WH1), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

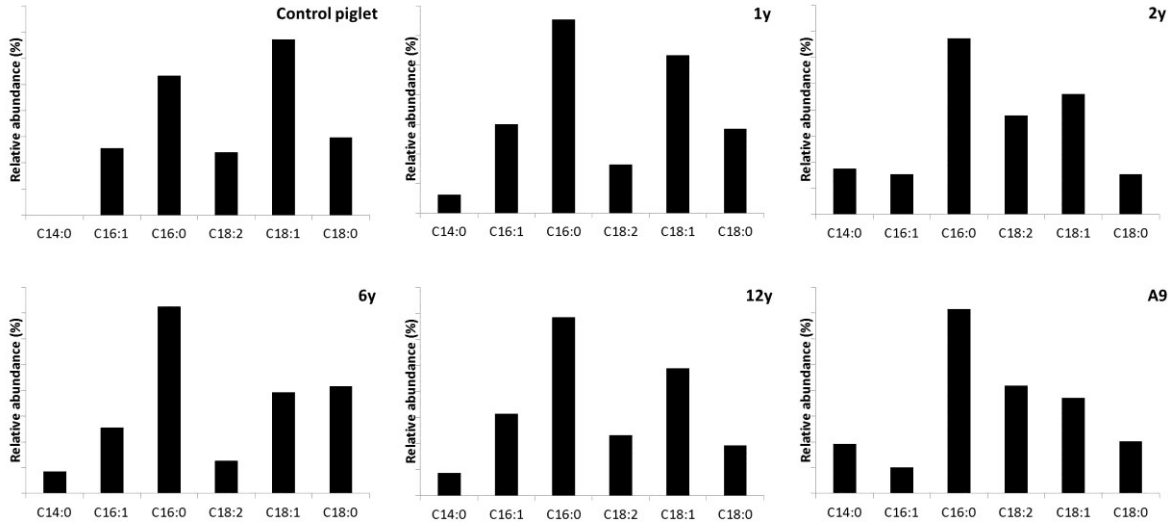


Figure 5:12. Fatty acid distributions for West Heslerton un-coffined burial (WH2), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

Fatty acids for Folkton range from C_{14}–C_{28}, both graves have distributions dominated by long chain fatty acids (FLK1, 2.16 x 10^{-2} – 8.30 x 10^{-2} mg/ mg TE and FLK2, 1.74 x 10^{-3} – 1.44 x 10^{-2} mg/ mg TE) the short chain fatty acid abundances being much lower (FLK1, 8.17 x 10^{-3} – 1.10 x 10^{-2} mg/ mg TE and FLK2, 1.25 x 10^{-4} – 1.63 x 10^{-3} mg/ mg TE). The dominant long chain fatty acid $C_{26,}$ typical of plant lipids (Baker, 1974, Eglinton and Hamilton, 1967, Bull et al., 2000b, Bull et al., 2000a). Unlike West Heslerton, long chain fatty acids abundances for both graves in Folkton are of a similar range, reflecting the high lipid content of the soil matrix and the nature of the site. Distributions for the short chain fatty acids of both FLK1 (Figure 5:13) and FLK2 (Figure 5:14) contain unsaturated fatty acids, though FLK2 lacks both $C_{14:0}$ and $C_{16:1}$ fatty acids. Overall distributions for both FLK1 and FLK2 differ to those expected for porcine adipose tissue, with varying levels of alteration to the fatty acid distributions occurring across the sample set. FLK1 shows distributions with low abundances of C_{18} components relative to $C_{16:0}$, with the greatest extent of degradation/alteration apparent for $C_{18:unsaturated}$ moieties. Comparison of FLK1 distributions with that of the control piglet, following normalising to $C_{18:0}$, reveals loss of all components with $C_{16:0}$ showing the least change, reflecting either additional input or formation via β-oxidation and associated reduction. Short chain distributions for FLK2 (Figure 5:14) reveal higher levels of degradation/loss than occurred in FLK1. The absences of $C_{16:1}$ and $C_{14:0}$ in FLK2 may result from the absence of a coffin and the high water table inducing their loss due to their low octanol: water partition coefficients. Comparison with the control piglet shows that the distributions all reflect losses, suggesting extensive degradation or alteration. Overall comparison of FLK1 and FLK2 indicates that greater preservation occurred in the coffin (FLK1) suggesting protection/retention of the remains as seen by the loss of $C_{14:0}$ and $C_{16:1}$ and lower abundances of short chain fatty acids in FLK2.

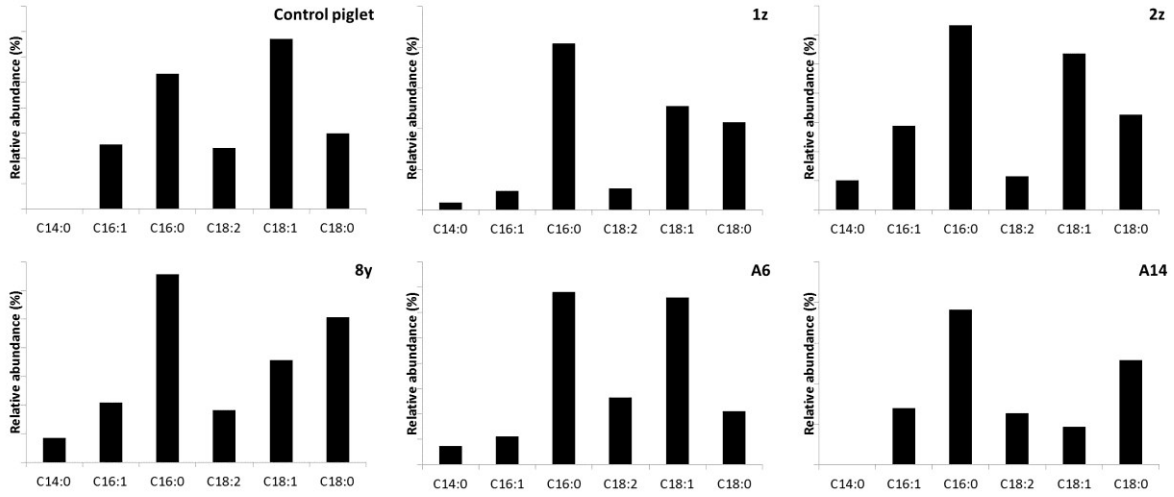


Figure 5:13. Fatty acid distributions for Folkton coffined burial (FLK1), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

