The fate of lipids in archaeological burial soils

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Abstract

Geochemical techniques have been developed in order to systematically analyse soils collected from several burial sites. Through the extraction and GC analysis of burial soils organic signatures that inform an understanding of the burial environment, including aspects relating to the individual or culture in which they lived, have been obtained. GC analysis revealed that the profiles of several lipid signatures found in the burial soil samples, including *n*-alkanes, *n*-alkanals, *n*-alkanols, fatty acids and steroids, have shown similarities to signatures relating to degraded human adipose tissue and bacterial signatures. The abundance of lipid signatures increases around the skeletal remains, particularly the gut region, which suggests that these components are present because of the remains. Fatty acids and steroids have been typically found in the analysis of tissues from mummies and bog bodies and are also present in adipocere, although the oxidative degradation products (hydroxy fatty acid and diacids) that have also been observed in these tissues have been absent during this study. However fatty acid reduction products such as *n*-alkanes, *n*-alkanals and *n*-alkanols have been observed in several of the graves suggesting that the remains can undergo microbial reduction in the soil.

In addition several specific signatures that are exclusive to only a few samples from the remains have provided information on the nature of the burial. The presence of coprostanol in the burial samples has the potential to infer on the individuals last meal, while the presence of specific resin acids within the soil provides information on the materials used to construct a coffin. The presence of pine diterpenoids observed in a burial from Iceland has suggested that materials, such as pine, were imported at the time as pine trees are not native to Iceland. Another specific plant signature, Hopenone *b,* has further revealed information about the nature of the burial inferring that the plant Cypress Spurge (also known as grave yard weed) was present within close proximity to the grave.

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Author's Declaration

I hereby declare that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously at this or any other university.

CHAPTER 1. INTRODUCTION

1.1. The study of archaeological human remains

Human burials have interested archaeologists for many years. They provide a link to our ancestors and provide information on morbidity (Rathbun, 1987; Jamieson, 1995; Jordana *et al*., 2009), disease (Brothwell, 1943; Rathbun, 1987; Waldron and Cox, 1989; Mays *et al*., 2006), burial rituals (Jamieson, 1995; Bromberg and Shephard, 2006) and past cultures (Brothwell, 1961; Kelley and Angel, 1987). Inferences regarding morbidity and disease are mainly derived through the condition of the bones, such as the presence of lesions and stresses. For example analysis of skeletal remains from Pazyryk warriors showed evidence of perimortem injuries on the cranial vault of an individual, the wound a sharp tangential mark with bevelled edges enabled the authors to suggest that death was due to a sharp force trauma to the head (Jordana *et al*., 2009). Examples of disease have also been observed through of skeletal analysis; Rathbun (1987) examined 37 individuals from a slave cemetery in South Carolina where sieve-like pitting was observed on the bone of several individuals suggesting that many of the slaves suffered from anaemia. Rickets is another disease that can be indentified readily through analysis of bone, features such as porosity of the bone and deformities in inadequately mineralised bone are used to identify the disease (Mays *et al*., 2006). In the above cases where disease has been determined inferences regarding the individual's diet could be drawn as both anaemia and rickets can indicate a deficiency within diet (iron and vitamin D, respectively). Burial rituals and cultural inferences are often determined from the positions of the remains, examples include Christian burials where generally the body is laid supine and positioned east to west (head to the west) and Muslim burials, which tend to be buried in a northeast-southeast direction with the remains laid on either their back or to the right side of the back with heads facing towards Mecca (Rohn *et al*., 2009). All the information inferred from the examples has been obtained from visual signals observed in the burials.

A select number of exceptionally well preserved remains have been subjected to extensive studies as they held the potential to provide comprehensive information on the individual and their culture. Examples include those of the iceman Ötzi (Barfield, 1994) Lindow man (Evershed and Connolly, 1988) and a number of Egyptian mummies (Gulaçar *et al*., 1990 ; Buckley *et al.,* 1999). Ötzi was found in the Alps

and is thought to have died over 5000 years ago (Barfield, 1994). Clothing and many artefacts were found with Ötzi, providing evidence of the culture at the time and their nature indicating that he was self-sufficient and equipped for a variety of situations. These artefacts included a long bow and arrows for hunting and 'birch-fungus' polypores attached to a leather strap, most likely used as an antimicrobial medical treatment. Lindow man was a bog body found near Manchester, who is thought to be around 2000 years old. Examination of the remains revealed a twisted sinew around the neck and injuries to the head (Stead and Turner, 1985). Analysis by forensic pathologists indicated that the man met a gruesome death by being struck on the head with an axe like object, garrotted and finally having his throat cut (Stead & Turner, 1985). The visual analysis of the Lindow man remains not only provided evidence on morbidity; the death of Lindow man has also been linked to a ritual killing, thus giving information on the culture at the time of Lindow man's death (Hutton, 2011). Egyptian mummies show exceptional preservation due to the extensive preparation of the body and the use of embalming resins. Hence, examination of Egyptian mummies has provided insights into the burial practices involved in the mummification process. Autopsies carried out on mummies have revealed evidence of the removal of organs and the removal of the brain through the nasal cavity (Aufderheide, 1999). In the cases cited here, in addition to the visual analysis, significant information has been gained from detailed chemical analysis of organic residues from the remains.

1.2. Organic matter from human remains

The main classes of organic matter found in animal tissues are proteins, carbohydrates and lipids (Evershed, 1993; Dent *et al.*, 2004). Given that proteins and carbohydrates are considerably more susceptible to chemical and microbial attack than are lipids, they have lower preservation potential (Evershed, 1993). Hence, lipids are the most commonly recovered compound class from archaeological materials, such as potsherds (Eglinton and Logan, 1991), and human remains (Forbes *et al*., 2002). Lipids are often described as compounds that are insoluble in water but are soluble in organic solvents and are the most common class of medium-

sized molecule produced by living organisms (Evershed, 1993). They comprise several groups; glycerides, phospholipids, sterols and fatty acids, all of which have a variety of structural and physiological roles amongst organisms.

Fatty acids are major constituents of both triacylglycerols and phospholipids, the former of which are the major components of fats (Reynold and Cahill, 1965; Dent *et al*., 2004) whereas the latter serve as major components in cell membranes (Mead *et al*., 1986). Typically fresh human adipose tissue contains C18:1 and C16:0 fatty acids with smaller amounts of C18:0, C16:1 and C14:0 (Brockerhoff 1965). The saturated components are reported to be the major products in degraded adipose tissue from both dry and wet environments (Figure 1.1; Forbes *et al.,* 2002; Forbes *et al.,* 2005).

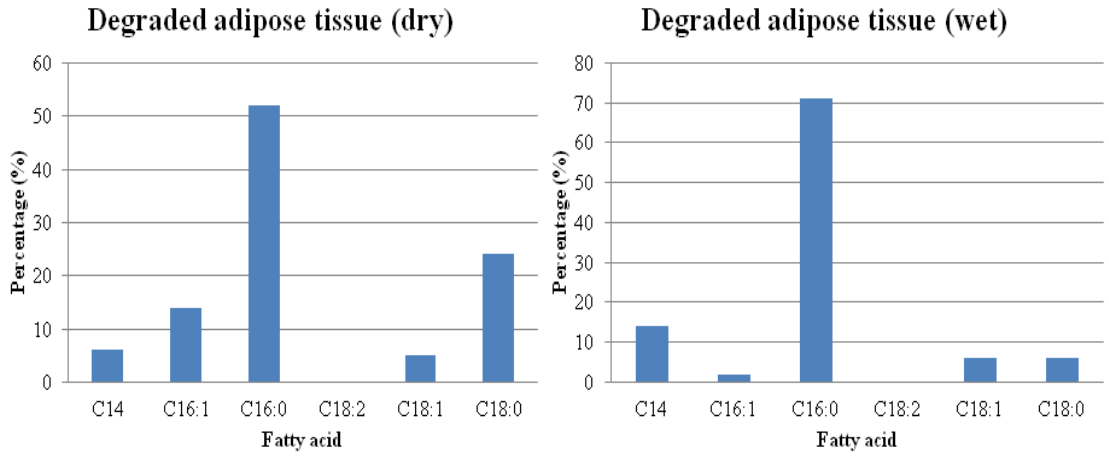

Figure 1.1: Distributions of fatty acids in degraded adipose tissue from dry and wet environments (taken from Forbes *et al*., 2002). Originally in colour.

Degradation of human remains involves various microorganisms associated with the body in addition to bacteria and fungi that migrate from the soil and air (Dent *et al.,* 2004). Thus, fatty acids derived from bacteria could be present within burial soils as well as components derived directly from the human remains. Bacteria contain relatively high amounts of C16:0, C16:1 and C18:1 fatty acids in addition to *iso*- and *anteiso*-acids in the range C15-C18 (Rezanka and Sigler, 2009).

Sterols associated with higher animals include cholesterol and coprostanol; cholesterol is a major animal sterol and is a major structural component of cell membranes (Jones *et al*., 1999) whereas coprostanol is formed from cholesterol by the actions of the intestinal microflora of a wide range of higher animals including humans (Bethell *et al*., 1994; Evershed *et al*., 1999). Due to the formation of coprostanol being a common process in the gut of higher animals it is regularly used as an indicator for the presence of faecal material (Bethell *et al*., 1994).

Lipids have been observed in sediments that are millions of years old (Poynter and Eglinton, 1991) their survival being due mainly to their hydrophobicity and resistance to degradation. The relatively low solubility of lipids in water limits their bioavailability to degrader microbial communities and they tend to aggregate and to associate with soil organic matter, providing a physical barrier to microbial attack (Eglinton and Logan, 1991). While lipids have been recovered frequently from archaeological materials in recent times, their identification in sediments and use as biomarkers by geochemists has a long history (Eglinton and Calvin, 1967; Philp, 1985) and provides the foundation for environmental reconstructions.

1.2.1. Biomarker concept

Biomarkers are compounds whose structures and/or stereochemistry provides an unambiguous link to a known biological precursor (Eglinton and Calvin, 1967; Philp, 1985). The use of biomarkers in archaeology is now an established technique, and analysis of lipids (Evershed, 1993, 2008) has been widely used throughout archaeology, including the use of cholesterol to provide information on the paleodiet of Roman soldiers through analysis of sediment samples from a feature identified as a cesspit (Knights *et al*., 1983). The value of biomarkers can be realised based either on the recognition of particular diagnostic components, such as coprostanol being used as an indicator for faecal pollution (Bethell *et al.,* 1994; Leeming *et al.*, 1996), or by computing ratio/index values for combinations of components. One such index is the widely used odd-over-even preference index for *n*-alkanes, which enables recognition of contributions from plant leaf waxes (Eglinton and Hamilton, 1967). To enable the biomarker concept to be used to its full potential the links between the

biomarker and the biological precursor need to be fully understood, therefore the general transformation pathways of organic components and the conditions under which particular components occur needs to be considered.

1.2.2. Transformation of biological molecules

Degradation of TAGs can take place *via* biochemical hydrolysis, releasing free fatty acids (Alford *et al.,* 1971). The double bonds of unsaturated free fatty acids can also undergo oxidation and hydrogenation, both of these processes lead to increased proportions of saturated fatty acids, decreasing the proportion of unsaturated components. Different oxidation processes have been presented in the literature and have been proposed as routes for the transformation of fatty acids in archaeological potsherds (Hansel *et al.,* 2011) and in the analysis of slab-lined pits from Norway (Heron *et al*., 2010), whereas hydrogenation has been observed in the breakdown of fatty acids in human remains (Forbes *et al.,* 2003, Forbes *et al*., 2005).

One oxidation pathways of TAGs involves the formation of vicinal di-hydroxy fatty acids, which are most likely formed through reactions of unsaturated fatty acids with hydroperoxides formed from autoxidation of other lipids (Hamilton *et al.,* 1997) via an epoxide intermediate (Figure 1.2: Frankel *et al*., 1980; Hansel *et al.,* 2011). Another oxidation route is through hydration, oxidative cleavage and ω-oxidation to form di-carboxylic acids. Hydrogenation reactions involve the removal of the double bond by the addition of hydrogen to yield saturated fatty acids (Forbes *et al.,* 2003; Fiedler and Graw, 2003; Forbes *et al.,* 2005; Notter *et al.,* 2008).

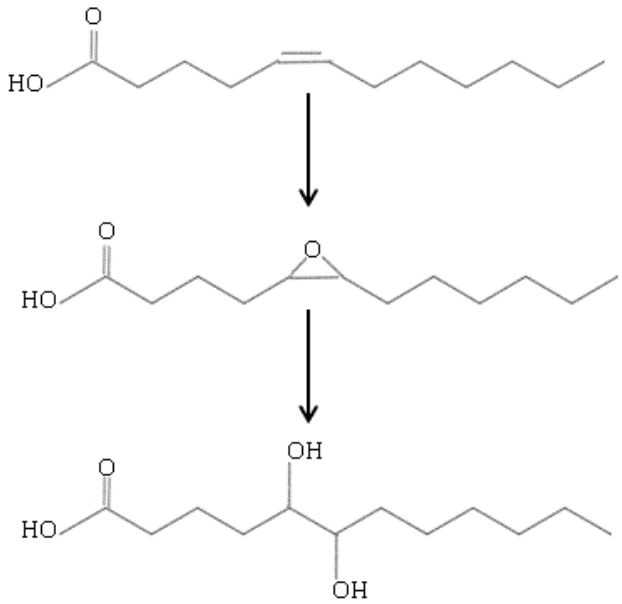

Figure 1.2: Oxidative pathway with epoxy intermediate.

Experimental work has shown that microbial reduction of fatty acids to *n*-alcohols can also occur via *n*-alkanal intermediates (Riendeau & Meighen 1985). Several laboratory experiments have established reduction pathways of fatty acids to *n*-alcohols mediated by different bacterial strains. Naccarato *et al*. (1972) grew *Escherichia-coli* (*E-coli*), which occur in the guts of higher mammals, under anaerobic conditions and identified two different pathways for *n*-alcohol synthesis from analysis of lipid extracts. One of the routes had no dependency on oxygen, and involved the reduction of fatty acid precursors via *n*-alkanal intermediates. In the experiments, *n*-alkanals were found in levels of 0.126-0.163 μg/ 100 mg total lipid extract (cf. fatty acids 0.636-0.820 μg/ 100 mg and alcohol 0.136-0.152 μg/100 mg total lipid extract). Another bacterium, *Clostridium butyricum*, which occurs in soils also showed reduction of fatty acids to *n*-alkanals and *n*-alcohols when subjected to anaerobic growth conditions (Day, 1978). Further reduction of the *n*-alcohols could lead to the formation of *n*-alkanes (Figure 1.3).

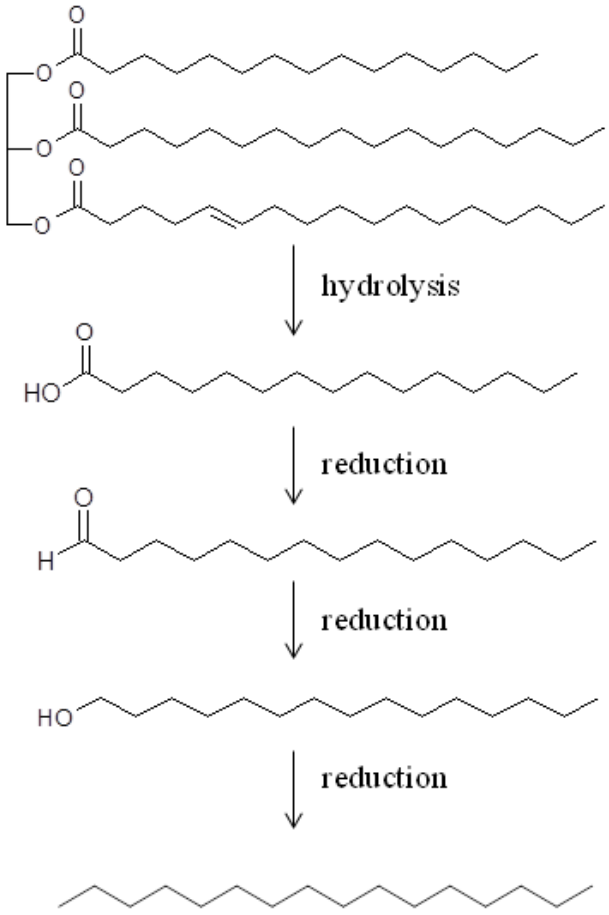

Figure 1.3: Putative pathway for the transformation of TAGs to *n*-alkanes, modified from Naccarato *et al*. (1972) where the reduction of tracer fatty acids to alcohols in the presence of a cell-free preparation of *E coli* and Day, (1978), where the biosynthesis of *n*-alkanals and alkanols by the bacterium *Clostridium butyricum* are discussed.

Although the operation of reductive pathways of fatty acids have not been recognised widely in the archaeological literature, the oxidative pathways have been observed in the analysis of tissues taken from mummies (Gulaçar *et al*., 1990) and bog bodies (Evershed and Connolly, 1988).

Cholesterol can, on its own, be an indicator of diet and the presence of its degradation products can infer additional information about archaeological remains. Cholesterol is known to degrade to 5α- and 5β-cholestanols (the latter is also known

as coprostanol) with 5α-cholestanol being the most thermodynamically stable component and the one normally found in sediments as a degradation product of cholesterol (Leeming *et al*., 1996). Coprostanol (5β-cholestanol) has been mentioned previously as a product of the breakdown of cholesterol in the mammalian gut whereas 5α-cholestanol has been found as the main degradation product of cholesterol in the environment (Bull *et al*., 1999a). The formation of coprostanol in the gut has been shown to occur through two different pathways; one *via* ketone intermediates (Rosenfeld *et al*., 1954; Ren *et al*., 1996), the other pathway *via* direct reduction of the double bond (Rosenfeld and Gallagher, 1964; Ren *et al*., 1996). The formation of 5α-cholestanol in the environment is also understood to involve the formation of ketone intermediates (Figure 1.4; Bull *et al*., 1998). The studies around the conditions for the formation of coprostanol have mainly focused on the bacteria involved (Rosenfeld *et al*., 1964; Björkhem and Gustafsson, 1971; Martin *et al*., 1973; Eyssen *et al.*, 1974; Leeming *et al*., 1996 Ren *et al*., 1996). Martin *et al.* (1973) compared the gut bacteria of humans, dogs, chickens and rats (all of which have coprostanol present in their faeces) to that of pigeons whose faeces does not contain traces of coprostanol or the coprostanone intermediates. The study inferred that anaerobic gram negative flora, present in the human gut but not in the pigeons, could be responsible for the formation of coprostanol (Martin *et al.*, 1973). Laboratory studies by Ren *et al.* (1996) involved the isolation of coprostanol after the anaerobic incubation of ^{14}C labelled-cholesterol and *Eubacterium coprostanoligenes* in growth medium. This study further inferred that an anaerobic bacterium, in this case *Eubacterium coprostanoligenes*, was responsible for the formation of coprostanol via a stanone intermediate (Ren *et al*., 1996). The majority of studies on the mechanism have focused on demonstrating that an anaerobic bacterium is involved in the transformation, consideration of the conditions in the gut has not been discussed. The conditions in the gut are quite unique in terms of pH and temperature, which could be part of the factor effecting the formation of coprostanol over the formation of the more stable 5α-cholestanol.

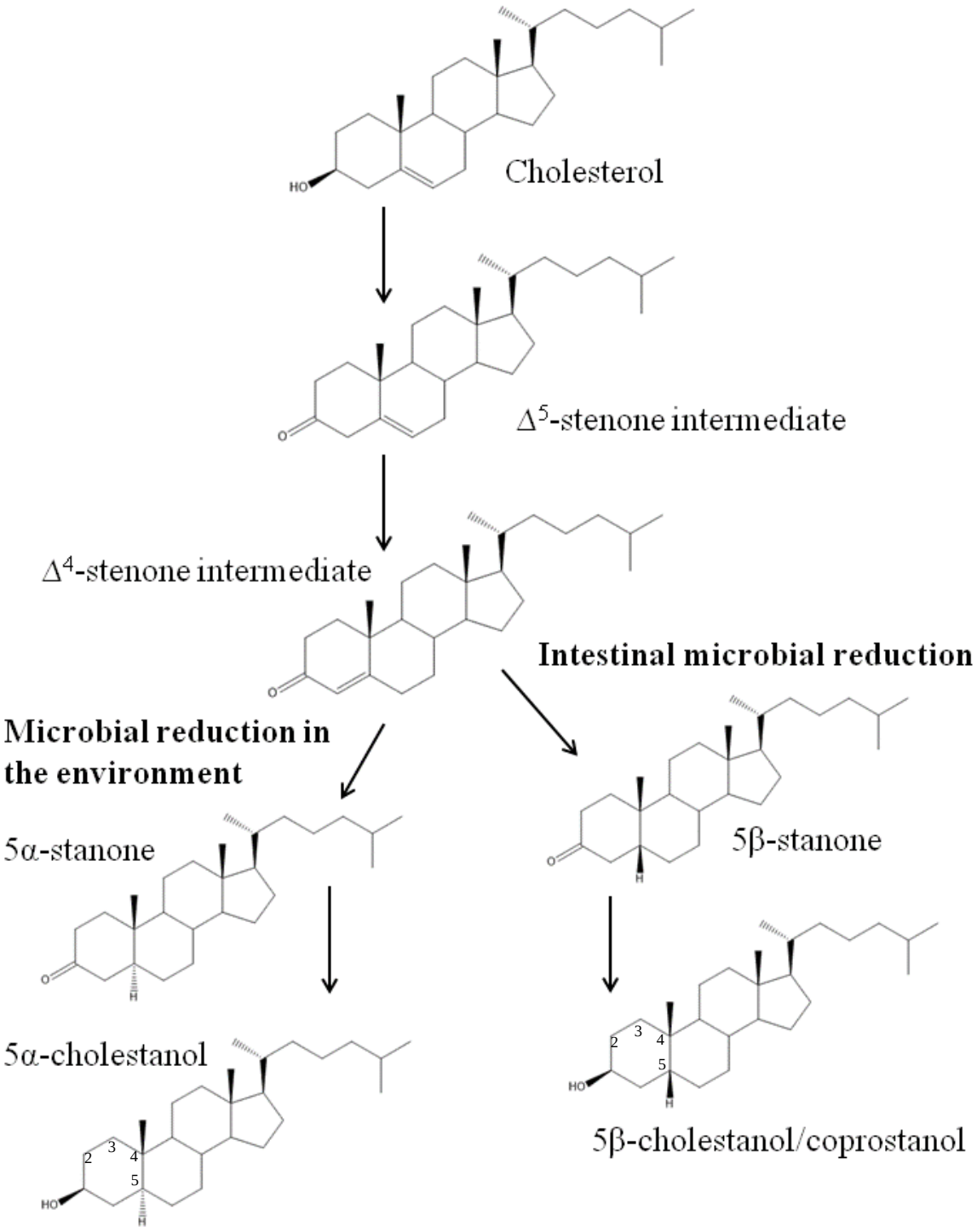

Figure 1.4: Degradation pathways of cholesterol in the environment and the guts of higher animals.

Further transformation of stanols to form sterenes has been suggested for the early digenetic transformation of sterols in sediments or in the water column of aquatic environments under anoxic conditions (see for example van Kaam-Peters *et al.* 1998). Further reduction of sterenes can lead to the formation of fully saturated steranes (Killops and Killops, 2008). Sterenes have been observed in immature sediments and in the water column and surface sediments of aquatic environments whereas steranes have only been observed in immature sediments (Mackenzie, 1982).

1.2.3. Effects of burial environment on the degradation of biological material

The degradation rates and transformation pathways of body tissues and organic materials within graves are dependent upon many factors. Some factors relate to the burial environment, including the soil type which can affect the composition and activity of the soil microbial community (Haslem and Tibbett, 2009; Tumer *et al*., 2013). In an experimental-based study extending over 6 months, Tumer *et al.* (2013) buried a total of thirty-two pig limbs into loamy, clayey, organic and sandy soils. Once lifted the limbs were subjected to visual analysis and their mass loss was recorded along with entomologic analysis. Tumer *et al*. (2013) found that decomposition of pig limbs was more extreme and the limbs had a greater mass loss in loamy and organic rich soils than in sandy and clayey soils. Extremes of pH can restrict/arrest microbial growth (Haslam and Tibbett, 2009); typically, acidic (pH 3.0-5.5) and alkaline soils (> 7.5) are dominated by fungal communities, whereas bacteria thrive in more neutral (pH 5.5-7.5) soils (Haslam and Tibbett 2009). Oxygen availability also plays a key role in the mechanisms of degradation of organic matter (Dent *et al.,* 2004). Oxygenation levels and degradation rates are often linked with the hydrodynamics of the burial environment. Water movement through the grave brings with it oxygen, nutrients and bacteria, promoting the breakdown of organic matter (Schoenen and Schoenen, 2013). By contrast, where anoxic conditions become established, for example, in waterlogged soils, the activities of certain microorganisms and the oxidative degradation of organic molecules may be inhibited. Fiedler *et al.* (2009) reported the presence of anoxic conditions affecting a

child coffined burial interred in an environment that had experienced fluctuating groundwater levels. They associated the formation of adipocere with periods of anaerobic conditions. The preservation of bodies in waterlogged anoxic environments has also been observed through the discovery of bog bodies (Evershed and Connolly, 1994).

The mode of burial (e.g. presence/absence of a coffin) also affects the decomposition of the remains and studies have found that bodies that are buried in coffins can have an effect on the decomposition of the remains (Mant *et al.,* 1987; Forbes *et al*., 2005). Mant *et al*. (1987) analysed 150 exhumed remains from war graves in Germany, chemical analysis wasn't available at the time therefore the extent of degradation was determined through gross changes. Observations of the remains determined that the bodies that were in the coffins decomposed more rapidly than the bodies that were buried in the soil. Studies by Forbes *et al*. (2005) confirmed the observations by Mant *et al*. (1987), through experimental burials. Adipose and muscle tissue was taken from pigs and placed; directly in the soil (control), a coffin lined with plastic and satin (from a funeral home), a coffin with no lining and plastic bags and buried in a moist and anaerobic soil environment (Forbes *et al*., 2005). The study determined that decomposition of the adipose and muscle tissue was greater with the samples that were in the coffined environments. The author suggested that the decomposition occurred quicker in the coffins because of a slightly more aerobic environment created within the coffin (Forbes *et al.,* 2005), this idea was evidence by the presence of fungi with the coffins. The coffins in the experimental burials by Forbes *et al*. (2005) were nailed and sealed shut but the coffins in the other burials analysed by Mant *et al*. (1987) were loosely fitting but it is possible that even the loosely fitting coffins still could create an aerobic environment around the body.

Mant *et al*. (1987) studied remains through observations of gross changes whereas Forbes *et al*. (2005) could a much more in depth analysis on the changes in the tissue composition through chemical analysis. The chemical analysis will therefore be able to determine what is occurring on a molecular scale so even slight changes can be observed and more accurate conclusions can be made about the extent of degradation.

1.3. Chemical analysis

The specificity and excellent detection capabilities of techniques such as gas chromatography (GC) and liquid chromatography (LC) make them suitable for use in archaeological studies to analyse chemical signatures that can augment the interpretation. When used in conjunction with mass spectrometry (MS) as a detection system complex mixtures can be separated chromatographically and structure assignments of individual compounds can be made from their mass spectra. Such capabilities are essential for the study of biomarkers (Evershed, 1993).

Other techniques that have been employed in the study of organic archaeological remains include Raman spectroscopy, carbon isotope analysis and infrared (IR) spectroscopy. For example, Raman spectroscopy has been used to provide a non-destructive method for analysing chemical signatures from pottery (Smith and Clark, 2004), while IR spectroscopy has been used to analyse skin tissue from the iceman Ötzi (Bereuter *et al.*, 1997). These spectroscopic methods were used as chromatography-mass spectrometric methods are destructive whereas both spectroscopy methods are non-destructive. In archaeology it can be important to preserve artefacts or in some cases only very small samples can be obtained and size may be considered limiting for methods such as GC-/MS. Nevertheless, GC-MS is a very important tool that provides excellent detection capabilities for various chemical markers and has been proven invaluable in the analysis of archaeological samples (Gulaçar *et al.*, 1990; Evershed and Connolly, 1988; Pollard and Heron, 2008). Spectroscopic methods do not have the specificity of GC/MS and exact structures of individual compounds cannot be determined. Other sources, such as soil, could potentially provide diagnostic information from burial without compromising the artefacts obtained from the burial sites.

Analysis of various isotopes have also given further insight into the nature of burials, strontium isotope analysis can provide indications of diet (Lambert *et al.*, 1982; Price, 1994) and also the underlying geology in which the individual spent their time (Bentley, 2006). Carbon and nitrogen isotopes from bone collagen have also been used to study diet and also to discriminate different dietary sources such as terrestrial, marine and freshwater inputs Stott *et al*. (1999). In both cases animal

feeding experiments were used to help identify different diets from analysis of human remains from coastal and in land sites.

Organic geochemical techniques have been widely used in archaeology to identify lipid residues. The application of GC/MS and LC/MS in the analysis of chemical signatures associated with archaeological human remains has focused mainly on mummified remains and the analysis of the body tissues and materials associated with intentional mummification (Buckley and Evershed, 2001; Buckley *et al.,* 2004). In addition, some forensic studies of recent burials have included analysis of soil surrounding the interred remains to exploit the decomposition of body fats to detect the presence of a body (Bull *et al.,* 2009).

1.3.1. Mummies

Mummies are defined as human remains found with preservation of non-bony tissue (Lynnerup, 2007). Mummification can occur naturally, mainly in arid or cold environments and can result from intentional actions developed to preserve human remains (Lynnerup, 2007). This can include the removal of organs, use of embalming techniques developed to preserve the body or by deliberately leaving the body in an arid environment to preserve it (Lynnerup, 2007).

Various studies have reported the analysis of body tissues and wrapping materials from mummified corpses of several Egyptian (therefore non-natural) mummies to determine the preparations employed in the mummification ritual and the transformation of the body tissues post-mortem (Gulaçar *et al.,* 1990; Buckley *et al*., 1999; 2001). These studies identified specific chemicals present in the preparations applied during mummification and provide insight into the compositions of the organic signatures preserved within the transformed soft tissues of the mummies. Gulaçar *et al.* (1990) observed the transformation of lipids in the tissues (skin) of a 4000 year old Nubian mummy. Notably, intact signatures of fatty acids, sterols and bile acids were found even in tissues that had undergone advanced decomposition. The tissues of the mummies revealed high levels of fatty acids with maxima at C16:0/C18:0 and C22:0/C24:0 and significant contributions from *iso*- and *anteiso*-

acids in the range C15-C18. Gulaçar *et al*. (1990) observed that the unsaturated fatty acids C16:1, C18:1 and C18:2, were almost completely removed in the mummified remains, suggesting that degradation of unsaturated fatty acids had occurred and the fatty acid contribution was similar to degraded adipose tissue (Forbes *et al*., 2002). The presence of the *iso*- and *anteiso*-branched acids suggests an input from bacteria, which could be responsible for the degradation of the fatty acids (Rezanka and Sigler, 2009). The authors inferred that the low levels of unsaturated fatty acids are a consequence of a significant extent of microbial degradation (Gulaçar *et al*., 1990); it is likely that the microbial degraders were responsible for the lack of TAGs and unsaturated fatty acids through hydrolysis and oxidation. By contrast, the unsaturated C18:1 fatty acid was found to be a dominant component, occurring together with the saturated C14, C16 and C18 fatty acids, in two Pharoanoic mummies analysed by Buckley *et al.* (1999), suggesting them to have undergone less degradation than the mummies analysed by Gulaçar *et al.* (1990). Nevertheless, Buckley *et al.* (1999) still found evidence of bacterial input associated with the Pharoanoic mummies through the presence of *iso and anteiso* branched fatty acids (Buckley *et al.,* 1999) indicating that bacterial degradation also occurred, though to a lesser degree.

The Nubian and Pharoanoic mummies showed evidence of oxidation of fatty acids through the presence of mono- and di-hydroxy fatty acids and di-carboxylic acids (Gulaçar *et al*., 1990; Buckley *et al*., 1999). In both cases the samples were dominated by C16 and C18 hydroxy fatty acids, which are likely to have formed by the oxidation of C16:1 and C18:1 unsaturated fatty acids. The di-carboxylic acids from the Nubian mummy ranged from C7-C18, while in the Pharoanoic mummies the di-carboxylic acids showed a narrower range, from C6-C12. All of the di-carboxylic acids identified could also result from the oxidation of the double bond in the fatty acids of the adipose tissues. In both cases, therefore, the tissue has undergone degradation of the fatty acids of the adipose tissue.

Tissues from the mummy analysed by Gulaçar *et al*., (1990) included a sample taken from the interior of the skull, two intra-thoracic samples and a skin sample from the abdomen area, all of which contained high levels of cholesterol. Samples of concretions from the intra-thoracic area contradicted the findings from other tissues

as cholesterol was absent and coprostanol and 5β-stanones were present. As coprostanol is produced in the gut its occurrence in the thorax is highly unusual. The authors suggest that the presence of coprostanol could be due to the migration of intestinal microflora to the thorax with the transformation of cholesterol to coprostanol being affected by the decomposer microbial community after death (Gulaçar *et al*., 1990).

Coprostanol was not observed in the mummies analysed by Buckley *et al*. (1999), though cholesterol was detected along with three steroidal hydrocarbons (cholestene, cholesta-3, 5-diene and cholesta-3, 5, 7-triene; the position of the double bond in cholestene was unknown) in the tissue/bandage of the Khnum Nakht mummy. The presence of sterenes in mummy tissues suggests that dehydration transformation pathways can operate within the mummified tissue.

1.3.2. Bog bodies

Bog bodies are essentially a distinct type of natural mummification, usually found in peat bogs in northern Europe (Stead *et al.*, 1986). It is understood that the bodies may have been placed in bogs as a form of punishment, placed there after punishment or in some cases may be the remains of people who entered the bog accidentally (Stead and Turner, 1985). Peat bogs are waterlogged organic rich soils which form in areas with very poor drainage. Through slow decomposition, the high organic content results in an environment containing very little oxygen, inhibiting aerobic bacterial degradation of organic matter including body tissues (Stead *et al.*, 1986). Peat bogs are also acidic environments and so are inhospitable for many bacterial species. Polyphenols, which are mainly found in plants, are thought to be the main tanning agents that aid the preservation of organic matter in peat bogs (Stankiewicz, 1997). Painter, (1991) proposed that a polysaccharide found in the cell walls of Sphagnum moss, known as sphagnan, was the tanning agent in the peat bogs. Although polysaccharides are not normally tanning agents, sphagnan contains several carbonyl groups which could react with free amino acid groups in collagen, the free amino acid groups being formed by the hydrolysis of collagen under the acidic conditions in the peat environment. Painter (1991) also suggested that a

compound identified as sphagnol, present in the ssphagnum mosses within the peat bogs, acts as an antimicrobial agent which again prevents microbial degradation of the skin tissue.

Owing to the good preservation of tissues in peat bogs, bog bodies have proved to be good subjects in which to study degradation pathways of body tissues. Examination of the skin of Lindow man, a bog body found in North West of England, revealed the presence of lipids such as fatty acids (Evershed and Connolly, 1988) and sterols (Evershed and Connolly, 1994). Fatty acids analysed from muscle tissue taken from the thigh (C12-C20) were very similar in composition to those associated with regular muscle although the unsaturated components were in lower abundance than their saturated counterparts, most likely due to oxidative degradation in the early stages of submersion in the peat bog (Evershed and Connolly, 1988). Analysis of the fatty acids associated with the peat itself revealed a composition having a greater range (C12-C30); the presence of the higher molecular weight (HMW) fatty acids in the peat but not in the muscle suggested that there was no migration of fatty acids from the peat to the muscle tissue (Evershed and Connolly, 1988)

Further studies by Evershed and Connolly (1994) examined sterols present in the muscle from the thigh (as in previous study) and skin tissue taken from the abdomen. The sterols included cholesterol as well as its' degradation products cholestanone and cholestanols. The muscle tissue sterols were dominated by cholesterol accompanied by lesser amounts of the degradation products 5α-cholestanol and the intermediate 5α-cholestanone. The skin tissue also contained cholesterol, 5α-cholestanol and 5α-cholestanone but had high levels of coprostanol and its ketone intermediate. The 5α- and 5β-cholestanols and their intermediate cholestanones were absent from the peat samples, suggesting that the degradation pathways leading to their formation were operating exclusively in the skin and muscle tissue (Evershed and Connolly, 1994). The presence of the 5β-cholestanols was attributed to the migration of gut flora after death, similar to the interpretation of Gulaçar *et al.* (1990), who also found coprostanol in the tissues of mummies.

Evershed and Connolly, (1994) also observed the steroidal ketone intermediate products in the tissues of the bog bodies, whereas these intermediates were not

observed during analysis of the tissues from the Nubian mummy examined by Gulaçar *et al*. (1990). It has been suggested that the presence of the ketones in the bog body tissues was due to the process of reduction not having reached such an advanced stage of transformation as in the mummy tissues. The mummies and bog bodies have revealed degradation that has occurred over thousands of years, albeit in well preserved conditions. Transformation of lipids over a shorter period of time is revealed through forensic studies of corpses and graves.

1.3.3. Forensic studies

Analysis of recent graves, ranging from corpses interred for just a few days in the forensic studies carried out by Bull *et al*. (2009) to remains of up to 12 years (Forbes *et al*., (2003) has focussed mainly on understanding the factors involved with the formation of adipocere. Adipocere is a solid soap-like substance formed from the decomposition of body fats. It is found most often in moist and anoxic burial conditions (Fründ and Schoenen, 2009). The formation of adipocere involves free fatty acids, hydrolysed from TAGs that conjugate with Ca^{2+} and Mg^{2+} ions forming insoluble soap. The presence of bacteria and water have been shown to be a necessary condition for the formation of adipocere (Vass *et al.*, 1992: Takatori, 1996), though adipocere has been observed in a grave in an arid and dry surrounding (Mant, 1987). The latter is interesting as the presence of water is important in the bacterial formation of hydroxy-fatty acids and oxo-fatty acids (oxidation products of unsaturated fatty acids) which have been thought to be essential for the formation and stabilisation of adipocere (Takatori, 1996).

The main components of adipocere are fatty acids formed by the hydrolysis of triacylglycerols (TAGs), the dominant structures in human adipose tissue (Forbes *et al.*, 2003). Forbes *et al.* (2002, 2004, 2005) have provided a detailed discussion of the compositions of the adipocere fatty acids formed from degraded adipose tissue in the early stages of decomposition. The distributions are dominated by C14:0, C16:0, C16:1, C18:0 and C18:1 saturated and unsaturated fatty acids. Hydroxy-fatty acids and keto-fatty acids are also found in the adipocere (Adachi *et al*., 1997). The same fatty acids and hydroxy- fatty acids have been reported from the skin and muscle

tissue of Lindow man (Evershed and Connolly, 1988) and the skin tissues of mummies analysed by Gulaçar *et al*. (1990) and Buckley *et al.* (1999), although the mummies analysed by Gulaçar *et al*. (1990) did not contain the unsaturated fatty acid components. This suggests that the fatty acids present in adipocere have undergone similar decomposition processes to the fatty acids in the adipose tissue, and involving oxidative transformation.

It is difficult to determine the soil conditions required for adipocere formation as there are no systematic studies and much of the literature surrounding conditions that favour the formation of adipocere is contradictory (Fiedler and Graw, 2003). Forbes *et al.* (2002) demonstrated that the proportions of the different fatty acids in adipocere can depend on the type of soil in which they occur. The study involved samples taken from cemeteries and forensic studies in southern Australia, with grave burial durations ranging from 5-55 years. There soils were collected from dry and wet environments although some of the graves that were considered to be dry were found to be flooded on exhumation. The study showed that the transformation pathways for TAGs and fatty acids differ depending on the soil type and that the proportions of unsaturated components can indicate the extent of degradation in the soil. It should be noted, however, that the presence of water in some of the 'dry environment' graves on exhumation indicates that the results and their interpretation may be compromised: it is likely that water passed through the graves periodically over the burial duration (Forbes *et al.*, 2002).

Bull *et al.,* (2009) analysed a white substance (identified through microscopy as adipocere) in soil from a temporary grave that was part of a crime scene. The adipocere and soil samples from the grave were compared with adipose tissue taken from the victim. The results confirmed the white matter as adipocere and indicated that the lipids in the soil were consistent with the lipid profile of the adipose tissue. The study provides evidence of products from a decomposing human corpse entering the soil. In that case it can be proposed that lipids relating to body decomposition entered the soil and hence can be present in burial soils. Analysis of the soils surrounding human remains has the potential to reveal signatures associated with the corpse. Soils can contain substantial amounts of organic matter from other sources, and these components are likely to be the major components seen during analysis.

1.4. Soil organic matter (SOM)

Soil organic matter (SOM) is a major pool of carbon in the biosphere. The size and composition of the pool can vary depending on the environment and the organic input from biota (Post *et al.,* 1982). Many studies have been undertaken to understand changes in SOM, whether they be driven by natural processes such as environmental change (Post *et al.,* 1982; Jaffe *et al.,* 1996; Pancost *et al.,* 2002; Xie, 2003) or by human activities such as land use (Jarde *et al.,* 2009; Bull *et al.,* 1999a). Several studies have employed the biomarker concept to understand these changes at the molecular level (Simpson *et al.,* 1998; Sturt *et al*., 2004; Stefens *et al.,* 2007). SOM comprises plant, animal and microbial residues (van Bergen *et al,*. 1998) and it is estimated that 99% of the organic resources that undergo decomposition in the terrestrial environment are plant derived (Carter *et al.,* 2007). It is evident, therefore, that burial soils will contain lipids from sources such as plants and that in some soils these may represent a dominant proportion of the soil lipid profile.

A wide variety of natural products have been observed in soils, with sources ranging from plants to bacteria and fungi (Eglinton and Hamilton, 1967; Zelles, 1997; Ruess *et al.,* 2002). Distinct from lipids associated with buried animal remains a number of lipids form part of the background soil matter. Thus, distributions of *n*-alkanes with chain lengths in the range C23-C33 and displaying an odd-over-even predominance are typical of the protective epicuticular waxes of higher plants (Eglinton and Hamilton, 1967; Jambu *et al.,* 1991; Jansen *et al.,* 2008). Distributions of *n*-alkanols ranging from C18-C30 with an even-over-odd predominance are also typical of components of higher plant waxes (Eglinton and Hamilton, 1967). The presence of *n*-alkanals and *n*-alkanones are also typical of plant waxes, *n*-alkanals range from C20-C30 with an even-over-odd distribution whereas *n*-alkanones range from C20-C30 with an odd over even carbon number distribution (Eglinton and Hamilton, 1967). The proportions of the different amounts of lipid can depend upon the type of plant, for example sugar cane has a proportion of only 10% of *n*-alkanes present in the leaf wax whereas *Cotyledon orbiculata* leaf wax is almost entirely made up of *n*-alkanes (Eglinton and Hamilton, 1967).

Not only can signatures vary through different plant input but van Bergen *et al*. (1997) took part in experiments at Rothamsted, UK to determine if lipid signatures varied due to changes in land use. The experimental samples were taken from the Rothamsted site, a site that has been used for experiments for around 150 years, where detailed records on the use of the site were available. Three areas were used for the study where vegetation had included woodland, grassland and mixed herb and shrub areas. The study revealed that different types of vegetation resulted in different lipid profiles that were prominent in the *n*-alkane and *n*-alkanol components, where different maximums were observed (e.g. grassland vegetation displayed a maximum at the C26 *n*-alkanol).

The experiments results have been further used to determine the formation of ancient soil deposits in Orkney, UK as grass turves (Bull *et al*., 1999). In addition to determine the origin of soil deposits the results have been used to infer that there were changes to land use (from woody to grassland) in sediments taken from a small lake in Yorkshire, UK (Fisher *et al*., 2003). The experiments show that even a slightest changes in the soil use can be evident in the analysis of lipids and that these changes have been observed on the archaeological scale (Bull *et al*., 1999; Fisher *et al*., 2003)

Phytosterols are common in plants and can include sitosterol, stigmasterol and campesterol (Goad, 1983). As with cholesterol the phytosterols can undergo degradation to form 5α-and 5β-stanols, the former of which are formed in the environment and the latter in the gut of higher animals. The presence of ethylcoprostanol (the degradation product of sitosterol in the gut) usually suggests faecal waste from omnivores (Leeming *et al.*, 1996), although 5β-stanols from phytosterols have been observed in 2000 year old human coprolites (Lin *et al*., 1978). Therefore the presence of 5β-sitosterol could indicate a diet of vegetable and could be present in the gut area of the remains. Some plant biomarkers may, therefore, relate to the remains or could be present due to items in the burial, such as plant decorations, resins used for embalming or coffin wood.

Embalming resins have been observed in samples taken from mummies and have included *n*-alkanes, sterols, diterpenoids, and hydroxy wax esters relating to plants,

wood resins and bees wax (Maurer *et al*., 2002; Buckley *et al*., 2006). Wrappings taken from a mummified cat displayed evidence of beeswax through the presence of wax ester (C40-C50), hydroxy wax esters (C42-C54) and *n*-alkanes (C25-C33). These signatures have also been observed in the resin material collected from a Roman mummy and were likely to reflect the use of beeswax in embalming materials (Maurer *et al*., 2002). In addition to evidence of beeswax both samples taken from the mummies included a series of diterpenoids that were typical of pine resins (Maurer *et al*., 2002; Buckley *et al*., 2006). The wrappings from the mummified cat included a high abundance of dehydroabietic acid and 7-oxohydroabietic acid (Buckley *et al*., 2006) whereas the resin from the Roman mummy contained dehydroabietic acid and 7-oxohydroabietic acid put other diterpenoids didehydrobietic acid and abietic acid (Maurer *et al*., 2002). Dehydroabietic acid, didehydrobietic acid and 7-oxodehydroabitic acids are all derivative of abietic acid and typical of pine resins (Maurer *et al.*, 2002). Abietic acid is found in fresh pine wood so it would be expected the more degraded the pine material the lower the levels of abietic acid (Colombini *et al.*, 2003). The resin acids have also been observed in soils samples collected from funnel shaped pits dating back to the Roman period, their presence suggested that these pits were used for pine resin production (Hjulström *et al*., 2006). Therefore these resins can be located in soil samples and not just in physical artefacts such as wrappings and resins.

It is evident, therefore, that burial soils will contain lipids from sources, such as plants that in some soils these may represent a dominant proportion of the soil lipid profile.

1.4.1. Mobility of lipids in soils

Lipids are generally considered hydrophobic, being insoluble or sparingly soluble in water, and thus are likely to remain in situ. Although this is dominantly the case, lipids can be subject to transport *via* leaching in soil water, root movement and soil erosion (Hedges and Oades, 1997). Fatty acids are sparingly soluble in water due the polar nature of the carboxyl group, enabling their leaching *via* percolating soil water. The low molecular weight (LMW) fatty acids have smaller octanol:water

coefficients than high molecular weight (HMW) lipids, indicating that the partial solubility in the soil water phase is more pronounced for LMW *vs* HMW fatty acids. Water movement through the soil can also supply oxygen and so can promote the degradation of lipids (Eglinton and Logan, 1991), resulting in degradation products such as di-carboxylic acids as discussed in Section 1.2.2 , components that generally exhibit even greater solubility in water than the precursor lipids. This is due to the polar nature of the carboxyl group and the reduction in the length of the hydrocarbon chain, producing lower hydrophobicity: the shorter the hydrocarbon chain the more water soluble the fatty acids.

Examples of erosive transport have been observed in laboratory studies of the fate of livestock-derived organic matter (Lloyd *et al.,* 2012), to determine how the organic matter moves through the soils and if it could leach into groundwater, which could cause contamination. The authors used a lysimeter containing packed soil and slurry was uniformly placed at the top. Rainfall was simulated during the experiment for 4 hours to determine how organic matter moved through the soils. The analysis found no lipids in the leachate samples suggesting that the lipids were retained in the soil though it was suggested that water movement could cause erosion of the soil therefore displacing the lipids to lower soil layers.

Water can also cause erosion that can re-distribute the smallest, least dense soil particles, which are generally rich in organic matter (Gregorich *et al*., 1998). Redistribution of soil particles is known to occur down slope and can redeposit particles at the bottom of the slope where organic matter can concentrate. Wang *et al*. (2014) used rainfall experiments to determine the effect of erosion down a slope. The experiments were carried out in stainless steels flumes. The flume was split into three zones where soil was collected. Organic carbon levels at the top of the slope were depleted whereas the carbon in the deposition zone at the base was increased after four rainfall events. Field experiments by Zhang *et al*. (2006) confirm that transportation can occur down a slope. The experiment involved the collection of samples 5 m apart down a field that was along a hill, analysis of these samples determine that carbon levels increased down the slope with the highest levels of carbon located at the bottom of the slope (Zhang *et al*., 2006) .

Mobility will vary depending on the chemical nature of each particular compound, which governs both its water solubility and the nature and strength of its association with the mineral and/or organic phases of the soil. As discussed in Section 1.3.3, analysis of a temporary grave revealed that organic signatures from both fresh and decomposed adipose tissue were found in the upper and lower soil of the grave site, showing that organic matter migrated through the soil during the early stages of body decomposition and remained in situ even after the body had been removed (Bull *et al.*, 2009). All factors need to be considered with regards to the sampling of archaeological burial soils, with the view to recovering signatures relating to the organic materials interred within the grave.

1.5. Analytical Techniques

1.5.1. Extraction

The analysis of lipids has become an established technique in the discipline of organic geochemistry, with many methods having been employed for extraction and identification. Lipids are commonly extracted using organic solvents either using Soxhlet apparatus, sonication or through automated systems such as accelerated solvent extraction (ASE) systems. As lipids are generally hydrophobic and miscible in organic solvents, an organic solvent mixture is usually used to extract lipids from matrices including soil and pottery. The solvent composition 9:1 DCM:MeOH (v/v) was employed in the preliminary Soxhlet extraction of soils during a pilot study (Tongue, 2008) and has also been used previously in the ASE of lipids from soils. The solvent mixture 9:1 DCM:MeOH (v/v) was used as it is above the azeotropic composition making solvent removal easier following extraction

ASE combines high temperatures and pressures to extract compounds from a matrix using liquid solvents. The high temperature is an important factor during the extraction process as it increases the solubility of the analytes (Hubert *et al.*, 2000), while it can also disrupt the strong interactions between the extractable components and the matrix (Richter *et al.*, 1996). The high temperatures also decreases the viscosity of the solvent, allowing faster diffusion rates and better penetration through

the matrix particles and increasing extraction efficiency (Richter *et al.*, 1996). High pressure is used to ensure that the solvent stays in liquid form at temperatures above the boiling point of the solvent or solvent mixtures (Gierielewicz-Możajska *et al.*, 2001). The use of the high pressure also ensures that solvent is forced into areas of the matrix that would not be normally reached under atmospheric conditions, again increasing extraction efficiency.

1.5.2. GC analysis

Volatile lipids can be analysed by GC, a chromatographic technique widely used to effect the separation and analysis of low molecular mass compounds. GC separates the volatile components in mixtures of organic compounds by boiling point and by differences in the interaction of analytes with a stationary phase and a mobile phase (carrier gas, e.g. hydrogen, helium or nitrogen). A GC instrument typically consists of an inlet (through which the sample is introduced), an oven containing a column and a detector (e.g. flame ionisation detector (FID) or mass spectrometer). For capillary GC the column is most commonly constructed from fused silica (typically of sub mm diameter and several tens of metres in length). The inner surface of the capillary is coated with a stationary phase, usually a non-volatile polymeric liquid/gum (e.g. dimethylpolysiloxane). Samples are rapidly injected into the system *via* a syringe, either directly into the column (on column injectors) or into a heated port where the gas is vaporised and then split to allow only a small amount to enter the column (split/splitless injectors). The analytes are swept through the column by the carrier gas where they are separated. Analytes are separated by their boiling points and their partitioning between the stationary and mobile phases, with more volatile components generally moving through the column quicker than less volatile components. The wide choice of stationary phase chemistries available enables selection of one that best exploits the strength of the interaction with the analytes to be detected. Often the temperature of the column oven is increased during the analytical run in order to elute all of the analytes from the column in a reasonable time and to limit peak broadening through diffusion.

1.5.2.1. FID detection

Flame ionisation detectors (FID) are commonly used in GC to detect organic compounds. The separated compounds enter the detector and are combusted in a hydrogen fuelled flame. The analytes are ionised at the high temperature of the flame and the carbon ions so produced are attracted to a collector electrode situated above the flame. The ion current generated at the collector is amplified and converted to a signal.

1.5.3. Mass spectrometry

Mass spectrometry (MS) is an analytical technique that can measure the mass-to-charge ratio (*m/z*) of gas phase ions. The analytes are converted to gas phase ions *via* one of a variety of different ionisation processes. The ions are separated according to their *m/z* by a mass analyser and the number of ions is measured by a detector coupled to a computer, which processes the data to form a spectrum (de Hoffman and Stroobant, 2007).

Mass spectrometers can also be coupled with GC to act as a detector. As each separated analyte leaves the GC, it enters the mass spectrometer where it is ionised and its spectrum is detected. Coupling these techniques allows for compound identification for particularly complex mixtures.

1.5.3.1. Electron ionisation

Electron ionisation (EI) is commonly used to obtain the gas phase ions required for analysis in mass spectrometry. The technique is applicable to gas phase/volatile organic molecules. The energies involved in EI can induce extensive fragmentation, sometimes leading to the absence of molecular ions in the spectra. As such the technique is typically referred to as a hard ionisation technique. The presence of fragment ions is particularly useful in lipid analysis, many lipid structures being quite similar and requiring the specificity conferred by the presence of multiple fragment ions to enable component identification. Detailed analysis of the molecular

ion and the fragmentation patterns allows, in many cases, for precise structural identification.

EI employs a heated filament source which gives off electrons that are accelerated towards an anode (Figure 1.5). The sample is introduced in a gas flow directed perpendicular to the electron pathway. Close passage of highly energetic electrons causes fluctuations in the electric field of the neutral analytes inducing ionisation through the abstraction of an electron. The ionisation depends on the chemical structure of the analyte and the energy of the electrons. An electron energy of 70 eV gives the maximum extent of fragmentation for organic molecules as it matches the typical bond strength for most organic molecules.

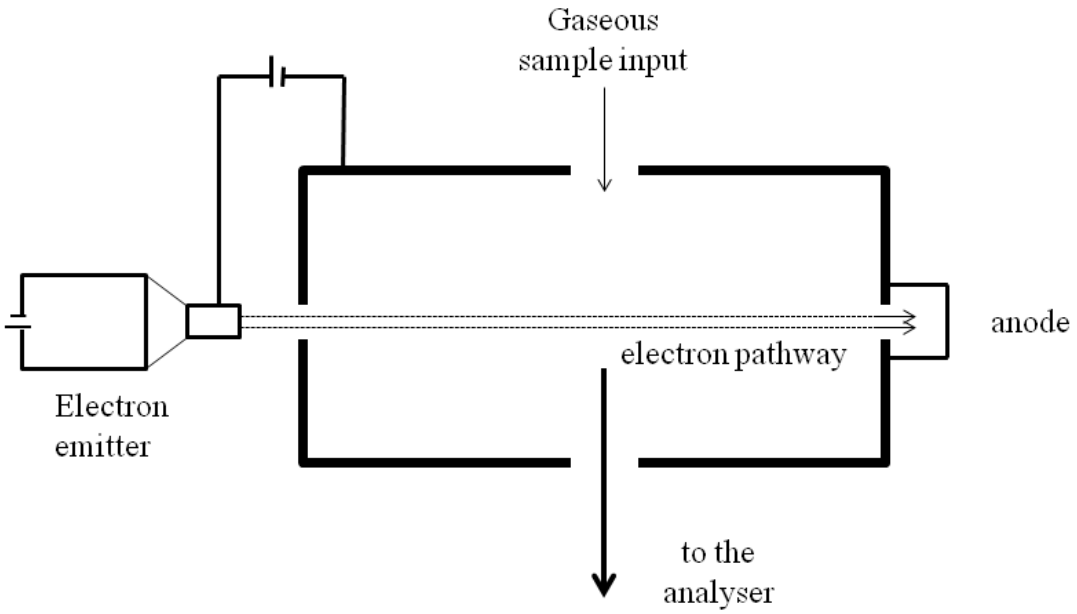

Figure 1.5: Electron ionisation source from a mass spectrometer.

1.6. InterArChive

The InterArChive project combines archaeological and chemical analytical methods and sampling techniques to develop and test a research strategy that can be used to maximise the evidence obtained from graves, enhancing their interpretation. Most of the examples of chemical analyses performed on archaeological samples discussed previously involved direct analysis of archaeological remains such as bone (Evershed *et al.*, 1995), body tissue, mummy wrappings (Buckley *et al*., 1999) or grave goods taken from burials.

Preliminary work on several graves from Eindhoven (Tongue, 2008) was performed on samples of soil collected from the skull, pelvis and feet. Organic residues extracted using a Soxhlet apparatus were separated into fractions. GC analysis of the fractions revealed that trace levels of organic matter were present and that these differed according to the sampling location. Owing to problems with contamination of some of the extracts, the results were not interpreted fully.

1.6.1. Sampling strategy

The InterArChive project proposed two sampling strategies; low intensity (similar to that used in the preliminary study) and high intensity designed to ensure that the information collected from grave soils was maximised. The strategies were developed to target areas around the remains that are likely to yield the most information about the body and its contents. It was envisaged that the sampling locations would provide information on body tissue decay (maximised around the torso), last meals (sampling from the gut area) as well as features of the burial ritual, such as evidence of coffin, shroud and pre-burial treatment. The low intensity sampling provides a basic overview from burials where multiple samples could not be obtained. The sampling strategy incorporates collection of three controls (C1, non-grave sediment; C2, upper grave fill; C3, lower grave fill, above the level of the skeletal remains) to provide information on the background soil organic matter and composition for comparison with the sampling points. The four different sampling points around the skeletal remains (Figure 1.6) are the skull (to look for signatures

from the scalp and adornments), pelvis (gut and faecal remains), feet (coffin, skin and footwear) and hands (adornments and inclusions). This sampling strategy represents the minimum number of samples to be collected for chemical analysis. It includes collection of replicates of undisturbed soil from each point for micromorphological analysis.

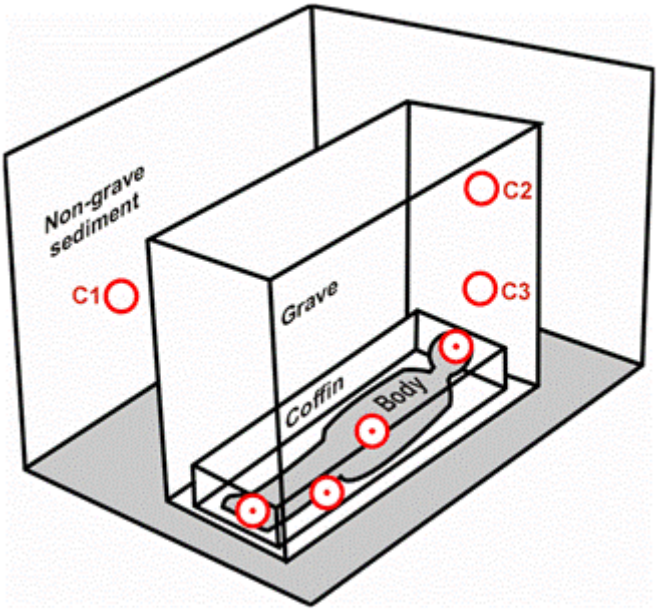

Figure 1.6: Low intensity sampling points and positions of the controls outlined by the InterArChive sampling strategy. Originally in colour.

The high intensity sampling strategy involves collection of a greater number of samples for chemical analysis and is aimed at providing a much more detailed investigation of the grave soils. The same three controls as in the low intensity sampling are collected (Figure 1.6), along with samples from 17 specific locations around the skeletal remains (Figure 1.7). The strategy was developed to obtain soil samples adjacent to the remains and from as close as possible to the bone and any associated micromorphological samples (collected according to the low intensity sampling strategy; Figure 1.6). Both sampling strategies (low and high intensity) allow for the collection of additional samples in cases where exotic features of the grave are observed during excavation. Additional samples are given a unique and

incremental number (preceded by the letter ‘A’ to denote an additional sample). Such samples would include grave goods, recognisable remains of fabric, body tissue and wood.

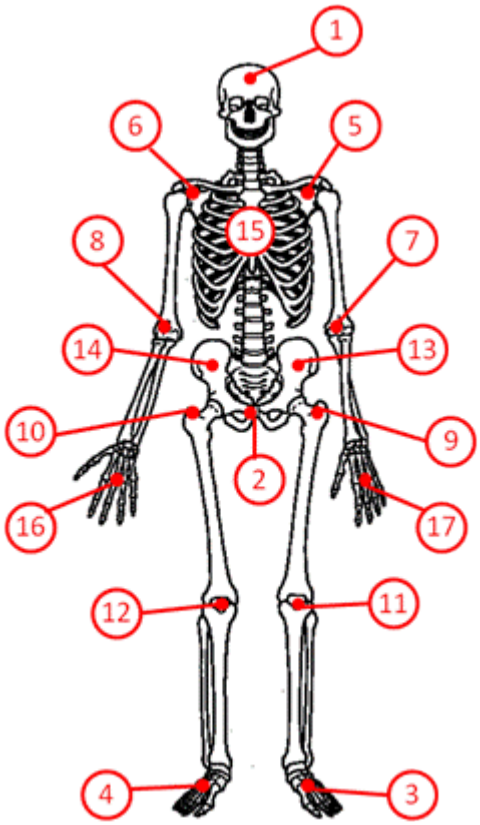

Figure 1.7: High intensity sampling of the skeletal remains. Originally in colour.

1.6.2. Aims

The overall aim of the work discussed in the thesis was to develop and test and approach for obtaining organic signatures from burial soils that inform archaeological understanding of the burial, encompassing aspects relating to the individual and/or the culture in which they lived.

Specific objectives were:

- to develop and test an analytical strategy for obtaining and analysing extractable organic signatures from burial soils and distinguishing signatures associated with the buried remains from the soil background. Previous

investigations of burials have focused mainly on visual remains (muscle, skin and wrappings), hence the value of organic signatures in the surrounding soil is not presently known. An essential requirement of the strategy was that samples were collected in a consistent manner so that results from different graves and different grave sites were comparable.

- to identify GC-amenable compounds in grave soils that relate to: (i) the burial and (ii) the background soil organic matter and to assess their origins and, where possible, draw inferences about the transformation pathways through which they survive; notable compounds discussed in the literature in relation to archaeological human remains include fatty acids and sterols.
- to evaluate the variability in the organic signatures in grave soils as a function of their anatomical position and vertical position in relation to the remains in order to: (i) assess the viability of the sampling approach proposed for the InterArChive project and (ii) identify locations where signatures specific to the burial are best preserved and determine if the InterArChive sampling strategy can provide a comprehensive overview of the grave.
- to assess evidence for the transport/mobilisation of organic matter within burial soils and evaluate if transport processes impact on the lipid contents and distributions.
- to identify the significance of specific signatures associated with particular buried human remains and assess their potential in revealing features of the burial and/or archaeology: It can be expected that some burials will contain unusual signatures that relate to particular practices or treatments of the remains.

CHAPTER 2. EVALUATION OF ANALYTICAL METHODS

2.1. Introduction

The aim of this chapter is to develop and test organic geochemical methods for the extraction, processing and analysis of grave soils to ensure that they are appropriate for the analysis of the InterArChive samples There are many sources of lipids in soils (such as soil flora and fauna; van Bergen *et al.,* 1997) and that the abundance of lipids derived from an interred body will diminish over time and may represent a relatively small component of the total lipid present. Therefore an evaluation of the extraction and samples handling techniques to be used for the InterArChive project in order to maximise the amount of information retrieved from the grave soils is required. In addition, the large number of samples that are to be processed needs to be considered, necessitating a systematic approach which can be automated and efficient.

2.1.1. Sample storage

Sample collection has previously been discussed in Chapter 1 but it is important to consider the mode of storage of these samples in order to preserve their integrity. Initially samples were collected in Whirlpack bags as they provide a compact means of storage while preserving the samples. The Whirlpack bags were then stored in the freezer to arrest the growth of mould and microorganisms.

2.1.2. Accelerated solvent extraction (ASE)

Preliminary work for the InterArChive project involved analysis of grave soils from Eindhoven, Netherlands, sampled at low intensity and extracted using a Soxhlet. Soil (3 g) was extracted using 9:1 (v/v) dichloromethane (DCM):methanol (MeOH) for 48 h (Tongue 2008). The method is time consuming, has limited reproducibility, is unsuitable for processing graves sampled at high intensity (average 22 samples, each sample requiring two day extraction), and uses a large amount of solvent. Hence, for the InterArChive project, an alternative extraction method was required to reduce time and solvent consumption.

ASE combines high temperatures and pressures to extract compounds from a matrix using liquid solvents. The high temperature is an important factor during the extraction process as it increases the solubility of the analytes (Hubert *et al.*, 2000), while it can also disrupt the strong interactions between the extractable components and the matrix (Richter *et al.*, 1996). The high temperatures also decreases the viscosity of the solvent, allowing faster diffusion rates and better penetration through the matrix particles and increasing extraction efficiency (Richter *et al.*, 1996). High pressure is used to ensure that the solvent stays in liquid form at temperatures above the boiling point of the solvent or solvent mixtures (Gierielewicz-Możajska *et al.*, 2001). The use of the high pressure also ensures that solvent is forced into areas of the matrix that would not be normally reached under atmospheric conditions, again increasing extraction efficiency.

The ASE method shortens extraction times considerably and decreases the amount of solvent required (Richter *et al.*, 1996; Giergielewicz-Mozajska *et al.*, 2001). A comparison of ASE and Soxhlet for the extraction of lipids from soils has been carried out previously (Jambu *et al.*, 1978; Jansen *et al.*, 2006). The study examined the extraction, from soils, of organic compounds of a wide range of polarities and suggested that ASE gave better efficiencies, reduced the volume of solvent used and decreased total time for extraction per sample (Jambu *et al.*, 1978; Jansen *et al.*, 2006). The ASE system is an automated process allowing samples to run sequentially, thus further decreasing processing time for batches of samples (Jansen *et al.*, 2006). Other studies have reported yields comparable with Soxhlet for the recovery of petroleum hydrocarbons and polycyclic aromatic hydrocarbons from various matrices such as sewerage sludge, urban dust and marine sediment (Richter *et al.*, 1996). Similarly, comparable yields have been reported for extraction of phenols from sediments (Dean *et al.*, 1996) and polycyclic aromatic hydrocarbons from contaminated soil (Saim *et al.*, 1997). Studies have shown good reproducibility among the recovery of different lipid components from soils with a relative standard deviation of between 2-6% being reported (Wiesenberg *et al.*, 2004).

ASE is compatible with a wide range of solvents and solvent mixtures. The solvent composition 9:1 DCM:MeOH was employed in the preliminary Soxhlet extraction (Tongue, 2008) and has also been used previously in the ASE of lipids from soils.

This solvent mixture was used for the work described here to ensure the method is comparable with other studies. Furthermore the solvent mixture is above the azeotropic composition making solvent removal easier following extraction.

Elevated temperatures have been shown to increase extraction efficiency (Richter *et al*., 1996). A temperature of 100 °C has been used previous in the extraction of soils (Gierielewicz-Mozajska *et al.,* 2001; Richter *et al.,* 1996) and gave good recoveries of lipids.

2.1.3. Fractionation

The total extracts from Eindhoven were very complex making it difficult to assign individual peaks. Total extracts can be simplified by fractionation, which separates groups according to polarity. Separating into distinct fractions reduces the number of components in each fraction, reducing the potential for co-elution and thereby making the chromatograms easier to analyse. Column chromatography has been widely used to separate lipids based on their polarity (Jansen *et al.*, 2008; Pitcher *et al*., 2009).

Column chromatography involves loading the extract onto a polar sorbent (silica gel); the lipid components interact with the sorbent differently depending on their polarities. Solvents, of increasing polarities, are passed through the column and components that have a greater affinity for each solvent or solvent composition over the sorbent are eluted from the column with that composition.

Preliminary work utilised columns constructed from glass Pasteur pipettes plugged with DCM washed cotton wool and loaded with silica gel (60 G). Four solvent compositions, hexane, 1:1 hexane:toluene, 4:1 hexane:ethyl acetate and 1:1 DCM:MeOH were used to elute four different fractions; hydrocarbons, aromatic hydrocarbons, medium polar (alcohols, sterols) and high polar (fatty acids; Tongue 2008).

2.1.4. GC

2.1.4.1. GC preparation

Derivatisation of alcohols and fatty acids (found in the medium polar and polar fractions, respectively) is required in order to make the components amendable to GC analysis. Fatty acids and alcohols are known to cause several issues in gas chromatography. Their high polarity decreases their volatility and makes them subject to thermal instability and losses in the chromatographic system. Polar oxygenated functional groups also have a tendency to form hydrogen bonds, which can form strong bonds to the stationary phase. These issues can be overcome by protecting the polar groups by conversion to a less polar substituent. Two commonly used derivatisation methods are methylation of fatty acids using diazomethane (Wells, 1999) and silylation of fatty acids or alcohols using N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA).

The hydrocarbon and aromatic fractions did not require derivatisation and were directly transferred to GC autosampler vials in DCM (100 µl). The polar fractions were methylated by dissolving in a small amount of DCM and adding freshly prepared diazomethane in diethyl ether (5 drops; 5 min reaction time) before drying under a gentle stream of nitrogen. The polar and mid polar fractions were further silylated by adding BSTFA (containing 1% TMCS; 100 µL), and pyridine (4-6 drops) and heating at 70 °C for 90 min. Both fractions were reduced to dryness under nitrogen prior to being reconstituted in DCM (100 µL) and analysed by GC.

2.1.4.2. GC-FID

GC has been widely used in the area of geochemistry to analyse lipids from soils (van Bergen *et al.,* 1998; Kogel-Knabner, 2002) and can readily separate the complex mixture of lipids that are typically recovered from soils. As discussed in 1.4.1.2 separation of the lipid components is a result of their differing chemical properties, which include volatility and their interaction with the stationary phase of the column.

Preliminary GC separations for the InterArChive group used GC equipped with a FID detector and a DB1 column, with the carrier gas (He) flow. The GC method was set to run for a total of 90 min (Tongue 2008).

2.1.4.3. Fast GC

GC-FID analysis of the total extracts of the soils and of the separated fractions is very time consuming. Sampling from a particular grave can accumulate over 20 samples, including the 17 sample points around the body and three control samples (also the possibility of some additional samples: Chapter 1). Each sample point is extracted and fractionated to generate a total extract and four fractions, generating a total of up to 100 analyses for one grave. Using a 90 min run time this would equate to over 150 h of GC run time and it can be expected that some of the samples do not contain a detectable amount of organic matter. Thus, a rapid GC method is required as a screening method to enable all of the samples to be processed. Hence, a strategy was required to allow each of the fractions to be screened using a fast GC method, enabling selection of extracts or fractions containing suitable analyte levels for further analysis using GC-MS.

Fast GC offers a reduction in analysis time while not compromising efficiency and resolution. This is achieved using short, narrow columns and direct column heating. Various different authors have defined rapid GC using a variety of different criteria. Dagan and Amirav (1996) defined rapid GC using a ‘speed enhancement factor’ (SEF), which is an increase in speed obtained due to a shorter column length and the carrier gas linear velocity increase, while van Deursen (2000) classified it using the size of the half peak width. These two factors were reviewed by Matisova & Domotorova (2003) and have been summarised in Table 2.1.

Table 2.1: Classification of Rapid GC (Matisova and Domotorova, 2003).

Type of Rapid GC	Analysis Time	Peak width at half height	SEF*
Fast	Minutes	1-3 s	5-30
Very Fast	Seconds	30-200 ms	30-400
Ultra Fast	Sub-seconds	5-30 ms	400-4000

*The SEF is calculated based on the time taken for a total run on the fast GC compared with a run on the 'regular' GC.

Regular GC capillary columns usually have an internal diameter of around a 0.1 mm and a length of 25-60 m; fast GC columns typically have an internal diameter of around 100 µm and a length of between 10 and 15 m. This decrease in length allows for a rapid analysis time, but the shorter column results in a decrease in efficiency. By decreasing the column internal diameter (ID) the number of theoretical plates increases as the height of the theoretical plates is decreased. Direct column heating is essential for fast GC, allowing the column to be heated and cooled rapidly, enabling rapid temperature programming. This is achieved by heating the column by means of heating wires wrapped around the column rather than via an oven.

2.2. Results and discussion

2.2.1. Sample storage

Analysis of some of the soil samples collected directly into Whirlpack bags revealed them to contain large amounts of plasticiser contamination, chiefly a C22:1 fatty amide. In order to determine whether these compounds were leaching from the

Whirlpack bags, a section of the plastic was extracted with dichloromethane. GC analysis revealed the extract to contain C22:1 fatty amide and confirmed the Whirlpack bags to be the source of the plasticiser contamination. Note this contamination did not include C16 and C18 fatty acids as previously thought. To protect samples from contamination it was important to ensure that the soil did not come into contact with the bags. Accordingly all future samples were collected in foil that had been baked in the Pyroclean oven (450 °C) and then stored in the Whirlpack bags. Analyses of extracts from samples collected in foil did not show any contamination from the Whirlpack bags.

2.2.2. ASE

In order to validate the ASE conditions for the extraction of lipids from grave soils, a sandy loam from St. Mary's church, Edinburgh was selected for testing. In order to ensure maximum recovery of soluble organic matter the ASE method included 3 extraction cycles with static time set to 5 min (Richter *et al.*, 1996). To ensure absence of contamination a blank extraction was performed prior to the soil extraction. Replicate (n = 3) test soil samples (3-5 g) were ground and sieved (< 200 µm), accurately weighed into 5 ml ASE cells and extracted with DCM:MeOH (9:1 v/v) for 5 min at 100 °C. All parameters are listed in Table 2.2.

Table 2.2: List of parameters for the ASE method

ASE parameters	Setting
Extraction temperature	100°C
Extraction cycle time	5 min
Extraction pressure	1500 psi
Static time	5 min
Number of cycles	3
Rinse volume	50%
Purge time	60 s

GC analysis of the blank extract showed that lipid components from previous extractions were present in the cells and that general cleaning was not sufficient to remove all traces of organic components. Further blank extractions of cells were carried out in order to demonstrate that the residues were removed by subjecting the cells to a rinse cycle using the ASE prior to packing. GC analysis of the extracts found that no further lipid components were present and that a blank extraction of the cells was sufficient to remove all residual lipid components. Subsequently, therefore, prior to use, all cells were subjected to a blank extraction to complete the cleaning process.

Literature reports indicate that increasing extraction temperature increases extraction efficiency (Richter *et al.*, 1996). Although GC analysis of soil extracts obtained using temperatures above 100 °C (100-175 °C in 25 °C intervals) showed better extraction efficiency, the profiles revealed an increase in the amounts of plasticisers (see Figure 2.1 for examples) in the total extracts (Dr. Matt Pickering, pers. comms).

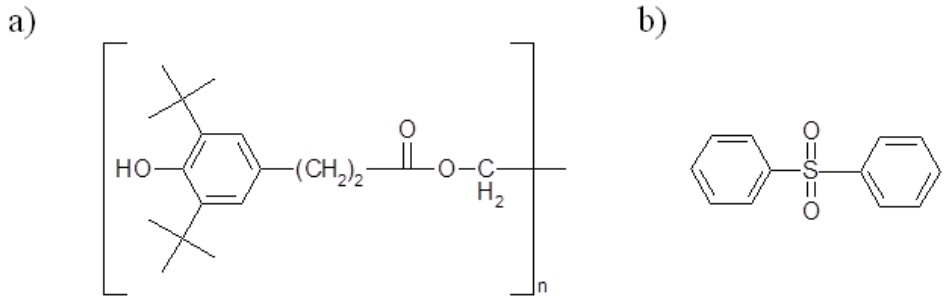

Figure 2.1: Example of plasticisers found in samples from the ASE; a) IRGANOX, b) diphenyl sulfone.

2.2.3. Fractionation

Small scale fractionation and sample cleanup procedures often employ solid phase extraction (SPE) cartridges. These cartridges are made of plastic and, accordingly, carry a risk of introducing foreign components (e.g. plasticisers) into the sample. It was, therefore, necessary to carry out the fractionation process using glass apparatus

which could be cleaned by heating to 450 °C. Small scale column chromatography is often performed in glass pipettes packed with sorbent as in the preliminary work (Tongue 2008). These columns are not ideal as the elution is slow and the flow is difficult to control. To increase efficiency, new glass columns were designed that could be used with a vacuum manifold in order both to allow control over the flow rate and to ensure consistency in the fractionation process between samples. The fractionation process on the new columns was evaluated to ensure good recovery and separation of distinct compound classes. Each standard (1 mg) was dissolved in DCM (10 ml). The hydrocarbon standard stock solution contained hexadecane (C16; **1**), octacosane (C28; **2**) and octatriacontane (C38; **3**). The aromatic standard stock solution contained phenantherene (**4**) phenyl heptadecane (**5**) and chrysene (**6**). The mid polar stock solution contained tetradecanol (C14; **7**), octadecanol (C18; **8**) and cholesterol (**9**). The high polar standard stock solution contained dodecanoic acid (C12; **10**), hexadecanoic acid (C16; **11**) and docosanoic acid (C22; **12**).

The same amount of silica and solvent was used as employed in preliminary work on grave soils the laboratory (Tongue, 2008). The columns were 90 mm long with an internal diameter of 10 mm and contained a sinter above the outlet (Figure 2.2). The sinter was covered with a plug of DCM washed cotton wool, added to reduce the potential for blockage of the sinter. The column was loaded with dry silica gel (60 G) and was equilibrated with hexane (3 bed volumes). The test sample, comprising several standards of varying polarity, hydrocarbons (non polar), aromatic hydrocarbons, medium polar and high polar, dissolved in 250 µl of DCM, was loaded onto the column, the column was eluted with solvents/solvent mixtures of increasing eluotropic strength and each eluent was collected.

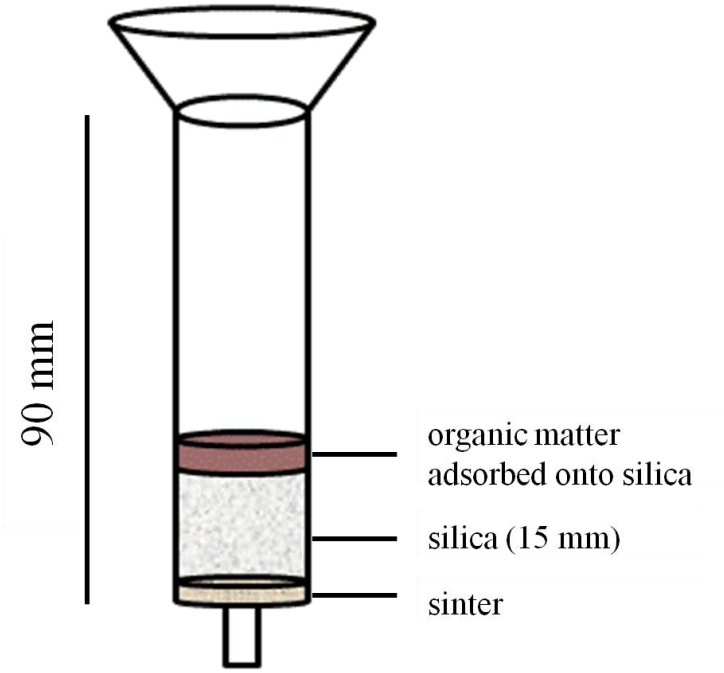

Figure 2.2: Schematic of column used for fractionation. Originally in colour.

GC analysis of the fractions showed that there was incomplete separation of components of the differing compound classes. The aromatic components eluted in the hydrocarbon fraction and high polar components eluted in the medium polar fractions. No components were present in the aromatic and high polar fractions. This suggested either that the solvent system needed to be altered or that the polarity of the solvent used to load the total extract onto the column may have led to components not being retained by the stationary phase, causing them to migrate and elute earlier than desired. The latter was investigated by adding the sample to a small amount of stationary phase and evaporating the solvent before adding this to the top of the preconditioned column. Adsorbing the sample directly onto the silica would ensure that the components of the extract were able to interact fully with the stationary phase and would avoid altering the eluotropic strength of the mobile phase. The column was prepared and conditioned as before and a small amount of silica (0.05 g) was added to a vial (3 ml). The test mixture in DCM (250 µl) was added to the vial containing silica, the top of the vial was plugged with DCM washed cotton wool and solvent was removed on a rotary vacuum concentrator (Christ RVC; 25 °C; 1500 rpm; 6 min), leaving the sample impregnated on the silica. The column

was equilibrated with hexane (3 bed volumes; under light vacuum) and the silica containing the test sample was loaded onto the column. The column was eluted with each of the four eluent solvent compositions as described previously and the eluents were collected, dried and reconstituted in DCM (100 µl).

GC analysis of the fractions revealed that there was good separation of the different compound classes present in the mix of the medium polar and high polar components (Figure 2.3). Separation of the hydrocarbon and aromatic fractions was incomplete, phenyl hexadecane (**5**) eluting with the hydrocarbon components. This can be explained by the presence of the aliphatic hydrocarbon chain in phenyl hexadecane presenting a greater challenge to separation than the polyaromatic components phenantherene (**4**) and chrysene (**6**) which lack aliphatic moieties. Hence, the C-16 hydrocarbon chain rendered the molecule sufficiently hydrophobic to elute with the hexane mobile phase, causing phenyl hexadecane (**5**) to behave more like the hydrocarbon components. Hence, aromatic molecules having a significant aliphatic component (e.g. a long hydrocarbon chain) can be expected to elute in the hydrocarbon fraction.

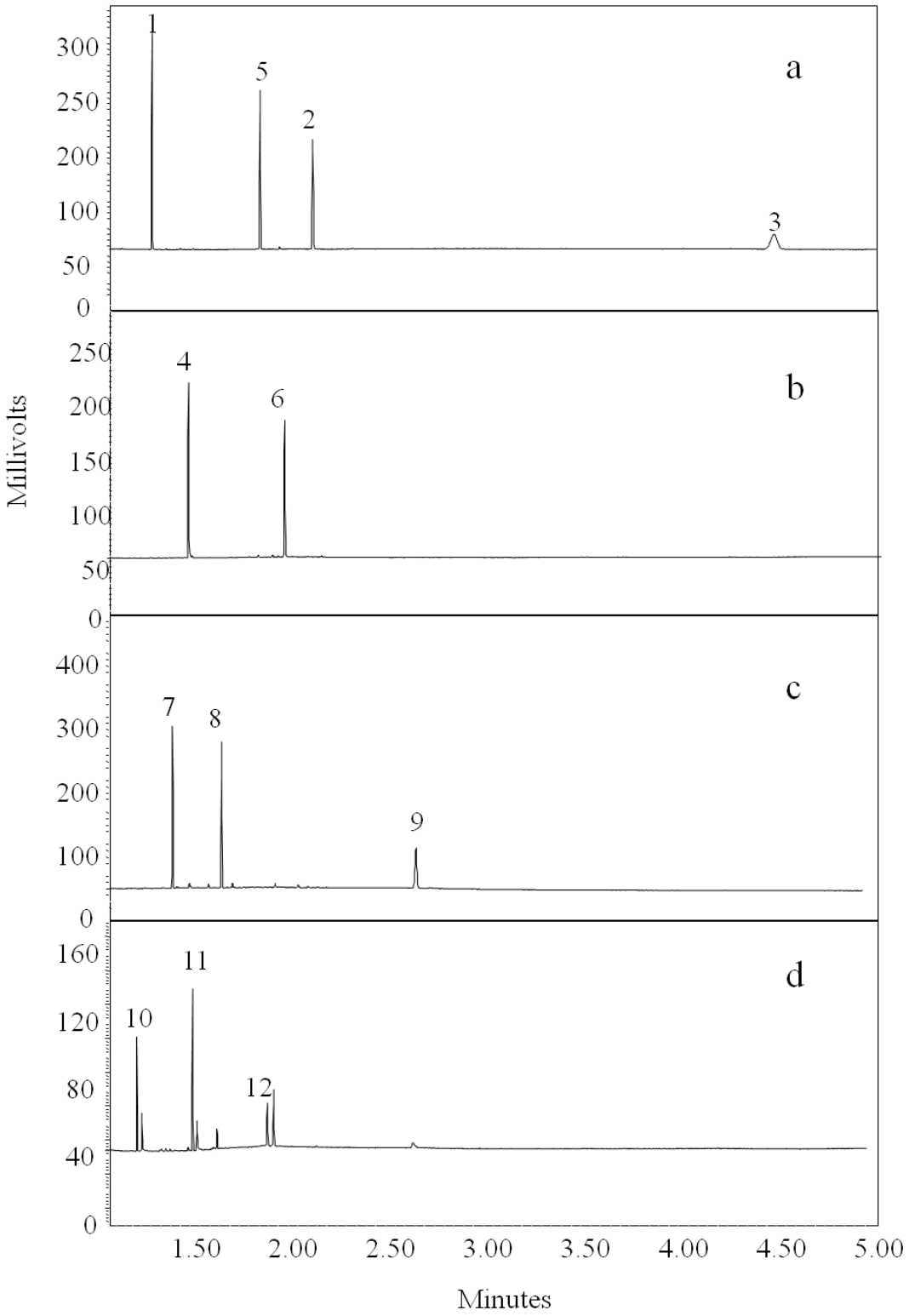

Figure 2.3: Partial fast GC-FID chromatograms of the various fractions collected from column chromatography of a mixture of standards: a) hydrocarbon fraction, b) aromatic fraction, c) medium polar fraction, d) high polar fraction. The peaks numbers represent the standards discussed in Section 2.2.3.

The modified sample loading approach allowed fractionation of the mixture of standards. Before it could be used for routine processing of grave soil extracts it was necessary to test it on natural mixtures of soil lipids. Accordingly, total extracts from grave soil sampled from a site in Hungate, York were fractionated using the same method. GC analysis of the fractions revealed that the abundance of the C16 and C18 fatty acids (eluting in the high polar fraction) increased relative to their abundance in the total extract indicative of contamination. As the glassware is rigorously cleaned at high temperatures this suggests that C16 and C18 fatty acid contamination may derive from the silica. Accordingly, the preparation of the column was modified to incorporate an initial wash with DCM:MeOH 1:1 (v/v) followed by a second wash of DCM prior to conditioning with hexane. The DCM:MeOH 1:1 (v:v) wash ensures that impurities are eluted from the silica prior to the sample being added, while the DCM wash removes of the remaining impurities, while also providing a less polar intermediate wash between the DCM:MeOH 1:1 and hexane.

Pure silica samples were fractionated using the new wash steps followed by the established fractionation method, each wash and fraction was collected and analysed by GC. Analysis of the fractions showed C16 and C18 components were present in the first DCM:MeOH 1:1 (v:v) wash sample, the second was sample contained a trace amount of C16 and C18 components suggesting that most of the impurities from the silica had been removed in the first wash. Analysis of the fractions showed that no impurities were present and that the washes had successfully removed all the impurities (Dr Matt Pickering, pers. comms).