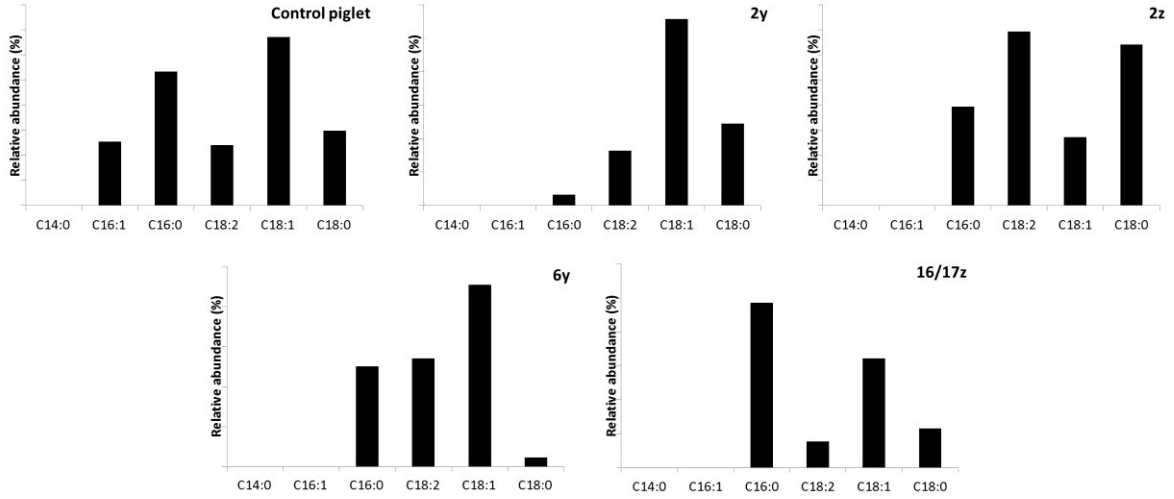


Figure 5:14. Fatty acid distributions for Folkton un-coffined burial (FLK2), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

The fatty acids in the Heslington East grave soils range from C_{14}–C_{30}, and are mainly dominated by long chain fatty acids from HE1(3.09 x 10^{-3} – 4.45 x 10^{-2} mg/ mg TE) with a small set of samples in which short chain fatty acids dominated (8.70 x 10^{-4} – 1.80 x 10^{-2} mg/ mg TE). The HE2 samples show the reverse trend with short chain fatty acids dominating (3.84 x 10^{-3} – 2.22 x 10^{-2} mg/ mg TE) over the long chain components (1.19 x 10^{-3} – 1.16 x 10^{-2} mg/ mg TE). Distributions of long chain fatty acids of both graves have C_{26} predominant and are of plant origin (Baker, 1974, Eglinton and Hamilton, 1967, Bull et al., 2000b, Bull et al., 2000a). The short chain fatty acid distributions for HE1 (Figure 5:15) and HE2 (Figure 5:16) both differ greatly to that of the control piglet, with a dominance/greater abundance of $C_{16:1}$ and $C_{16:0}$ evident in the majority of samples. These two fatty acids were found to be in a greater abundance in controls from the burial, suggesting them to originate from the soil. Comparison of the distributions from Heslington East

with the control piglet distributions normalised to $C_{18:0}$ shows an overall increase in abundance of all components. This increase, along with the elevated levels of $C_{16:1}$ and $C_{16:0}$, suggests significant input from the soil environment. As the abundances of short chain fatty acids for both graves are similar it may suggest that the fatty acids in the graves derive mainly from the soil and that the fatty acids from the remains have been heavily degraded/incorporated into the soil environment.

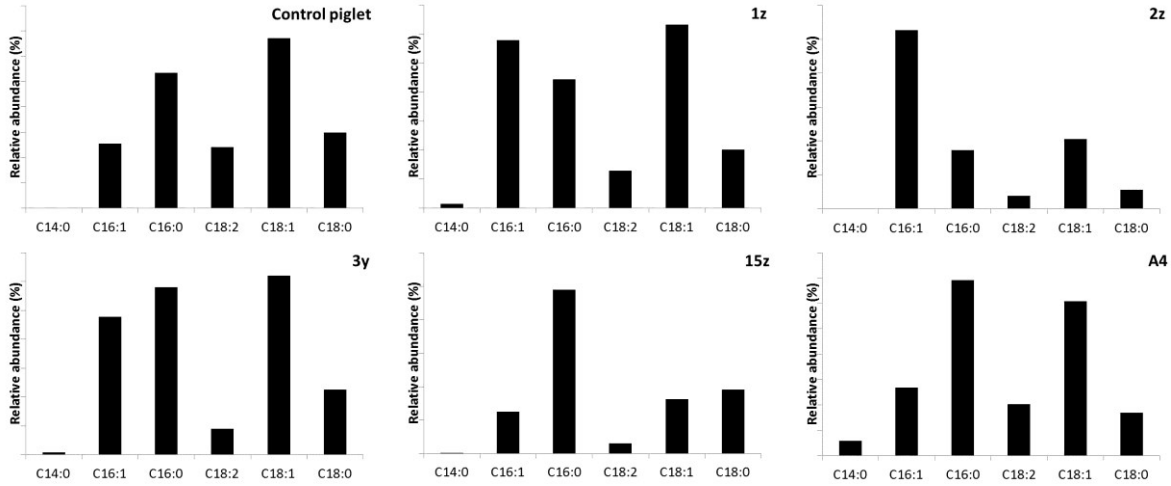


Figure 5:15. Fatty acid distributions for Heslington East coffined burial (HE1), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

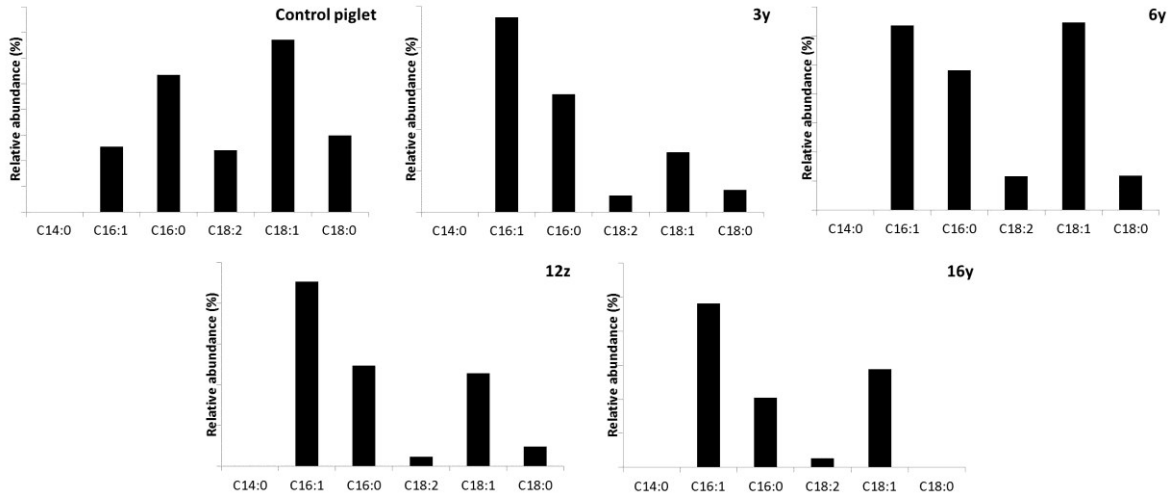


Figure 5:16. Fatty acid distributions for Heslington East un-coffined burial (HE2), showing distribution expected for porcine adipose tissue and the varying distributions throughout the samples.

Branched chain fatty acids, signatures of bacterial activity (Zelles, 1999) were found in all grave fill with both burials from West Heslerton showing branched chain $C_{15:0}$ and $C_{17:0}$ *iso* and *anteiso* fatty acids in the burial matrix and lower levels in the controls. Heslington East graves showed the presence of C_{15} *iso* and a*nteiso* fatty acids with higher abundance levels in the coffin samples of HE1, and similar abundance levels in the controls and the samples of HE2. Folkton graves differ in

that C_{15} *iso* and *anteiso* as well as C_{17} *iso* fatty acids were present. Abundances for the branched chain fatty acids from Folkton show the coffin samples (FLK1) to contain lower abundances than the corresponding controls, with FLK2 samples having equivalent abundances to the controls. The higher abundance levels associated with the remains suggest bacterial activity associated with the decomposing remains (Pickering et al., unpublished).

Mass balance for all graves (Figure 5:17) show TAG abundances to be higher in samples from coffined graves, with clear shifts towards fatty acids for un-coffined remains. The differences in the distributions in the graves suggests that the more accessible nature of the un-coffined remains enabled higher levels of degradation with particular increase in degradation in graves that had increased water levels.

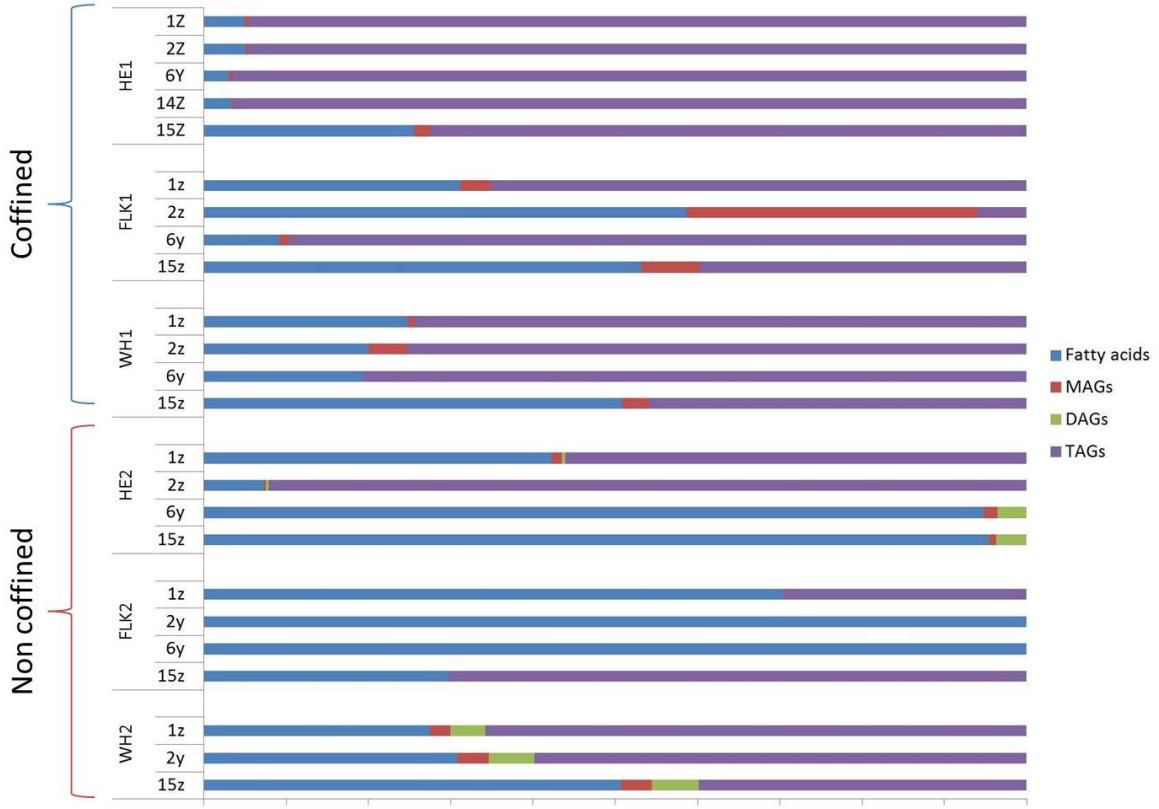


Figure 5:17. Mass balance for both coffined and un-coffined graves from West Heslerton, Folkton and Heslington East of triacylglycerol degradation products through to free fatty acids.

Long chain *n*-alkan-2-ones (C_{25}-C_{29}) were present in the controls and burial soils at West Heslerton, Folkton and Heslington East and are attributed to plant matter (Eglinton and Hamilton, 1967). FLK1, however, also showed the presence of short chain (C_{16} and C_{18}) *n*-alkanals that were not detectable in either the un-coffined grave FLK2 or the controls. Their presence suggests a reducing environment in the coffined grave (Chapter 2 and Chapter 4) as well as an environment conducive to the protection of these molecules from further degradation.

5.4 Conclusion

Elemental analysis and total organic carbon content, in particular, show greater organic contents in samples from around coffined remains than from the controls. Average TOC throughout all sets of remains for West Heslerton show a clear difference between the coffined and non-coffined remains. Folkton shows very little difference between the samples, reflecting the high organic carbon content of the soil. The soils from Heslington East show higher TOC contents in un-coffined remains reflecting greater input into the grave due to its accessible nature and the high through put of water.

All six graves sampled show clear signals from *n*-alkanes of a plant origin (Eglinton and Hamilton, 1967) with similar distributions and abundances for the controls and samples suggesting that the signatures are directly related to the organic plant matter in the soil. The WH1 and WH2 soils show clear evidence for the introduction of the *n*-alkanes, with similar levels in the control and un-coffined samples and lower abundances in the coffined samples. By contrast *n*-alkanols appear to be present only in the coffined graves; this may reflect the protective nature of the coffin allowing for the preservation of *n*-alkanols via retention in the soils.

The range of fatty acids and abundance levels of the short chain components are consistent throughout all graves. Long chain fatty acid abundances are similar for Folkton and Heslington East, yet the graves of West Heslerton differ, with WH1 showing similar abundances to Folkton and Heslington East and WH2 showing much higher abundances. This difference, as mentioned earlier, suggests that, in the case of West Heslerton, the coffin has a major effect on the preservation of signatures in the grave. The majority of samples show distributions dominated by long chain fatty acids typical of plant matter, with WH1 and HE2 being the only exceptions. The WH1 soils show similar levels of short and long chain fatty acids, supporting the prospect of the coffin providing a unique environment for degradation, probably by preventing water movement and subsequent loss of fatty acids as is suspected to have occurred in WH2. The soils from HE2 showed dominance of short chain fatty acids, signatures that also matched the distributions in the controls, suggesting that these fatty acids were most likely incorporated through water transport. The dominance of long chain fatty acids in HE1 lends further support to this interpretation, suggesting that the coffin prevented alteration of the signatures, providing a protective barrier to degradation/alteration.

Comparison of all of the normalised burial soil distributions with that of the control piglet show significant reductions in the abundances of fatty acids except $C_{16:0}$. These changes in distribution suggest that the reduction and loss of fatty acids occurs through water movement. The short

chain distribution from WH2 (Figure 5:12) shows similar features to that of WH1 with increased relative abundances of $C_{16:1}$ and $C_{16:0}$ being much greater than could be expected from the pathway involving β-oxidation and associated reduction as was discussed in earlier chapters. The increases in $C_{16:1}$ and $C_{16:0}$ match the short chain fatty acid distributions of the controls from the grave fill, which had $C_{16:1}$ and $C_{16:0}$ in high abundance, suggesting that the elevated levels of these components related to the soil background. Folkton and Heslington East show these signatures to be low for the former and high for the latter. The low levels in Folkton, may suggest loss due to water movement and microbial activity, with the increase in Heslington East showing additional input into the grave, possibly due to water movement.