The fractionation method was tested on a different grave soil extracts. Total extracts of grave soil sampled from a site in Leith, Edinburgh were fractionated using the devised column chromatography method. GC analysis of the fractions revealed good separation between the different component groups, indicating that the method was suited to the fractionation of total extracts.

2.2.4. GC analysis

2.2.4.1. Derivatisation

Inspection of the GC chromatograms from the previous fractionated standard mixture revealed that, in addition to the desired fatty acid methyl esters (FAMEs), the high polar standard fractions contained a significant amount of the corresponding trimethylsilyl esters (TMS) were present. This observation suggests that the fatty acids were not fully methylated by diazomethane and that the residual free fatty acids were silylated, implying insufficient reaction time, an insufficient amount of diazomethane or possibly that the solvent used to dissolve the sample hindering the reaction.

To investigate the cause of the incomplete methylation of fatty acids in the high polar fractions, the high polar standards were dissolved in several different solvents; DCM, ethyl acetate, ether and acetone. The solvents were chosen as the lipid components exhibit good solubility in them. To determine which solvent was most appropriate for the derivatisation reaction the high polar fractions were methylated with diazomethane and silylated with BSTFA to derivatise any un-reacted fatty acids. Analysis by GC indicated that the methylation reaction worked best for the samples dissolved in acetone, these samples exhibited the greatest reduction in trimethylsilyl components. Thus, acetone was selected for use in dissolving samples for methylation.

The preparation of diazomethane involves production of gaseous diazomethane followed by trapping in cold diethyl ether. Diazomethane is allowed to accumulate for at least 2 h before use. As a consequence there is poor control over the amount of diazomethane produced and how much is added to the samples during methylation, although it is believed to be added in excess. To observe the effect of the amount of diazomethane on the methylation the high polar standards were dissolved in acetone and 50, 100, 150 and 200 µl of diazomethane were added. The samples were silylated with BSTFA and analysed by GC. No difference was observed in the fatty acid trimethylsilyl ester abundances relative to the methyl ester counterparts indicating that the amount of diazomethane added does not affect the derivatisation.

To investigate the effect of reaction time on the completeness of methylation the high polar standards were methylated over reaction times of 5, 10 and 15 min, after which the samples were analysed by GC. Increasing the reaction time to 15 min methylated all of the fatty acids present in the sample. The methylation time was, therefore, increased to 15 min and the sample was dissolved in a small amount of acetone before the addition of diazomethane.

In addition to the FAMEs and fatty acid silyl esters (FASEs) a number of additional peaks were present in the high polar fractions. To determine the source of the additional peaks a blank sample of acetone was derivatised with diazomethane as in the previous methods, but was not silylated, and was analysed by GC. The chromatogram of the blank contains several peaks due to contaminants from the diazomethane solution. It is, therefore, important to derivatise a blank sample alongside grave soil extracts.

Despite the tests detailed above, incomplete methylation was observed during analysis of grave soil extracts. This could be due either to the concentration of fatty acids being very high in some samples or possibly to some components in the samples limiting the availability of the diazomethane (e.g. by destroying it). TMS diazomethane has been used as an alternative derivatisation agent (Podlech 1998). As this reagent does not require preparation and can be purchased, there is greater control over the amount of reagent introduced to the sample. Samples were dissolved in 2:1 DCM:MeOH (v:v; 100 µl) and derivatised with TMS diazomethane (20 µl) prior to silylation and analysis by GC. This led to complete methylation of the fatty acids and eliminated the presence of the fatty acid silyl esters formed from incomplete derivatisation.

2.2.4.2. GC-FID

The GC method used in the preliminary work (Tongue 2008) was transferred to a Thermo Trace GC Ultra equipped with a Thermo Triplus autosampler and a FID detector. Notably, the column used, (DB 1) gave good separation of the lipid components but did not give good peak shape for the more polar components. The chromatography of the polar components was improved by using a DB5 column

(0.32 mm I.D x 60 m; 1.0 µm film thickness; A.J. and W Scientific) with the same oven programme and flow settings.

The GC methods allow analysis of a wide range of carbon chain lengths (C12- C38) to ensure that we are getting a full scope of compound that are present in the soil. The low molecular weight components (C12- C20) have been previously observed in the tissues of bog bodies (Evershed and Connelly, 1994) and mummies (Gulaçar, 1990) and components with these chain lengths could link to degraded tissues from the remains while longer chain components could include cyclic components such as cholesterol and coprostanol (C27), the latter of which has been found in the tissues of bog bodies (Evershed and Connelly, 1994) and has been used as a faecal marker (Bull *et al.,* 2009). The range of components will also allow for the analysis of the much larger components that can be found in bees wax (components can include components with chain lengths of C30 and above; Tulloch, 1969). Bees wax has been known to have a number of uses including art, medicine and religious rituals (Regert *et al*., 2001; Garnier *et al*., 2002) and has also been found in ancient pottery (Heron *et al*., 1994; Evershed *et al*., 1997)

2.2.4.3. Fast GC

Fast GC columns enable faster ramp speeds than conventional GC columns, allowing shorter run times to be achieved. A fast GC method was created using the same starting temperature (70°C) and the same end temperature (320°C) as for the regular GC method and with the same injector and detector temperatures. Given that fast GC run times are normally less than 10 min an initial ramp rate of 180°C min^{-1} was chosen as a starting point, giving a run time of around 5 min. This method gave good separation but poor resolution of the peaks. Hence, a range of ramp rates was investigated to determine the optimum value. Methods with ramp rates of 90, 75, 60, 45 and 30°C min^{-1} were evaluated to achieve the best balance between resolution and short run-time. The method using the 90°C min^{-1} ramp enabled good separation of peaks and gave resolution comparable with the other ramp rates while still giving a short overall run time (7.5 min). Accordingly, this method was used to screen fractions. Partial chromatograms of medium polar fractions from the grave soil from

Hungate, York show the differences between the run times while still showing the same separated components (Figure 2.4).

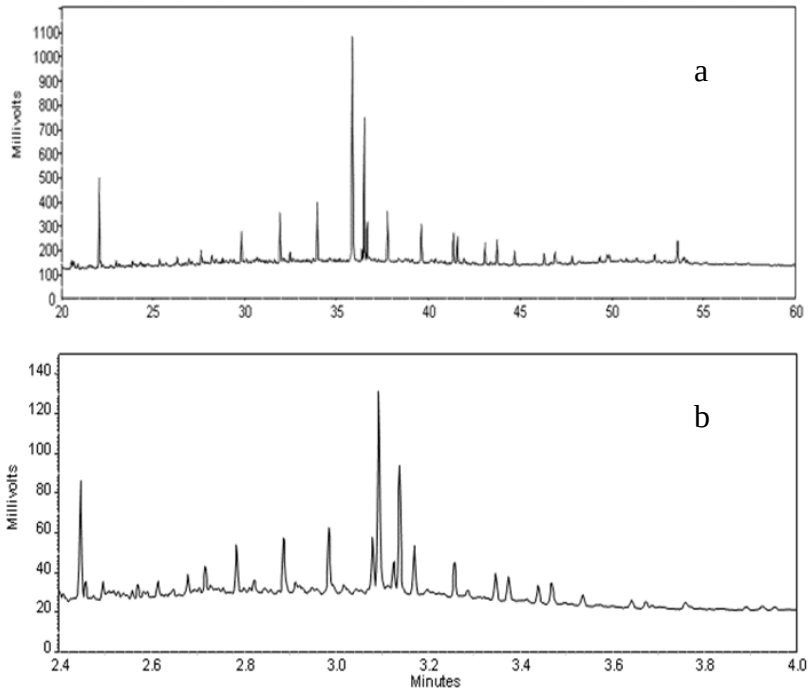

Figure 2.4: Partial GC-FID (a) and fast GC-FID (b) chromatograms of a medium polar fraction from test soil from Hungate, York.

2.3. Conclusions

The techniques discussed provide a systematic approach to the analysis of the high quantity of samples required for the InterArChive project. As the abundance of lipids related to the burial may be low, compared with lipids from other sources, it is important that contamination of the samples is kept to a minimum to ensure that we are able to observe lipids at these low levels. Contamination has been reduced in several of the analysis steps by storing the samples in aluminium foil, performing blank extractions of the ASE cells prior to loading the samples and by pre-washing the silica during the column chromatography.

The method has been designed to enable all samples to be extracted and analysed using the exact same conditions to ensure that all samples undergo exactly the same treatment so samples from different areas of the remains, from different graves and

from different grave sites can be compared to each other. The method uses automated sieving, automated extraction process, automated solvent removal and GC and GC-MS analysis so that the whole method can be repeated using the same technique and further samples can be analysed in the same way and compared to the results discussed in the thesis.

Using the ASE enables for a much quicker extraction process and also allows the extraction process to be much more controlled with conditions being programmed and automated to ensure that many samples can be processed together and that they are extracted under the same conditions. The ASE system also allows for a smaller amount of solvent to be used in the extraction process. The GC method allows for the analysis of a wide range of components that could be present due to the remains and that have been known to have been found in an archaeological context. The fast GC provides a good screening method for the samples, cutting the analysis time by 80 min and allowing a larger amount of samples to be screened within a shorter space of time. The range of samples analysed also reflects what is being analysed in the other GC method.

CHAPTER 3: COMPOSITION AND DISPLACEMENT OF LIPIDS IN SOILS FROM IRON AGE AND ROMAN BURIALS

3.1. Introduction

The work carried out on this chapter will focus on the lipid components present in grave soil, assess their possible origin and deduce whether they are associated with the presence of buried human remains. In addition the study will focus on how lipids might be transported within the burial environment and whether water movement has an impact on the overall lipid contents and distribution of specific lipid classes.

The three burials chosen for this study have all in the past been subjected to significant water movement; the Heslington East site was located in line with a natural spring, the Basly site was quite rocky allowing for water to flow freely through the burial and the Thessoloniki site was located within a harbour, where fluctuating groundwater was a feature throughout the excavation site.

3.2. Heslington East (HE09)

3.2.1. Site description and sampling

The Heslington East site is situated on the outskirts of York and was excavated in September 2009 during the development of the new Heslington East campus at the University of York. The excavation was a community based project bringing together local people, York archaeological trust (YAT), On Site Archaeology and the University of York archaeological department. The excavation has provided evidence for much activity on the site over a long period of time ranging from the Mesolithic through to the Roman period (Neal 2012). The soil matrix is a heterogeneous mixture of sand, silts and gravels of glaciofluvial origin (Neal 2012). The site has a deep, free draining soil, that is highly permeable and has a high water storage capacity (Cranfield Soil and Agrifood Institute, 2013) and the grave site was situated along the line of a natural spring across a gentle south facing slope (Neal 2012). The results discussed here are from HE09 713 726, one of three Roman burials found at the site.

The grave contained the supine skeletal remains of a mature adult female (46yr+; Figure 3.1), orientated north to south, with hands placed over the pelvis and with the feet together (Holst 2010). The grave was partially surrounded by large stones lining the edges. The bones were generally in poor condition and the skeleton approximately 60% complete (Holst, 2010).

Figure 3.1: Grave HE09 713 726 after excavation and during the period of sampling. Grave was a supine burial with the hands placed across the pelvis (photograph by Brendan Keely). Originally in colour.

High intensity sampling was carried out in accordance with the InterArChive sampling strategy (Chapter 1; Table 3.1; Figure 3.2).

Table 3.1: Samples collected from grave HE09 713 726, including sample numbers and the locations from which they were collected.

Sample number	Sample position
C1	Control from edge of the excavation site
C2	Control from top of the grave fill
C3	Control from just above the remains
1	Skull
2	Pelvis
3	Left foot
4	Right foot
5	Left shoulder
6	Right shoulder
7	Left arm
8	Right arm
9	Left pelvis
10	Right pelvis
11	Left knee
12	Right knee
13	Left hip
14	Right hip
15	Chest
16	Right hand
17	Left hand
A1	Under cranial region
A2	Under the mandible
A3	Left cheek bone
A4	Right cheek bone
A5	Left side of the jaw
A6	Right side of the jaw
A7	Under sacrum
A8	Below right pelvic wing
A9	Within pelvis
A10	Abdomen below stomach
A11	Bone fragments at top of the legs

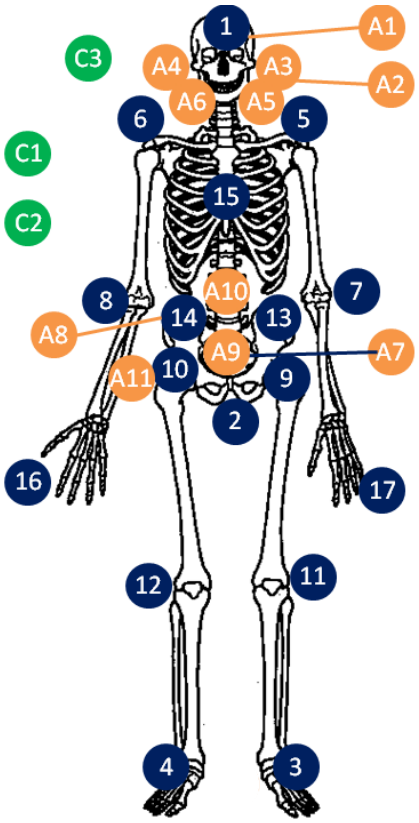

Figure 3.2: Samples taken from grave HE09 713 726, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive high intensity sampling strategy (Chapter 1) are labelled in blue and additional samples are labelled in orange. Originally in colour.

3.2.2. Results and Discussion

3.2.2.1. Bulk soil analysis

Analyses of the total abundance levels of carbon, hydrogen, nitrogen, sulfur (CHNS) and total organic carbon (TOC) were performed to provide a baseline characterisation of the organic material present in the soil. The absence of detectable nitrogen in all of the samples from grave HE09 713 726 (Figure 3.3) indicates an absence or extremely low levels of proteinaceous material preserved in the burial soil and in the control samples. The similarities in the total carbon and TOC values (Figure 3.3) suggest that the soil also contains very little inorganic carbon.

Taking into account the error that has been observed (~0.1%) from the EA all sampling points exhibit similar levels of TOC to the controls (~0.5%) with the exception of sample A11 (~1.5%). Notably, this sample was found to contain fragments of bone during sample processing. Archaeological bone often retains a significant organic component (Collins *et al.,* 2002), thus, inclusion of bone-derived organic carbon could explain the samples higher TOC value for A11. All samples exhibited hydrogen contents which greatly exceed the maximum hydrogen content possible for fully saturated organic matter. Moisture can be excluded as a source as the samples were dried thoroughly prior to analysis. The high hydrogen values, therefore, most likely reflect hydrogen liberated by the de-watering and/or dehydroxylation of clay minerals during the analyses. There was no detectable sulfur in the soil samples.

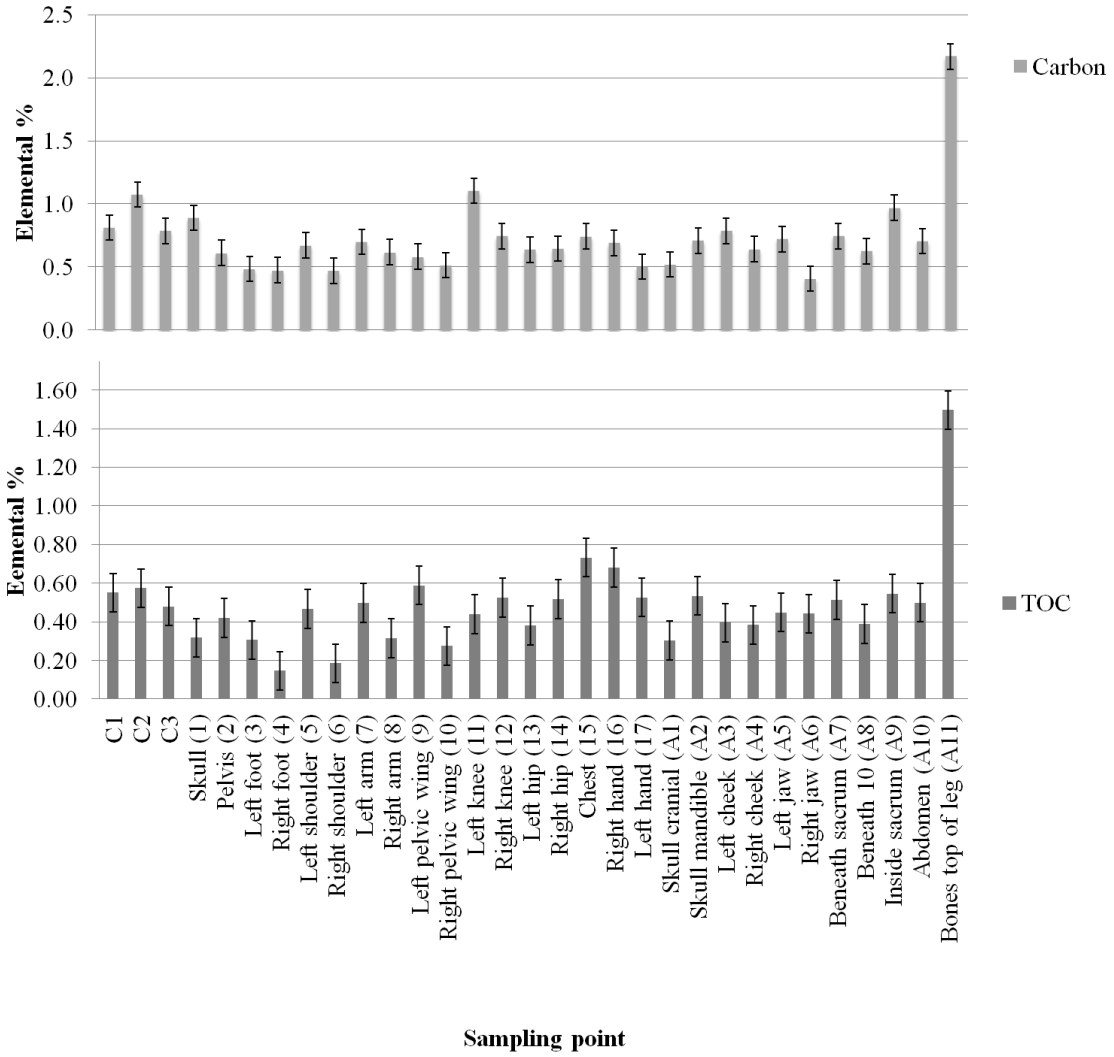

Figure 3.3: Bulk carbon and total organic carbon (TOC) contents of soils from HE09 713 726. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

The extractable organic matter contents vary across the samples (Figure 3.4). Samples 4-6, 8-11 A1 and A7 contained greater amounts of extractable organic matter than the controls whereas all other sampling points contained similar or lower amounts of extractable organic matter to the controls. The right foot (4), right shoulder (6), the left pelvis (9) and the left knee (11) contained the highest solvent extractable organic matter contents. Notably the right foot sample (4)

shows a high level of extractable matter compared with the left foot (3) sample. The higher content of extractable organic matter in the pelvic sample (9) may reflect the enhanced retention of the products of body decomposition by virtue of the protective bowl shape of the pelvis. The left shoulder (5), right arm (8), right pelvis (10), beneath skull (A1) and beneath sacrum (A7) also contain a relatively high amount of extractable organic matter, albeit not as much as points 4, 6, 9 and 11. The sampling points displaying extractable organic matter greater than the controls show patterns across the body. Higher levels of extractable organic matter are found around the lower regions of the pelvis and at the shoulders and beneath the pelvis and skull.

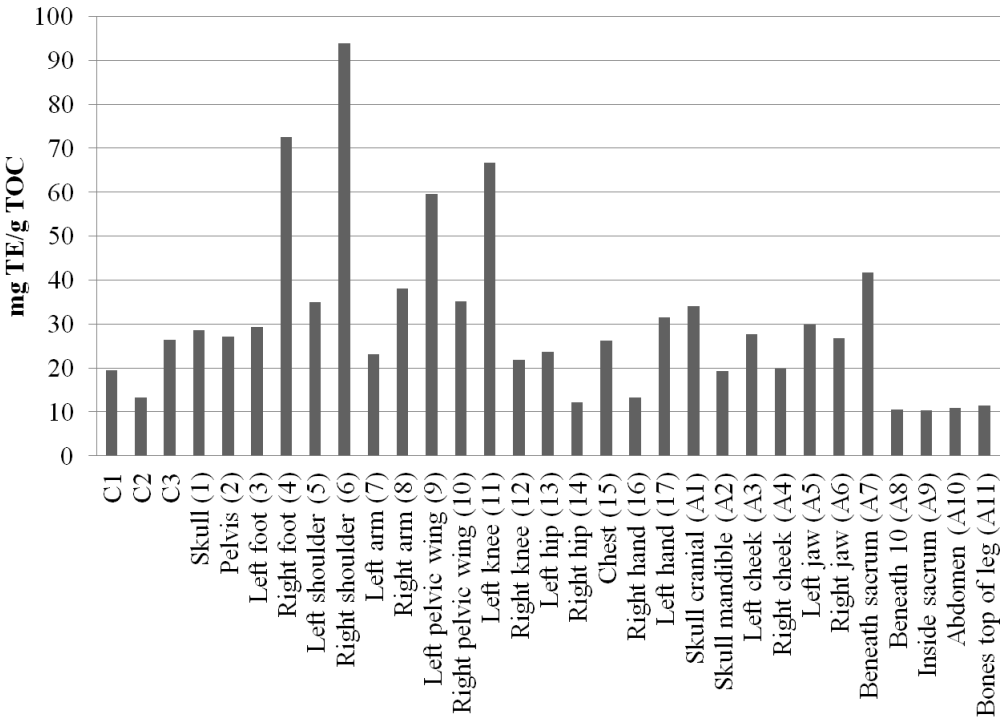

Figure 3.4: Solvent extractable organic matter in soil samples from HE09 713 726 normalised to total organic content.

3.2.2.2. Total extracts

GC analysis of the total lipid extracts revealed complex lipid distributions. For the purpose of comparison the total extracts were fractionated into four distinct fractions: hydrocarbon; aromatic hydrocarbons; medium polar; and high polar. Each of the fractions was analysed by fast GC in order to observe any differences in the distributions between the sampling points.

3.2.2.3. Hydrocarbon and aromatic fractions

The GC chromatograms of the hydrocarbon fractions show fairly simple distributions of *n*-alkane components that differ slightly from the distribution of

the control sample C1. The *n*-alkanes were identified through MS; Figure 3.5 shows an example mass spectrum for the *n*-C31 alkane, the molecular ion is present at *m/z* 436. The fragmentation pattern also shows a series of fragments that are 14 mass units apart representing a loss of $(CH_2)_nCH_3$.

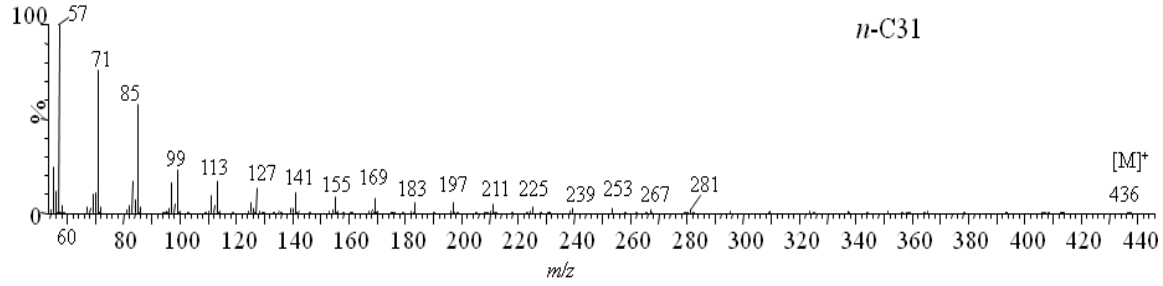

Figure 3.5: Example of an *n*-alkane (*n*-C31) mass spectrum.

All samples show a bimodal distribution of *n*-alkanes ranging from *n*-C23-C33 relating to plant material (Eglinton & Hamilton, 1967; Jambu *et al.*, 1991; Jansen *et al.*, 2008). Control C1 shows a maximum at *n*-C29 (Figure 3.6a), whereas all of the other sampling points display a maximum at *n*-C31 (Figure 3.6b). A maximum at *n*-C29 has been noted in soils from areas of woodland vegetation whereas a maximum at *n*-C31 has been noted in soils from grassland areas of (Salasoo, 1987; van Bergen *et al.*, 1998). The difference in the alkane maximum between the top soil and the soil of the grave fill suggests that the vegetation at the site in recent times is different to that present at the time of burial i.e. a change from mainly grassland to one with a greater input from woodland-type vegetation.

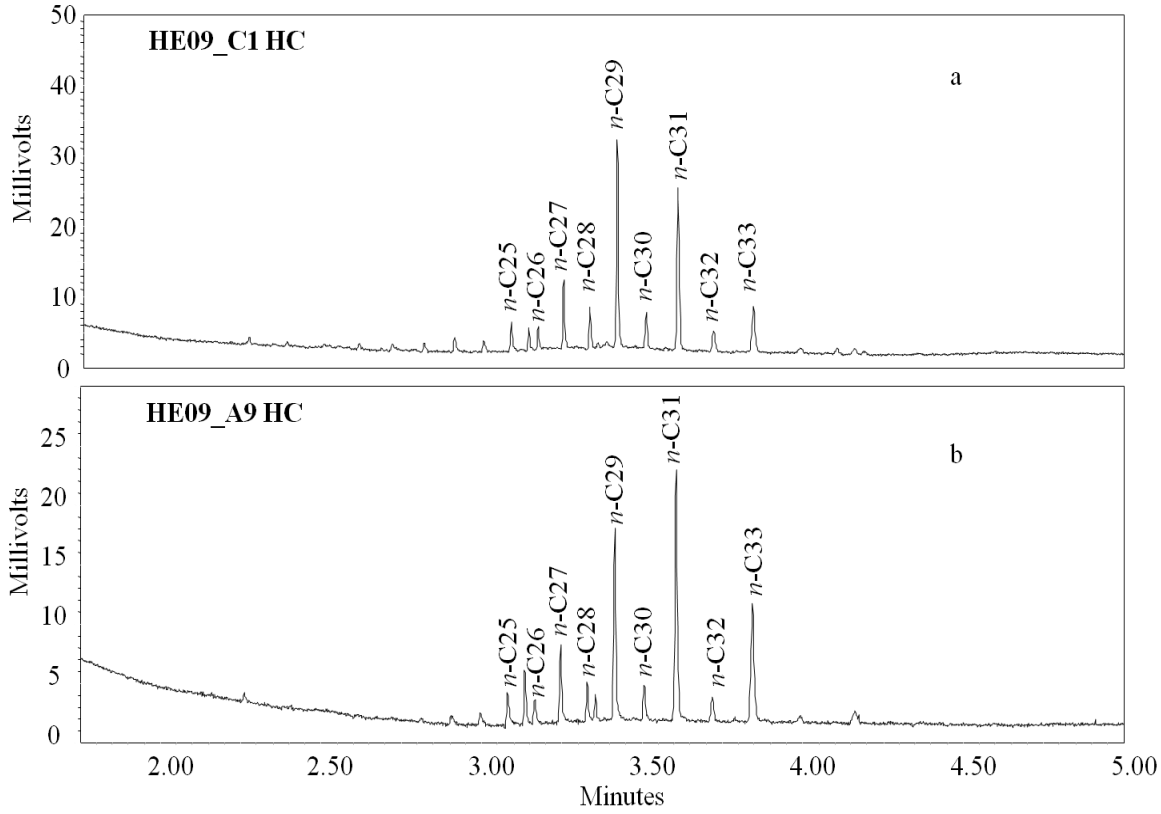

Figure 3.6: Partial GC chromatograms of the hydrocarbon fractions of control C1 (a) and additional sample A9 (b), typifying the two distinct classifications of lipid distributions observed in all sample points for grave HE09 713 726.

3.2.2.4. Medium polar fractions

All samples contained a series of *n*-alkanols and *n*-alkanones representing a plant source (Eglinton & Hamilton, 1967). The alcohols (analysed as their trimethylsilyl esters) were identified by MS with Figure 3.7 showing an example of a typical alcohol mass spectrum for the C26 alcohol. The molecular ion is observed at *m/z* 440 with fragments diagnostic of TMS alcohols present at 75 and 103 representing loss of the trimethylsilyloxy group and β-cleavage of the carbon chain, respectively. A cluster of peaks are also present each showing a loss of 14 which represents a loss of $(CH_2)_n$.

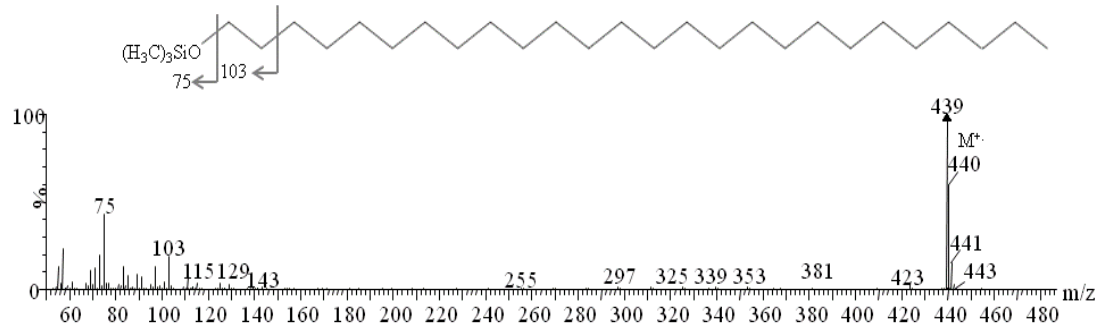

Figure 3.7: Example of an alcohol (C26) mass spectrum.

The *n*-alkanones were identified through MS, Figure 3.8 shows an example of an *n*-alkanone mass spectrum taken from one of the Heslington East samples. The molecular ion is identified at *m/z* 422 and there is a base peak present at *m/z* 59; this base peak represents the Mclafferty rearrangement that alkyl ketones undergo (Figure 3.9) involving the migration of the γ-hydrogen followed by β-cleavage (Budzikiewicz *et al*., 1966). There is also a cluster of peaks present each showing a loss of 14 which represents a loss of $(CH_2)_n$.

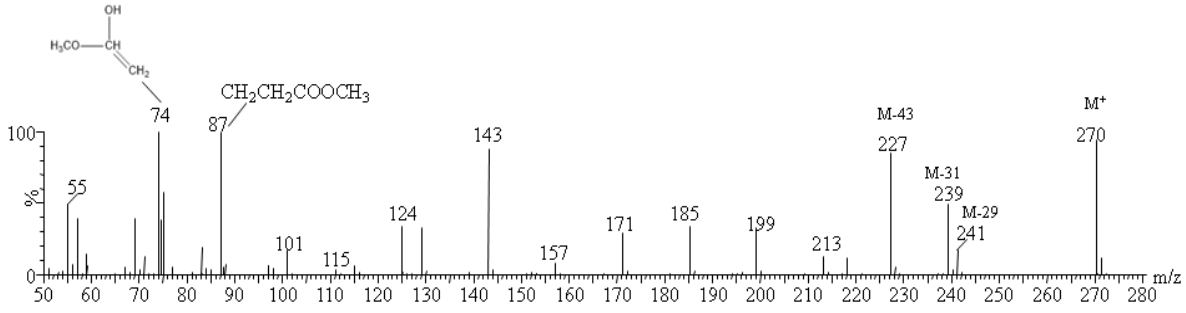

Figure 3.8: Example of an alkanone mass spectrum.

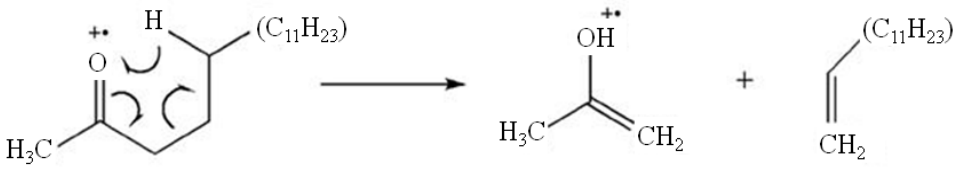

Figure 3.9: Mclafferty rearrangement of an alkyl ketone (Budzikiewicz *et al*., 1966).

The *n*-alkanols present in the extracts ranged from C22-C30 with a maximum at C26, typical of grassland vegetation (van Bergen *et al.,* 1998) and the *n*-alkanones displayed an odd over even predominance ranging from C23-C29, which is typical of plant waxes (Eglinton and Hamilton, 1967). As the components are present in the control samples they most likely reflect a component of the background soil organic matter.

3.2.2.5. High polar fractions

All samples contained a series of HMW (>C20) and LMW (<C20) fatty acids, analysed as their methyl esters (FAMEs). The fatty acids were identified by MS; Figure 3.10 gives an example of a C16 fatty acid found in one of the samples from Heslington East. The molecular ion was identified at *m/z* 270 with a base peak at *m/z* 74; the base peak is produced by a Mclafferty rearrangement involving migration of the γ-hydrogen to the carboxyl oxygen followed by β-cleavage (Tornabene *et al*., 1967), which is similar to the rearrangement undergone by ketones (Figure 3.9). The fragments at *m/z* 241 and 227 represent the expulsion of ethyl and propyl groups respectively and the fragment at *m/z* 239 represents a loss of the methoxy group. Other fragments separated by 14 *m/z* units show successive cleavage of the carbon chain.

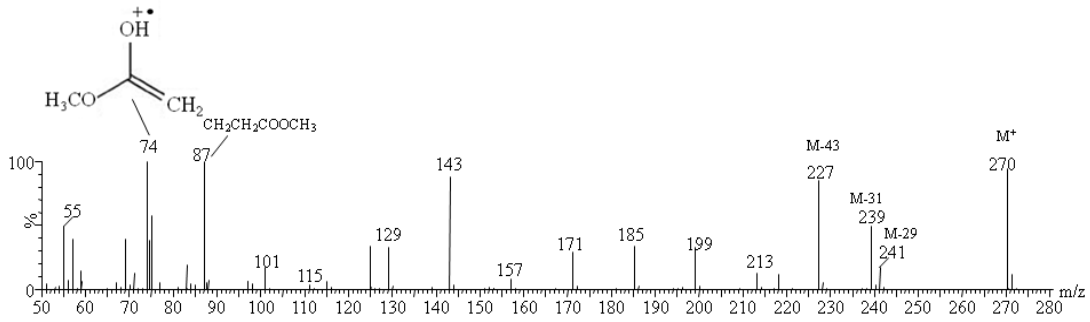

Figure 3.10: Example of a fatty acid mass spectrum (C16).

The HMW fatty acids (Figure 3.11) are known to be related to plant waxes (Eglinton and Hamilton, 1967) while LMW fatty acids, have a number of possible sources including; animal tissues, fungi, bacteria and to a lesser extent plants (Forbes *et al.*, 2005; Ruess *et al.*, 2002; Zelles, 1997; Eglinton & Hamilton, 1967). C18:2 FAs, were not present in the samples suggesting fungal inputs do not dominate (Ruess *et al.*, 2002). Bacteria, which contain relatively high amounts of C16:0, C16:1 and C18:1 fatty acids (Rezanka and Sigler, 2009), could be a source of these lipids (Figure 3.11), C16:1 in particular being scarce in animal and plant sources. Notably, branched fatty acids known to be associated with bacterial lipids, which have also been observed in tissue samples from mummies (Gulaçar *et al*., 1990; Buckley *et al*., 1999), were absent from the soil extracts, suggesting that bacteria were not a dominant contributor to the FA signatures. It is worth noting, however, that work carried out by Zelles (1997) on the fatty acids from cell membranes of several types of bacteria showed that several gram negative bacterial species did not contain any branched fatty acid components. Therefore, the fatty acid signatures (in particular the C16:1 component) may still imply bacterial activity and that the bacteria present in the soils differs from that detected in the mummies.

The samples also contain significant amounts of the C14 FA that is not normally abundant in plants, fungi or bacterial input in soils. Fresh human adipose tissue contains triacylglycerols based primarily on C18:1 and C16:0 fatty acids with

smaller amounts of the C18:0 and C14:0 components (Brockerhoff 1965). Notably, the C14:0 components have been observed in preserved tissue samples of mummies (Buckley *et al*., 1999) in addition to been determined as a component that is found in adipocere (~8 %: Forbes *et al.,* 2005), suggesting that the C14 component is still present in degraded adipose tissues. The C16 and C18 FA components are also reported to be major products in degraded adipose tissue, present in similar proportions to the C14-C18 components observed in the samples (Forbes *et al.,* 2005). It is possible, therefore, that the fatty acids, in particular the C14 fatty acid, reflect an input from body tissues. In light of the abundance of the C16:1 FAs, however, the lipid profiles are likely to contain a bacterial overprint.

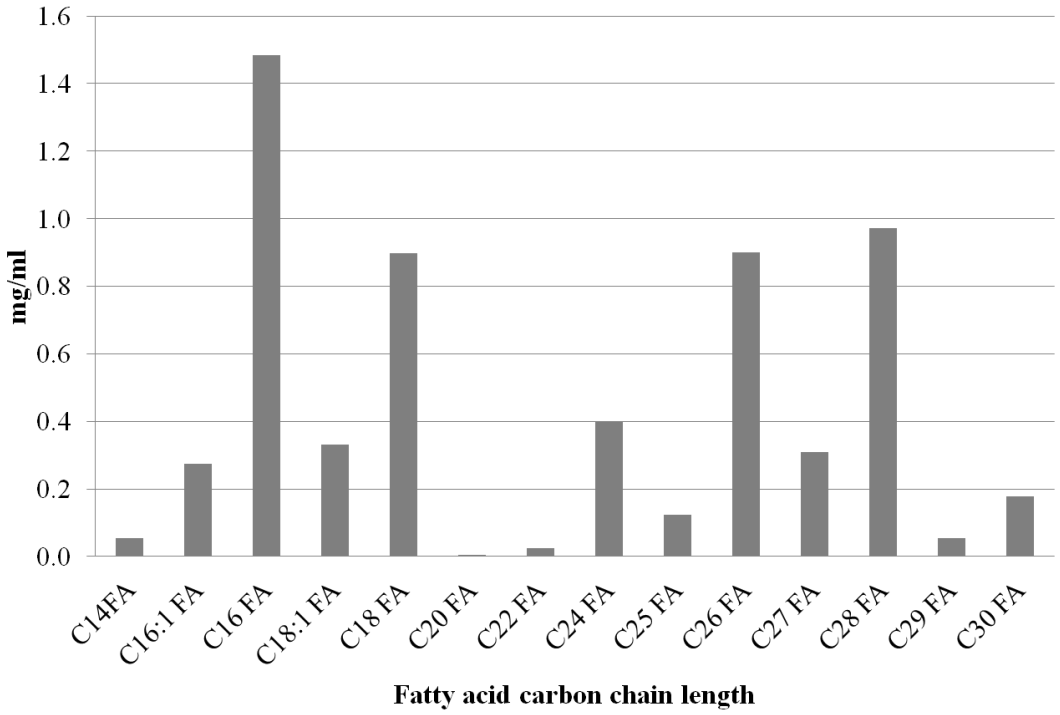

Figure 3.11: Example of fatty acid distribution from sampling point C3, typical of all samples from HE09 713 726.

The abundances of the HMW fatty acids relative to the LMW fatty acids differ among the samples. Higher ratios of LMW:HMW fatty acids (Figure 3.12) are

indicative of higher inputs from bacteria and/or degraded adipose tissue as opposed to plant-derived material such as that naturally present in the soil (Dent *et al.*, 2004; Zelles, 1997). Positions showing the highest ratios of LMW:HMW fatty acids occur around the pelvic area (9, 10, A7, A8 and A10) and at the feet (3 and 4), suggesting that there is a greater bacterial and/or degraded adipose tissue distribution in these areas. The pelvic area of the human body typically has the largest proportion of adipose tissue and contains large amounts of endogenous gut bacteria. After death there are few barriers to keep these bacteria in place and the human tissues act as a growth medium (Goff, 2009). Thus, the bacteria break down the fatty tissue, which may account for the highest LMW fatty acid content being observed in the samples from the pelvis. However this does not account for the elevated areas of LMW fatty acids in the samples taken from the feet as the feet area would have lower levels of fatty tissue and bacteria content than compared to the pelvic region.

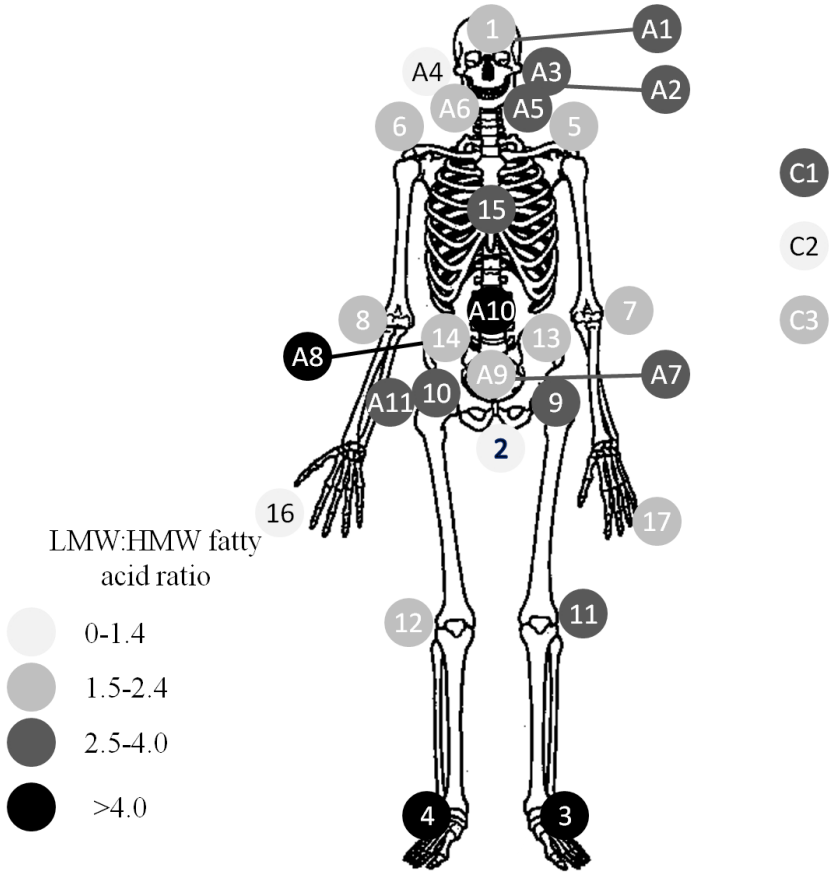

Figure 3.12: Summary of the ratios of LMW:HMW fatty acids from the various sampling points around the body. The key reflects differences in the ratio indicated by the different shades of grey.

The elevated levels of LMW fatty acids in the feet could be present due to something being placed in the lower part of the burial, although if this was the case a different fatty acid profile would be expected for the feet samples, which is not the case. The fatty acid profiles at the feet are similar to the profiles observed in the other samples around the remains. It can therefore be inferred that the organic material that was originally positioned at other parts of the remains may have been redistributed to the foot end of the burial. Redistribution of soil is known to occur through water movement though the soil (Gregorich *et al*., 1998; Wang *et al*., 2014) and soil that is present on a downward slope can be significantly affected by the redistribution of soil particles (Zhang *et al*., 2006).

As previously mentioned the burial was in line with a natural spring and was located on a downward slope therefore water from this spring may have flowed through the burial and redistributed the soil from its original position, with the soil and its associated organic matter migrating towards the foot end of the burial. This would be similar to what Zhang *et al*. (2006) and Wang *et al*. (2014) observed in their field and laboratory experiments where soil particles from the top of a slope were transported and redistributed at the bottom of the slope where levels of organic carbon increased. But a coffin base could provide a barrier and hence cause retention of transported soil/organic matter around the foot area, however, there was little physical evidence (wood or nails) to suggest that this burial was a coffin burial.