Branched chain fatty acids (bacterial markers) were present in all of the graves. Their higher abundances in samples around the remains suggest that bacterial activity contributed to the degradation of remains. Triacylglycerols also show higher abundances in the coffined remains, with significant alteration in the TAG distributions compared with that of the control piglet. Short chain *n*-alkanals in FLK1 suggest a reducing environment and a lack of presence in FLK2 suggest that the coffin environment provides protection to these molecules.

6 Overview and further work

6.1 Conclusions and discussion

Clear aims and objectives were set out at the start of this body of work to address the overarching theme 'The fate and preservation of lipids in grave soils from archaeological and experimental burials'.

I. To determine if signatures from body decay survive in burial soils and can be distinguished from the background soil signature.
II. To identify possible signatures relating to organic materials that may not have physically survived.
III. To understand factors of the burial environment that effect degradation and preservation.
IV. To determine the role of coffins in influencing preservation and degradation of the buried remains.
V. Construct degradation pathways and timelines.

6.1.1 Determination of signatures from body decay and background soil signatures

6.1.1.1 Signatures from the soil background

Soils naturally contain organic matter from the degradation of plant matter. Analysis of the controls and sample soils of the majority of the graves show some degree of incorporation of these signatures in the grave. The piglet burials in the two defined matrices clearly show the ingress in the grave fills and burial soils closely associated with grave goods, of organic molecules from degraded plant organic matter. Thus, *n*-alkanes, *n*-alkanols and fatty acids were present in samples from the controls and grave goods. In Fewston, the *n*-alkane and *n*-alkanol distributions are both typical of plant matter, with the controls and grave soils for all single inhumations containing signatures that reflect this background soil organic content. Similarly, the mass burials from Fromelles and Mechelen also exhibit *n*-alkane and *n*-alkanol distributions. All graves sampled at West Heslerton, Heslington East and Folkton show clear signals of *n*-alkanes of plant origin (Eglinton and Hamilton, 1967). The similarity in the distributions and abundances in the controls and samples suggest that the signatures represented plant organic matter.

6.1.1.2 Signatures originating from the remains

The release, to the soils, of signatures from the remains has been clearly demonstrated through the use of a burial matrix that is free from background signatures from the soil organic matter. Thus, signatures from the remains were identified in all of the graves, occurring at very different

abundance levels. The major components in the soils that derive from the remains were triacylglycerols, fatty acids and cholesterol. Although Haymarket was dominated by signatures relating mainly to the soil organic matter, the other graves showed clear inputs of organic matter from the remains. Triacylglycerols, the major components of adipose tissue, were preserved to varying degrees. The experimental piglet burials showed that triacylglycerols in the soils exhibit significant differences relative to the distributions of adipose tissue, with preservation of the saturated components being more pronounced for piglet L (limestone matrix) and unsaturated triacylglycerols being preserved better for piglet S (sand matrix). The differences in the TAG distributions indicate that significant differences in their degradation pathways exist as a consequence of the different burial environments. Some of the archaeological graves showed similar profiles with TAG distributions in Mechelen and Fewston showing degradation of triacylglycerols to a level in which the saturated components dominated, indicating their more resistant nature.

Fatty acid distributions in the soils of all mass grave samples show clear evidence of inputs from the remains. Fromelles samples showed distributions expected for adipose tissue degraded in a water logged environment, the presence of C_{18} 10-OH fatty acid in the samples being a key indicator, and the signatures matching those reported by Forbes (2002). Mechelen mass grave samples and samples from the single grave from the same site (GR422) show fatty acid distributions that suggest degraded adipose tissue, consistent with a dry environment due to the lack of C_{18} 10-OH fatty acid. Furthermore, whereas the dominant fatty acid of fresh adipose tissue is $C_{18:1}$, both the degradation experiments (Forbes et al., 2002) and soil sample distributions from Mechelen and Fromelles were dominated by $C_{16:0}$. Notably, a mass burial from Ridgeway in Dorset also contained signatures from adipose tissue, in that case the distribution contained only $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids, with $C_{18:0}$ dominating (Pickering et al., 2014).

The short chain fatty acids present in Fewston reflect heavily degraded adipose TAGs with higher levels of oxidation/conversion than were evident in Fromelles and Mechelen. The high degree of alteration and degradation of the short chain fatty acids for Haymarket and the similarities with the distributions from the Ridgeway mass grave suggest either that the conditions in both graves were conducive to increased levels of degradation or that these graves are the oldest sampled that age may be a significant factor influencing the extent of degradation of the signatures.

Fatty acid distributions of piglet S and piglet L shows that the majority of samples from piglet S are similar to that of the control piglet, reflecting what would be expected from hydrolysis of porcine triacylglycerols to form free fatty acids. This suggests that hydrolysis played a major role in

degradation. Piglet L differed greatly from the controls and the majority of samples from piglet S, suggesting either that a different mode of degradation/alteration occurred, for example β-oxidation and associated reduction, or that hydrolysis of TAGs occurred and the distributions were altered subsequently *via* differential solubilisation resulting from the percolation of water through the soil.

Cholesterol together with its transformation products, coprostanol and 5α-cholestanol, reflect contributions from the human remains and of alteration products formed before and after burial. Analysis of steroidal components tentatively allowed for the general assessment of the last meals before death. Individuals from Fromelles were showed to have had a high cholesterol intake whereas Mechelen samples showed profiles consistent with low cholesterol or high cholesterol high plant content.

6.1.2 Signatures from non-surviving organic materials

The unique environments that mass graves provide, due to the large quantities of organic remains interred, enhances the potential for the survival of organic signatures and hence investigations based on their analysis. Textiles from the head and pelvis of SK1525B from Fromelles produced signatures dominated by squalene and ambrein, both of which occur in sperm whale oil. The presence of these components in the grave is attributed either to the presence of a waterproofing agent on the textile or, perhaps more likely, a degraded canister of rifle oil amongst the remains. Soil sample A3 from Mechelen mass grave yielded a blue dyestuff believed to originate from the textile at this location, despite the textiles remains not yielding any dyestuff. The extract contained indigotin, originating from either woad or indigo, which was most likely from a textile dye. The occurrence of the dye in the soil though not in the remains of the textile in the grave most likely reflects the exclusion of the dye from the textile fibres during their mineralisation. This example illustrates very clearly the potential for organic analysis of the burial matrix to provide information relating to the occupant(s) of a grave.

Single inhumations also provided unexpected signatures from organic molecules. Resin acids were found to dominate all of the grave soils from Fewston, providing insight into the burial practices. Graves SK277, SK331 and SK334 showed resin signatures from the coffins, which indicate them to have been of gymnosperm (*pinacea*) origin. A resinous aggregate from SK334 showed strong signatures of thermal degradation products of wood resins, indicative of a pine pitch wood treatment. Abietic acid, absent in SK408, suggested the coffin to be angiosperm in origin, lending support to the judgement of the excavator that it was of oak construction (Buglass, 2010).

6.1.3 Factors of burial environment that effect degradation and preservation

6.1.3.1 Movement of organic signatures through the soil profile

Analysis of the two graves with controlled matrices enabled direct inputs from sources external to the burial to be traced and the alteration of the signatures from the degrading remains to be identified. This investigation also yielded unexpected information on the movement of organic molecules in the soil profile. Analysis of the grave fills showed the migration of soil organic matter derived *n*-alkanes, *n*-alkanols and long chain fatty acids to the samples above the coffin lid. Due to their absence from the initial burial matrices, the lipid contents in the soils clearly results from inputs after burial. The presence of leaf litter on the overlying exposed soil surface at the time of sampling and the higher contents of the plant derived signatures with depth in the fills suggest them to have migrated through the soil profile from the top. This result implies that transport through the grave fill was probably mediated by water percolation. The hydrophobic nature of *n*-alkanes and *n*-alkanols implies that they moved in association with soil particles (Clemente et al., 2011). The absence of *n*-alkanes and *n*-alkanols in the soils from the upper coffin fill also indicates that the coffin lid acted as a barrier to movement of soil particles. This movement of lipids is also evident in the archaeological graves: in Fromelles the highest levels of $C_{14:0}$ fatty acids were found in samples that had been exposed to increased water flow through the opening of the initial sondage. Graves from Fewston also reflected a direct correlation to water movement with graves in the north, which were free draining, exhibiting extensive degradation and the waterlogged graves in the south exhibiting much better preservation. Hence, the study provides clear indications that water movement and the hydrology of burial sites strongly influence the levels of degradation and preservation of organic remains in graves.

6.1.3.2 Effects of hydrology

The effects of hydrology are apparent throughout the sites examined. Greater preservation is evident in graves located in areas of waterlogging, with the hydrology appearing to be a key factor in their preservation. Graves from the southern part of Fewston and the mass graves from Fromelles and Mechelen all experienced periods of waterlogging, leading to high levels of preservation. By contrast, earlier studies demonstrated that Ridgeway, a mass grave site, showed unexpectedly high degrees of degradation. The location of the site on a chalk escarpment can be interpreted to be a key factor in the extensive degradation of the organic matter. The highly porous nature of the bedrock will have allowed for rapid percolation of water through the site, and consequent inflow of oxygen to aid degradation. Haymarket, which shows similar distributions to Ridgeway, may also have experienced significant fluctuations in water levels, leading to degradation of the organic matter distributions in an analogous manner to Ridgeway.

The intra site analysis both for Fewston and for Fromelles shows the graves to have differing degrees of degradation that reflect their different hydrological states. In the case of Fromelles, greater levels of preservation and less extensive transformation of the signatures from the remains were evident in Grave 4, which was heavily waterlogged and contained significantly fewer root systems than Grave 3. Thus, the proximity of Grave 3 to the wood appears to have significantly influenced the water flows and consequent oxygenation of the grave environment. Similarly, graves from Fewston which were situated on the northern slope and contained a porous soil were more extensively degraded than those from the southern part of the graveyard. Hence, the lipid abundances in Fewston were significantly lower in the northern graves than in the southern part of the graveyard coinciding with the high water table in the latter graves.

The distributions of fatty acids in Fromelles and Mechelen differed greatly from those for Ridgeway, with more extensive degradation being evident at Ridgeway (Pickering et al., 2014). The burial environment of Ridgeway, a shallow pit on a chalk escarpment, excludes waterlogging from being a dominant feature in the environment. Fromelles was heavily waterlogged during sampling, and the signatures suggest that the site has been waterlogged over time whereas Mechelen is situated on a wet sandy ridge in the flood plain of the river Dijle. The TAG distributions from the three environments suggest distinction in the transformation pathways due to the differences in hydrology. The more waterlogged graves contain triacylglycerols with Mechelen showing evidence of degradation and Fromelles being dominated by cleaved/oxidised short chain triacylglycerols (currently unidentified). By contrast, no TAGs were present at Ridgeway (Pickering et al., 2014).

The effect of water on the grave profiles is not only evident in the levels of degradation. Haymarket *n*-alkane profiles revealed low abundances in the controls and higher levels around the remains, consistent with observations for the single inhumation from Mechelen (Chapter 2) and the mass grave from the same site. The higher *n*-alkanes abundances around the skeletal remains suggest that the remains themselves may have acted as barriers to the movement of water, inducing the sedimentation of fine particles around the bones, as suggested for the textiles in Fromelles.

6.1.4 The effects on the fate of organic matter of coffined versus non-coffined burials

Comparison of the coffined vs non-coffined piglet burials from the experimental burials situated at the three sites with active matrices show clear differences between coffined and non-coffined remains both from the bulk elemental analysis and from the detailed molecular level organic

signatures. The average TOC values for West Heslerton and Heslington East show distinct differences between the sites and each site also reveals differences between the coffined and non-coffined burials. Organic signatures from West Heslerton show clear evidence for the incorporation of soil derived *n*-alkanes into the grave, with lower abundance levels in the coffined samples than in the controls and the un-coffined samples. The absence of *n*-alkanols from all but the coffined grave at West Heslerton reflects the protective nature of the coffin, the *n*-alkanols not being lost through uptake by flora/fauna or removal by water movement. In addition, the differences in retention of *n*-alkanes and *n*-alkanols in the soils reflect their differential residence times in the dynamic soil environment. Long chain fatty acids abundances also reflect the protective nature of the coffin, the greater abundances in the un-coffined graves revealing the exclusion of this soil organic matter derived components. Triacylglycerols also show better preservation in coffined burials, revealing the importance of the greater exposure in un-coffined burials to degraders and groundwater to the fate of organic matter in burials.

6.1.5 Sequence of degradation

Triacylglycerols are known to liberate free fatty acids *via* hydrolytic degradation, forming diacylglycerols, monoacylglycerols and ultimately producing glycerol. This process appears to have occurred in all graves and the mass balances of the acyl and fatty acid components reveal different extents of degradation among the graves. Graves from the northern edge of the Fewston graveyard contain distributions dominated by fatty acids whereas those from the southern edge are dominated by triacylglycerols, suggesting a greater level of preservation. The piglet burials also showed different extents of degradation with piglet S exhibiting higher proportions of fatty acids than piglet L, which contained higher proportions of triacylglycerols. The triacylglycerol distributions indicate that unsaturated TAGs are preferentially degraded by comparison with their saturated counterparts. The lack of evidence for reduction of the fatty acid moieties suggests preferential hydrolysis. Archaeological and experimental graves were found to contain oxidised triacylglycerols as well as components thought to bear short acyl moieties and presumed to be formed by cleavage in the acyl groups of unsaturated TAGs. This suggests that, although unsaturated fatty acid moieties appear not to be reduced when bound to a glycerol backbone, oxidation and cleavage occur, most likely in the early stages of degradation as signatures in the graves suggest that anoxic conditions were present in many of the burial environments.

The generation of anoxic conditions in graves is attributed to the consumption of oxygen as degradation of organic matter ensues (Dent et al., 2004). Clear evidence of a reducing

environment was apparent in the burials from Mechelen and graves from Fewston, both sites containing short chain *n*-alkanals (C_{16}-C_{18}) in the extracts. These compounds are formed *via* reduction of fatty acids and are highly susceptible to oxidative transformation; hence, their survival in the grave soils suggests that anoxic conditions persisted. Both piglet S and piglet L (defined matrices) also contained C_{16} and C_{18} *n*-alkanals and the absence of lipid signatures from soil organic matter enable the source of the *n*-alkanals to be firmly attributed to the remains. Geochemical studies have suggested that formation of *n*-alkanes in soils and sediments occurs from the degradation of fatty acids via *n*-alkanols (Ratledge, 1984). Green (2014) proposed *n*-alkanal formation in the graves to occur as part of the degradation pathway from triacylglycerols and proposed that further degradation leads to the production of *n*-alkanes. The confirmation of the source of *n*-alkanals in the grave, and finding of both short chain *n*-alkanes and *n*-alkanols by Green (2014), strongly suggests the operation of transformation pathway by which triacylglycerols lead ultimately to *n*-alkanes.