3.2.3. Conclusion

The burial from Heslington East shows evidence of lipids that could be associated with the presence of the buried human remains. The high polar fraction contained the saturated fatty acid components C14:0, C16:0 and C18:0 along with their unsaturated counterparts C16:1 and C18:1, all of these components have been observed in fresh adipose tissue as well as degraded adipose tissue (Brockerhoff, 1965), tissue from mummies (Gulaçar *et al*., 1990; Buckley *et al*., 1999), muscle tissue from bog bodies (Evershed and Connolly, 1988) and are the main components of adipocere (Forbes *et al*., 2005) inferring that these components are present due to degradation of the corpse. The abundance of the LMW fatty acids compared with the plant related HMW fatty acids are elevated in samples collected from around the gut region, which would be an area where higher proportion of the body's fat tissue would be located. Therefore it can be inferred that the LMW fatty acids signatures can be associated with the presence of human remains within a burial and that the abundance of the components will be increased around the gut region of the remains.

The high levels of LMW fatty acids at the feet suggest that organic material from around the remains has been redistributed and migrated to the feet. The redistribution of the organic material is likely to be due to the movement of water through the burial. The presence of a natural spring that passes along the burial and location of the burial on a slope may have provided conditions that would allow for the transportation and redistribution of soil particles which were eventually deposited it at the foot end of the burial

3.3. Basly (LCB)

3.3.1. Site description and sampling

Basly is a small town located in the Normandy region of France. The excavation site, originally discovered by a farmer, was run by the French Ministry of Culture. Excavation during the first season (2010) uncovered a Bronze Age settlement including human burials. During the second season a series of graves were identified close to the settlement. Excavation of the grave site uncovered a total of thirteen burials dating from around the 10^{th} to 11^{th} century. Sampling took place during the 2011season.

Figure 3.13: View of the 10th to 11th century grave site at Basly looking east (photograph by Brendan Keely). Originally in colour.

The results discussed here are from grave LCB 2121 (Figure 3.14), which was a supine burial of an adult male. The remains were positioned with the arms by the sides and the feet positioned close together. Samples were collected from 17 sampling points from around the remains along with three controls C1-C3 in accordance with the InterArChive sampling strategy (Chapter 1). There was very little in the way of organic soil and the parent material was mainly Jurassic limestone with a free-draining calcareous soil. Samples taken from grave LCB are presented in Table 3.2 and Figure 3.15.

Figure 3.14: Grave LCB 2121 before sampling took place. Skeletal remains are supine and the arms are place at the side of the remains (photograph by Brendan Keely). Originally in colour.

Table 3.2: Samples collected from grave LCB 2121, including sample numbers and the locations from which they were collected

Sample number	Sample position
C1	Control taken from edge of the grave
C2	Control taken above the rib area
C3	Control taken next to the skull
1	Skull
2	Pelvis
3	Left foot
4	Right foot
5	Left shoulder
6	Right shoulder
7	Left arm
8	Right arm
9	Left pelvis
10	Right pelvis
11	Left knee
12	Right knee
13	Left hip
14	Right hip
15	Chest
16	Right hand
17	Left hand
A1	Inside the skull
A3	Sternum, between the vertebrae
A5	Under the last four vertebrae
A7	Sacrum

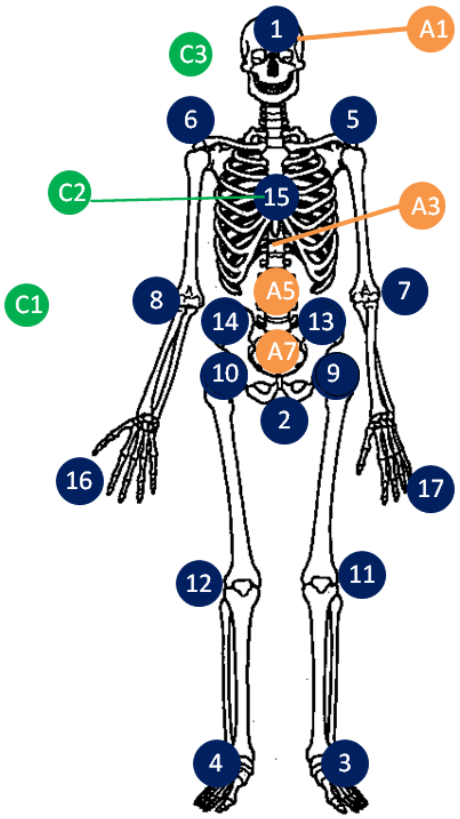

Figure 3.15: Samples collected from LCB 2121, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive high intensity sampling strategy are labelled in blue and additional samples are labelled in orange. Originally in colour.

3.3.2. Results and Discussion

3.3.2.1. Bulk soil analysis

Elemental analysis of the samples revealed that the total carbon contents of the samples ranged from around 6.5-9.5% (Figure 3.16) with total organic carbon values in the range 1.0-3.5%, suggesting the presence of a large amount of inorganic carbon (carbonate) in the samples. The levels of organic and inorganic carbon are greater than for the samples from the Heslington East site. The presence of inorganic carbon was evident in the graves during sampling in the form of large amounts of limestone fragments in the grave (Figure 3.14). The soils showed low amounts of nitrogen with similar values being recorded at all

sampling points (~ 0.2%) and there was no detectable sulfur in the soil. All samples exhibited hydrogen to carbon ratios which greatly exceed the maximum hydrogen content possible for fully saturated organic matter. As with Heslington East, the high hydrogen values most likely reflect hydrogen liberated by the de-watering and/or dehydroxylation of clay minerals during analysis.

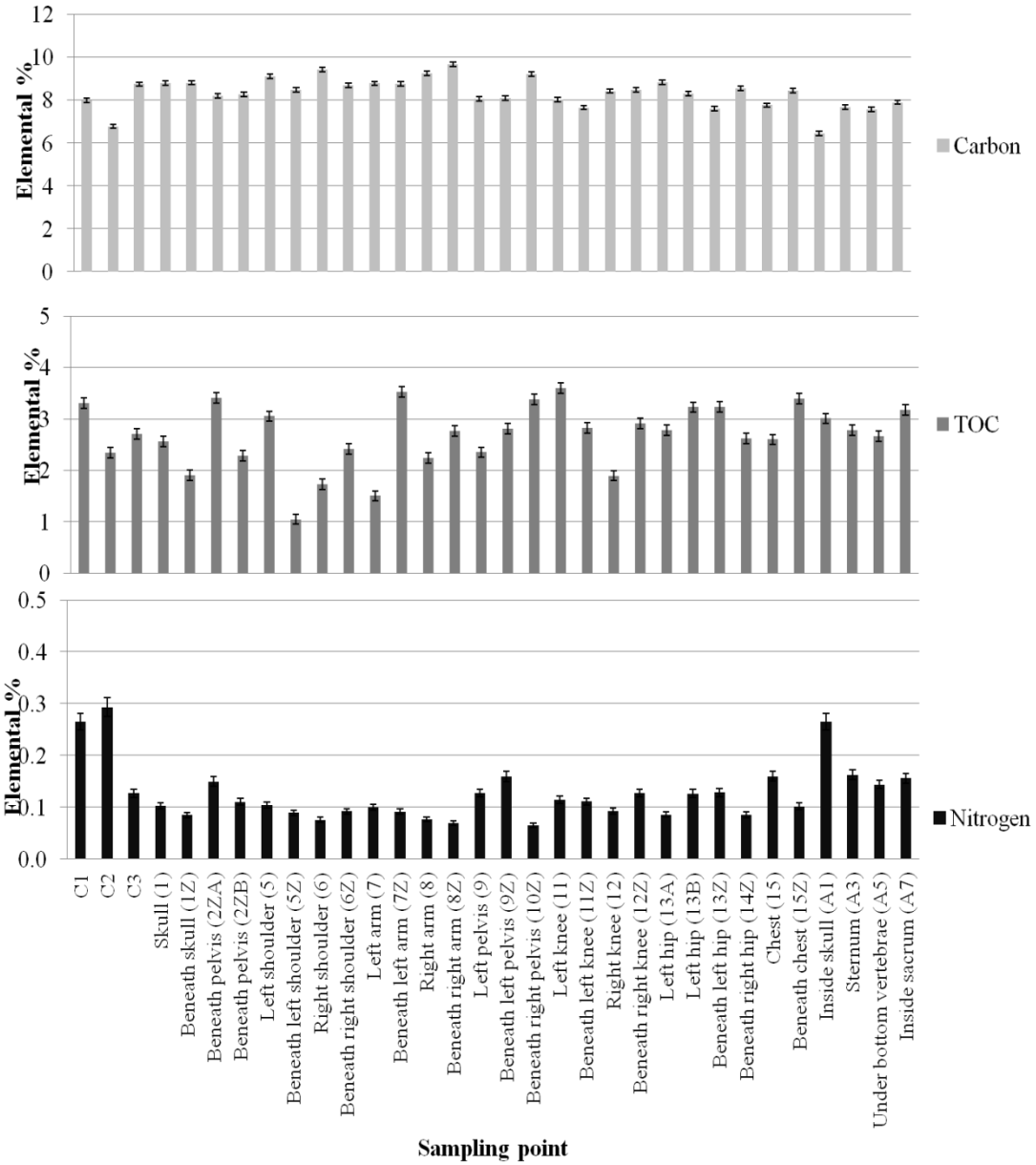

Figure 3.16: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soil samples from LCB 2121. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

The weight of total lipid extract normalised to TOC (Figure 3.17) reveals lower proportions of extractable organic matter than for Heslington East. Notably, the grave soils from Heslington East had lower TOC levels than those from Basly. The higher levels of TOC and the lower levels of extractable organic matter suggest that a greater proportion of the organic matter present in the samples from the Basly site is polymeric and therefore not extractable. Polymeric material in the soil could include the biopolymers suberin and cutin; suberin is found in the protective layers of plant roots, whereas cutin is found in the wax layers of plant leaves and bark (Kolattukudy, 1975). Roots were observed in the top layers of the soil and it is possible that degraded root material is present in the lower layers of the soil which would suggest the polymeric material could be suberin. Other polymeric materials found in rot material include lignin and cellulose which could also contribute to the high levels of organic carbon. Notably, the micromorphological analysis of the soils found a large amount of root material (Carol Lang unpublished results). All sampling points gave similar or lower amounts of extractable organic matter than the control samples with the exception of sampling points from the left arm (7), inside the skull (A1) and the sternum (A3), which all exhibit higher levels of extractable organic matter than the control samples. The amount of extractable organic material in the controls show a decrease as the position of the controls moves closer to the remains, therefore the top soil is likely to have a vegetation cover which decreases through the soil layers.

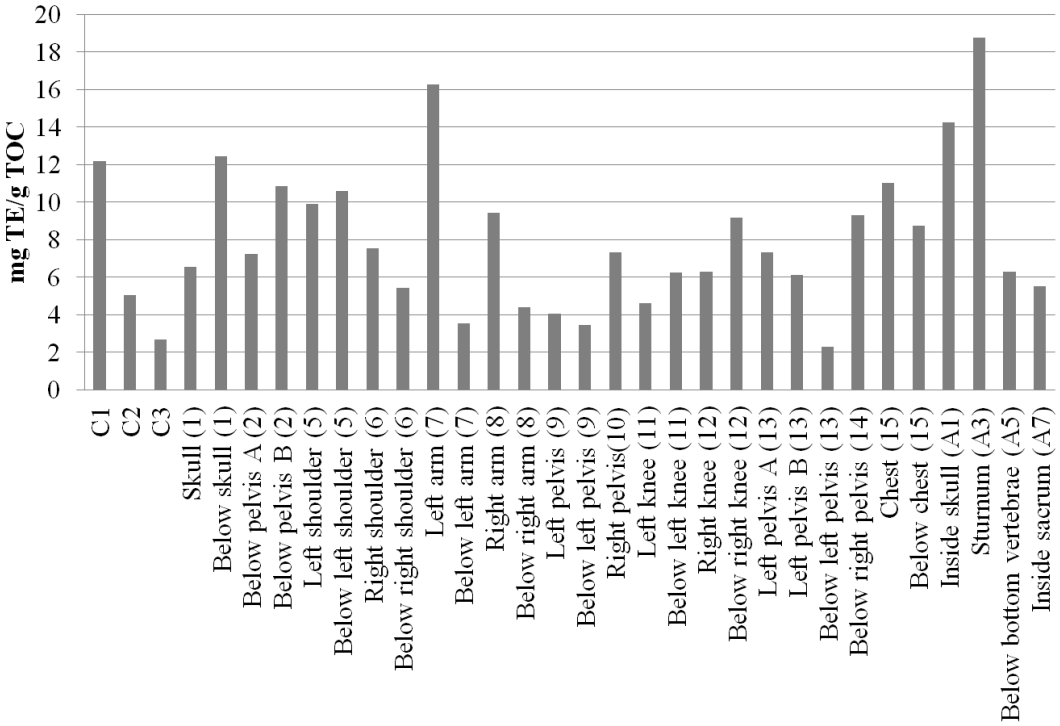

Figure 3.17: Solvent extractable organic matter in the soil samples from LCB 2121 normalised to total organic carbon content.

3.3.2.2. Total extracts

GC analysis of the total lipid extracts showed very low levels of extractable lipids. The GC chromatograms of sampling points under the skull (1), right shoulder (6), right arm (8), left hip (9), under the left knee (11), under the right knee (12) and under the lumbar vertebrae (A5) contained no detectable lipid components. The other sampling points displayed similar lipid components to those present in the C1 and C2 samples.

The control samples and samples that contained detectable components contained *n*-alkanes and *n*-alkanols relating to plant wax material. As the components relating to plant waxes are present in the control samples it is likely that they are part of the background soil organic matter.

In addition to HMW fatty acids, the C1 sample also showed the presence of LMW fatty acids (C16:0 and C18:0). The LMW fatty acids were accompanied by their unsaturated counterparts (C16:1, C18:1 and C18:2) along with C16 branched chain components. The branched chain components included fatty acids with the branched methyl group present on the penultimate carbon (iso) and the ante-penultimate carbon (anteiso). Identification of the branched carbons are very similar to straight chain fatty acids (Figure 3.5) when analysed my MS but they can be identified through the M-15 peak, which related to the loss of the methyl branched group. These peaks, however, were only present in the C1 sample and are therefore likely to represent material present within the top soil and have no relevance to the background organic matter deeper in the grave.

The extracts contained stigmasterol, sitosterol and campesterol which are all sterols present in plants (Bergmann, 1953; Jambu *et al.*, 1993). Cholesterol, a major animal sterol (Jones *et al.*, 1999), was identified at low levels in the C1 and C2 controls and in all other of the sampling points apart from C3. All the sterols were identified using MS, Figure 3.18 shows an example of a cholesterol TMS derivative and its typical mass spectrum. The molecular ion is seen at *m/z* 458, with a loss of 15 *m/z* units representing a loss methyl from the trimethylsilyl group. The loss of 90 *m/z* units represents loss of the trimethylsilyloxy group (Diekman and Djerassi, 1967).

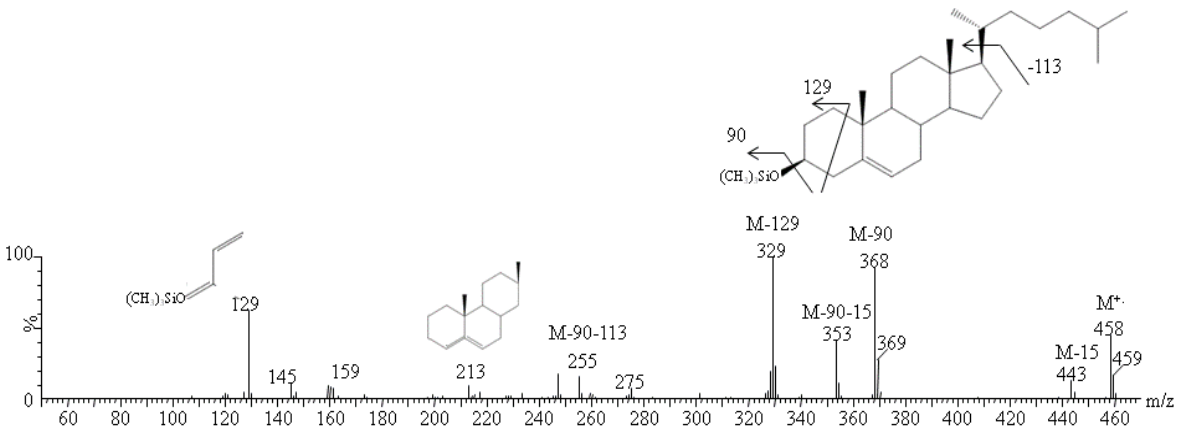

Figure 3.18: Example of cholesterol mass spectrum.

The other sterols mentioned show losses of 90 and 129 *m/z* units as seen in the cholesterol mass spectrum (Figure 3.18) but due to their differing side chains the loss of 113 that is seen for cholesterol is not observed in the other sterols. Campesterol has an additional methyl group in its side chain and therefore the mass chromatogram will show loss of 128 *m/z* units (Figure 3.19). Both sitosterol and stigmasterol have an additonal ethyl group on the side chain but stigmasterol also has a double bond present in the side chain therefore sitosterol and stigmasterol display losses of 142 and 140 *m/z* units, respectivly (Figure 3.19).

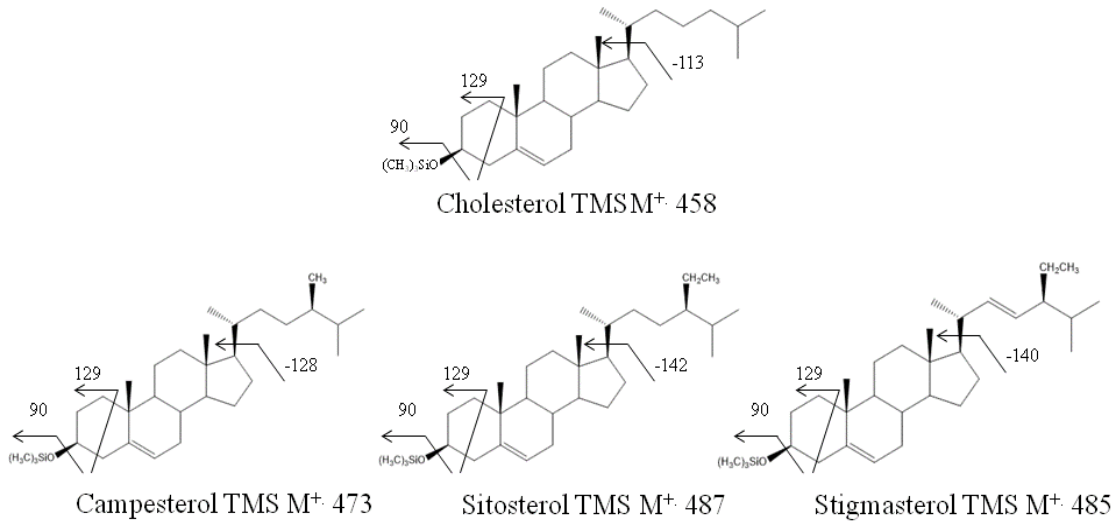

Figure 3.19: Structure of cholesterol and other plant sterols showing molecular ions and typical mass losses during mass spectrometry.

The C3 control sample contained fewer components than C1 and C2 and the components that were present were in lower abundance. Furthermore, C3 did not contain any of the plant sterols that were seen in C2 and C1, or the fatty acid components that were present in the C1 and C2 extracts. This suggests that the fatty acids and plant sterols are mainly associated with the top soil and are not a significant component of the lower grave fill.

There was little variation amongst the different sampling points, with the lipid components relating to plant derived material or not being detected suggesting that there is no particular input that can be ascribed to the buried remains. There is very little in terms of extractable lipid components present within the soils analysed and that is also reflected in the amount of extractable organic matter (3.2.2.1) that was calculated.

The low levels of extractable organic matter could be due to the transportation and redistribution of soil particles to lower levels within the burial owing to the absorption of lipids to clay particles. Several laboratory and field experiments have proven that water movement is responsible for the transportation of small soil particles on a downward slope (Zhang *et al*., 2006; Wang *et al*., 2014). Although the graves at the Basly site were not on a slope the grave was cut into well draining limestone rock, which would have provided good drainage and allowed for the smaller soil particles and associated organic matter to be readily transported downwards away from the grave. Such movement of organic matter away from the skeletal remains would account for some of the sampling points displaying little or no lipid components in the chromatograms.

The C2 and C3 samples contain similar components to that of C1 (top soil) but in lower abundance levels, suggesting that the input to the soil was similar over time but that the degradation of organic matter has, over time, reduced the levels of organic matter in the lower layers of the soil where the C2 and C3 samples were collected.

3.3.3. Conclusion

There is very little in terms of extractable lipids present in the soil, this is reflected in the GC analysis of the total extracts in addition to the levels of extractable organic matter observed in the bulk soil analysis. The organic carbon levels were similar to that seen in Heslington East, however, this was not reflected in the levels of extractable organic matter suggesting that the organic carbon present in

the soil is non-extractable and likely to relate to suberin and lignin biopolymers found in plant roots (Kolattukudy, 1970).

The low levels of extractable organic carbon and lipid components observed are likely to be due to the nature of the burial site; the site was mainly rocky material which would allow for the water borne transportation of the smaller soil particle to the deeper soil levels. These smaller soil particles are generally rich with organic matter (Gregorich *et al*., 1998) and would account for the significant loss of organic matter which could be related to the interred remains within the soil. Therefore water movement can have a significant effect on a burial and can be the cause of the removal of a significant amount of organic material.

3.4. Thessaloniki

3.4.1. Site description and sampling

Thessaloniki is a large city situated on the north mainland of Greece that has a natural harbour opening onto the Aegean Sea. The cemetery excavation was organised by the Greek Ministry of Culture and was located at the city's rail station and part of the metro system. The excavation in 2010 (Figure 3.20) involved one section of the rail line and contains both Hellenistic and Roman burials (Acheilara, 2010). The site was situated on a Holocene calcaric fluvisol type of soil, which is usually found in lake or marine deposits (Ghilardi *et al.*, 2008).

Figure 3.20: Archaeologists at work at the Thessaloniki site (photograph by Brendan Keely). Originally in colour.

The grave discussed here is Tφ182, a Hellenistic burial (c. 250-300 BC) containing an adult male (Figure 3.21). The remains, arranged in a supine configuration, were complete and exhibited good bone preservation. There was no evidence that a coffin had been present but grave goods, in the form of two ceramic vessels and two bottles, were present to the left hand side of the grave. The feet were quite close together, in an upright position and the hands placed by the side of body possibly suggesting a shrouded burial. Evidence suggestive of organic deposition below the sacrum was recorded during sampling.

Figure 3.21: Grave NSS Tφ182 an adult male supine burial with hands placed down the side of the remains (photograph by Brendan Keely). Originally in colour.

High intensity sampling of the remains was carried out, 17 samples were taken from around the remains using the sampling strategy set out by the InterArChive team (Figure 3.22; Table 3.3). Controls C2 and C3 were also collected along with four additional samples; under the skull (A1), under the right iliac blade (A2), under the chin (A3) and under the sacrum (A4). Sample C2 was collected from the subsoil above the abdominal area and sample C3 taken from just above the abdomen, level with the exposed feet.

Table 3.3: Samples collected from grave NSS Tφ182 , including sample numbers and the locations from which they were collected

Sample number	**Sample position**
C2	Control from subsoil above abdomen
C3	Control from just above abdomen
1	Skull
2	Pelvis
3	Left foot
4	Right foot
5	Left shoulder
6	Right shoulder
7	Left arm
8	Right arm
9	Left pelvis
10	Right pelvis
11	Left knee
12	Right knee
13	Left hip
14	Right hip
15	Chest
16	Right hand
17	Left hand
A1	Under the skull
A2	Under iliac blade
A3	Under the chin
A4	Under the sacrum

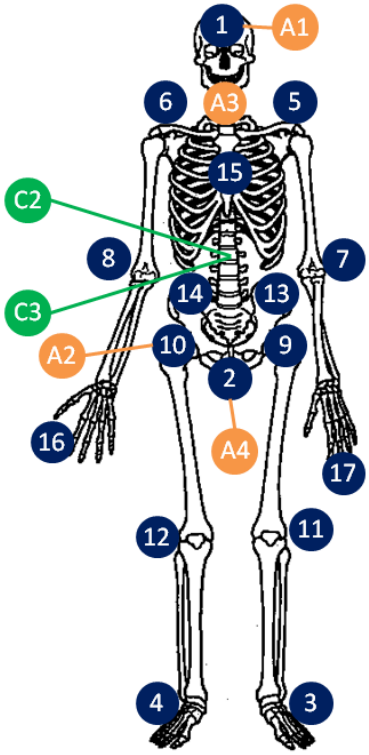

Figure 3.22: Samples taken from Thessaloniki grave NSS Tφ182, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive sampling strategy are labelled in blue and additional samples are labelled in orange. Originally in colour.

3.4.2. Results and Discussion

3.4.2.1. Bulk soil analysis

Elemental analysis was performed on the soil samples from grave Tφ182 (Figure 3.23). Analysis of each sampling point shows that all of the samples and controls have no detectable nitrogen present (Figure 3.23). The samples display very similar levels of TOC (~0.2%) at all points around the skeletal remains and in the controls and are lower than those of Heslington East and Basly (~0.5 and ~5% respectively). All samples exhibited hydrogen to carbon ratios which greatly exceed the maximum hydrogen content possible for fully saturated organic matter most likely reflecting hydrogen liberated by the de-wetting and or

dehydroxylation of clay minerals during the analyses. There was no detectable sulfur in these soil samples.

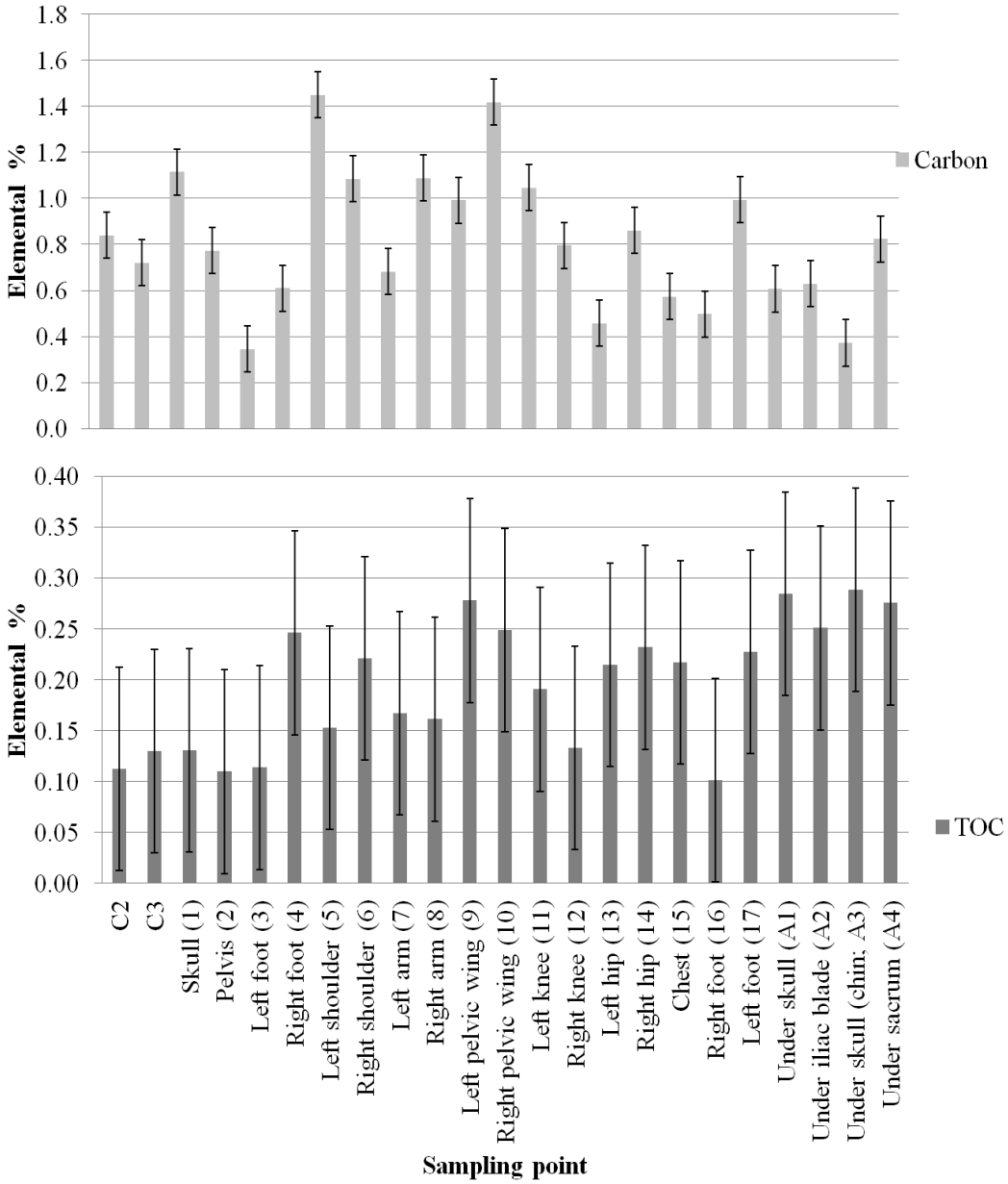

Figure 3.23: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soil samples from NSS Tφ182. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

The weight of the total lipid extracts normalised to the TOC content (Figure 3.24) shows that the majority of samples have similar amounts of extractable organic matter to the control samples and the samples extracted from the Basly site, with the exception of samples from the skull (1), pelvis (2), the shoulders (5 and 6), right arm (8), left pelvic wing (9), left knee (11) and the right hand (16), which yielded higher levels. High levels of organic matter are observed across the top half of the torso and skull, suggesting a greater concentration of organic matter around the upper half of the remains. Notably, the chest sample (15) contains relatively low amounts of organic matter (similar to the controls). This may be due to the sample having been taken from above the sternum where the bulk of the organic matter derived from the body would most likely have translates downwards under the influence of gravity and water movement..

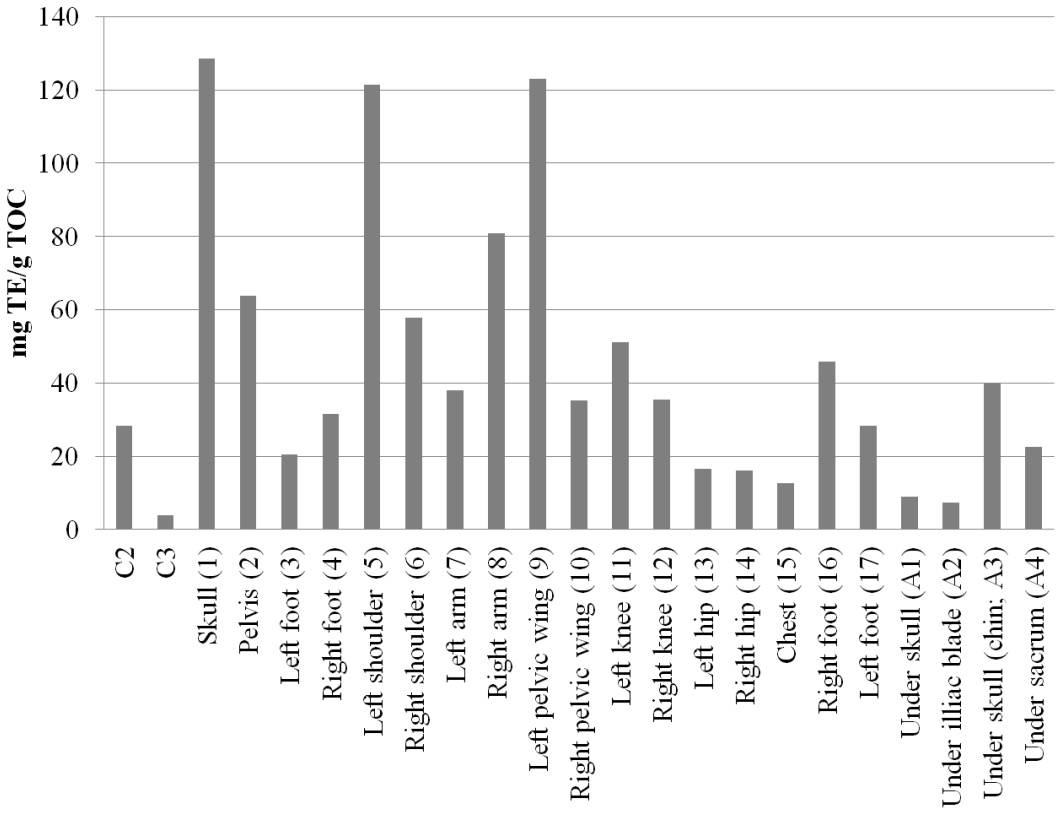

Figure 3.24: Solvent extractable organic matter in the soil samples from Tφ182 normalised to total organic carbon content.

3.4.2.2. Total extracts

GC analysis of the extracts showed them all to possess broadly similar profiles. In order to analyse the samples further the total extracts were first fractionated and analysed in accordance with the InterArChive protocols.

3.4.2.3. Hydrocarbon and aromatic fractions

The GC chromatograms of the hydrocarbon fractions show variations in the lipid distributions with samples exhibiting two distinct distributions. Sampling points from the right arm (8) and the chest (15) show no detectable hydrocarbons (Figure 3.25; red). Sampling points C2, C3, pelvis (2), the right knee (12) and right pelvis (14) contain a series of *n*-alkanes (C18-C33; Figure 3.25, dark blue), the early eluting components comprising chain lengths in the range C18-C22 with an even-over-odd chain length predominance (Figure 3.25). The later eluting components, with chain lengths in the range of C23-C33, have a unimodal distribution dominated by odd carbon chain members maximising at C29. The later eluting components are present at considerably lower abundance levels than the earlier components. Sampling points 1, 3-5, 9-11, 17, A1, A2 and A4 display similar components to those of C2, C3, 2, 12 and 14 but the distributions have higher relative abundances of LMW (C16-C22) *n*-alkanes (Figure 3.25; light blue). The remaining grave soil samples exhibit distributions which contain high levels of the LMW (C14-C20) *n*-alkanes, with *n*-C16 being the most abundant component and *n*-C20 being the least abundant (Figure 3.25; green).

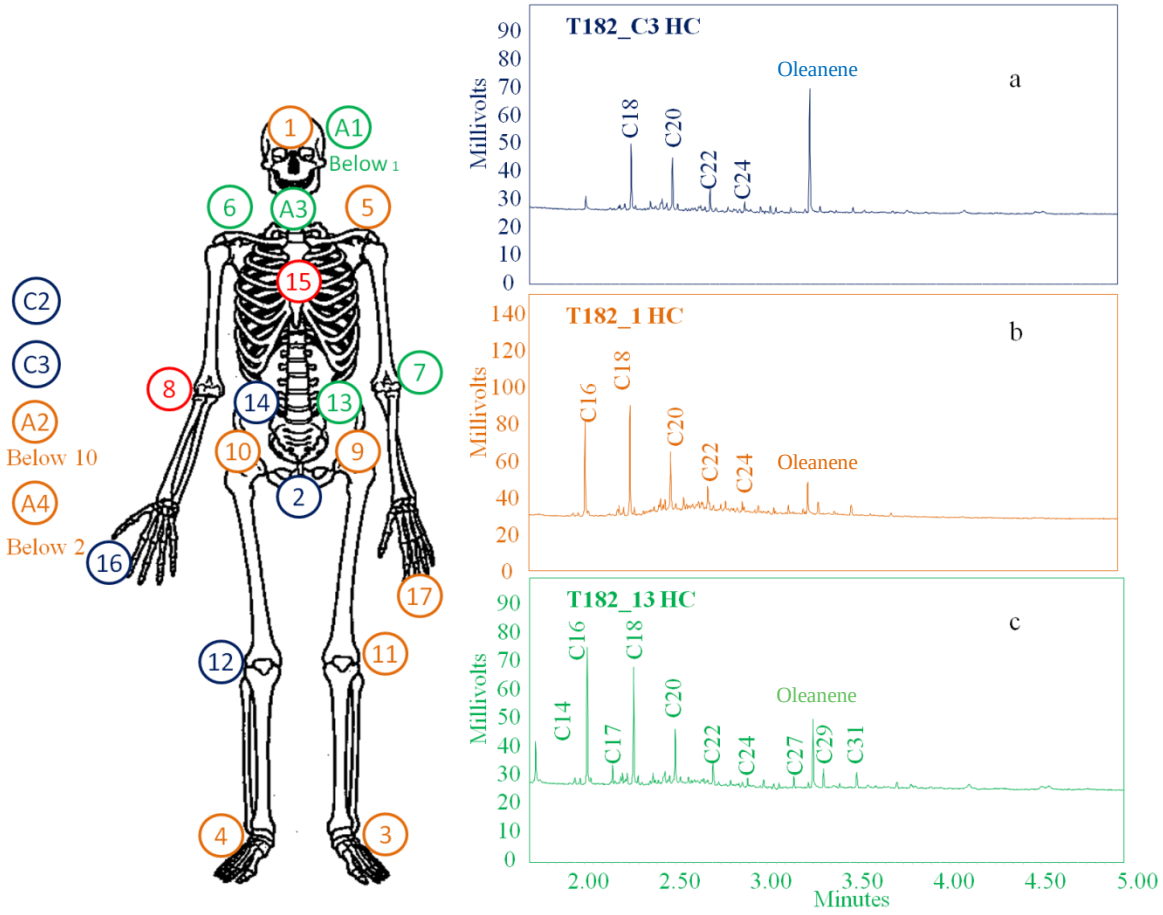

Figure 3.25: GC chromatograms of the hydrocarbon fractions and classification of the sampling points in grave NSS Tφ182 according to their distinctive *n*-alkane profiles. GC chromatograms are from sampling points a) C3, chromatograms displaying similar distributions are labelled in blue on the skeleton image, skull (1); b), chromatograms displaying similar components are in orange and the left hip (13); c) chromatograms displaying similar distributions are labelled in green on the skeleton image, which typify three distinct classifications into which the lipid extracts from all sample points for grave Tφ182. Sampling points labelled in red show no detectable hydrocarbon components. Originally in colour.

The hydrocarbon fractions for all sampling points also displayed one large peak at around 3.3 min. GC-MS analysis of the fractions allowed this component to be identified as olean-13-ene (Figure 3.26).

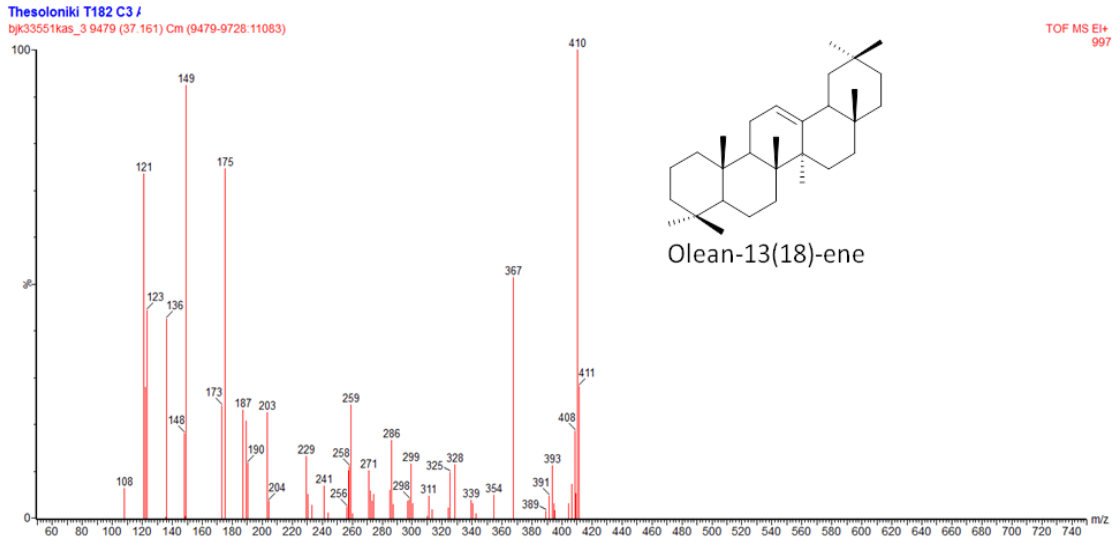

Figure 3.26: Mass spectrum of component eluting at 3.3 min, olean-13(18)-ene. Originally in colour.

Distributions of the HMW *n*-alkanes suggest a plant source but the LMW *n*-alkanes have not been described as significant components of plants. The LMW *n*-alkanes are occur in bacteria (Ladygina *et al*., 1996) but could also be formed through the microbial reduction of free fatty acids C14-C18 (Naccarato *et al*., 1972; Day, 1978). C14-C18 fatty acids, from which these *n*-alkanes could be formed, have been observed in degraded human tissues (Evershed and Connolly, 1988; Gulaçar *et al*., 1990; Buckley *et al*., 1999). The relative abundances of the microbial input were greater at sampling points C3, 1, 3-5, 7, 9-11, 16-17 and A1-A4, suggesting that these components are likely to have derived from the degradation of body tissues. The C3 was collected from just above the abdomen quite close to the remains so it could contain material that does related o the burial

The low levels of later eluting components indicate a low contribution from plant-derived organic matter. In addition, high levels of olean-13(18)-ene were identified in all of the samples. Olean-13(18)-ene is a triterpenoid formed from the bacterial degradation of β-amyrin, a higher plant triterpenoid (Killops and Killops, 2005; Rullkotter *et al*,. 1994). Olean-13(18)-ene formation has seemed to

favour lacustrine-fluvial/deltaic sediments (Adedosu *et al*., 2010; Eneogwe *et al*., 2002), the site was based on a natural harbour and the soil type was similar to that found in lake or marine deposits (3.4.1). It is therefore possible that the presence of olean-13(18)-ene is likely to be a component of the background soil organic matter as it is abundant in similar levels throughout all of the sampling points.

3.4.2.4. Medium polar fractions

The extracts contain low levels of plant-derived components such as n-alkanols and sterols that represent the soil background organic matter.

3.4.2.5. High polar fractions

The GC chromatograms of the high polar fractions display similar distributions at all of the sampling points with a dominant contribution from saturated C14:0-C18:0 fatty acid and unsaturated C16:1 and C18:1 FAMEs. Notably, some fatty acid silyl esters (FASEs) were present in low levels due to incomplete methylation of fatty acid components during treatment with diazomethane and subsequent silylation of remnant free acids with BSTFA. In addition to the free fatty acid esters, a C18 mono acyl glycerol (MAG) was identified, occurring in low abundance. The MAG could represent a degradation product of cell membrane phospholipids or triacylglycerols via hydrolytic cleavage of their acyl moieties and/or phosphate head groups. The LMW FAMEs, FASEs and MAGs are present in the controls, suggesting that the form part of the soil background.

All samples apart from C2 contained cholesterol. Cholesterol is a major animal sterol (Bull *et al*., 1999a), though it occurs to a lesser extent in plants (Jones *et al*., 1999). Cholesterol has been used as a marker for human activity (Bethell *et al.*, 1994) and it has also been found in archaeological bone (Evershed *et al.*, 1995), the tissue of mummies (Buckley *et al.*, 1999) and bog bodies (Evershed and Connolly, 1994). Its absence from the C2 control suggests that the cholesterol is not a significant component of the background soil organic matter and may

originate from the human remains. The presence of cholesterol in C3 could be due to the proximity of this control (taken from just above the abdomen on a level with the feet) to the skeletal remains or indeed to dispersed remains within the soil of the graveyard.