Alteration of the fatty acid distributions from that expected for fresh adipose tissue is clearly evident in all graves, suggesting the reduction of unsaturated fatty acids to form their saturated counterparts. Changes in the distributions appear in most cases to be microbially mediated, with the presence of branched chain fatty acids providing evidence for microbial activity. The evidence of increased levels of $C_{16:0}$ fatty acids in the grave soils compared with those of adipose tissue components excludes water movement as a major contributor to alteration of the distributions with the most likely cause being microbially mediated β-oxidation and associated reduction.

6.1.6 Variation in sampling position

A large number of sample positions were analysed in accordance with the InterArChive protocol, in particular for Haymarket and the experimental burials. Potential variations between positions that were expected to be similar (shoulders, elbows and knees) allows for the identification of external sources of organic matter (11 and 12 for Haymarket differ due to contamination) and different degradation pathways. Analysis of total organic carbon for positions 5 and 6 (shoulders) from Haymarket showed a difference between 5y and 6y whereas position 5z shows similar TOC contents to 6y. Long and short chain fatty acids at these positions also differ, with 6y showing a distribution similar to that of 5z for the long chain fatty acids, and all positions differing for the short chain distribution. Such variation between the distributions suggests differing degrees of alteration and degradation of the organic signatures. Of particular interest are the differences between samples adjacent to and those below the level of the remains. Elbow positions for Haymarket (7 and 8) show the same distributions for the short chain fatty acids, and different

distributions of the long chain fatty acids. Recognition that the long chain fatty acids reflect the background soil organic matter indicates that the distributions reflect different sources. By contrast, the similarities in the distributions of short chain fatty acids suggest similar extents of degradation and contributions from the remains at the two positions. Analysis of the experimental burials positions 1y and 1z show the abundance levels of the majority of short chain fatty acid distributions are greatest in the (z) position (below the remains).

6.1.7 Recommendations for sampling protocol

Mass graves pose a problem with regards to their sampling due to the complex nature of the burial. The standard InterArChive sampling protocol focuses on individual skeletal remains whereas, the remains in mass graves, are usually intertwined, overlapping or in very close proximity, reducing the available sampling positions. Analysis showed distinct information can be gleaned from a variety of positions. Thus, the best sampling protocol for mass graves would be to sample from the pelvic regions of the remains with further sampling from the head, feet and positions that show unexpected features.

The analysis of single inhumations, however, should conform to that of the high intensity InterArChive protocol where possible. Where full sampling is not possible the recommendations for mass graves should be followed. Of particular interest and value is the sampling of controls and overlaying vegetation to allow for background signatures to be accounted for in the profiles relating directly to the remains. The practice used for the sampling of controls for both Haymarket and the piglet graves is the preferred method, with three samples taken across two planes. In the case of a preserved coffin a third plane should be sampled below the coffin lid. Samples should also be taken were possible directly below the remains as these positions show greater abundances of lipid residues.

6.2 Further work

The analysis and investigation of the fates of lipids in grave environments using both archaeological and experimental graves has posed a series of further questions for investigation. Analysis of triacylglycerols showed the presence of short chain and oxidised triacylglycerols resulting from degradation. The methods available at the time of analysis and the multitude of structural possibilities did not allow for the identification of these components. Further analysis and determination of these triacylglycerols is required as information on the degradation pathways and selectivity of the distribution is likely to provide considerable insight into the degradation processes and redox status in the graves. This will require the synthesis of known triacylglycerols followed by degradation experiments under highly controlled conditions. In

addition, development of new analytical methods will be required in order to separate and characterise the components.

Further studies regarding the transformation pathways of fatty acids should be targeted to provide definitive identification of the sources of components (*n*-alkanals). Significant insights into their origins could be revealed using stable isotope analysis. The application of stable isotope analysis to triacylglycerol degradation products from the soils of graves, looking at shifts in $\delta^{13}C$ to understand the mode of degradation, biotic or abiotic, will allow for greater knowledge into the known pathways of degradation. Analyses of $\delta^{2}H$ and $\delta^{18}O$ signatures will allow for the establishment of direct comparison with the triacylglycerols of the remains and for the determination of lipids in the soils that are linked to degradation of human adipose tissue, thereby enlightening the source(s) of the components.

The use of controlled matrices in burials provided a considerable amount of high quality information and insight into grave environments and the fates of lipids. Further experimental burials could provide a greater level of understanding, with controlled burials targeted at providing information on the effects of different matrices, hydrology and degradation pathways. In particular a pseudo kinetic study, with collection of samples over a series of time periods and during periods of waterlogging would reveal valuable insights into the significance of the lipid distributions in grave soils. Alongside experimental burials, laboratory based degradation experiments utilising sterile controlled matrices could provide evidence for the effects that individual soil matrices have on degradation. Experimental setups could also be developed to examine the impacts of water movement the amount and nature of lipid signatures lost from the soil environment due to hydrological flows and lipid solubility.

7 Experimental

7.1 General procedures

This chapter deals with the experimental procedures used in the analysis of all samples collected from the archaeological and experimental burials. The methods used were set out previously by (Green, 2013). All solvents were of HPLC grade to avoid contaminants. Reagents and standards were all of the highest purity available. Glassware used was baked at 450 °C for 6 h to ensure the removal of all organic residues, using a Pyro-Clean oven (Barnstead/Thermolyne Pyro-Clean Trace).

7.2 Sampling

Samples were collected from all graves by following, as closely as possible, the sampling protocol developed for the InterArChive project (Figure 7:1, a, b);(Usai et al., 2013). Samples from the experimental burials were collected at key points that could be associated, anatomically, with the sampling positions for human remains (Figure 7:1, c).

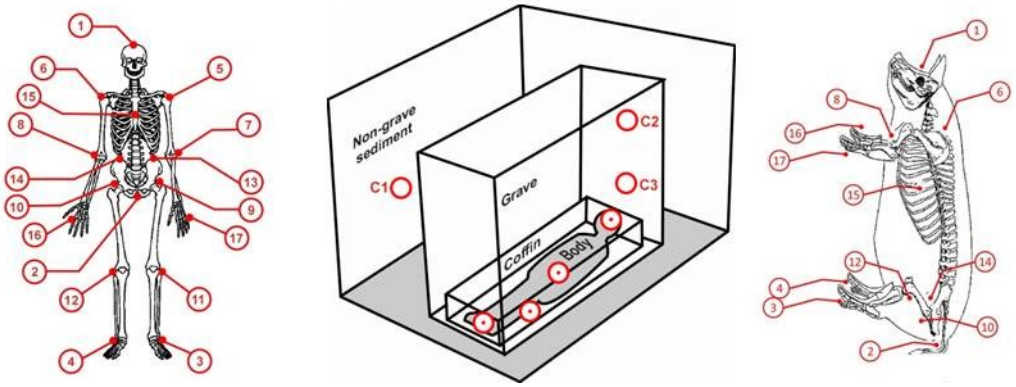


Figure 7:1. a) Locations of the 17 positions sampled around the body using the high intensity sampling protocol developed for the InterArChive project. b) Locations of the controls (numbers prefixed with C) for all grave fills and sampling positions around the skeletal remains as defined for the low intensity sampling protocol developed for the InterArChive project. c) Locations of the positions sampled for experimental piglet burials.

For high intensity sampling, 17 positions were sampled at key points that could be easily identified in the grave according to the skeletal structure (Figure 7:1, a). At these positions samples were taken either above (x), adjacent to (y) or below (z) the skeletal remains. If the remains were compromised by the nature of the grave, low intensity sampling was favoured with samples taken from only the head (1), pelvis (2), feet (3 and 4) and hands (16 and 17). In addition to samples from around the remains, controls from the site (C1, non-grave fill) and grave fill were also collected (C2, upper grave fill and C3, lower grave fill), in order to determine any background signatures from the soil. In developing the sampling protocol for the experimental burials the closest representation to the high intensity sampling for the human skeletal remains was sought

(Figure 7:1, c). As the prescribed sampling positions did not always encompass all interesting features within the graves additional samples were collected (labelled A#). To prevent contamination, all samples were collected and wrapped in baked aluminium foil (450 °C, 6 h) prior to storage in geochemical sample bags. Samples were stored in a cool box on site and were immediately frozen at -20 °C on return to the laboratory.

7.3 Sample preparation

Samples for analysis were first freeze dried by placing the untreated frozen sample on a glass petri dish in a freeze dryer (Thermo Heto PowerDry PL3000). Samples were dried for up to 6 h at approximately 1 hPa, depending on the nature of the soil and water content with freeze drying stopped on the complete removal of ice. The dried bulk sample was ground in a pestle and mortar before sieving through, 1000 µm, 400 µm and 200 µm sieves. The sieves were agitated using a sieve shaker (Endecottes Octagon Digital) for 5 min. The 200 µm fraction was collected for organic analysis and stored in a pre-cleaned glass vial at -20 °C prior to use (other fractions were stored for possible further analysis).

7.4 Elemental analysis

Total organic carbon (TOC), total carbon, hydrogen, nitrogen and sulfur (CHNS) analysis was performed on soil samples (10-20 mg) using a Thermo Flash 2000 elemental analyser fitted with a MAS 200 auto-sampler. Samples for CHNS and TOC were combusted at 900 °C in a quartz reactor tube containing granules of copper oxide and electrolytic copper wire (Marco et al., 2001). Samples for TOC measurement were treated with 2 drops of 6 M HCl to destroy inorganic carbon (carbonates; (Columbo and Baccanti, 1990)) and placed on a preheated hotplate (80 °C) for 6-8 min to remove excess HCl prior to analysis. Samples for CHNS analysis were combusted in folded tin foil capsules (8 x 5 mm) to exclude air and those for TOC analysis were combusted in folded silver foil capsules (8 x 5 mm). A series of standards where run prior to analysis with sulfanilamide (2-3 mg) used as a calibration and cystine (2-3 mg) as a check for the calibration/measurement drift.

7.5 Sample extraction

7.5.1 Soil samples

Extraction of soils was carried out using accelerated solvent extraction (ASE; (Jansen et al., 2006, Quenea et al., 2012)) in pre-cleaned ASE cells, with ASE cells being cleaned by blank extracting. Each sample (3-6 g) was extracted three times with dichloromethane: methanol (DCM:MeOH) (9:1, *v/v*) for 5 min at ~ 1500 psi and 100 °C (3 x 5 mL washes). The solvent was removed using a

rotary vacuum concentrator (RVC: Christ, room temperature, rotations: 1310 min^{-1}, 1.45 h), transferred to pre-weighed vials, dried and the mass of extract determined. Total extracts were split into two equal portions, one for analysis and one for chromatographic separation.

7.5.2 Adipose tissue

Fresh adipose tissue was extracted in a method adapted from (Hassan, 2010). Pork fat (≈ 5 g) was extracted in a covered flask containing DCM:MeOH (9:1 *v/v*) for 24 h, at room temperature. The solution was filtered and the solvent removed under vacuum in a rotary evaporator. The dry extract was dissolved in hexane (5 mL) and washed with water (4 mL x 2). The organic layer was collected, filtered through a plug of DCM washed cotton wool, and dried before storing at -20 °C.

7.5.3 Dyed textiles

Samples that were thought likely to contain dyestuffs were extracted using a method adapted from (Koren, 2006). A small amount of textile/soil (≈ 50 mg) was extracted for 5 min using hot dimethylformamide (DMF). The solvent was decanted and removed under a gentle flow of nitrogen. The extract was dissolved in DCM:MeOH and analysed by UV-vis spectrophotometry to determine absorbance prior to HPLC-UV-MS analysis.

7.6 Fat degradation experiments

Extracted pork fat (≈ 0.2 g) in deionised water (25 mL) containing ≈ 10.0 g of soil matrix and 1.0 g KOH was stirred gently under an atmosphere of nitrogen for 24 h either at room temperature or at 60 °C. On completion, the organic matter was extracted by liquid: liquid extraction using 10 mL aliquots of hexane (×3) with each aliquot washed with water. The extract was dried under nitrogen and derivatised as mentioned in Section 7.8.2 before analysis.

7.7 Bile acid analysis preparation

A portion of the total extract was dissolved in DCM:Isopropanol (2:1, *v/v*) and passed through a pre conditioned aminopropyl solid phase extraction cartridge. Elution with DCM:isopropanol (2:1, *v/v*) led to the collection of a neutral fraction, with a further acidic fraction collected on the elution of 5% acetic acid in methanol. The neutral fraction was dried under nitrogen and stored at -20 °C. The acidic fraction was subjected to a urea reduction as described in (Bull et al., 1999a) with the sample dissolved in hexane:acetone (2:1, *v/v*) and agitated during the addition of a saturated urea solution in methanol. On formation of a precipitate the solvent was removed under nitrogen and DCM used to re-suspend the Urea precipitate. The DCM was pipetted and passed through a cotton plug to remove urea crystals and ensure the collection of the acidic cyclic fraction which was derivatised for analysis by GC as detailed in Section 7.8.2.

7.8 Fractionation and derivatisation

7.8.1 Fractionation

Fractionation was carried out in small scale glass columns (10 mm i.d., height 90 mm) packed with a bed of silica gel 60 (1.5 cm; activated at 120 °C for 6 h; (Ruiz-Gutierrez and Perez-Camino, 2000)). Samples were dried onto a small amount of silica and added to the top of the column, elution was carried out with successive portions of hexane (3.5 mL), hexane:toluene (1:1 *v/v*, 3.5 mL), hexane:ethyl acetate (4:1 *v/v*, 3.5 mL) and DCM:MeOH (1:1 *v/v*, 3.5 mL) to obtain the apolar (AP), low polarity (LP), medium polar (MP) and high polar (HP) fractions, in turn.