There is very little variation amongst the sampling points from around the remains and in the controls in terms of the fatty acid profiles and their abundance, suggesting that the lipid markers present in the samples are related to the soil background matter. However, the LMW fatty acid profiles are very similar to the fatty acid profiles that have been observed in tissues from mummies (Gulaçar *et al*., 1990; Buckley *et al*., 1999), bog bodies (Evershed and Connolly, 1988) and adipocere (Forbes *et al*., 2005) and these profiles are generally not observed for plant sources, therefore these signatures may relate to the presence of the remains. The presence of the LMW fatty acids in all of the samples, including the controls, may be due to the displacement of the fatty acid components, which has been previously observed in the Heslington East grave. However, the movement of water in that grave was on a downward slope with organic matter being transported towards at the feet end of the burial where there was a higher concentration of organic matter. It is known that groundwater levels have fluctuated over the history of the site, inducing changes in the soil in the lower levels of the graveyard where the earlier burials occur. Therefore the Thessoloniki burials were subjected to water moving up and down through the burial soils thus likely providing a more significant dispersion of the small soil particles and associated organic matter throughout the burial.

Notably, micromorphological analysis was carried out by members of the InterArChive archaeology team on samples from C2, C3, skull, pelvis hands and feet. The presence of clay coatings has been observed throughout the micromorphological samples within this grave (Helen Williams unpublished results). The presence of clay coatings in soils may arise from a process of alleviation, which is controlled by the movement of water (Gunal & Ransom 2006). The micromorphological evidence for water movement provides further

support for fluctuation in the level of the water table. Consequently, the redistribution of organic components through movement of substantial amounts of water through the grave soil is the most likely explanation for the similarities in the abundance levels throughout the grave

Such hydrodynamic processes cause movement of fine sediment grains, hence the organic material associated with the sediment could also be translocated (Lloyd *et al.*, 2012). Furthermore, although lipids are generally hydrophobic some fatty acids are sparingly mobile in water due the polar nature of the carboxyl group. The LMW fatty acids have smaller octanol-water partition coefficients than HMW lipids which would lead to higher relative abundances of the LMW fatty acids in dissolved organic matter. It is possible that the changes in water level may partly explain the presence of the LMW fatty acids in the control samples. Fluctuations in water levels may have carried organic matter derived from the body upwards as well as downwards which could contribute to the complicated nature of the high polar samples.

Notably a Roman grave analysed from the same site (Tφ178; InterArChive unpublished results; Figure 3.27) displayed less complicated extracts and distinct differences in the total extract profiles of the samples around the skeletal remains. This particular grave included a tiled base, which could have impeded vertical fluctuations in the water level within the grave, thus reducing the movement of organic material within the grave itself. The remains had also been encased in mortar, which may have further prevented movement of the organic matter through water movement.

Figure 3.27: Grave Tφ178 from the Thessaloniki site. The grave has a tiled floor, which could prevent the fluctuation of water within this grave (photograph by Brendan Keely). Originally in colour.

3.4.3. Conclusion

The GC analysis of the hydrocarbon fractions has shown an increase in LMW *n*-alkanes in samples collected from around the remains. These distributions are not generally present in plant sources as we have previously seen in other soils from Heslington East but are known to be typical of bacteria. The *n*-alkanes include C14, C16 and C18 chain lengths which could also be formed from the microbial reduction of the fatty acids C14, C16 and C18 that are major components of degraded adipose tissue. The higher levels of LMW *n*-alkanes are focussed around the skeletal remains and likely reflect their origin from the remains.

Water movement is a feature within this burial site and, through micromorphological analysis, it was determined that it affected this particular burial. Continuous water fluctuation could transport soil particles throughout the burial thus transporting soil around the remains and controls, unlike the Heslington East burial where re-deposition of the soil particles concentrated in one particular area. Therefore, the analysis of this grave suggests that fluctuation

of groundwater has dispersed soil particles and associated organic matter within the grave.

3.5. Conclusions

In this study several GC amenable compounds have been observed within the soil, a high proportion of which are related to plant-derived material naturally present within the soils (as indicated by occurrence of these components in the controls). A significant overprint of background soil organic matter is to be expected for the analysis of grave soil samples as opposed to preserved tissue samples from mummies or bog bodies, for example. However, in both the graves form Heslington East and Thessoloniki there are high proportions of LMW fatty acids including saturated components with C14-C18 chain lengths and unsaturated C16:1 and C18:1 components.

The distributions of these fatty acids are not associated plant sources and are similar to those which have been observed in extracts from the tissues of mummies (Gulaçar *et al*., 1990; Buckley *et al*., 1999), bog bodies (Evershed and Connolly, 1988) and are the major components in adipocere (Forbes *et al*., 2005). Fatty acids are known to be formed through the hydrolysis of TAGs that are in the major lipid component of adipose tissue (Forbes *et al*., 2005), therefore these free fatty acids are likely to be largely formed from the degradation of fatty tissue. In addition some of the components, such as C16:0, C16:1 and C18:1, are known to be related to bacteria activity. These components are therefore likely to be present within the soil due in part to the microbial degradation of fatty tissue from the remains. Notably, the samples taken from Heslington East display an increase in the abundance of LMW fatty acids around the remains, particular the pelvic region. The pelvic region has a greater abundance of fatty tissue and this is a position where significant bacterial input from the gut microflora is expected, which would explain the elevated levels of components relating to fatty tissue and bacterial activity. This further provides evidence that these LMW fatty acids are present due to the interred human remains.

Evidence of bacterial activity was also observed for the Thessaloniki burial studied: the hydrocarbon fractions contain bacterially-derived *n*-alkanes in the range C14-C18. As with the fatty acids observed in samples taken from the Heslington East grave these LMW *n*-alkanes increase in abundance around the remains and in particular the pelvic region. Therefore this suggests that the LMW *n*-alkanes are associated with the interred remains.

The main aim of this Chapter was to investigate the effect of water movement on the organic residues preserved in grave soils. Water movement was a common feature throughout each of the burials within this Chapter though it affected the levels of extractable organic matter in different ways, which reflects the unique nature of each of the burials. The samples collected from the Basly site showed very little in terms of extractable organic matter in all of the sampling points. This was likely to do grave being cut into well draining limestone where smaller soil particles could be easily transported through the grave with rainwater, thus removing high levels of organic material away from the remains and allowing organic material from the top soil to be deposited around the remains. This would account for the little variation observed in lipid signatures between the samples taken around the body and the controls taken from the upper soil layers.

The Heslington East burial showed an increase in levels of fatty acids relating to the remains around the feet area. The burial was on a slope and along the line of a natural spring, which may have allowed water to flow through the burial. The water movement is likely to have been responsible for the transportation of small soil particles and associated organic matter through the burial and increase in the levels of fatty acids deriving from the remains at the feet.

The Thessoloniki burial showed little variation within the fatty acid distributions, this has been attributed to the redistribution of soil particles and organic matter due to fluctuating ground water levels that were feature of this burial.

GC analysis of the soil samples revealed components that are likely to be associated with the buried remains can be found within burial soils and that these

components can increase in certain samples close to the remains, in particular the pelvic regions. The study also illustrates that water movement can have an effect on the organic matter through transportation and re-deposition of organic matter depending on the nature of the burial. Fluctuating waters appears to redistribute soil amongst all the sampling point whereas burials that are positioned on a slope can be subject to erosion where soil can be deposited at the base of the burial, if a feature of the burial acts as a barrier, or as in laboratory experiments at the base of the slope. In addition, for burials that are located within rocky well draining environments organic matter associated with soil particles can be readily lost through erosion.

CHAPTER 4. DISTRIBUTIONS AND FATE OF LIPIDS IN MEDIEVAL AND 16TH CENTURY BURIALS.

4.1. Introduction

This chapter focuses on the variability of components based on their anatomical location and vertical position (i.e. above and below) to the remains. In addition, the chapter will focus on specific signatures that relate to the buried remains and how they can reveal information about the burial. The burials discussed here have been chosen as they have all been subjected to high intensity sampling, with one of the graves having samples collected from above and below the remains. All of the graves were buried in sandy soils and were chosen to ensure that the similar soil types were analysed and compared.

The three burial sites include; a 13th century infant burial from Syningthwaite Priory (North Yorkshire), a 16th century Christian burial from Edinburgh (Scotland), and a 14th century Christian burial from Mechelen (Belgium). The burial from Mechelen was extensively sampled, including samples from above and below the skeletal remains, taken with the intention of determining which of the two sampling locations yield the strongest signatures related to the interred remains. The infant was a very young child (less than 18 months) and has been included to determine whether its smaller size and different anatomical features impact on the signatures recovered from the soil.

4.2. Syningthwaite (CGS09)

4.2.1. Site description and sampling

An excavation was carried out at Syningthwaite Priory in August 2009 (Figure 4.1). Syningthwaite is located between Wetherby (3 miles) and York (8 miles) and is the site of a former priory, which dated from around the 11th-17th century. The site is currently the location of a farm and holiday cottages.

Figure 4.1: Excavation at Syningthwaite Priory, facing south (photograph by Brendan Keely). Originally in colour.

The fields to the west of the farm complex contained features including pits and gullies dating back to medieval times, which relate to the occupation of the priory. The burials were found to the south-east of the farm in a pasture area that had been left uncultivated for 50 years. The soil had several different layers; the top layer was mainly a sandy loam (with root litter present), the layer below was more of a silty clay loam and the bottom layer, in which the burials were found, was a deep sandy clay soil. Three graves in total were found at the site, two adults and one infant, the relative positions of which are shown in Figure 4.2. The grave discussed in this chapter is G2, the infant burial.

Figure 4.2: Relative positions of the burials of two adults and an infant at Syningthwaite priory, discovered during excavation in August 2009 (photograph Brendan Keely). Originally in colour.

The skeletal remains of the infant were largely intact, though the hands, lower arms and feet were absent, either through degradation or displacement during excavation. Given the positions of the legs and arms which were tight into the body, it is thought likely that the individual was buried in a shroud. The infant was 62 cm in length from shoulder to toe and was located at a depth of approximately 76 cm below the surface. The bones were intact, but were soft and friable. High intensity sampling of soil from around the skeletal remains was carried out, with samples in the locations of the missing limbs being taken according to where the arms and feet were estimated to have been situated within the grave (Figure 4.3; Table 4.1).

Table 4.1: Samples collected from grave CSG09 G2, including sample numbers and the locations from which they were collected.

Sample number	Sample position
C2	Control from subsoil above the abdomen
C3	Control from above the skull
1	Skull
2	Pelvis
3	Left foot
4	Right foot
5	Left shoulder
6	Right shoulder
7	Left arm
8	Right arm
9	Left pelvis
10	Right pelvis
11	Left knee
12	Right knee
13	Left hip
14	Right hip
15	Chest
16	Right hand
17	Left hand
A1	Skull contents
A2	Skull contents
A3	Sacral area
A4	Eye socket
A5	Skull contents including signatures that could be worm casts
A6	Right side of the skull
A7	Nasal area
A8	Beneath skull
A9	Left side of the skull and cheek
A10	Skull contents

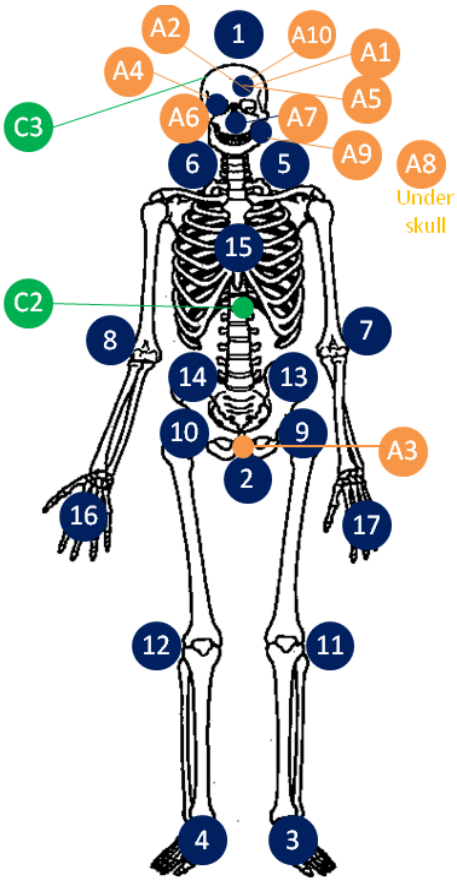

Figure 4.3: Samples taken from Syningthwaite grave CGS09 G2, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive sampling strategy are labelled in blue and additional samples are labelled in orange. Originally in colour.

4.2.2. Results and discussion

4.2.2.1. Bulk soil analysis

Elemental analysis of the soils from grave G2 revealed all samples to have % nitrogen contents ranging from 0.07-0.22% (Figure 4.4) that were generally higher around the skull area (A4, A5, A7 and A10). Although most of the sampling points show similar TOC contents (0.3-1.3%), sample A5 from inside the skull exhibited a slightly elevated value (1.8%). This suggests that there is more organic carbon material inside the skull area whereas all other soils have very similar organic carbon content to the controls.

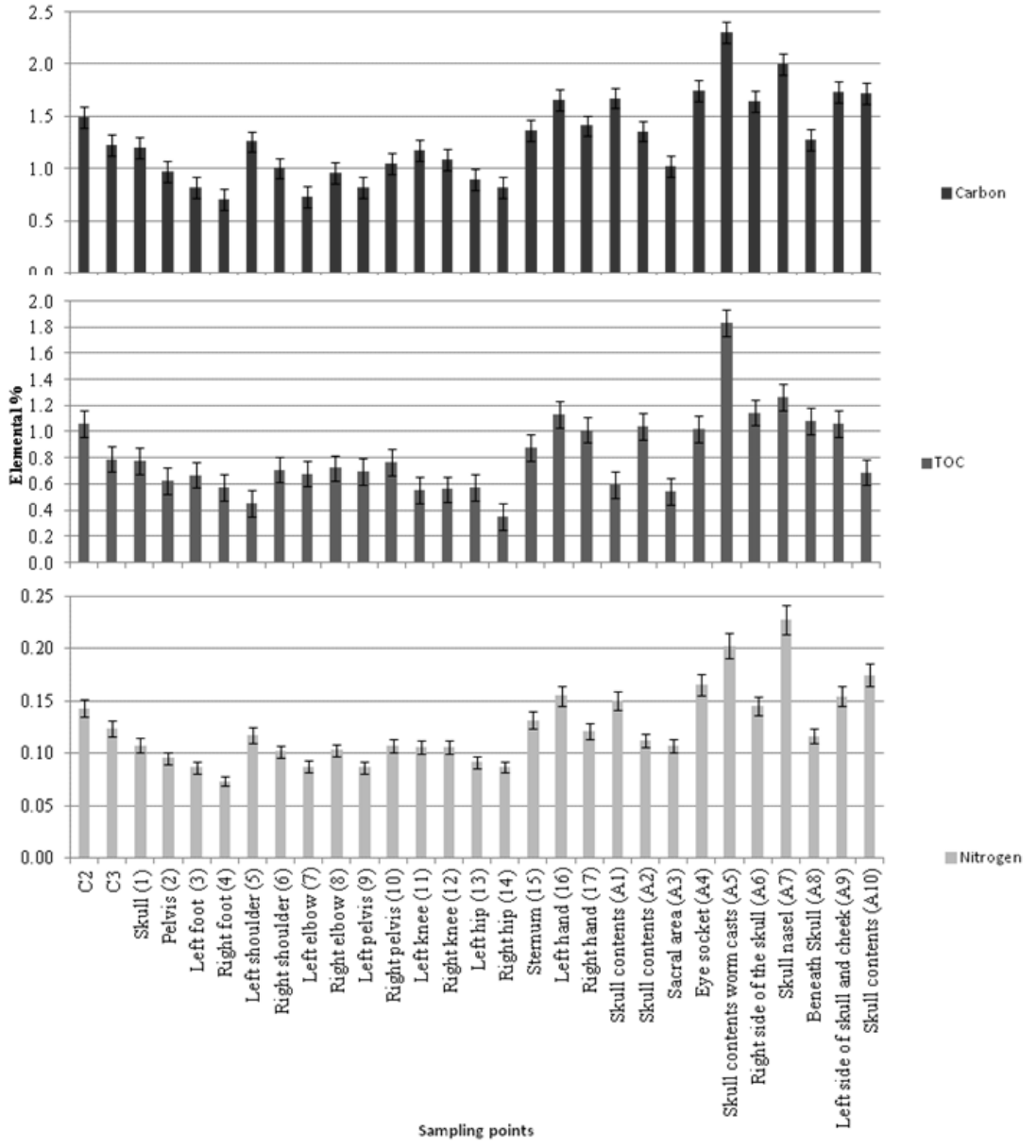

Figure 4.4: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soils from CGS 09 G2. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

The mass of total extract normalised to the TOC content shows that sampling points 1, 10, 11, 16, A5, A7 and A8 all have similar amounts of extractable organic matter to the control samples, whereas all the other positions have higher levels (Figure 4.5) The pelvic samples (13 and 14), and the skull contents (A10), contained the highest proportion of solvent extractable organic matter. The skull contents (A10) contains

higher levels of extractable organic matter than other sampling points from inside the skull, it was noted during sampling of the remains that the soil sample A10 had a different texture compared to the surrounding soil. At this stage in the analysis, it is not possible to provide an explanation for the greater proportion of extractable organic matter in A10 by comparison with the other samples from within the skull. Notably, sample A5 from inside the skull contained a low proportion of extractable organic matter, indicating this sample to comprise a greater proportion of polymeric (solvent insoluble) organic matter relative to the other sampling points.

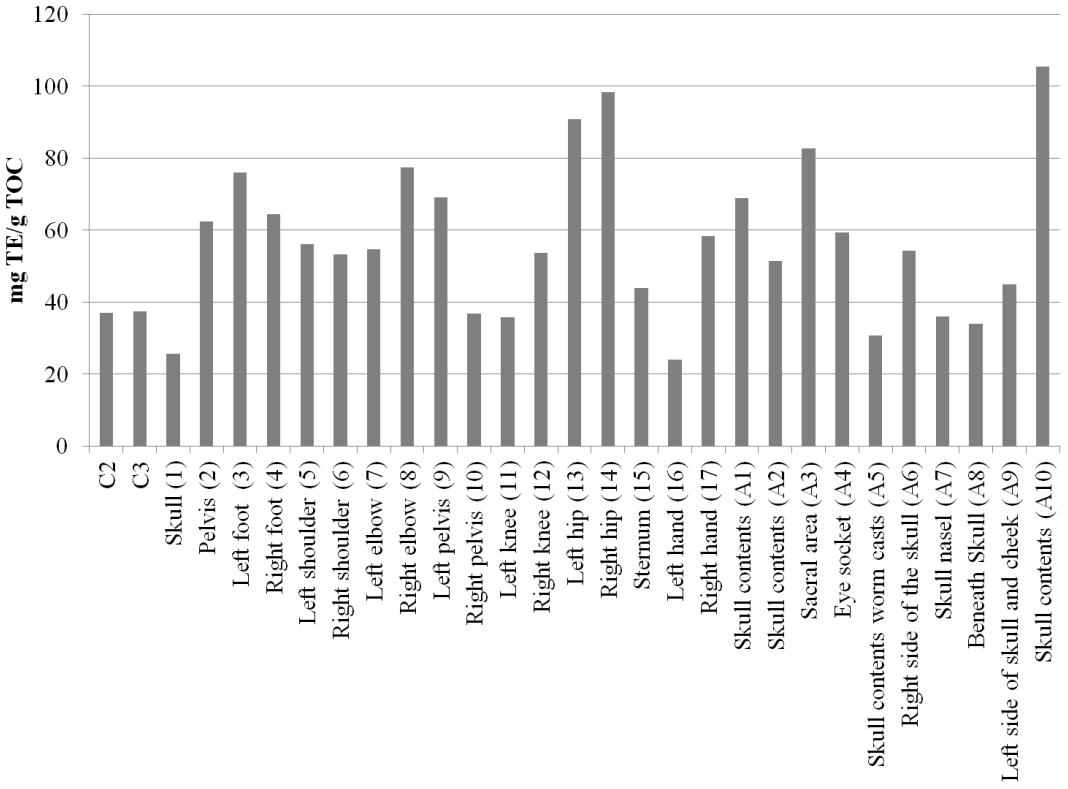

Figure 4.5: Solvent extractable organic matter in the soils from CGS09 G2 normalised to total organic carbon content.

4.2.2.2. Total extracts

GC analysis of the total extractable organic matter showed the samples to contain complex mixtures of lipids (Figure 4.6). The sampling points can be separated into three groups according to similarities in their lipid profiles. Sampling points 1-3, 5-7, A1, A2, A4 and A10 share similar profiles of components to the controls (C2 and

C3), the extracts being dominated by fatty acids and *n*-alkanols. The fatty acids elute in two distinct groups: LMW fatty acids that are ubiquitous among plants, animals, fungi and bacteria (Eglinton and Hamilton, 1967; Kolattukudy, 1970; Zelles, 1997; Ruess *et al.*, 2002) and HMW fatty acids, which exhibit distributions typical of plants (Eglinton and Hamilton, 1967). In addition to free fatty acids, C16:0 and C18:0 fatty acids were present as acyl moetites in monoacylglycerols (MAGs), which most likely represent degradation products of cell membrane phospholipids or TAGs (Fiedler and Graw, 2003). The extracts also contain a series of *n*-alkanols typical of higher plant waxes (Eglinton & Hamilton, 1967; Ambles *et al.*, 1991; van Bergen *et al.*, 1998; Jansen *et al.*, 2008) .

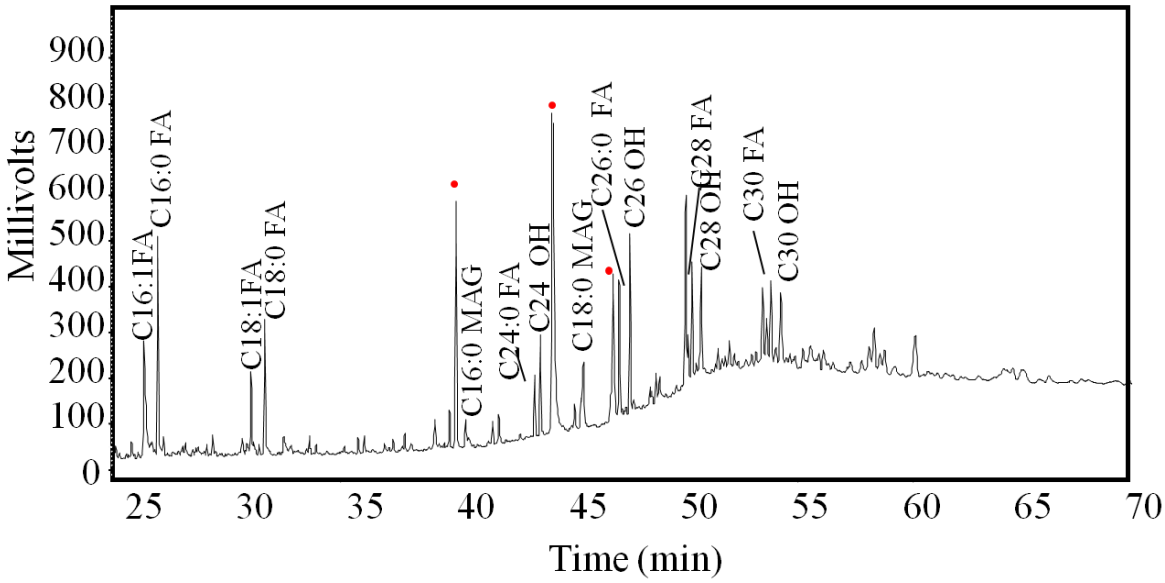

Figure 4.6: Partial GC-FID chromatogram of the total extract from the left knee (11) from CGS09 G2, displaying the different components present; FA = fatty acids, MAG = monoacylglycerol and OH= *n*-alkanol. The peaks labelled with a red dot represent plasticiser contamination from the Whirl Pack sample bags. Originally in colour.

Sampling points from the skull (1), right foot (4), right arm (8), left pelvis (9), hands (16 and 17) and nasal area of the skull (A7) contain similar levels of LMW fatty acids to the controls (~ 1 mg/g TOC), but the proportion of the C16:0 and C18:0 fatty acids are elevated (Figure 4.7; > 2 mg/g TOC). Straight chain *n*-alkanols in these

samples are consistent with profiles for plant waxes. At sampling points 4 and 8, the C18:0 fatty acid was more prominent than the C16:0 fatty acid. By contrast, the opposite situation was noted for sampling points 9-17 and A5-A9, with the C16:0 fatty acid being the more prominent of the two components.

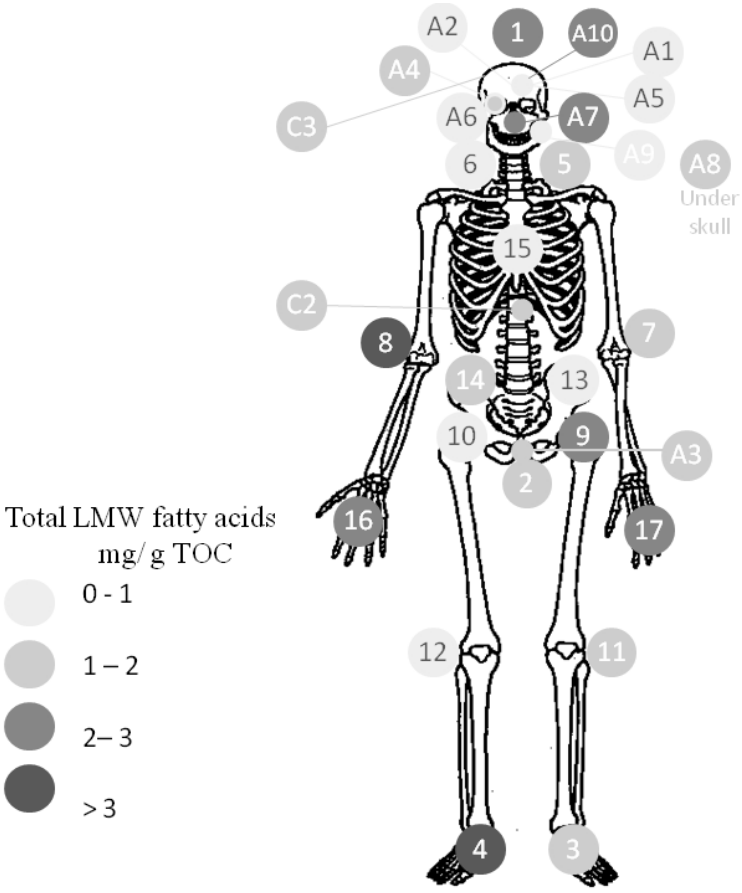

Figure 4.7: Distributions of LMW fatty acids among the sampling points collected from CGS09 G2.

In order to observe the different lipid distributions more clearly and reveal masked components, fractionation was performed on the extracts to separate groups of lipids according to their polarity.

4.2.2.3. Hydrocarbon and aromatic fraction

Sampling points C2, C3, 1-4 and 6-8 contain *n*-alkanes showing a bimodal distribution (Figure 4.8). The samples all have a lower proportion of LMW *n*-alkanes

(C14-C20) than the HMW (> C20) *n*-alkanes (Figure 4.8 green chromatogram), suggesting that there is a greater contribution from plant derived material than bacterial sources. The remaining soil samples contain higher levels of the LMW *n*-alkanes (Figure 4.8; blue chromatogram), with *n*-C16 the most prominent and *n*-C20 the least prominent. The prominence of LMW *n*-alkanes is most likely due to a contribution from the reduction of fatty acids originating from the body and/or bacterial input (Ladygina *et al*., 2006). The samples labelled in blue also contain a series of plant-derived *n*-alkanes, albeit less prominent than for the controls, ranging from *n*-C27 to *n*-C33 and centred on *n*-C31, with an odd over even predominance (Eglinton & Hamilton 1967).

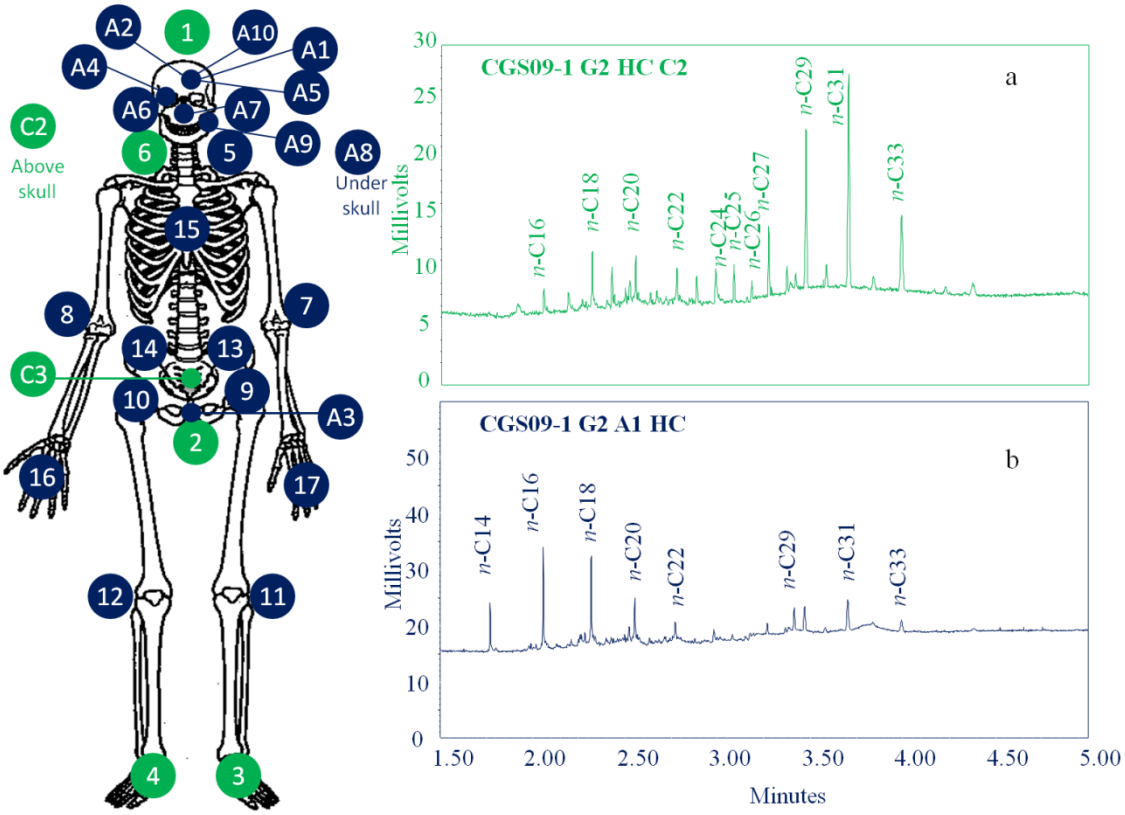

Figure 4.8: Distributions of hydrocarbons at different locations on the skeletal remains. Points labelled in green show GC-FID chromatograms similar to C2 (a). Points labelled in blue show chromatograms similar to position A1 (b). Originally in colour.

The increase in the abundance of LMW *n*-alkanes is generally observed for the samples that are closest to the torso, which is likely to contain a greater proportion of adipose tissue. Sample (1) taken next to the skull is the only sample from the skull area that has a low abundance of LMW hydrocarbons. All of the other skull samples were taken closer to the skull or from inside the skull, suggesting that the increase in LMW *n*-alkanes observed could be present due to inputs from microorganisms associated with the remains and possibly involved with the degradation of the body tissues. The other sampling points, displaying lower proportions of microbiallly-derived *n*-alkanes, correspond to the controls, the pelvis (2; between the legs) and the left shoulder (6).

No components were detected within the aromatic fractions from any of the sampling locations, suggesting that no significant aromatic related biomarkers were present within the soil.

4.2.2.4. Medium polar fractions

All soil samples, including the controls, contain a series of even carbon chain *n*-alkanols ranging from C26-C30, typical of plant alkanols (Eglinton & Hamilton 1967), as well as the plant sterols campesterol, stigmasterol and sitosterol. In addition, small amounts of cholesterol and 5α-cholestanol were identified, the latter being a common early degradation product of cholesterol. Cholesterol is primarily an animal sterol (Evershed 1993), though it can also be found as a minor component of plant lipids (Jarde *et al.*, 2009). 24-ethylcoprostanol, a degradation product of sitosterol formed in the gut of most higher animals (Leeming *et al.*, 1996), was also present in the samples. As it is present in the controls, it is most likely a component of the background soil organic matter. Given that the land was used for farming and the persistence of sterols in the environment, it is more than likely that the presence of the 24-ethylcoprostanol derives from grazing animals or manuring of the fields.

4.2.2.5. High polar fractions

All sampling points contain C16:0 and C18:0 fatty acids, and their unsaturated C16:1 and C18:1 counterparts. Significant contributions from C16:0 and C18:0 MAGs were also recognised, the latter two components being present in an approximate 1:1 ratio. The MAGs were present in all positions, except for the left foot (3), right pelvis (10), right knee (12) and right hip (14). The fractions from samples C3, the right foot (4) and the right arm (8) each show larger contributions of C16:0 and C18:0 MAGs than the other positions. All samples show similar fatty acid distributions to the controls (Figure 4.9), which are similar to the profiles reported for adipose tissue (Forbes *et al*., 2005). Although they have the same distributions, the abundance of these fatty acids increased in samples from the skull (1), right foot (4), right arm (8), left pelvis (9), hands (16 and 17) and nasal area of the skull (A7; Figure 4.9).

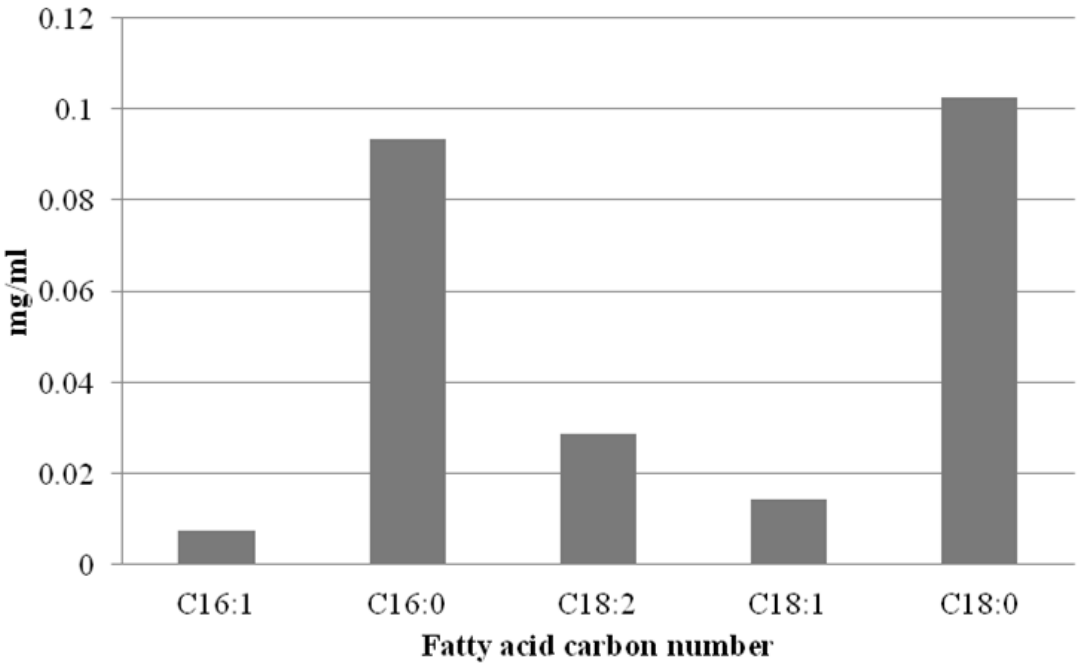

Figure 4.9: Fatty acid distributions in the C2 control sample from grave CGS09 G2.

4.2.3. Conclusions

The extracts from the infant burial G2 from Syningthwaite were dominated by components present in the controls, showing little in terms of variation amongst the

different sampling points and the controls. This could be due to a low amount of organic input from the burial itself, due to the small size of an infant's body.

The hydrocarbon fractions show some variation amongst the sampling points and controls as there is an increase in the abundance of LMW hydrocarbons in most samples from around the remains, compared to the controls. LMW *n*-alkanes are generally bacterially derived which suggests that there has been an increase in bacterial inputs to the soil as a result of the presence of the remains. There is little variation, however, in the lipid extracts across the remains that could be related to anatomical position. Due to the small size of the infant remains, the sampling points were quite close together and can explain the similarities in lipid distributions observed between the sampling points.

The LMW fatty acid contents are increased for certain samples from the remains compared to the control. Furthermore profiles observed are very similar to that reported for degraded adipose tissue in a dry environment (Forbes *et al*., 2005). The presence of LMW *n*-alkanes are likely to represent degraded human adipose tissue through reductive microbial degradation of fatty acids. While these provide evidence for a reductive transformation of fatty acids relating to the body tissues there is no contribution to the extracts from the reduction intermediates (alkanones and alcohols) observed by Naccarato *et al*. (1972). The lack of intermediates could suggest that the free fatty acids may have fully degraded to *n*-alkanes.

The site from Syningthwaite show no specific signatures that could provide more information about the remains, however it does indicate that the degradation of the original remains likely to have involved a reduction pathway.

4.3. Edinburgh (ETCS08)

4.3.1. Site description and sampling

The Edinburgh site, located on Constitution Street in the north of the city, was excavated during works undertaken prior to the construction of a tram system serving Leith and Edinburgh city centre. The site was considered a highly sensitive

archaeological area from the start of the development due to it being situated within the medieval core of Leith and the 16th and 17th century town defence (Spanou 2010).

The excavation located numerous human burials under the street outside the existing walls of St. Mary's church. The present church lies on the old foundation of an earlier church dedicated to the Virgin Mary, which can be dated to 1483 (Spanou 2010). The discovery of human burials was unexpected as it was not thought that the graveyard had ever extended beyond its current walls. Their discovery demonstrated that the graveyard once extended further, and was covered when houses were built opposite the walls in the 18th century. It is also possible that the graveyard may have been split as early as 1560 when military buildings were constructed on the site (Spanou, 2010). The site contained numerous layers of closely packed burials. The grave discussed in this chapter is ETCS08 6B (Figure 4.10), which contained the remains of an individual male lying supine. The bones were dark brown in colour and bone preservation was very good. Notably, the skeleton was missing the right arm, no marks on the remains were present to suggest this was removed pre-mortem. Pipe work present on the right hand side of the skeletal remains (Figure 4.10) shows that there may have been disturbance of the burial to the right hand side of the skeleton.

Figure 4.10: Grave ETCS08 6B after sampling. Kubina tins for samples collected for micromorphological analysis are shown at the skull and left hand (photographed by Brendan Keely). Original in colour.

The grave was constructed within a feature interpreted as a large former dune and, as such, the burial matrix was very sandy. The grave was sampled according to the high intensity sampling strategy set out for the InterArChive project (Chapter 1). Owing to the proximity of the other burials on the site, it was impossible to obtain a C1 control sample. Furthermore, the proximity of other graves means that the C2 and C3 controls obtained could have been compromised by other burials (Figure 4.11; Table 4.2.)

Table 4.2: Samples collected from grave ETCS08 6B, including sample numbers and the locations from which they were collected.

Sample number	Sample position
C2	Control from edge of the burial by right femur
C3	Control from below the feet at the edge of the burial
1	Skull
2	Pelvis
3	Left foot
4	Right foot
5	Left shoulder
7	Left arm
9	Left pelvis
10	Right pelvis
11	Left knee
12	Right knee
13	Left hip
14	Right hip
15	Chest
17	Left hand

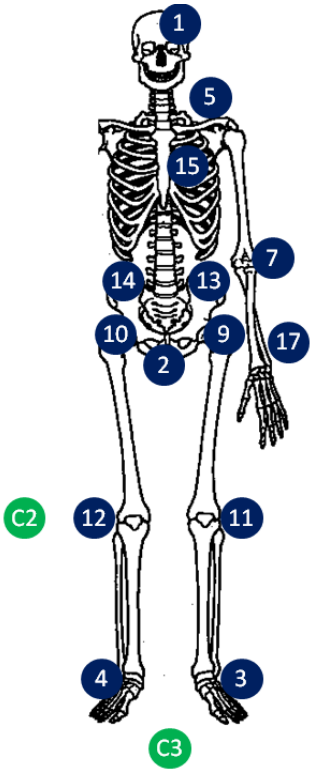

Figure 4.11: Samples taken from Edinburgh grave ETS08 6B, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive sampling strategy are labelled in blue. Originally in colour.

4.3.2. Results and Discussion

4.3.2.1. Bulk soil analysis

Elemental analysis showed there to be no measurable amount of nitrogen in the samples. All samples exhibit low carbon contents ranging between ~0.20-0.72%, of which organic carbon represents the major form of carbon (Figure 4.12). Notably, the C2 control had a large TOC content relative to most other samples analysed, and the only sample with comparably high TOC is from the left foot (3). The C2 was taken at the same level as the exposed skeleton outside the right edge of the grave cut; its high TOC content (0.6%) is anomalous and cannot be explained at present. The C3 sample and other remaining samples have lower TOC contents (0.2-0.4%) The left foot (3) shows the highest levels of TOC content of all of the sampling points from around the skeletal remains, though given the low contents of all samples this indicates only a slightly greater organic content at this location. All the other sampling points have very similar amounts of TOC around 0.2-0.4%. The total carbon (~0.2-0.7%) and TOC (~0.1-0.6%) values from grave 6B are similar to those of the grave soils from Heslington East (Chapter 3), which is also believed to have been affected by relatively free water movement.

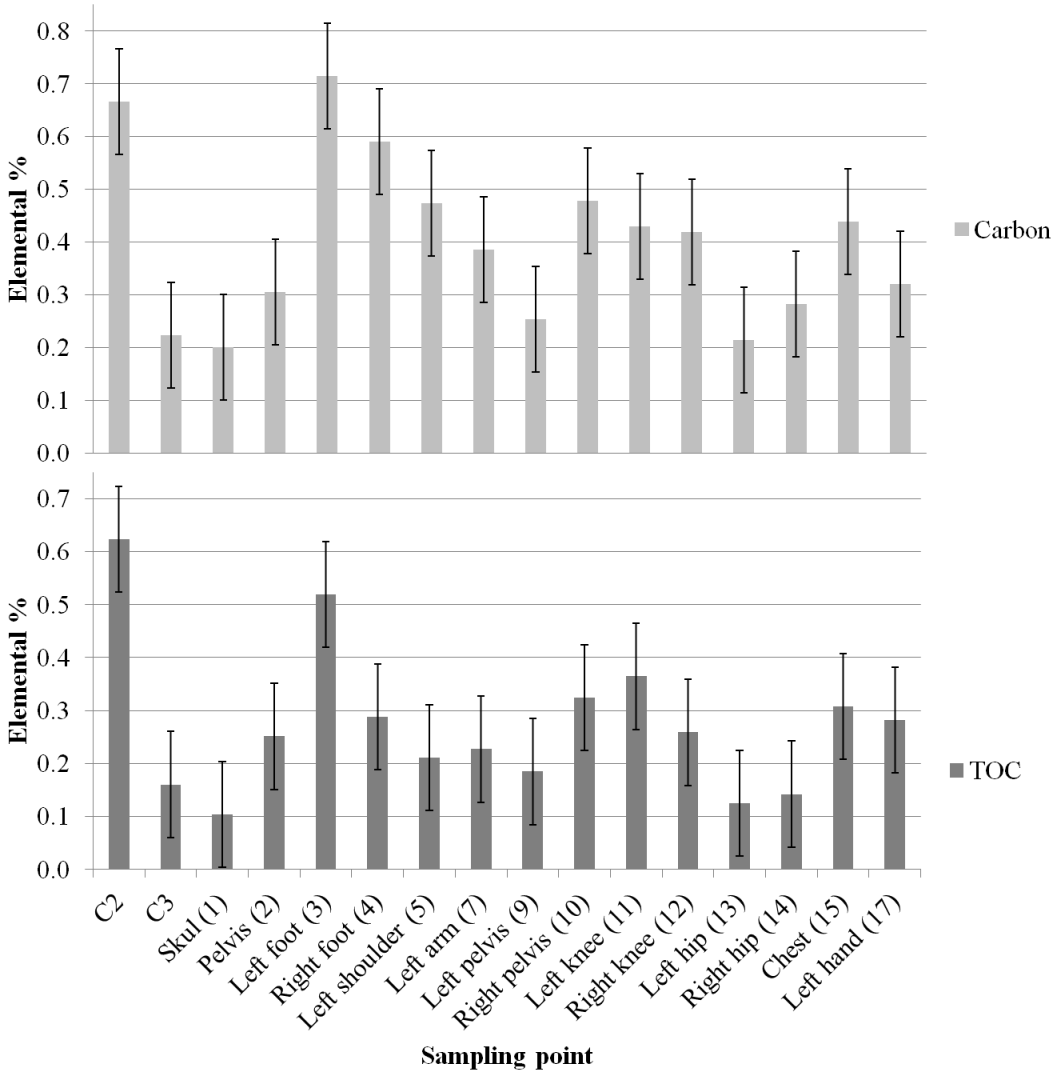

Figure 4.12: Bulk carbon and total organic carbon (TOC) elemental analyses of soil samples from ETS08 6B. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

Masses of solvent extractable organic matter normalised to TOC content for the samples (Figure 4.13) follow a broadly similar pattern to that seen for TOC content. Control C2 exhibits a similar amount of extractable organic matter to the left shoulder (5), left arm (7), left knee (11) and left hip (13), and has a greater amount than C3 and the head (1; Figure 4.13). All other sampling points show greater extractable organic matter content than the C2 control. The similarity in the extract yield for C2 to that for many samples from around the skeletal remains suggests that

the high TOC content in the control reflects a greater proportion of polymeric (solvent insoluble) organic matter than is present in the samples. C3 gave a low extract yield, similar to that of the head (1). Samples from the left shoulder (5), left arm (7) and left knee (11) contain similar amounts of extractable organic matter. Likewise, samples from the pelvis (2), right foot (4), left and right positions of the pelvis (9 and 10), right knee (12), right hip (14) and chest (15) show similar levels of extractable organic matter to each other. The left foot (3), which exhibited the highest TOC value of all of the samples from around the skeletal remains, also exhibits the greatest proportion of extractable organic matter. Samples with higher amounts of extractable organic matter appear to be associated with the pelvis (2, 9, 10 and 14), chest (15) and left hand (17). These samples are taken from the mid-line of the remains, with the exception of the hand, which was positioned quite close to sampling point 9 at the pelvis (Figure 4.10). The distributions suggest that the elevated levels of extractable organic matter are associated with the presence of the remains.

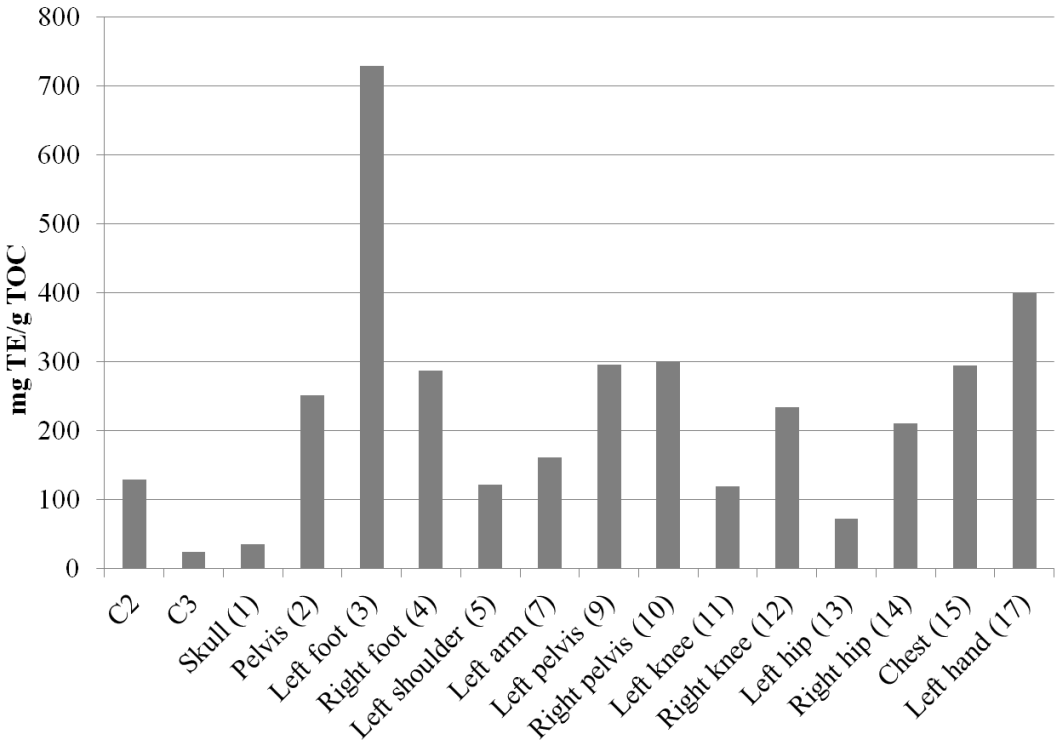

Figure 4.13: Solvent extractable organic matter in the soil samples from ETCS08 6B normalised to total organic carbon content.

4.3.2.2. Total extract

Analysis of the total extracts revealed chromatograms showing similar profiles for all samples, C16:0 and C18:0 fatty acids being the dominant components along with smaller amounts of their unsaturated counterparts. Further analysis of fractions was performed to provide a more detailed view of the differences between the sampling points.

4.3.2.3. Hydrocarbon and aromatic fractions

All samples contain a series of *n*-alkanes (C18-C33) showing a bimodal distribution (Figure 4.14) with contribution from plant material (Eglinton & Hamilton, 1967) and microbial activity (Ladygina *et al.*, 2006). Although higher plant-derived distributions of *n*–alkanes are common in soils (Carter *et al*., 2007), they were not observed in the C2 control and were generally only observed in low abundance around the skeletal remains. However, samples from the left foot (3), right of the pelvis (9), left knee (11) and left hand (17), had a higher abundance of the plant derived *n*-alkanes. Micromorphological analysis was performed on the C2 and C3 controls and samples from the skull, left elbow, left hand, right pelvis, left knee and left foot (Carol Lang, unpublished results). Of the samples studied, only the left hand and left foot revealed the presence of fragments of plant material, which could explain the presence of higher plant *n*-alkanes in the extracts from these samples.

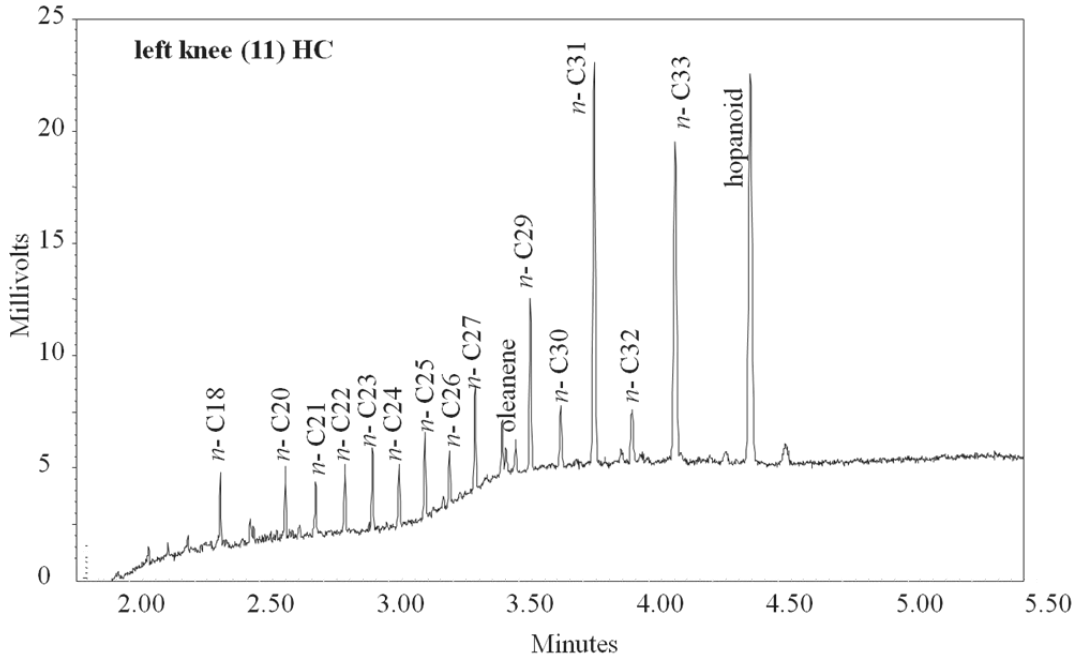

Figure 4.14: Fast GC-FID chromatogram of the hydrocarbon fraction from position 11 of ETCS08 6B, showing the bimodal distribution of *n*-alkanes typical of hydrocarbon fractions from the grave.

In addition to the *n*-alkanes, peaks at ~3.5 and ~4.5 min were observed in the GC chromatograms of the hydrocarbon fractions (Figure 4.14). The mass spectrum of the earlier eluting component obtained via GC-MS revealed it to be the higher plant-derived triterpene olean-13(18)-ene (discussed in Chapter 3), which can be formed from microbial transformation of β-amyrin and/or taraxerol (Killops and Killops 2005). The later-eluting peak exhibited a mass spectrum consistent with diploptene (Figure 4.15), having a molecular ion ($M^{+\cdot}$) at *m/z* 410 and major hopanoid fragment ions at *m/z* 189 and 191 (Evershed *et al*., 1995).

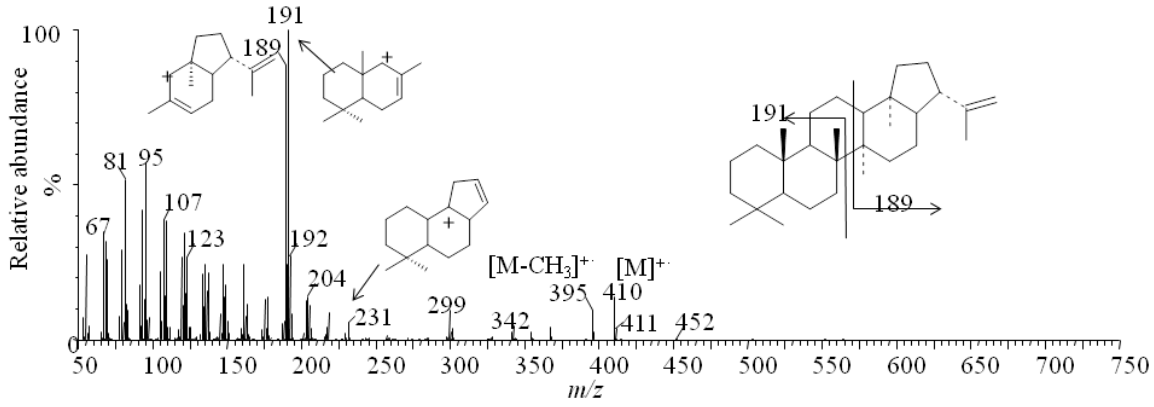

Figure 4.15: Mass spectrum of a peak eluting at ~4.5 min in GC, showing fragments consistent with diploptene.

Diploptene is known to be derived from ferns and bacteria (Ourisson *et al.,* 1979) and has also been observed during lipid analysis of bone (Evershed *et al.,* 1995). The authors proposed that the presence of diploptene could arise from microorganisms originating from the soil that have been involved in the decay of bone (Evershed *et al.,* 1995). In the case of 6B, diploptene is not present in the control samples and varies in abundance among the different sampling points showing a clear association with the remains (Figure 4.16; a). Most of the sampling points from around the skeletal remains display a similar amount of diploptene (0.01-0.09 mg/g TOC), although it is elevated in samples from the left foot (3; 0.176 mg/g TOC) and the knees (11 and 12; 0.342 and 0.315 mg/g TOC; Figure 4.16; a).

LMW bacterially derived *n*-alkanes (C18-C22) are present in higher abundance in samples from the left hand side of the remains and the feet (Figure 4.16; b). Notably, the left hand side had lower amounts of extractable organic matter than the right hand side of the skeletal remains. As mentioned in Section 4.3.1., the right hand side of the grave appears to have been previously disturbed as the right arm is completely missing. The disturbance and exposure of the grave on the right hand side of the skeleton may have caused the distributional differences apparent in the organic matter between the right and left hand sides. Thus, the left hand side of the remains may display signatures more closely related to the buried remains. The abundances of plant derived HMW *n*-alkanes (C23-C33) vary amongst the different points

around the skeletal remains (Figure 4.16; c) as do those of oleanene (Figure 4.16; d). All components appear to be elevated around the left foot area, further suggesting a concentration of organic matter at that position.

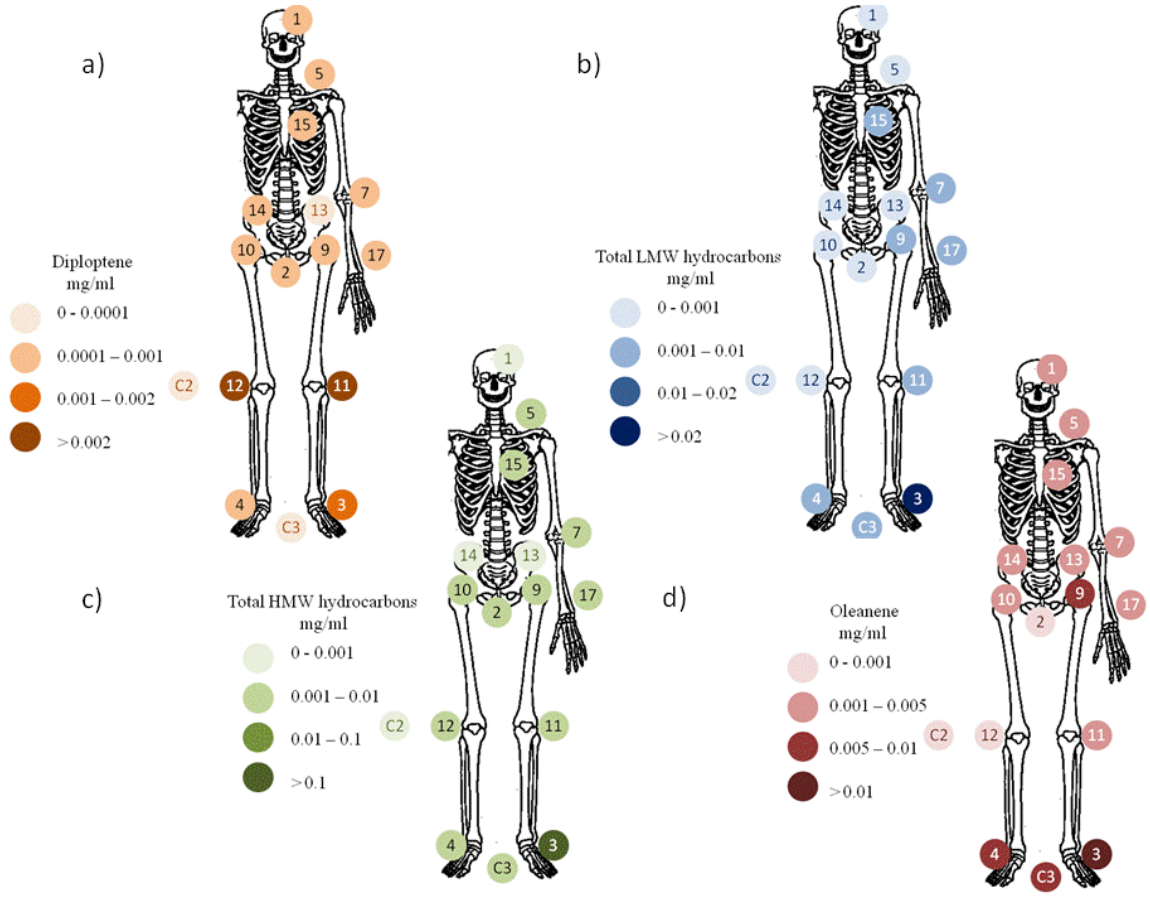

Figure 4.16: Distribution of a) Diploptene, b) LMW hydrocarbons, c) HMW hydrocarbons and d) oleanene from the various sampling point positions around the burial of ETCS08 6B. Originally in colour.

The GC chromatograms of the aromatic fractions did not contain any significant components.

4.3.2.4. Medium polar fraction

The medium polarity fractions contained very low levels of a series of *n*-alkanols (C24-C28) relating to higher plant waxes (Eglinton & Hamilton, 1967). GC-MS of the medium polar fraction revealed that a peak at around 5 min, observed in GC

(Figure 4.17), exhibits a mass spectrum consistent with diplopterol (Figure, 4.18; analysed as its trimethylsilyl ether).

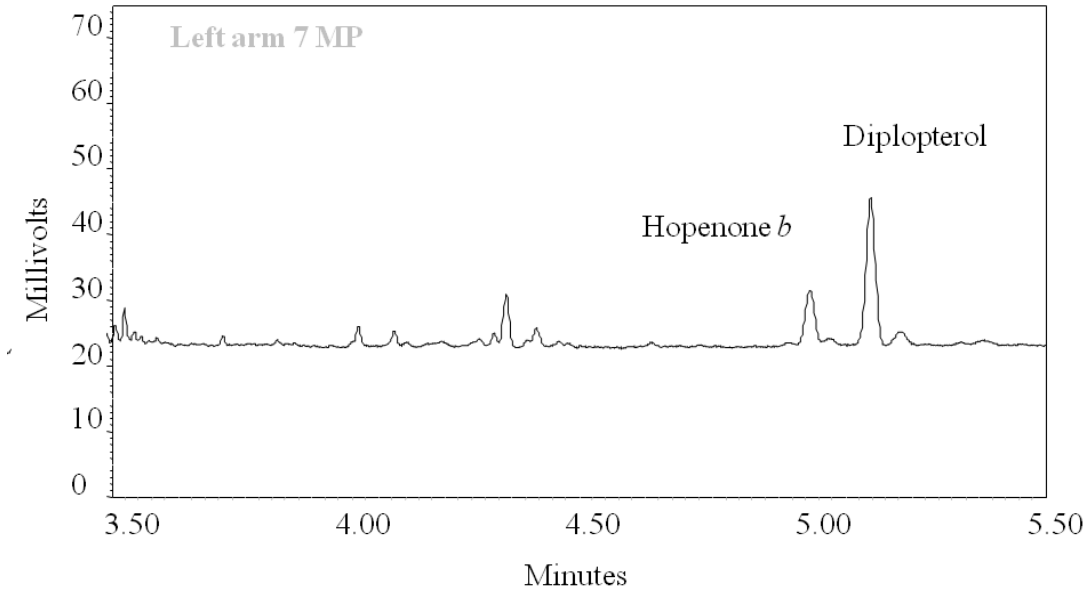

Figure 4.17: Partial GC-FID chromatogram of the medium polar fraction from the left arm (7) in ETCS08 6B.

Like diploptene, diplopterol is present in ferns and bacteria (Ourisson *et al.*, 1979). Due to the absence of an obvious correlation with the total abundance of plant *n*-alkanols around the remains (Figure 4.16), the diplopterol in these samples is more likely, therefore, to be of bacterial origin and possibly linked with microorganisms involved in the degradation of the remains, as suggested for diploptene (Ourisson *et al*., 1979; Evershed *et al.*, 1995).

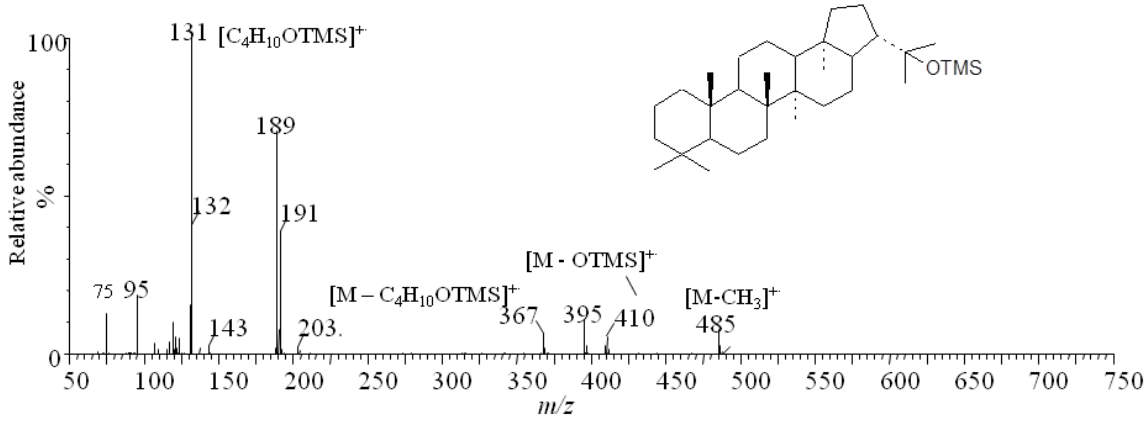

Figure 4.18: Mass spectrum of a component consistent with diplopterol, found in medium polar fractions from ETCS08 6B.

Small amounts of Hopenone *b* were also observed in the medium polar fractions. Hopenone *b* is the triterpenone counterpart of diploptene having a ketone group at carbon 3 (Figure 4.19).

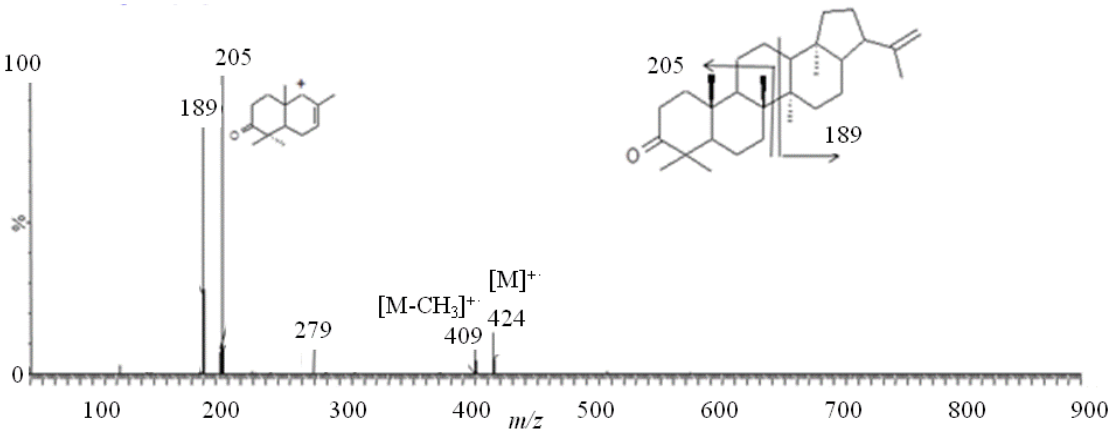

Figure 4.19: Mass spectrum of component consistent with Hopenone *b* found in the medium polar fractions from ETCS08 6B.

All three of the hopanoids (diploptene, diplopterol and hopenone *b*) appear to be present in greater abundance around the lower pelvis area and the knees (Figure 4.20). Diploptene (Figure 4.20; a) is present in higher levels in a greater number of

the sampling points than diplopterol (Figure 4.20; b) and hopenone *b* (Figure 4.20; c), especially towards the feet (3 and 4). The distributions of the three hopanoids across the different sampling points suggest that those of diplopterol and hopenone *b* are the most closely related, their abundances showing similar patterns with sample location. Both of the knee samples exhibit a high abundance of the hopanoids, whereas the C2 control, which was sampled adjacent to the knees, has very little of the hopanoids present. This suggests that there has been little migration of the lipids towards the edge of the grave.

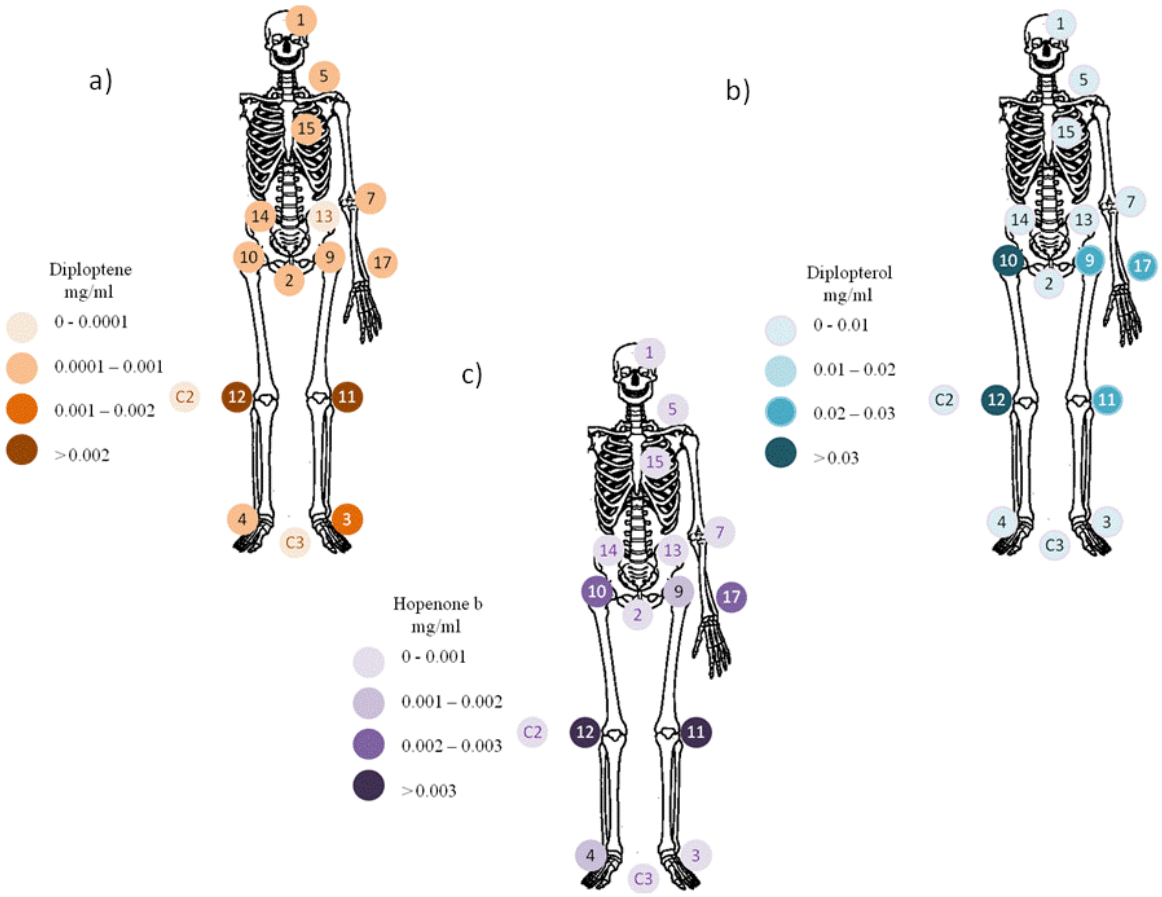

Figure 4.20: Distributions of a) diploptene, b) diplopterol and c) hopenone *b* in the different samples from around the skeletal remains. Originally in colour.

Hopenone *b* has not been reported widely in nature, but has been isolated from a few species of the plant genus *Euphorbia* (Koops *et al.*, 1991; Starratt, 1969). In particular, it is found in the species *Euphorbia cyparissias* (Starratt, 1969), also known as cypress spurge or graveyard weed (Royal Hortecutural Society, 2011)

given it is often present in country graveyards. The plant is known to grow in well-drained soil (Royal Hortecutural Society, 2011); the soil at Edinburgh, being sandy, would provide ideal conditions for the species to thrive. As mentioned previously, hopenone *b* and diplopterol appear to have similar distributions around the body (Figure 4.20), with increased amounts of the hopanoids found around the lower pelvis and the knees. Although diplopterol has yet to be found in *Euphorbia*, it has been found in ferns and bacteria (Ourisson *et al*., 1979). Diplopterol has also been found in pink-pigmented facultative methylotrophs (PPFMs) isolated from the surface (phylloplane) of several different plant species (Knani *et al.*, 1994; Holland, 1994). Although *Euphorbia* species were not among the plants examined for PPFMs, it remains possible that the PPFMs colonise the surface of cypress spurge. If so, and both hopanoids are from the surface of the plant, this may account for the similarity in the distributions of both hopenone *b* and diplopterol in grave soil (Figure 4.20). The sampling points with elevated levels of the hopanoids also have slightly elevated amounts of HMW *n*-alkanes relating to plant material (Figure 4.16). The signatures for plant material, including those potentially from cypress spurge, are quite localised (around bottom of pelvis and knees), suggesting that material was either placed in this location in the grave itself, or may have followed the ingress of soil associated with the collapse of the coffin. Cypress spurge is not known to be used as a floral tribute. Therefore, it is more likely that the plant material has come from top soil that has entered the grave after a collapse of the coffin lid.

4.3.2.5. High polar fractions

Analysis of the high polar fractions revealed that all of the samples contain a series of fatty acids (analysed as their FAMEs, Figure 4.21). By contrast, control C2 did not contain fatty acids. The profiles are dominated by LMW compounds comprising fatty acids ranging from C14:0 to C18:0, with only the even chain length fatty acids being present. In addition, the samples contain the unsaturated fatty acids C16:1, C18:1 and C18:2, present in varying abundances. The distributions of fatty acids from samples C3, skull (1), left arm (7) and left hip (13) are dominated by the saturated components C16:0 and C18:0 (Figure 4.21), and all other samples dominated by the unsaturated components C16:1 and C18:1 (Figure 4.21). The

unsaturated component C18:2 was present in all samples, but in very low concentrations. As discussed previously, sources of C14-18 fatty acids include plants, animals, fungi and bacteria (Zelles, 1997; Ruess *et al*., 2002; Forbes *et al*., 2005).

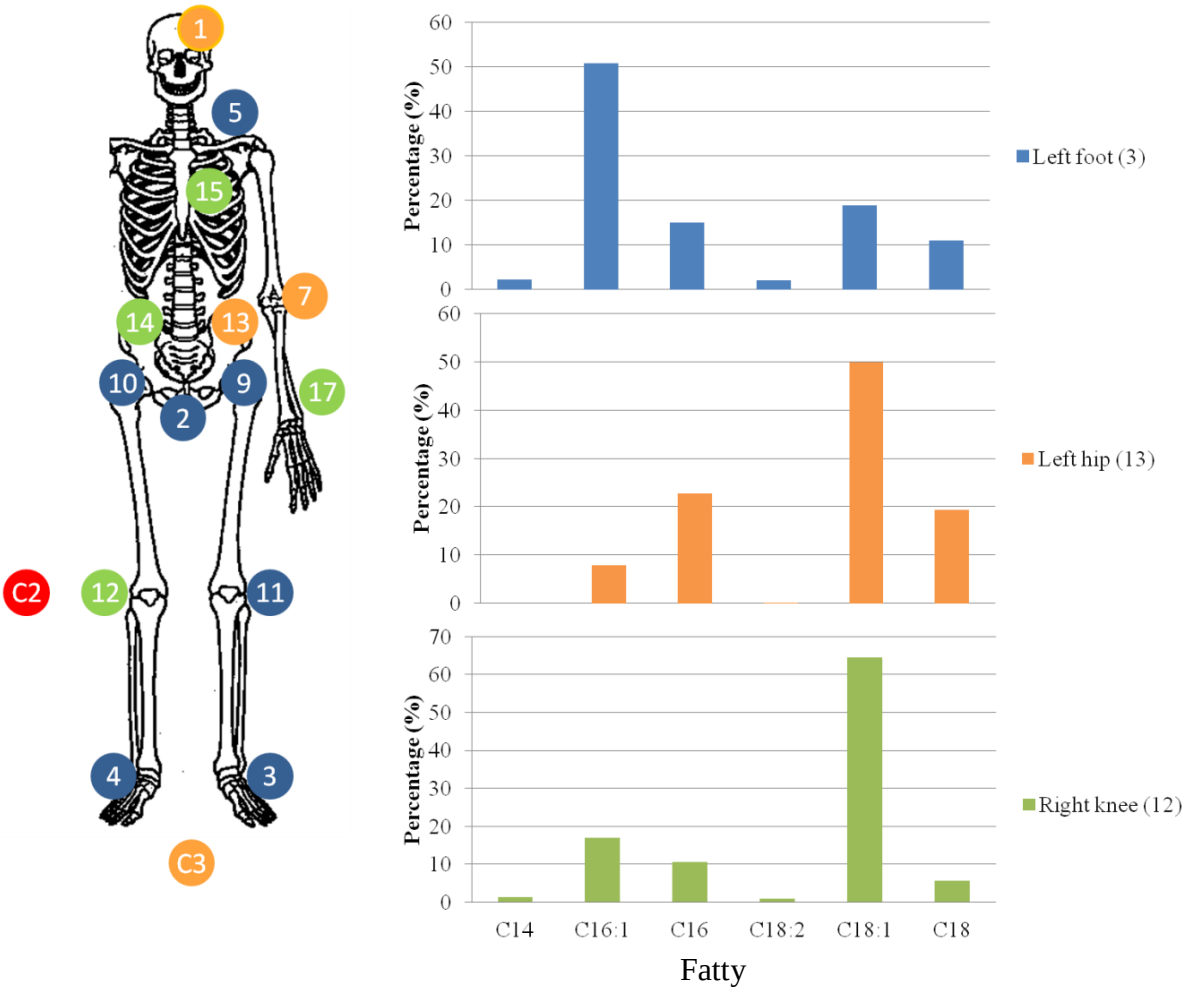

Figure 4.21: Distributions of fatty acids at different locations around the skeletal remains. Points labelled in blue show profiles similar to position 3 (top). Points labelled in orange show profiles similar to position 13 (middle). Points labelled in green show profiles similar to position 12 (bottom). Points labelled in red do not contain any detectable fatty acids. Originally in colour.

Plants contain predominantly C16:0 and C18:2 fatty acids. The low concentrations of C18:2 in the samples suggest either little contribution of plants to LMW fatty acid distributions in the grave, or, perhaps, that preferential degradation of unsaturated

fatty acids has occurred (Forbes *et al*., 2003; Hansel *et al*., 2011). Notably, however, plants also contain high molecular weight (HMW) fatty acids in the range C22-C34 (Zelles 1997), which do not feature in the distributions. Hence, a significant contribution to the fatty acid profiles from plants appears unlikely. Fungi contain predominantly C16:0, C18:1 and C18:2 FAs (Ruess *et al.*, 2002), whereas bacteria contain relatively high amounts of C16:0, C16:1 and C18:1 fatty acids (Rezanka and Sigler, 2009). Each could be a source of these lipids in the samples. Notably, none of the sources discussed above contain significant amounts of the C14 fatty acid. Some bacteria, however, contain C14 fatty acid at lower levels (~5% of total fatty acid), and could be a source for the lipid in the grave samples. Fresh human adipose tissue contains primarily C18:1 and C16:0, with smaller amounts of C18:0 and C14:0 (Brockerhoff 1965).The saturated members are reported to be the major products in degraded adipose tissue (Forbes *et al.*, 2002; Forbes *et al.*, 2005), exhibiting similar distributions to those seen in the grave samples (Section 1.2). The other samples (i.e. those labelled blue and green in Figure 4.22) show an increase in the proportions of C16:1 and C14:0 fatty acids relative to control C3. It is possible, therefore, that the fatty acids in the samples (in particular the C14:0 fatty acids) reflect an input derived from the body tissues. In light of the abundance of the C16:1 fatty acid, however, the lipid profiles are likely to contain a significant bacterial overprint.

There were no branched hydrocarbons observed in any of the samples that are known to be common within a bacterial input (Zelles *et al*., 1997) however the observation of diploptene in the samples suggests that there is a significant contribution from a bacterial source. The study in which diploptene was observed in the degradation of bone did not mention the presence of any branched fatty acids in the analysis (Evershed *et al*., 1995), therefore even though there are no branched components present the *n*-alkanes, fatty acid and diploptene does give evidence for the presence of bacteria in the burials.

The evidence of relatively little of plant input in these samples compared with other sites studied (Chapter 3) could possibly be due to the graves being covered, firstly by the construction of buildings in the 18th century, and later by a road being constructed over the top of the burials. Both might have prevented fresh input from

any overlying vegetation. It is also possible that only a small amount of vegetation was present due to the relative poor fertility of the sandy soil.

4.3.3. Conclusion

The Edinburgh grave showed significant variation amongst the components at different sampling points. The sampling points that showed significant contributions from different components were located on the left hand side of the remains.

Samples taken from around the gut region show an increase in fatty acids relating to bacteria and degraded human adipose tissues; this is likely to be due to that particular area being the area having the greatest amount of fatty tissue and would be the location of gut bacteria. The higher abundance of markers relating to remains in the samples from around the gut area suggests that these samples are likely to reveal more information about the remains.

This particular grave, however, has shown that the knee area has provided the most information about the nature of the burial. The samples taken from the knee area have shown an increase in signatures relating to bacteria (diploptene), in addition to the plant markers (diplopterol and hopenone *b*). These markers have provided an insight to the nature of the grave environment such as what bacteria and also what plant floras were present at the time of burial. Therefore, although the gut region is likely to provide a greater opportunity to the study of degradation of body tissue (e.g. adipose tissue), the other sampling points should not be dismissed as they can provide information on the nature of the burial environment.

Specific components have been observed within this burial, which have helped provide additional information on the burial environment; the presence of diploptene, a bacterial marker that has been previously found in ancient bone (Evershed *et al*., 1995), suggests that there is significant bacterial input around the remains and they are likely to be responsible for bone degradation.

Another specific component has given an insight into the burial environment; hopenone *b* suggests that the plant cypress spurge was present in the grave (Starratt

1969). As these markers are not present in the control and are unique to certain sampling points the material is therefore related to the grave. The identification of hopenone *b* in particular sampling points of the grave provides evidence that specific signatures that relate to certain features within the burial, which may have not necessarily been apparent just by observation, can be detected through chemical analysis of the burial soil. In this case the signatures have provided evidence of the specific plants present at the time of burial or the type of floral tribute that was placed in the burial. Cypress spurge or graveyard weed may have been part of the surrounding flora and become incorporated into the burial but although the plant is not commonly used for decoration it may have been possible that the plant could also have been picked to be used as a decorative offering for the burial.

4.4. Mechelen

4.4.1. Site description and sampling

The city of Mechelen is located in central Belgium and lies between Brussels and Antwerp. The excavation took place in the cemetery of the St. Rombouts' cathedral (Figures 4.22 and 4.23), the original chapter of which is known to have been founded between 972 and 1008 AD (Kinnaer 2010). The chapter, the only parish church in Mechelen, gained parish rights in 1134 and between 1134 and 1205 was the only church in the town, implying that all of the residents who died in Mechelen were buried in the cemetery surrounding the church (Kinnaer, 2010).

Figure 4.22: Tower of St. Rombouts' cathedral, Mechelen (photograph by Brendan Keely). Originally in colour.

In 1560, the original chapel was pulled down; the new chapel built in 1596 was, in turn, demolished in 1798. It is assumed that between 1000 and 1785 AD, people were continually buried in the cemetery of St. Rombouts' (Kinnaer, 2010).

Figure 4.23: Mechelen excavation site, showing several partially excavated burials (photograph by Brendan Keely). Originally in colour.

Over the period of its use (1000-1785 AD), the cemetery was extensively re-used for burials. Excavation revealed graves layered one on top of another and close together, with a lot of throw-in material (e.g. bones from disturbed graves) mixed in with the fill. As such, the C2 and C3 control samples could contain organic residues associated with disturbed burials. The grave focused on for this chapter is G422 (Figure 4.24), that of an adult female. The soil type varied throughout the site but was a sandy loam around this particular burial.

Figure 4.24: Grave MESW G422; supine burial of an adult female. Close to the right hand side of the skeleton, another grave has been excavated, highlighting the close proximity of the burials at the site (photograph by Brendan Keely). Originally in colour.

As there were several layers of graves within the site, it was impossible to collect C2 and C3 samples from above the remains. Accordingly, soil samples C2 and C3 were collected from positions adjacent to the grave. Soils from a full 17 sampling positions (Figure 4.25; Table 4.3) were also taken from positions adjacent to and below the bones, in accordance with the InterArChive sampling protocol (Chapter 1).

Table 4.3: Samples collected from grave G422, including sample numbers and the locations from which they were collected.

Sample number	Sample position
C2	Control taken from soil adjacent to the grave
C3	Control taken from lower soil adjacent to the
1Y and 1Z	Skull adjacent and below
2Y and 2Z	Pelvis adjacent and below
3Y and 3Z	Left foot adjacent and below
4Y and 4Z	Right foot adjacent and below
5Y and 5Z	Left shoulder adjacent and below
6Y and 6Z	Right shoulder adjacent and below
7Y and 7Z	Left arm adjacent and below
8Y and 8Z	Right arm adjacent and below
9Y and 9Z	Left pelvis adjacent and below
10Y and 10Z	Right pelvis adjacent and below
11Y and 11Z	Left knee adjacent and below
12Y and 12Z	Right knee adjacent and below
13Y and 13Z	Left hip adjacent and below
14Y and 14Z	Right hip adjacent and below
15Y and 15Z	Chest adjacent and below
16Y and 16Z	Right hand adjacent and below
17Y and 17Z	Left hand adjacent and below

*Y relates to samples taken adjacent and Z relates to samples taken below the remains.

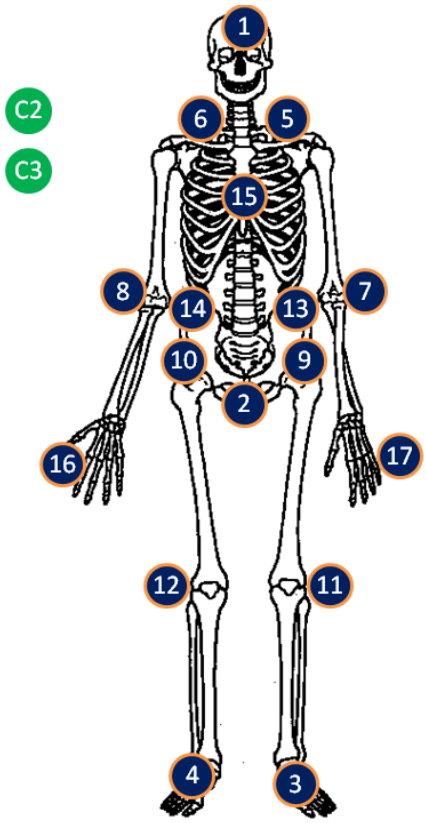

Figure 4.25: Samples taken from Mechelen grave G422 the control samples are labelled in green. Blue circles and orange circles represent samples taken adjacent to (Y) or from underneath (Z) the skeletal remains, respectively. Originally in colour.

4.4.2. Results and Discussion

4.4.2.1. Bulk soil analysis

Elemental analysis of samples from grave G422 reveals all of the samples (including controls) to exhibit no detectable nitrogen or sulfur. The TOC values for the samples were generally low (<0.7%), with most of the sampling points showing slightly higher levels of TOC than the controls (Figure 4.26). In some of the sampling points 6Y, 11Y, 13Y, 14Y, 1Z, 2Z and 6Z, the TOC content was measured to be greater than the total carbon content. The anomalous results could be due to the soils containing very low levels of organic carbon, amplifying the errors inherent in the analysis. With the exception of the left foot (3), the left pelvis (9) right knee (12) and the right hand (16), the sampling points adjacent to the remains have lower TOC contents than the samples taken from below the remains.