7.8.2 Derivatisation

Total extracts and MP and HP fractions were both methylated and silylated before analysis. Methylation was carried out in DCM:MeOH (2:1 *v/v*, 300 µL; methanol added to prevent formation of by-products) and trimethylsilyl diazomethane (TMS DAM, 20 µL) over 30 min (Schlenk and Gellerman, 1960, Hashimoto et al., 1981), after which samples were dried under a gentle stream of nitrogen. Silylation was performed by addition of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane (TMCS; 100 µL) and 5 drops of pyridine (added as catalyst due to its ability to act as an HCl acceptor in silylation reactions; Makita *et al.,* 1969). The solution was reacted for 1.5 h at 60 °C and, on completion; the derivatised extract/fraction was taken to dryness under a gentle stream of nitrogen.

7.9 External standards and quantification

A series of external standard solutions were produced for quantification of analytes in both GC and LC analyses. For GC quantification a series of hydrocarbon standards (0.02 – 0.09 mg ml^{-1}) containing *n*-C_{24} (Aldrich 97 %), *n*-C_{28} (Koch-Light), *n*-C_{30} (Koch-Light), *n*-C_{32} (Fluka Rurum) and *n*-C_{34} (Aldrich) were produced to run alongside GC analyses. Standards used for LC analysis were produced using the TAG *PPO* (1,2-palmitin-3-olein, Larodan AB) (0.01 – 0.20 mg ml^{-1}). In both cases calibration curves were generated by plotting concentration vs peak area.

7.10 Gas chromatography

7.10.1 Gas chromatography - flame ionisation detection

Gas chromatography (GC) analysis of the total extracts was performed on a Thermo Trace GC Ultra gas chromatograph fitted with a flame ionisation detector (FID) utilising a Triplus auto-sampler and ChromQuest 5.0 V3.2.1 software for the control of instruments, data acquisition and analysis. A DB-5 capillary column (60 m x 0.32 mm i.d., 0.25 µm film thickness) was used to

achieve separation with helium as the carrier gas (2 mL min^{-1}). Total extracts were dissolved in either 100 µL or 200 µL of DCM, depending on the concentration of the extract, and 1 µL of the solution was injected into the GC (splitless, temperature 280 °C). The oven was ramped from 70 °C to 130 °C at a rate of 20 °C min^{-1}, and to 320 °C at a rate of 4 °C min^{-1} where it was held for 40 min. Detection was carried out with the FID maintained at 330 °C. A series of external standards were run alongside the samples to enable quantification.

7.10.2 Fast gas chromatography

All fractions were analysed on a Thermo Trace GC Ultra configured with a fast GC column (TR-5, 10 m x 0.1 mm i.d., 0.1 µm film thickness), helium carrier gas at a flow of 0.5 mL min^{-1}, injector temperature at 280 °C and a split ratio of 1:100. The oven was programmed from an initial temperature of 50 °C (0.1 min hold time) to 330 °C (4 min hold time) at a rate of 90 °C min^{-1}, FID detector maintained at 330 °C. Data analysis was carried out using Chromquest software 5.0 V3.2.1 (Thermo, Hemel Hempstead, UK).

7.10.3 Gas chromatography – mass spectrometry

Samples selected for gas chromatography – mass spectrometry (GC-MS) were re-silylated (due to trimethylsilyl derivatives being susceptible to hydrolysis) and dissolved in the same volume of DCM as for GC analysis. Analysis was performed using an Agilent 7860A gas chromatograph equipped with a 7683B Series auto-sampler and coupled to a Waters GCT Premier time-of-flight mass spectrometer. A fused silica capillary column (Zebron, ZB-5, 30 m x 0.25 mm i.d., 0.25 µm film thickness) was employed and samples were injected via a split injection port (280 °C, split flow 1:5). The oven program was identical to that used for GC analysis and helium carrier gas was used at a flow of 1 mL min^{-1}. The MS transfer line was set to 300 °C and electron ionisation was employed with the energy set at 70 eV. Mass spectra were acquired over the range *m/z* 50-750 at a cycle time of 0.2 s. Spectra were produced using MassLynx V4.1 and identification was supplemented by comparison with the NIST 08 spectral library.

7.11 High performance liquid chromatography - mass spectrometry

7.11.1 Analysis of total extracts, medium and high polar fractions

Total extracts were analysed using a Dionex Ultimate 3000 rapid separation liquid chromatograph (control software, Chromeleon 6.8) system coupled to a Bruker HCTultra ETD II quadrupole ion trap mass spectrometer fitted with an atmospheric chemical ionisation (APCI) source. Analysis was performed according to the method of Hasan (2010). Briefly, separation was accomplished using two 3 µm Spherisorb ODS2 columns (150 x 4.6 mm i.d.) coupled in series, using a solvent

gradient system comprising acetonitrile, dichloromethane and ammonium acetate in methanol (0.01 M). APCI was operated in positive ion mode using the following settings: vaporiser temperature 450 °C, nebuliser gas (N_2) 50 psi, drying gas (N_2) flow rate 3 L min^{-1}, temperature 150 °C and corona discharge current 4 μA. Mass spectra were acquired over the range *m/z* 300-950. A triacylglycerol standard was run alongside samples for quantification purposes.

7.11.2 Analysis of dyestuffs

The analysis of extracted dyestuffs was carried out using an HPLC-UV-MS method modified from (Gulmini et al., 2013). Analyses were performed using a Dionex Ultimate 3000 RSLC system with an online diode array detector (DAD, λ 300-850 nm, resolution = 0.60 nm), coupled to a Bruker HCTultra ETD II quadrupole ion trap mass spectrometer fitted with an APCI source. Extracts were dissolved in DMF and separation achieved using the same column set up as described in Section 7.11.1. The binary solvent programme employed water and acetonitrile, both containing 0.15 % formic acid with the gradient running from 95 % water and 5 % acetonitrile to 100 % acetonitrile over 30 min (flow rate of 0.7 mL min^{-1}), followed by 10 min isocratic period (Gulmini et al., 2013). APCI was operated in positive ion mode using the following settings: vaporiser temperature 400 °C, nebuliser gas (N_2) 40 psi, drying gas (N_2) flow rate 8.5 L min^{-1} and temperature 300 °C, corona discharge current 4 μA and capillary voltage -4 kV. Mass spectra were acquired over the range *m/z* 50-500.

Abbreviations

AP	Apolar
APCI	Atmospheric pressure chemical ionisation
ASE	Accelerated solvent extraction
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
Da	Dalton
DAG	Diacylglycerol
DCM	Dichloromethane
EA	Elemental analysis
EI	Electron ionisation
FA	Fatty acid
FID	Flame ionisation detector
GC	Gas chromatography
HP	High polar
HPLC	High performance liquid chromatography
ID	Internal diameter
K_{ow}	Octanol: water partition coefficient
KOH	Potassium hydroxide
LC	Liquid chromatography
LP	Low polarity
$M^{+\bullet}$	Molecular ion
MAG	Monoacylglycerol
MP	Medium polar
MeOH	Methanol

MS	Mass spectrometry
m/z	Mass to charge ratio
SE	Silyl ester
SEM	Scanning electron microscopy
SPE	Solid phase extraction
TAG	Triacylglycerol
TE	Total extract
TMS	Trimethylsilyl
TOC	Total organic carbon
UV	Ultraviolet
(*v/v*)	volume/volume

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