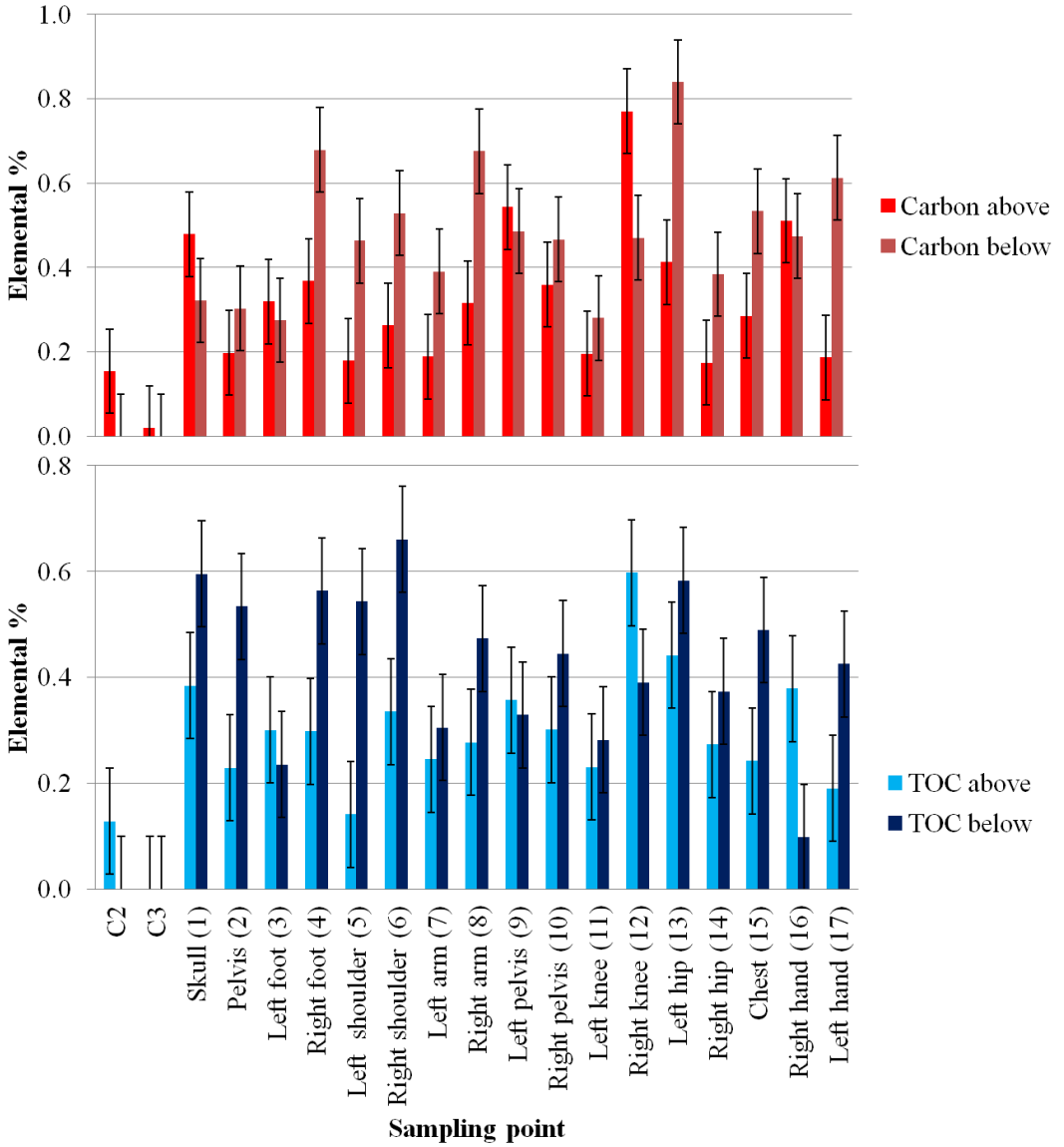

Figure 4.26: Bulk carbon and total organic carbon (TOC) elemental analyses of soil samples from MESW G422. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4). Originally in colour.

The mass of total extract normalised to TOC shows similar amounts of extractable organic matter (40-80 mg TE/g TOC) for sampling points taken from adjacent to the remains, with the exception of the skull (1), the left shoulder (5), the left arm (7), The left pelvis (9), the chest (15) and control C2, which are significantly higher (Figure 4.27). The amount of extractable organic matter in C2 is higher than for the C3 control, but is consistent with the difference in TOC contents of the two controls. The position of the C2 control, from the edge of the grave, means that it may have

been compromised by another grave due to the close proximity of the burials. Samples from beneath the pelvis (2Z, 9Z, 10Z and 13Z), left arm (7Z), the knees (11Z and 12Z) and hands (16Z and 17Z) all display higher levels of extractable organic matter than the corresponding positions sampled adjacent to the remains. Notably, the hands were positioned over the hips (Figure 4.27); hence, these samples are likely to incorporate organic matter associated with the pelvic area. The sample from below the right hand exhibits a significant amount of extractable organic matter, whereas the TOC levels are lower than those in the corresponding sample taken from adjacent to the right hand. This suggests that there is a much higher level of extractable organic matter below the hand than there is adjacent to it, where the presence of more polymeric material is implicated. The samples from the skull (1), feet (3 and 4), shoulders (5 and 6), right hip (14) and chest (15) positions exhibit lower extractable organic matter contents below than adjacent to the remains. The right hip (14) is the only position from the pelvic region to display lower levels of extractable organic matter in the sample taken from below the remains.

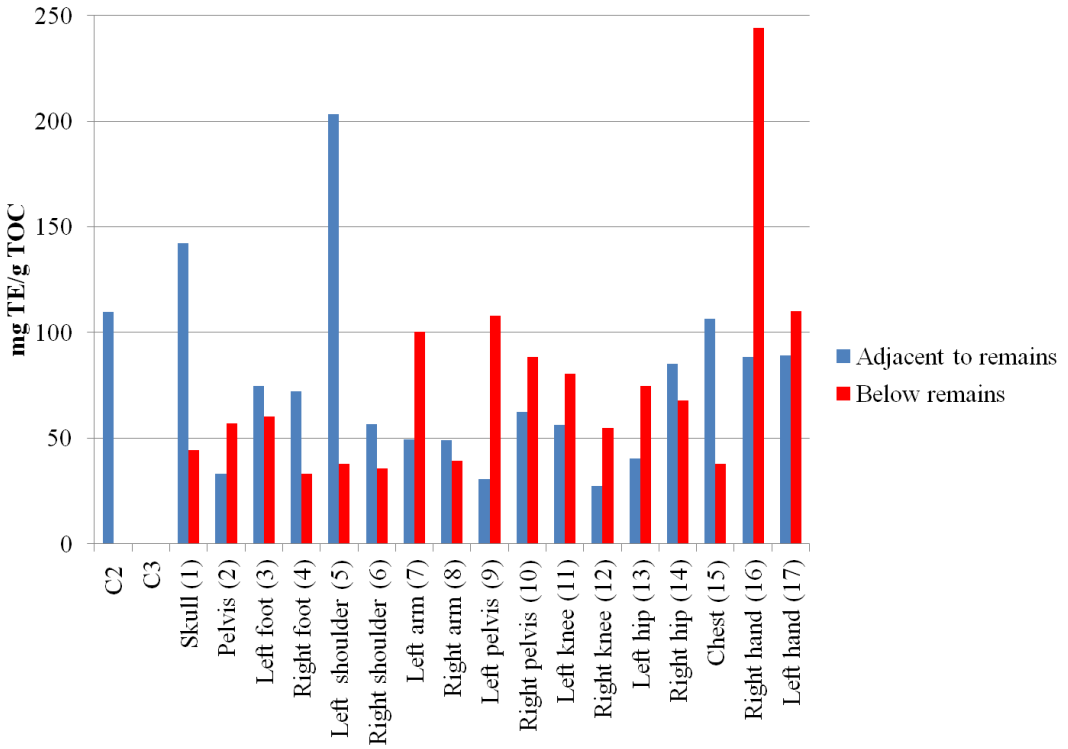

Figure 4.27: Solvent extractable organic matter contents of the soil samples from MESW G422 normalised to total organic carbon content. Originally in colour.

4.4.2.2. Hydrocarbon and aromatic fractions

No hydrocarbons were detected in samples from positions 4Y, 5Y and 15Y. Controls C2 and C3, and samples 1-3, 6-11 and 13-14 taken from adjacent to the skeletal remains, contain a series of *n*-alkanes showing a bimodal distribution (Figure 4.28 dark blue). The early eluting components (C18-C22; Figure 4.28) are consistent with *n*-alkanes relating to bacteria (Ladygina *et al*., 2006) and/or *n*-alkanes formed through reduction of free fatty acids from degraded adipose tissue. The later eluting components (C23-C33) indicate plant input (Eglinton & Hamilton, 1967; Jambu *et al.*, 1991).

Samples from the skull (1Z), the right foot (4Z), the shoulders (5Z and 6Z), the arms (7Z and 8Z), the left pelvis (9Z), the knees (11Z, 12Y and 12Z), the hands (16Y, 16Z and 17Z), exhibit similar distributions (Figure 4.28, green) to samples from the skull (1Y) the pelvis (2Y) the left foot (3Y), the right shoulder (6Y), the arms (7Y and 8Y), the pelvis (9Y and 10Y), the left knee (11Y) and the hips (13Y and 14Y) taken from adjacent to the skeletal remains (Figure 4.28, dark blue), but exhibit a higher relative abundance of early eluting, bacterially-derived components (C18-C22). The abundances of HMW *n*-alkanes are broadly similar (0.7-2.0 μg/g TOC) throughout all of the samples, however, the relative abundances of LMW *n*-alkanes do differ amongst the samples. Samples from the right knee (12Y) and all samples from below the remains with exception of the skull (1Z), left arm (7Z), left pelvis (9Z) and right knee (12Z) all display a higher relative abundance of LMW *n*-alkanes. The latter all had similar abundances of LMW *n*-alkanes to samples collected adjacent to the remains. The total abundances of the LMW *n*-alkanes (C16-C20) are greater than those of the plant *n*-alkanes in samples from below the remains, whereas the samples from adjacent to the remains have similar abundances for the LMW and plant *n*-alkanes. The LMW alkanes are most prominent in sampling points from below the pelvis (2), left foot (3), right pelvis (10), left hip (13), right hip (14) and left hand (17; Figure 4.28, light blue). Notably, positions 2, 10, 13 and 14 were all located around the pelvis. The interment of a body brings with it an input of bacteria, the majority of which resides in the gut region. In addition, a large proportion of the body tissue is distributed in the abdominal region which can provide sustenance for

degrading microorganisms (Dent *et al*., 2004). The LMW *n*-alkanes could also be present due to the reduction of the fatty acids (Park, 2005) released by decomposition of body tissues; the main fatty acids from the human body being C16 and C18 components. The C16 *n*-alkane increases in abundance significantly more than the C18 alkane in the samples from the pelvic region (2Z, 10Z, 13Z and 14Z) and the left foot (3Z), possibly indicating a contribution from an additional source. Further inputs from bacteria or degradation of fatty acids relating to adipose tissue are both possibilities.

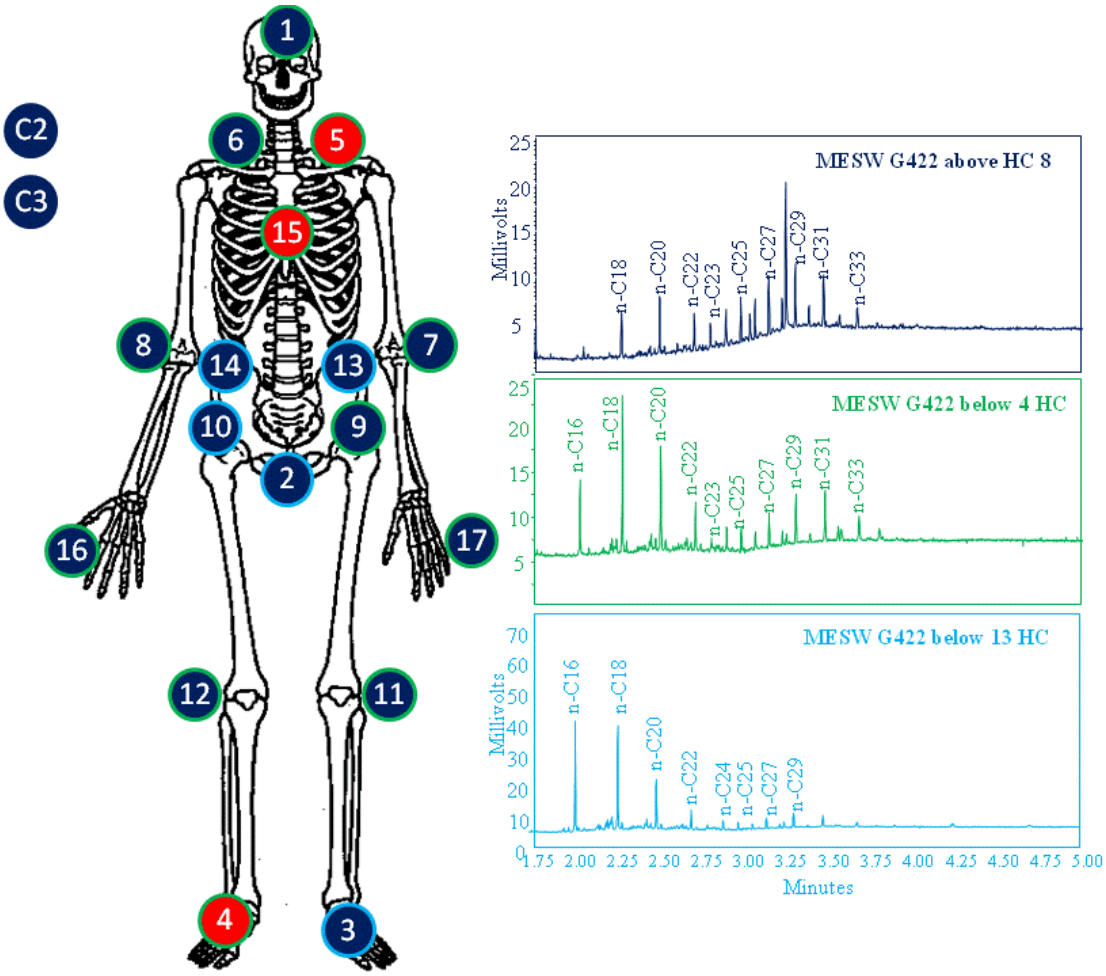

Figure 4.28: GC-FID chromatograms of the hydrocarbon fractions from sampling points 8Y (a), 4Z (b) and 13Z (c) from MESW G422, which typify the three distinct *n*-alkane distributions observed in the grave samples. Sampling positions are represented on the skeleton; the fill colour in each label represents the sample taken adjacent to the remains (Y) and the outline colour that taken from below the remains (Z). Points labelled in blue, green and orange have chromatograms similar to those of, in turn, 8Y, 4Z and 13Z. Points labelled in red represent chromatograms in which no hydrocarbons were evident. Originally in colour.

4.4.2.3. Medium polar fraction

The extracts from samples from beneath the remains at positions 5, 6 and 16 all contain significant amounts of C16:0 and C18:0 alkanals in an approximate ratio of 1:1 and C16:0, C18:0 *n*-alkanols, these being more prominent at the shoulders (positions 5 and 6) than at the right hand (16; Figure 4.29). The *n*-alkanals and *n*-alkanols were not present in either of the controls, or in samples that were collected adjacent to the remains suggesting that their presence is attributable to the interred body.

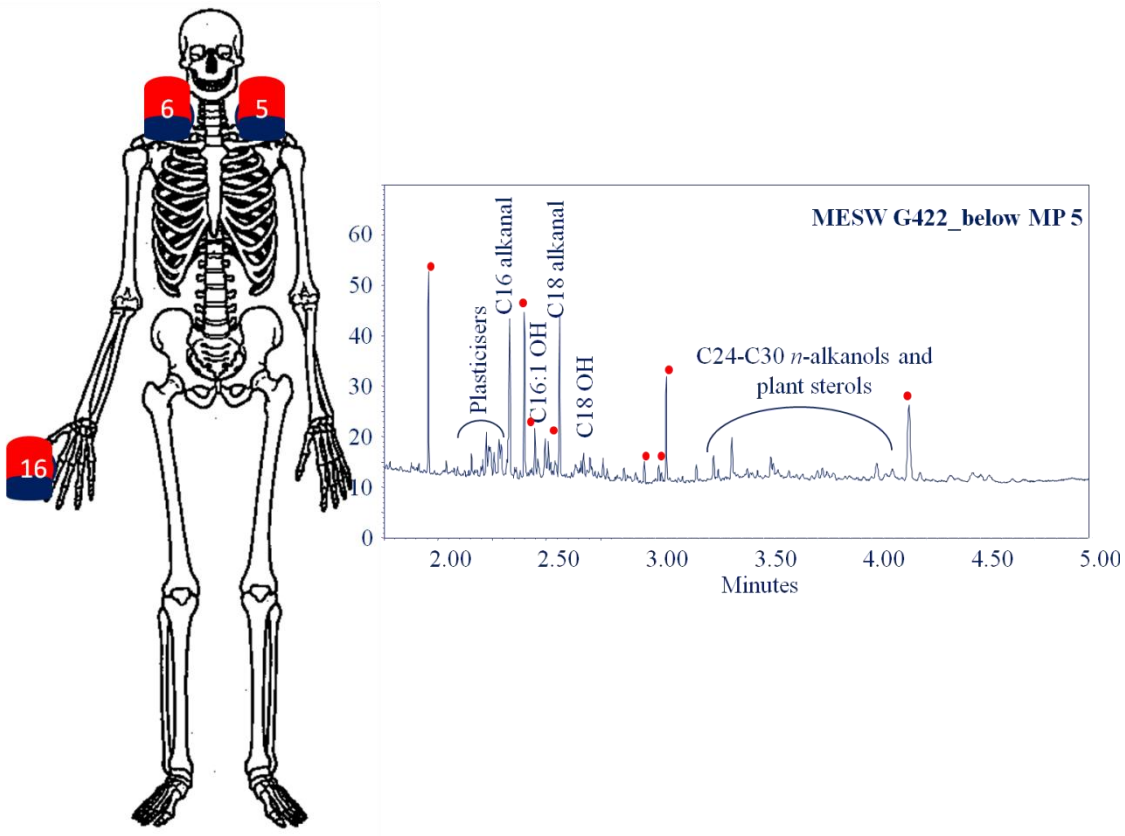

Figure 4.29: GC-FID chromatogram of the medium polar fraction from sample 5Z from MESW G422. Positions having similar distributions are indicated on the skeleton; the top colour in each label represents the sample taken adjacent to the remains (Y) and the bottom colour that taken from below the remains (Z). Points labelled in blue have chromatograms similar to that of 5Z. Peaks labelled with red dots in the chromatogram represent plasticisers introduced from the whirlpack bags and the ASE system. Originally in colour.

C16:0 and C18:0 alkanals are found in low levels in luminous bacteria (0.1-15 nmol/g cell measured in three different types of bacteria: Shimomura *et al.,* 1974) and can also be produced through the reduction of fatty acids (Day, 1978). It is possible that anaerobic conditions prevail within the Mechelen grave soil, providing an environment whereby free fatty acids from the hydrolysed TAGs in adipose tissue can be reduced, either by soil bacteria or bacteria from the gut region. Notably, C16 and C18 alkanals have also been isolated in rat, dog and bovine heart muscle (Gilbertson *et al.*, 1967). As the *n*-alkanals were observed in the upper part of the torso but not in the chest region in MESW G422, it is unlikely that the heart residing in the buried body was a source for. Long chain length (>C20) *n*-alkanals have been identified in the waxes of plants (Eglinton & Hamilton 1967), but there have been no reports of short chain *n*-alkanals such as C16:0 and C18:0 in soils. In addition to the *n*-alkanals, C16:0 and C18:0 alkanols are also present in samples from beneath the shoulders (5Z and 6Z) and the right hand (16Z). These components are also reduction intermediates for the degradation of free fatty acids (Park, 2005) and provide additional evidence that free fatty acids hydrolysed from TAGs in adipose tissue are being reduced under the conditions present within the burial.

The remaining sampling points exhibited similar chromatograms to those of the controls, each containing a range of C18-C30 HMW *n*-alkanols with C26 *n*-alkanol as the dominant component, a trait typical of soils from grassy areas (Bull *et al.,* 2000; vanBergen *et al.,* 1997).

4.4.2.4. High polar fractions

All of the soils (including the controls) display similar fatty acid profiles and feature contributions from C14-C18 LMW fatty acids. For all sampling points, the normalised profiles for the C14-C18 fatty acids (e.g. below the skull, 1Z; Figure 4.30) are similar to those reported for degraded adipose tissue (from a dry environment) and distinct from those pertaining to other possible sources, such as plants and fungi (Figure 4.30;. Although the typical signature is also present in C2 and C3, degradation products from adjacent or overlying burials may be present in

the controls and account for the resemblance of their fatty acid profiles to that of degraded adipose tissue.

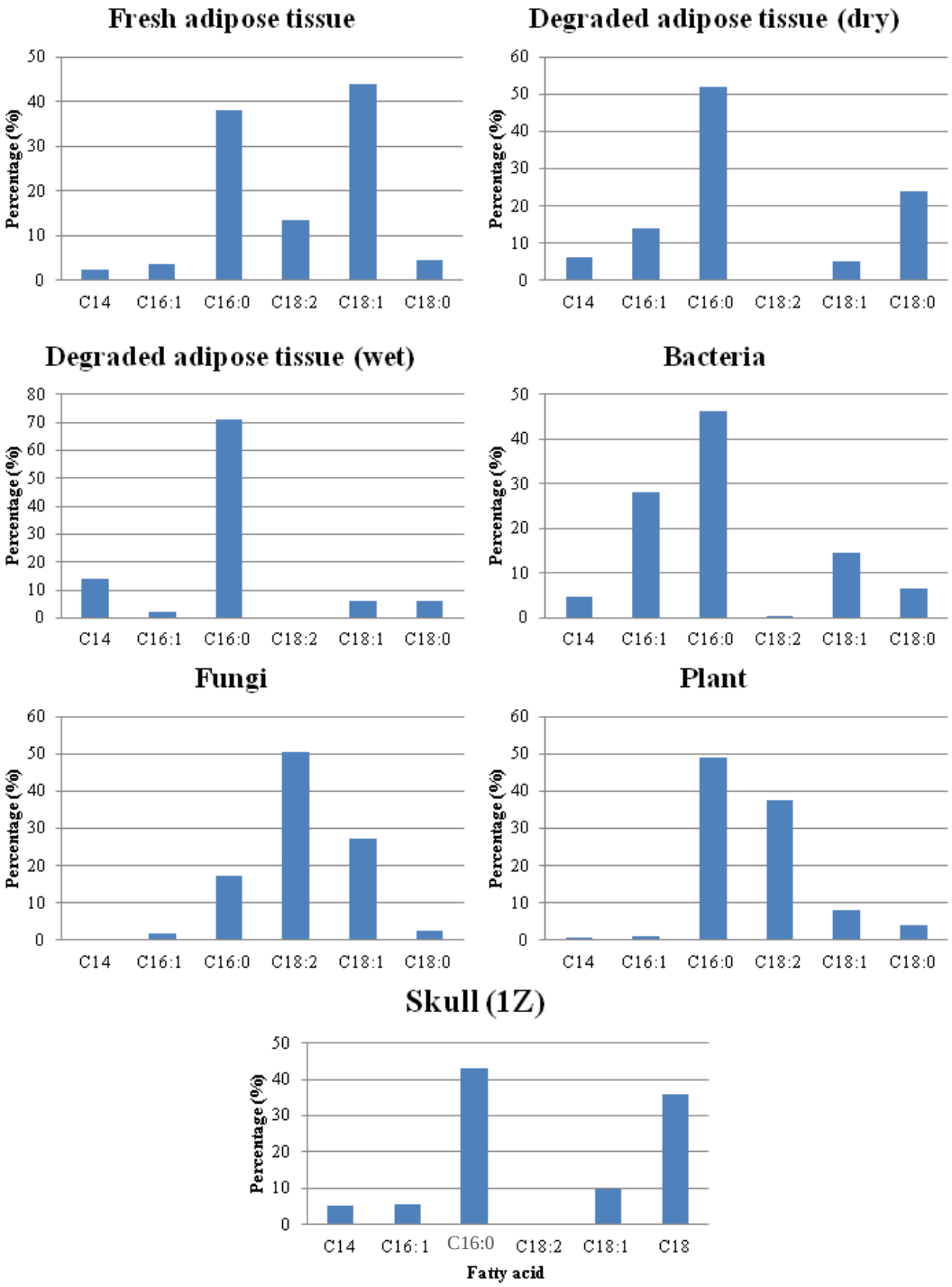

Figure 4.30: Typical profiles of fatty acids derived from different natural sources (adapted from Forbes *et al*., 2002; Forbes *et al*., 2005; Ruess *et al*., 2002; Zelles, 1997) and from extracts from grave MESW G422 (represented by the sample taken from below the skull, 1Z). Originally in colour.

In addition, the samples contained a series of fatty acids relating to plant waxes, and small amounts of cholesterol, stigmasterol and sitosterol. The ubiquity of the HMW fatty acids and sterols, including in the controls, indicate them to be present in the background soil organic matter. Although cholesterol, which is a major animal sterol, is found in the controls, in most samples it is found in greater concentrations in the samples collected from below the skeletal remains than in those collected adjacent to the remains (Figure 4.31). Exceptions are the samples from the shoulders (5 and 6), left arm (7), right knee (12) and right hand (16). The difference is more pronounced in the samples from the right foot (4), chest (15), hips (13 and 14), right arm (8) and left hand (17). Cholesterol has been used as a marker for human activity (Bethell *et al*., 1994) and has also been observed in the tissues of mummies and bog bodies (Buckley & Evershed 2001; Evershed & Connolly 1994), as well as bone (Evershed *et al*., 1995). The increase in cholesterol in samples below the body could indicate a higher input of material from the remains into the areas below the body. The positions showing an increase in cholesterol below the body are predominantly located around the chest and pelvic regions, which are areas where most of the body tissues (fatty tissue and organs) would be present. The feet areas also have higher cholesterol concentrations below the remains, although this could possibly be due to migration of lipids from the torso or contamination from another grave.

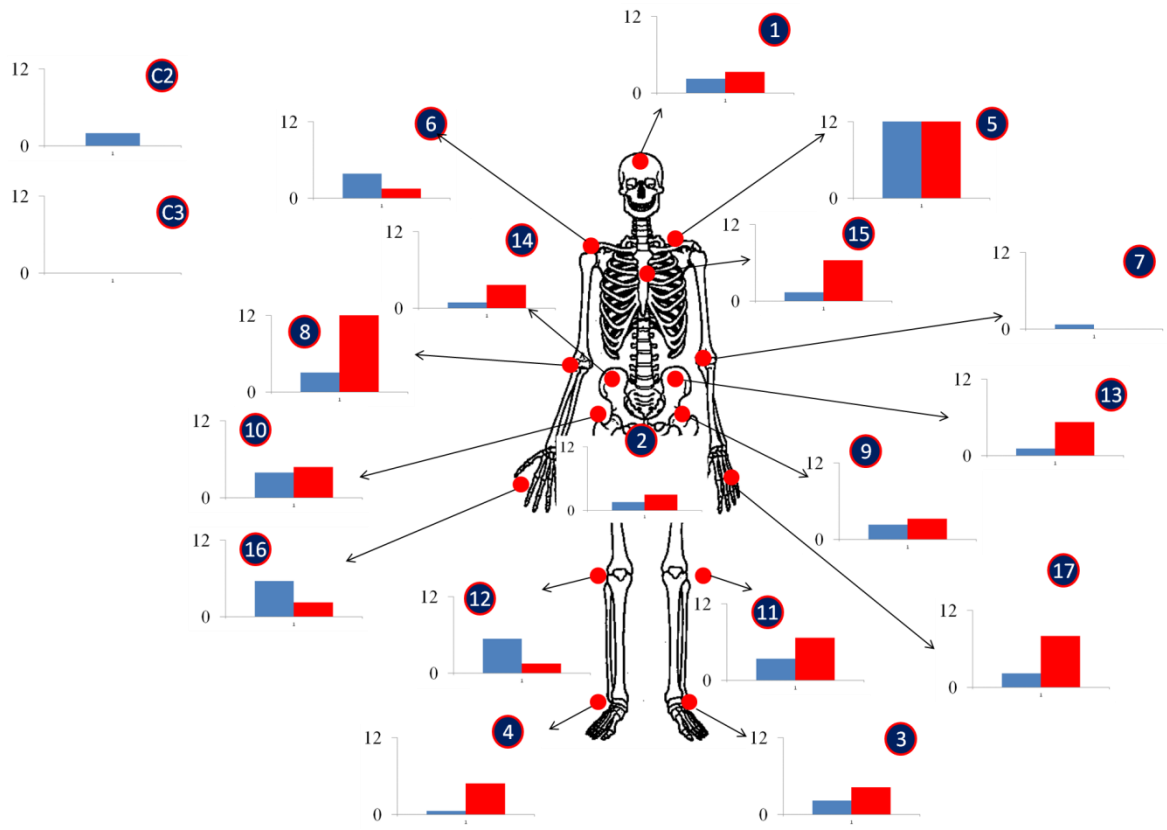

Figure 4.31: Cholesterol concentrations from the different sampling points from around the skeletal remains in MESW G422. The blue bar represents concentrations adjacent to the remains, and the red bar concentrations beneath the remains. Graph y-axis scales each range from 0-12 mg/g TOC. Originally in colour.

GC-MS of the sample from under the left hip (13Z) shows the presence of a small amount of coprostanol and 24-ethylcoprostanol (Figure 4.32). Coprostanol is formed by microbial transformation of cholesterol in the gut of most higher animals (Murtaugh & Bunch, 1967) and has been used to detect manuring (Bull *et al.*, 1999) and human activity (Bethell *et al.*, 1994). 24-ethylcoprostanol is formed by microbial transformation of sitosterol in the guts of higher animals (Leeming *et al.*, 1996) and has been used as a marker for herbivores. The steroidal components coprostanol and 24-ethylcoprostanol are signatures that are specific to higher animal activity and have both been observed in the faeces of humans (Leeming *et al.*, 1996), and have been known to reveal information on diet. An example of such was used to distinguish between the faecal waste carnivores and herbivores in waste waters (Leeming *et al.*,

1996). The author analysed the faces and waste of carnivores (cat), omnivores (human) and herbivores (cow and sheep) the presence of coprostanol was a major component in carnivore faeces whereas the human faeces found the presence of coprostanol and ethylcoprostanol and the herbivore faeces just showed the presence of ethylcoprostanol. No coprostanol or ethylcoprostanol could be detected following GC-MS analysis of the C2 sample or samples from adjacent to the hips (13Y and 14Y) or below the right hip (14Z). Thus, coprostanol and ethylcoprostanol appear to be localised to below position 13 and are likely to be present due to the remains, therefore, these components could be used to show that the individual had eaten a diet of meat and vegetables shortly before death.

Epicoprostanol was also detected below the left hip (13Z). The presence of the lipid in soil sediment has been attributed to further *in situ* microbial degradation of coprostanol (Bull *et al.*, 2002; Wardroper *et al.*, 1978), suggesting that significant degradation of coprostanol has occurred post mortem in the grave. The localised presence of coprostanol at position 13 suggests it to derive from the remains, and that isomerisation to epicoprostanol occurred over time. Epicoprostanol is known to have formed through the mediation of anaerobic bacteria (Leeming *et al.*, 1996) therefore it is likely that an anaerobic environment was established in the grave. Notably, hydroxy fatty acids or diacids, that have been found in the tissues of mummies (Gulaçar *et al.*, 1990), bog bodies (Evershed and Connolly, 1991) and adipocere (Forbes *et al.*, 2005), have been not been observed. Both of these components have been used as evidence for degradation of remains through an oxidative transformation pathway. As they are not present in the grave studied here it suggests that the body did not degrade through an oxidative transformation route. The occurrence of sterol reduction products, LMW *n*-alkanes and C16 and C18 *n*-alkanols and *n*-alkanals all provide evidence for an anaerobic environment having been established in the grave.

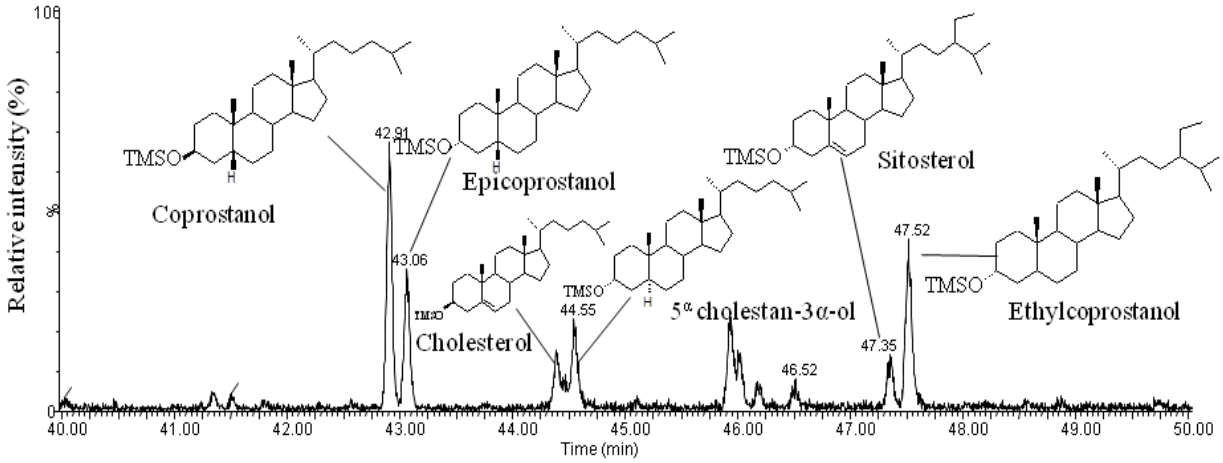

Figure 4.32: Partial mass chromatogram (*m/z* 215) of the high polar fraction from the sample below the left hip (13Z) in MESW G422 showing trimethylsilyl (TMS) derivatives of sterols.

4.4.3. Conclusion

The high intensity sampling from this particular burial has clearly shown differences between samples collected adjacent to and below the remains. Generally the samples that were collected from below the skeleton contain a greater abundance of markers relating to the remains.

Markers such as the LMW *n*-alkanes that relate to bacteria (Ladygina *et al*., 2006) and microbial reduction of free fatty acids formed from the degradation of adipose tissue (Park, 2005) are present in all of the samples along with plant related HMW *n*-alkanes. However, the abundance of these LMW *n*-alkanes increases in samples below the remains compared with the samples taken from adjacent to the same sampling location. The greatest abundance is found in samples that were collected from the gut region, similar to that seen in the Edinburgh grave (6B). By contrast, the abundance of HMW plant *n*-alkanes remains was fairly consistent for all of the samples. As the increase in LMW *n*-alkanes is more significant in the samples collected below the remains it suggests that markers relating to the body are more likely to be found in samples below the remains.

The steroidal component cholesterol, which relates to human activity (Bethell *et al*., 1994) is found in all the samples but as with the *n*-alkanes, cholesterol has a greater

abundance in most samples below the body. Other steroidal components that have been linked to human activity, such as coprostanol and 24-ethylcoprostanol (Leeming *et al*., 1996), were also found beneath the remains. Therefore, the additional sampling for this burial provides evidence that samples collected adjacent to did not. As with the Edinburgh grave, signatures relating to bacteria and/or degraded adipose tissue is increased around the gut region.

The steroidal components coprostanol and 24-ethylcoprostanol can reveal specific information relating to the interred remains. These compounds are specific to higher animal activity and have both been observed in the faeces of humans, where they can be used to reveal information on diet (Leeming *et al*., 1996). The components were only found below the left hip suggesting these are specific to the human remains and have the potential to reveal information about the diet of the buried individual. The presence of coprostanol and 24-ethylcoprostanol suggest that the individual that was buried could have potentially consumed a meat and vegetable diet in a short period before he died. However faecal material also contains levels of coprostanol that is related to non-dietary cholesterol therefore further analysis would be required to conclusively determine last meal and diet. The presence of epicoprostanol reveals that the coprostanol has been subjected to further degradation via anaerobic bacteria after the death of the individual within the soil. These signatures have been observed in faecal matter (Leeming *et al*., 1996) and have been used to detect manuring (Bull *et al*., 1998). Therefore the analysis of this grave illustrates the potential to observe such markers within soils, even if physical evidence of the material is not present within the burial

Several signatures within the burial have suggested that the grave environment was subject to anaerobic conditions; epicoprostanol is one of the signatures as it is known to be formed from coprostanol through anaerobic bacteria (Leeming *et al.*, 1996). Many of the graves studied have observed LMW *n*-alkanes and have seen significant increase in abundance of such components around the remains suggesting that these markers are related to the remains, either through bacterial activity (Ladygina *et al*., 2006) or the reduction of fatty acids relating to adipose tissue (Park, 2005). While the other graves discussed here have shown no traces of any reductive intermediates linking fatty acids to *n*-alkanes, C16:0 and C18:0 *n*-alkanols and *n*-alkanals which

could represent intermediates in the reduction (Park, 2005) were observed in the samples taken from below the shoulders and right hand from the grave from Mechelen. Notably, oxidation products of fatty acids such as hydroxy fatty acids and diacids (Gulaçar *et al.*, 1990; Evershed and Connolly, 1988; Forbes *et al*., 2005) have not been observed in any of the samples, therefore it can be inferred that degradation of the adipose tissue is unlikely to have been through an oxidative route. The presence of fatty acid reduction products (*n*-alkanals, *n*-alkanols and *n*-alkanes) and the lack of oxidative reduction products (hydroxy fatty acids and diacids) provide evidence that degradation of the fatty acids (that were hydrolysed from TAGs in the adipose tissue) occurred through a reductive pathway and that the remains were subjected to an anaerobic environment.

4.5. Conclusions

The first aim of the chapter was to determine the variability of components in different sampling points based on their anatomical and vertical position with respect to the skeletal remains. All of the graves discussed featured a sandy soil, which provides a consistent burial environment throughout the study in addition; the environmental conditions were quite similar for all of the graves as they were all located in northern Europe and will have experienced broadly similar weather and climatological conditions.

The Mechelen burial clearly shows the importance of sampling below the remains to provide the maximum information about the burial. This grave also suggests that degradation products from the body translate downwards through the grave under the influence of gravity/displacement by soil above or water movement, alternatively the bones may have protected the organic matter beneath them. Translation of degradation products downwards beneath the soil have been previously observed on analysis of soils of a temporary grave (Bull et al, 2009) and in leaching experiments carried out by Cassar *et al*. (2011); in which adipocere from a degrading pig carcass was found in the soil below the remains. Both the Mechelen and Edinburgh graves show that samples from gut region have a greater potential to retain signatures from the remains. However the analysis of the graves from Mechelen and Edinburgh all

infer that we should not be dismissive of other sampling points. The knee and hand region of the Edinburgh grave provided information on the nature of the burial environment (observation of diploptene and Hopenone *b*), whereas the shoulder and right hand in the Mechelen grave provided information on possible degradation routes of the remains (reduction products; *n*-alkanals, *n*-alkanols and *n*-alkanes). Even though very little was seen in variation the Syningthwaite burial should not be dismissed as it also showed an increase in abundance of LMW *n*-alkanes around the body compared with the controls. Therefore, despite their size, infant burials also have the potential to reveal signatures relating to the burial. Due to small nature of the remains, however, variation across the samples was limited which suggests that infant burials may require only limited sampling. All sampling points have the potential to reveal information about the burial but by sampling below the skeleton we may increase the potential of recovering chemical signatures that relate to the remains.

The aim of this chapter was also to focus on specific signatures that relate to the remains and what information they can reveal about the nature of the grave. Diploptene was observed in several samples taken from the Edinburgh grave. This compound occurs in bacteria and has been found in ancient bone (Evershed *et al*., 1995) where it was suggested to derive from bacteria responsible for the degradation of bone.

The Edinburgh grave also showed the presence of Hopenone *b* known to be found in a particular plant known as Cypress Spurge or grave yard weed (Starratt, 1969) as it was commonly found in graveyards. This type of plant was not known to be used for decorative purpose so may have became incorporated into the grave by accident during burial.

The specific signatures observed in the left hip sample collected from Mechelen included coprostanol and 24-ethylcoprostanol; both are microbial degradation products of cholesterol and sitosterol, respectively, in the gut of higher animals (Leeming *et al*., 1996). Signatures such as these have the potential to reveal information about diet of the individual (Leeming *et al*., 1996), as both 24-ethylcoprostanol and coprostanol are present this could suggest that the individual

consumed a diet of meat and vegetables before their death although the situation is complicated by contribution to the faecal material from non-dietary cholesterol. The sample also contains epicoprostanol, which is a degradation product of coprostanol by anaerobic bacteria, which suggests that post-mortem there is evidence of a reductive degradation route (Leeming *et al*., 1996).

The study of these graves therefore shows that analysis of the soil does have the potential to unearth specific signatures that reveal further information about the burial that may have not been revealed through observation of the grave alone. In all of the cases there was no physical evidence to suggest that either faecal material or plant material was present within the burial and only through analysis of the soil can these materials be revealed.

In addition to the previous aims, the study has also inferred a possible degradation pathway of organic matter within the graves. All of the sites have shown an increase in LMW *n*-alkanes, relative to the controls, suggestive of the reduction of fatty acids deriving from the remains (Day, 1978; Park, 2005). In addition no oxidative degradation products of fatty acids (hydroxy acids and diacids) that have been previously found in degraded tissues and post-mortem products (Gulaçar *et al*., 1990; Evershed and Connolly, 1988; Forbes *et al*., 2005) were detected in any of the samples analysed. The Mechelen burial provides the strongest evidence of a reductive pathway linking fatty acids to *n*-alkanes through the presence of potential reductive intermediates C16:0 and C18:0 *n*-alkanols and *n*-alkanals in samples from under the shoulders and the right hand. Degradation of organic matter via a reductive pathway provides evidence for anaerobic conditions within the burial.

These finding illustrate that the samples collected below the remains provide a greater potential of recovering components relating to the remains and that sampling from below the remains, where possible, would provide the most information from the grave. The graves also show that specific signatures can be found within the soil even if physical evidence is not present; these signatures have the potential to provide additional information regarding the diet of the individual and possible funerary decoration (or graveyard vegetation). In addition, the study has also revealed evidence for the operation of a reductive pathway of organic matter

degradation, which has not been previously observed in the analysis of degraded human remains.

CHAPTER 5. COFFIN BURIALS FROM NORTHERN EUROPE.

5.1 Introduction

The aim of the work described in this Chapter was to identify lipid components associated with burials and human remains, and to consider their possible transformation pathways post deposition. In addition, specific chemical signatures that could provide additional information regarding the nature of the burial ritual and burial environment were examined. The graves discussed in this Chapter were chosen as they all contained evidence of a coffin or of one having been present, through the presence of wood fragments and/or coffin nails. The study aimed to determine whether specific lipid signatures provide evidence for the presence of a coffin (which may be of particular use when visible evidence of a coffin has not survived) and the materials used in its construction.

Three burials were examined, from sites in Hofstaðir (Iceland), Sala (Sweden) and Thaon (Northern France). All of the burials showed evidence of a coffin having been present, usually through the evidence of wood fragments or coffin stains.

5.2 Iceland (HST-11)

5.2.1 Site description and sampling

The site of Hofstaðir in north-eastern Iceland was the location of a Viking settlement that was later used by Christians. Adult burials of Christian origin were found to the east of the church, with the women buried to the north, men to the south and child burials to the south of the church, although the locations by sex/age were not exclusive. Skeleton HSM-11-116 (Figure 5.1) was excavated from the Northern part of the cemetery during the 2011 season and is that of an adult female.

The burial soil was a histic andosol: andosols are soils rich in volcanic ash. They can hold large quantities of water, which can freeze and prevent drainage (Arnalds & Gretarsson 2004). Histic andosols occur in poorly drained areas, have organic contents of < 20% and pH values around 4.0-5.5, making them acidic

(Arnalds & Gretarsson 2004). The skeletal remains were in very good condition and were positioned supine with the hands crossed over the pelvic region.

Figure 5.1: Grave HSM-11-116 (photograph by Brendan Keely). Originally in colour.

Samples were collected from the burial in accordance with the InterArChive high intensity sampling strategy. One C2 and two C3 samples were collected from the grave fill. Control C2 was collected from above the abdominal region at a depth of 7 cm below the current soil surface and two C3 samples were collected, above the skull and one above the abdomen, at 36 cm depth. The remains were present 53 cm below the surface. In accordance with the InterArChive sampling strategy, samples were collected from 17 points around the remains, these soil samples were collected either adjacent to or below the remains (Figure 5.2; Table 5.1).

Table 5.1: Samples collected from HSM-11-116 including the sample numbers and the locations from which they were collected.

Sample number	Sample positions
C2	Control from above abdominal region (7 cm
C3	Control from above skull (36 cm depth)
C3	Control from above abdominal (36 cm depth)
1Y and 1Z	Skull (adjacent and below)
2Y and 2Z	Pelvis (adjacent and below)
3Y and 3Z	Left foot (adjacent and below)
4Y and 4Z	Right foot (adjacent and below)
5Y and 5Z	Left shoulder (adjacent and below)
6y and 6Z	Right shoulder (adjacent and below)
7Y	Left arm (adjacent)
8Y	Right arm (adjacent)
9Z	Left pelvis (below)
10Z	Right pelvis (below)
11Z	Left knee (below)
12Y and 12Z	Right knee (adjacent and below)
13Y and 13Z	Left hip (adjacent and below)
14Y	Right hip (adjacent)
15X	Chest (above)
16Z	Right hand (below)
17Z	Left hand (below)
A1	Black/brown stain above mandible
A2*	Possible coffin stain above thighs
A3*	Sample of wood above thighs
A4*	Sample of wood from next to right hip
A5	Beneath ribs right hand side
A6	Beneath the spine
A7*	Base of grave below shoulders
A8	Base of grave around sample 10
A9	Base of grave around sample 12

* Samples were collected for pyrolysis gas chromatography (Py-GC) analysis and will not be discussed fully in this Chapter. Y relates to samples taken adjacent and Z relates to samples taken below the remains.

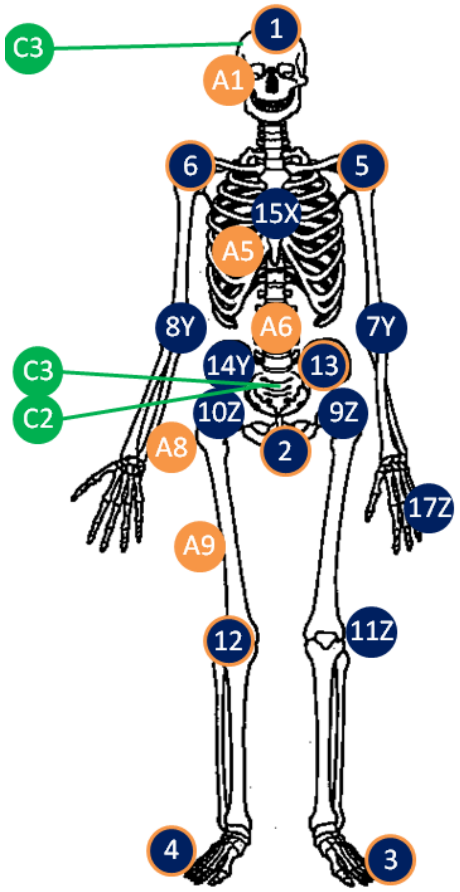

Figure 5.2: Positions of samples collected from grave HSM-11-116. Samples in green represent the controls. The blue represent InterArChive sampling strategy samples and the orange represents additional samples. Samples in blue with an orange outline represent more than one sample collected at that sampling point see Table 5.1. Original in colour.

5.2.2 Results and Discussion

5.2.2.1 Bulk soil analysis

Elemental analysis shows that the soil has a high TOC content (1-6%) and high levels of total carbon compared with other grave sites that have been analysed (Figure 5.3). The control C2 shows a low TOC level compared to total carbon, suggesting that the upper soil of the grave fill has more inorganic carbon content than that of the samples surrounding the remains. Samples from below the skull (1Z), the left hip (13Y), below the left hand (17Z), above the mandible (A1), coffin base below 10Z (A8) and coffin base below sample 12 (A9) have

significantly more organic carbon present than the controls. These samples were all taken from below the body and the hand sample 17Z was positioned above the pelvis in a similar region as 13Y. A1 was taken from around the skull, A8 and A9 were taken from the right hand side of the body and were of a darker brown shade, suggesting they may have incorporated coffin stains, the increase in organic matter may be due to material from a degraded coffin.

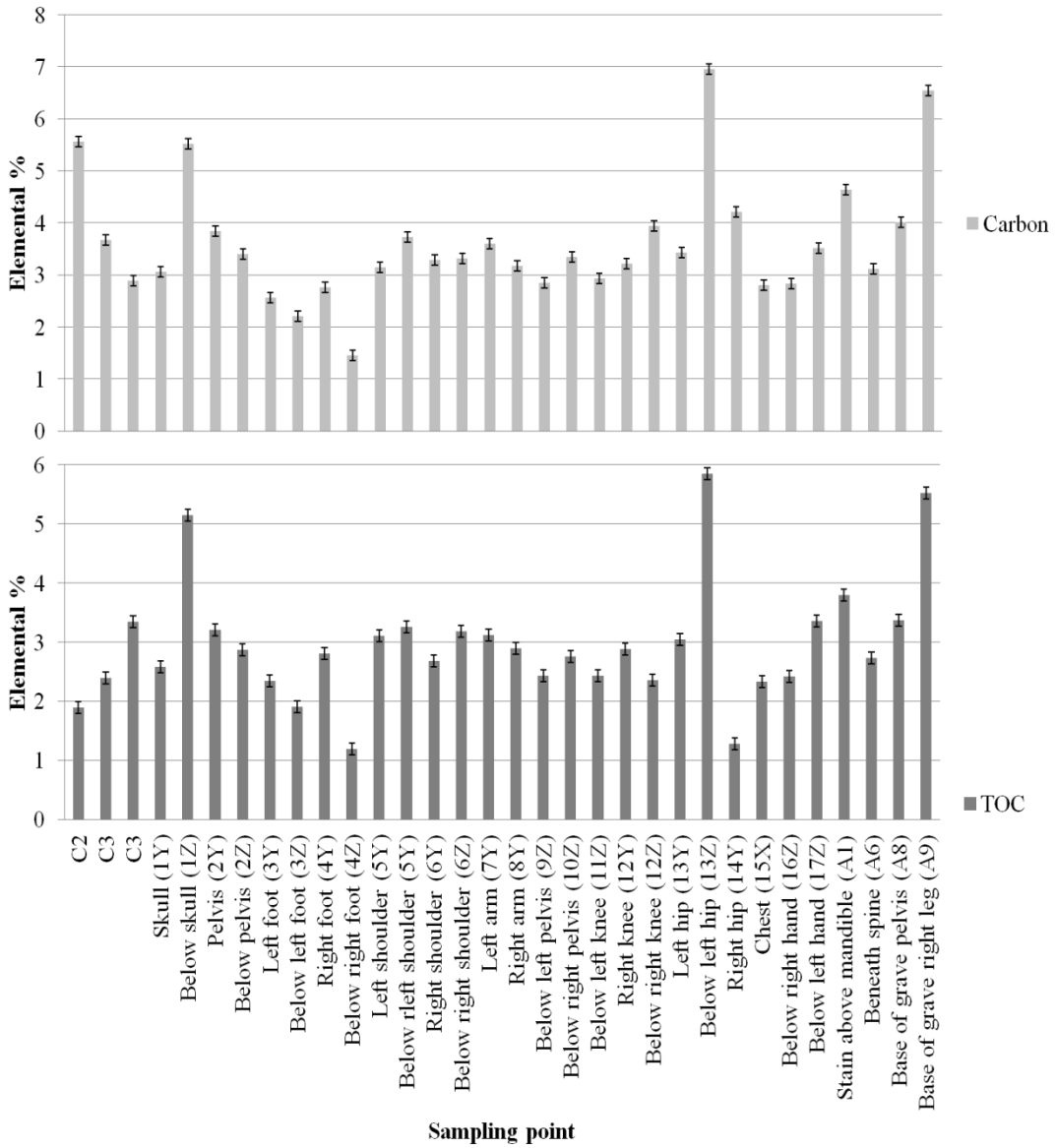

Figure 5.3: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soil samples from HSM-11-116. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

Sampling points from the C3, pelvis (2Y), right arm (8Y) and the left and right hip (13Y and 14Y) all contain elevated amounts of extractable organic matter compared to the other samples (Figure 5.4), which all have similar amounts of extractable organic matter (20-100 mg/g TOC). Notably the two C3 controls have differing amounts of extractable organic matter; this could be due to the positions that the samples were taken.

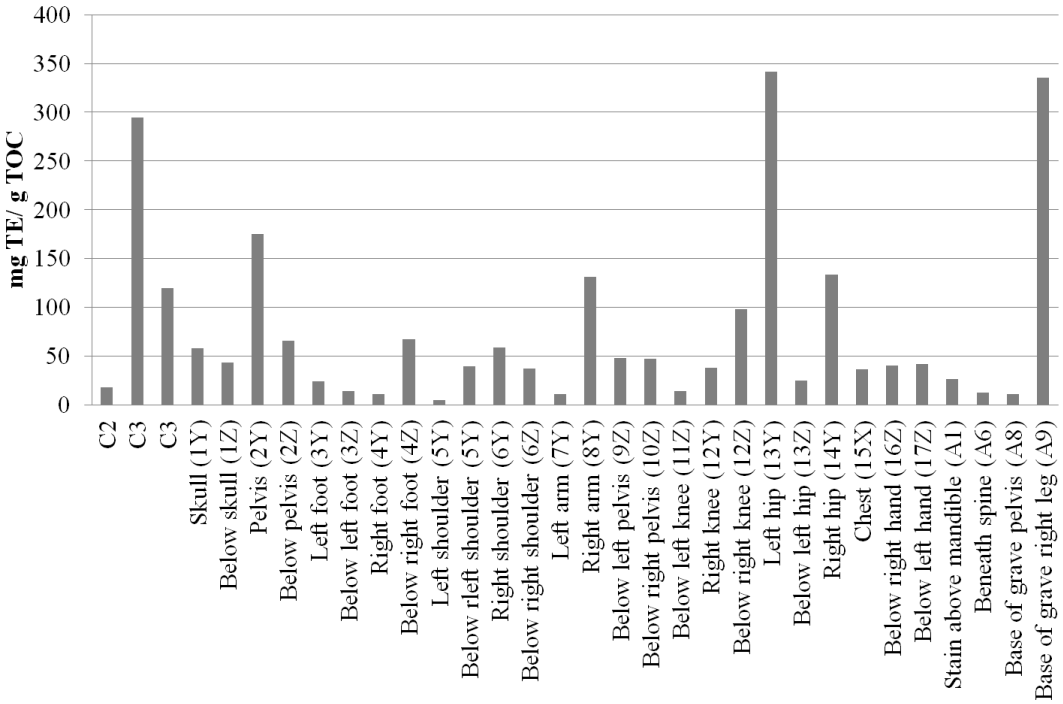

Figure 5.4: Solvent extractable organic matter in the soil samples from HSM-11-116 normalised to total organic carbon content.

5.2.2.2 Hydrocarbon fractions

The GC chromatograms of the hydrocarbon fractions for all sampling points exhibit the same hydrocarbon pattern showing a typical plant wax distribution (Eglinton and Hamilton, 1967; Jambu *et al.*, 1991) with a maximum at *n*-C27 (Figure 5.5). As these distributions occur in all of the sampling points this suggests that the signatures reflect the background soil organic matter and are not associated with the remains.

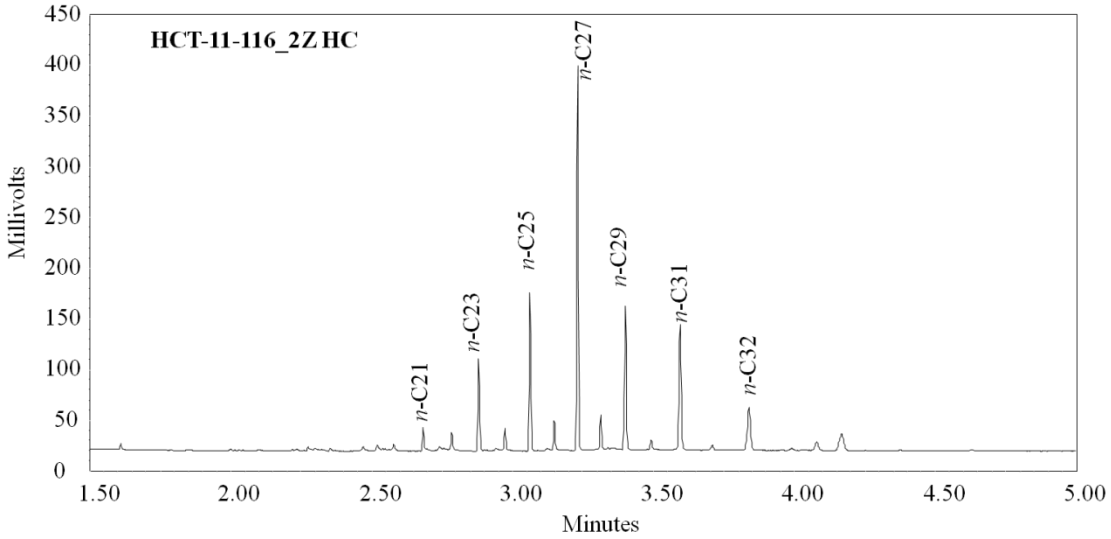

Figure 5.5: Partial GC-FID chromatogram of the hydrocarbon distribution from sampling point 2Z of grave HSM-11-116, which represents the hydrocarbon distribution from all of the sampling points.

5.2.2.3 Aromatic fractions

The aromatic fractions did not contain appreciable levels of aromatic compounds; however it did show the presence of *n*-alkanals and *n*-alkanone components which, owing to their polarity typically elute in this fraction. The aromatic fractions from C3, adjacent to the right foot (4Y), adjacent to the shoulder (5Y), adjacent to the left arm (7Y), under the left and right pelvis (9 and 10 Z) below the left and right knee (11 and 12 Z), adjacent to the left hip (13Y) and beneath the spine (A6), contain a number of *n*-alkanals and *n*-alkanones. All other sampling points contained no detectable components. The *n*-alkanones range from C21-C29 and displayed an odd over even distribution, the *n*-alkanals range from C21-C29 and shows an even over odd predominance both of the signatures is typical for higher plant waxes (Eglinton & Hamilton, 1967).

5.2.2.4 Medium polar fractions

The medium polar fractions of all samples contain distributions of *n*-alkanols with carbon chains ranging from C21-C30 and having an even over odd predominance (Figure 5.6), which is typical of leaf epicuticular waxes (Eglinton and Hamilton, 1967). Sampling points from the control above the abdomen (C3), adjacent and below the right foot (4Y and 4Z), adjacent to the left shoulder (5Y) below the right shoulder (6Z), the left arm (7Y), below the pelvis (9Z and 10Z), adjacent and below the knees (11Z, 12Y and 12Z), A5, A6 have an apparent maximum around C22 (Figure 5.6a) whereas other sampling points have a maximum at C26 (Figure 5.6b). The former distribution is typical of root input (Bull *et al.*, 2000) and the latter distribution is very typical of several types of leaf wax including grass and herbs (vanBergen *et al.*, 1997).

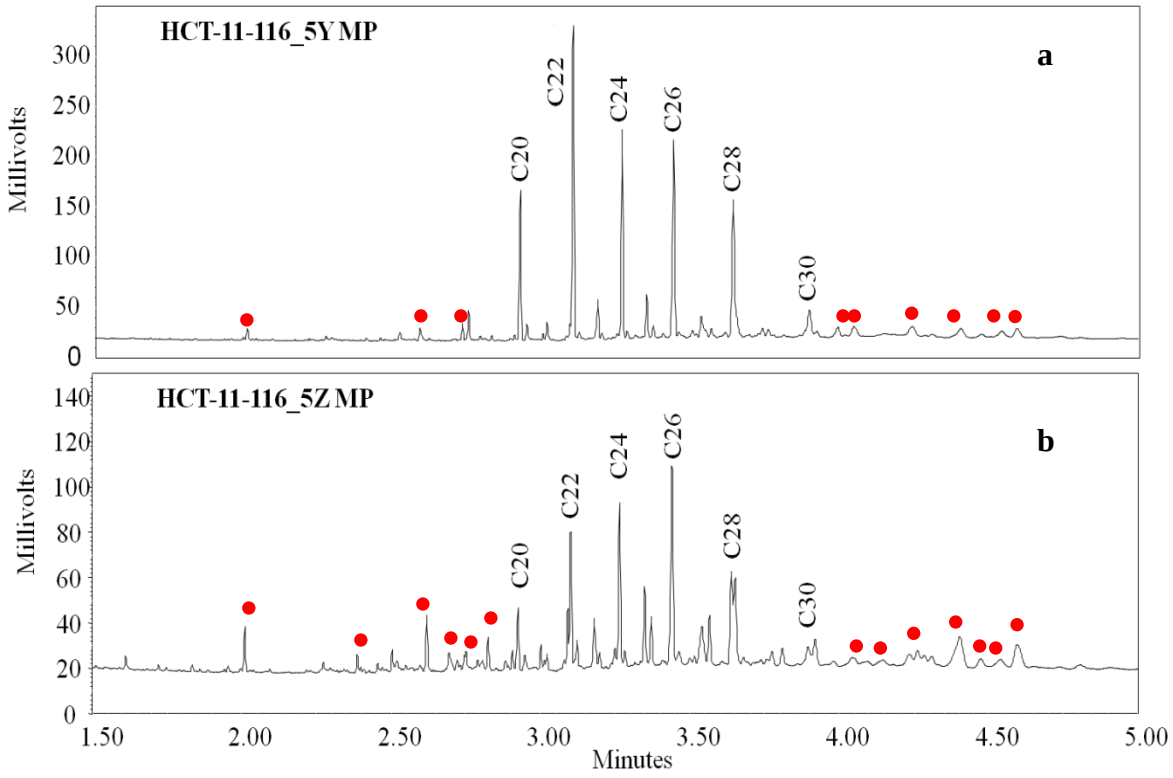

Figure 5.6: Partial GC-FID chromatogram of sample 5Y, which typifies the medium polar chromatogram for all samples collected from grave HSM-11-116. Red dots relate to plasticiser contamination (Chapter 2). Originally in colour.

Sterols including cholesterol, 5α-cholestanol, sitosterol, 24-ethyl coprostanol and stigmasterol were present in all of the samples. Sitosterol and stigmasterol are major sterols in plants whereas cholesterol is a major animal sterol, although it does occur to a lesser extent in plants. 5α-Cholestanol is the primary reduction product of cholesterol in the environment (Leeming *et al*., 1996). 24-Ethyl coprostanol is the reduction product of sitosterol within the gastrointestinal system of most higher animals (Leeming *et al.*, 1996). Sheep were kept on the site and their droppings were observed across the site which could provide a source for 24-ethyl coprostanol in the samples. As all these sterols are present in all of the samples with similar abundances they represent part of the soil background organic matter.

5.2.2.5 High polar fractions

The high polar fractions are dominated by a series of high molecular weight fatty acids (C20-C30) derived from plant sources (Eglinton and Hamilton, 1967) with very small contributions from C16:0 and C18:0 fatty acids and their unsaturated counterparts C16:1, C18:1 and C18:2. The LMW fatty acids have multiple possible sources including plants, animals, fungi and bacteria (Chapter 1). However the dominant components in the high polar fraction were the high molecular weight fatty acids and their abundances do not vary much throughout the samples. In addition the abundances of the LMW weight fatty acids are small so it is unlikely that they reflect an input from the remains.

Several ω-hydroxy fatty acids ranging from C22-C28, dominated by the C24 component, are also present in all of the samples. C22-C28 subunits linked as polyesters are a major component of suberin, a plant biopolymer associated with the exterior surface tissues, e.g. bark and roots (Kolattukudy, 1970). Microbial hydrolysis of the ester linkages in suberin can liberate ω-hydroxy fatty acids. Plant root systems are thought to be a significant contributors of ω-hydroxy fatty acids to soils (Bull *et al.*, 2000).

The GC-MS mass chromatogram of the high polarity fraction from below the skull revealed the presence of a number of cyclic diterpenoids (Figure 5.7) including pimaric acid, didehydrobietic acid, dehydroabietic acid and 7-oxo dehydroabietic acid, all detected as their methyl esters. In addition there is peak corresponding to the TMS ester of dehydroabietic acid presumable resulting from incomplete methylation of dehydroabietic acid with trimethylsilyl diazomethane.

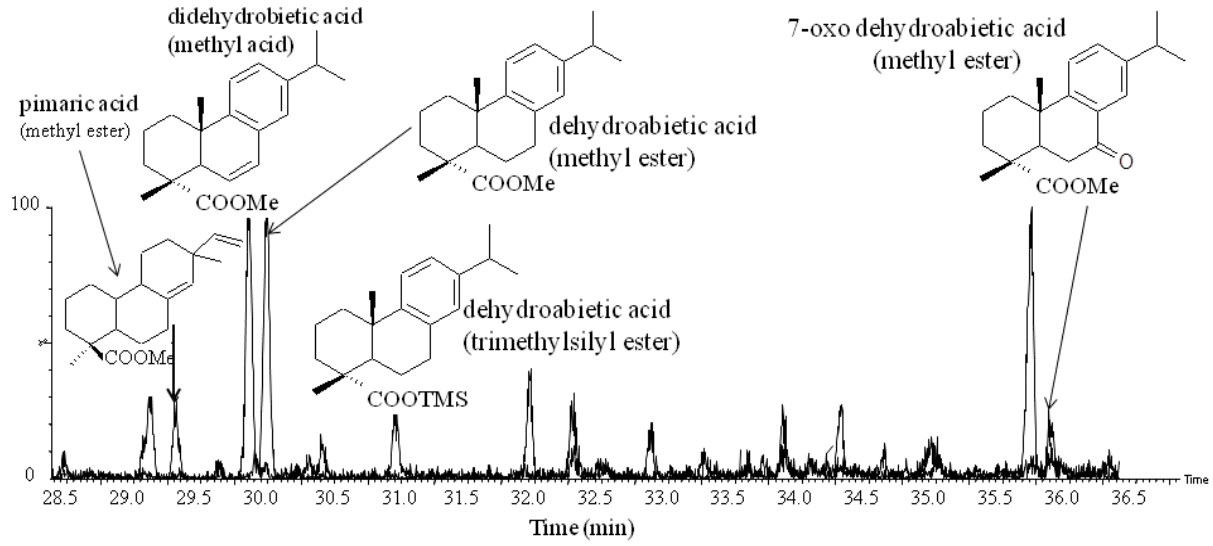

Figure 5.7: Combined partial mass chromatograms extracted for *m/z* 197 and *m/z* 257.

The diterpenoids were detected using MS, an example of the fragmentation of dehydroabietic acid TMS ester is shown in Figure 5.8 and further diagnostic fragments for the other diterpenoids are presented in Table 5.2.

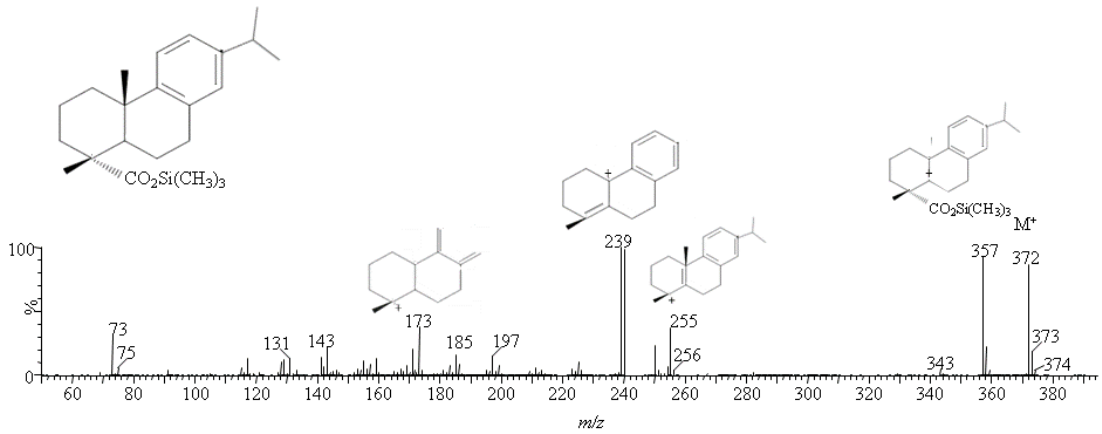

Figure 5.8: Mass spectrum of dehydroabietic acid TMS showing diagnostic fragments.

Table 5.2: Molecular ions (GC-MS) and diagnostic fragments for the diterpenoids identified.

Diterpenoid	**M^+**	**Diagnostic fragmentation *m/z***		
Abietic acid methyl ester	316	301	256	241
Didehydroabietic acid methyl ester	312	297	253	237
Dehydroabietic acid trimethylsily ester	314	299	255	239
7-oxo-dehydroabietic acid methyl ester	328	313	268	253

Abietic acid, pimaric acid and their degradation products are characteristic components of the resins that occur in the wood of the *Pinaceae* family (Colombini *et al.*, 2003; Robinson *et al.*, 1987) and are used as biomarkers for pine resin in aged wood (where the majority , if not all, of the abietic acid has typically been converted into dehydroabietic acid; Pollard & Heron, 1996). They have also been observed in soils that have been extracted from funnel shaped pits dating from the Viking to Roman age where the diterpenoids were considered to be related to pine tar production (Hjulström *et al.*, 2006).

Notably, the grave soil samples contain the compound 7-oxo-dehydroabietic acid, an oxidation product of dehydroabietic acid (Buckley *et al.*, 2004) that has been observed in the lipid analysis of wrappings that were taken from a mummified cat and was attributed to degradation of conifer resins (Buckley *et al.*, 2004). Abietic acid, which is typically the major component of fresh pine wood, was not present in the grave soil extracts (Colombini *et al.*, 2005); instead, high proportions of its degradation products dehydroabietic acid and didehydrobietic acid were present. It is, therefore, likely that abietic acid has been degraded completely. Pyrolysis GC analysis of small fragments of wood that were found in the grave revealed lignin derivatives consistent with gymnosperm wood (e.g. pine) supporting the findings from the solvent extractable diterpenoid components (Pickering *et al.*, 2011).

An origin for the resin components from the root systems of pine trees is unlikely given that trees of this kind, indeed any kind, are rare in Iceland (Arnalds &

Gretarsson, 2004). Furthermore, the resin acids were not detected in the C2 control though they were detected in the C3 controls. As the C3 samples were taken at a level just above that of the body, they could possibly contain material from the coffin lid. Accordingly, the presence of resin acids indicates the presence of coniferous wood (most likely a coffin) having been present in the grave.

The evidence for pine related material is of interest as pine trees are not indigenous to Iceland, which is described as non-vegetated or sparsely vegetated and has not changed much over time (Einarsson & Kristinsson, 2010). Icelandic vegetation is usually either grasses or mosses with birch and willow being the only natural trees to grow (Einarsson & Kristinsson, 2010). It is, therefore, likely that the pine was imported from neighbouring countries, such as the Scandinavian countries or Scotland, or was salvaged driftwood that had floated from neighbouring countries.

5.2.3 Conclusion

Bulk soil analysis shows high levels of organic matter in the burial soils. The majority of the organic signatures relate to root material and plant waxes. There appears to be very little in terms of organic material, such as LMW *n*-alkanes, fatty acids and sterols discussed in previous chapters that can be related to the skeletal remains. Consequently, little can be inferred about degradation pathways for organic material relating to the burial. The burial soil is a histic andosol, which can be prone to erosion (Arnalds & Gretarsson, 2004). An erosive environment could mean that soil containing the organic markers relating to the body has eroded over time. Such a process could explain the paucity of material relating to the remains and the similarities between the organic signatures around the skeletal remains and those of the host soil.

In spite of the paucity of organic signatures, specific markers that relate the burial have been observed in low abundance in the sample below the skull. These include pine resins, not previously observed in other burial sites, which could indicate the presence of a coffin. Small fragments of coffin wood were present within the burial and analysis of the soil (from samples below the skull) and Py-GC of the wood itself have determined it to be pine wood. Not only does this signature reveal the type of wood used for the burial but it also brings up questions relating to how the wood was transported to the site. As pine wood is not native to Iceland, the wood must have been either imported from other countries or reclaimed driftwood and was used to make the coffins. Hence, even though the grave showed little in terms of the human remains, the presence of specific signatures within the soil provides information on the nature of the burial.

5.3 Sala (RAA)

5.3.1 Site description and sampling

Sala is located in the Västmanland area of Sweden and is known for its silver mine that dates back to the 16th century (Zakrzewski and Burke, 1987). The soil is a podsol which is typically found under coniferous forests. The grave discussed here is 7464, which is that of a female aged in her early teens (Figure 5.9). The bones and coffin wood were well preserved, the latter allowing for collection of coffin wood samples. The body was laid supine with the arms crossed over the body. The skull was collapsed and there appears to be an ash deposit on the left side of the chest area. Samples were collected in accordance with the InterArChive high intensity sampling strategy (Table 5.3; Figure 5.10). A control (C3) was taken from the right side of the grave and 15 samples were taken from around the skeletal remains, the hands (samples 16 and 17 in the sampling protocol) were located across the pelvic area of the remains.

Figure 5.9: Grave RAA 7464 (photograph by Brendan Keely). Originally in colour.

Table 5.3: Sample collected from RAA 7464 including the sample numbers and the locations from which they were collected.

Sample number	Sample positions
C3	Control from right side of the grave
1Y	Skull
2Y	Pelvis
3Y	Left foot
4Y	Right foot
5Y	Left shoulder
6Y	Right shoulder
7Z	Left arm
8Z	Right arm
9Z	Left pelvis
10Z	Right pelvis
11Y	Left knee
12Y	Right knee
13Z	Left hip
14Z	Right hip
15X	Chest
A1	Coffin base adjacent to left femur
A2*	Coffin wood LHS top
A3*	Coffin wood LHS bottom
A4*	Coffin wood RHS top
A5*	Coffin wood RHS bottom
A6*	Coffin wood LHS middle
A7*	Coffin wood LHS middle
A8	Inside the base of the skull
A9	Below Skull

* Samples were collected for Py-GC analysis and will not be fully discussed in this Chapter. Y relates to samples taken adjacent and Z relates to samples taken below the remains.

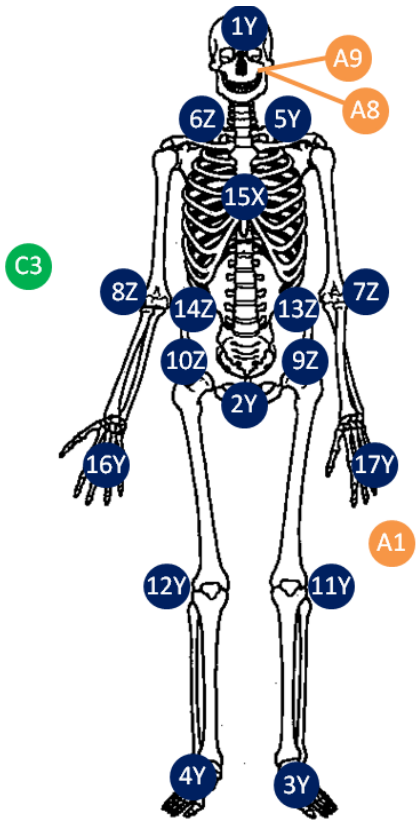

Figure 5.10: Samples taken from Sala grave RAA 7464, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive sampling strategy are labelled in blue and additional samples are labelled in orange. Originally in colour.

5.3.2 Results and Discussion

5.3.2.1 Bulk soil analysis

CHNS and TOC analysis was performed in order to indicate the abundance and form of organic material present in the soil. Elemental analyses of samples from grave RAA 7464 shows that they all have a small amount of nitrogen (0.05-0.7%) and that TOC levels are generally low compared with the amount of total carbon (Figure 5.11), indicating that the soil contains predominantly inorganic carbon. The sampling points from the skull (1), right foot (4), left hip (9), right hip (10), left iliac blade (13), right iliac blade (14), sternum (15), left hand (17) and under the skull (A9) all show significantly greater TOC levels than that seen in the

control (C3). The other samples show similar or lower TOC contents than the control sample (C3).

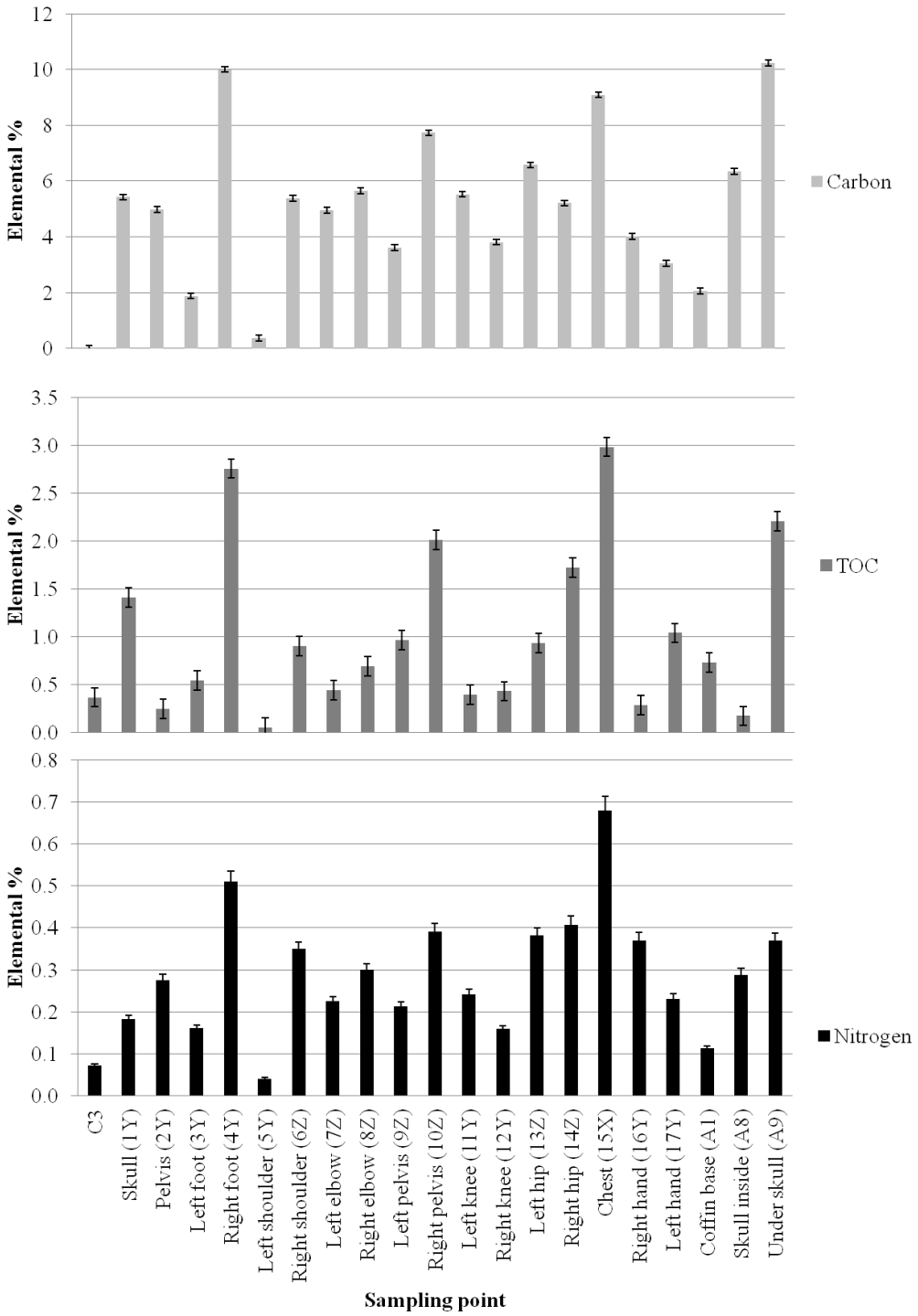

Figure 5.11: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soil samples from RAA 7464. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

Sampling points from the pelvis (2), left shoulder (5), left knee (11), right hand (16) and inside the skull (A8) contain the highest proportion of solvent extractable organic matter. All other sampling points have similar amounts of extractable organic matter (40-100 mg/g TOC). Notably the sample from inside the skull (A8) shows a much higher level of extractable matter than the other samples although it did have a low TOC content by comparison with the other samples, suggesting that this sample could contain less polymeric organic material.

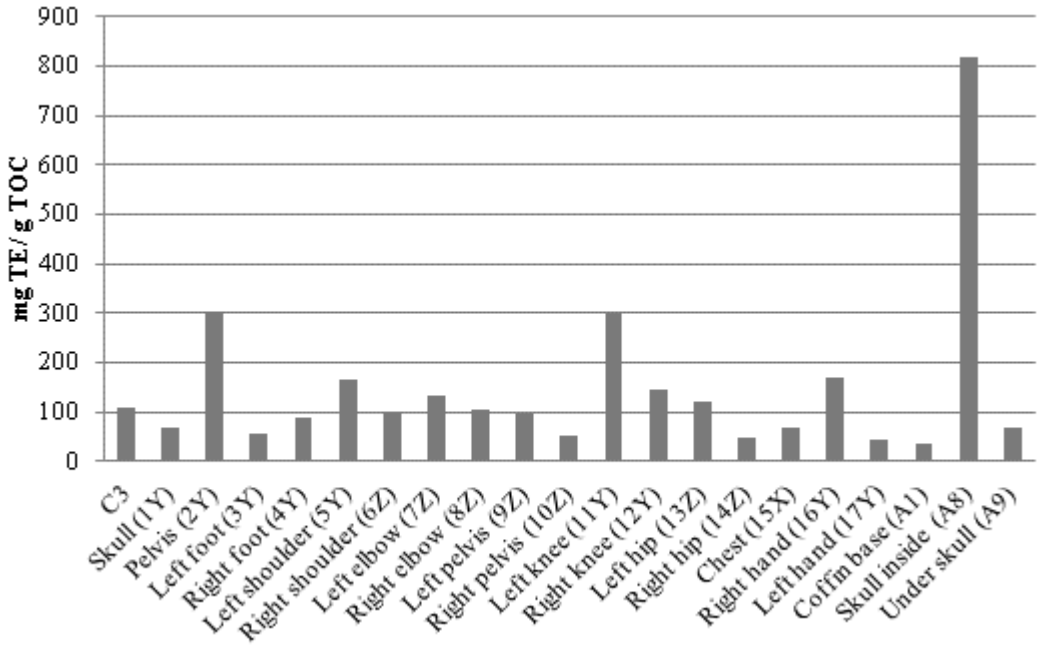

Figure 5.12: Solvent extractable organic matter in the soil samples from RAA 7464 normalised to total organic carbon content.

5.3.2.2 Total extracts

The GC chromatograms of the total extracts of the samples from around the skeletal remains were significantly different from that of the C3 control (Figure 5.13). The C3 control showed very little by way of components eluting before 35 min, whereas, all other samples showed a cluster of abundant components (Figure 5.13). In order to simplify the distributions the extracts were separated into four chromatographic fractions.

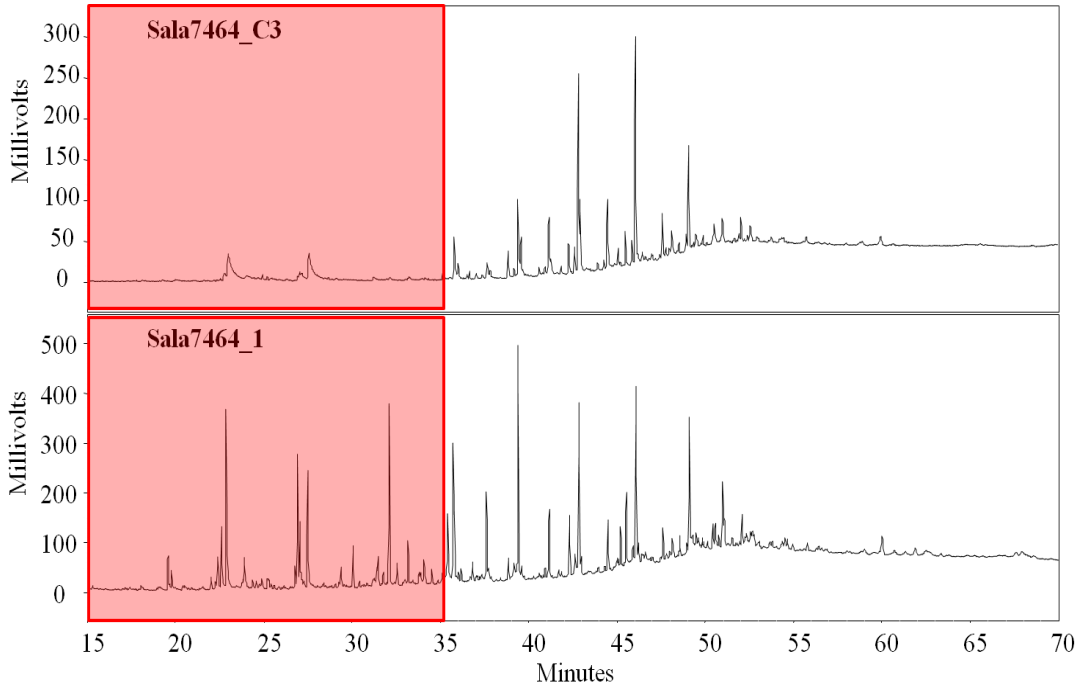

Figure 5.13: Partial chromatogram from the C3 control sample (top) and the skull (1; bottom), which typifies the total extract profiles for the sampling points from RAA 7464. Originally in colour.

5.3.2.3 Hydrocarbon and aromatic hydrocarbon fractions

The hydrocarbon fractions from all sampling points contain a series of *n*-alkanes ranging from C23-C33, the *n*-alkanes displayed a bimodal distribution with and odd over even predominance typical of plant waxes (Eglinton and Hamilton, 1967). The distribution maximum, at *n*-C31, reflects that of grassland as the predominant vegetation (van Bergen *et al.,* 1998). The *n*-alkane distribution is the same throughout the samples, including the control, suggesting that they reflect the soil background organic matter.

The aromatic fractions for all samples contain a series of ketones (C23-C29) having an odd over even predominance and a maximum at C27 and thus, which can be related to plant material (Eglinton and Hamilton, 1967).

5.3.2.4 Medium polar fractions

All samples contain a series of *n*-alkanols (C22-C30) with an even over odd predominance and a maximum at C24, characteristic of plant wax components (Eglinton and Hamilton, 1967). A maximum at C24 is typical of woodland soils (van Bergen *et al*., 1998).

In addition, the soil samples contained a series of plant sterols including stigmasterol and sitosterol, the former being present in high abundance throughout the samples. The transformation product 24-ethyl coprostanol, the major degradation product of stigmasterol in the gut of higher animals, was present in the samples (Leeming *et al*., 1996). Cholesterol and its degradation product 5α-cholestanol were present in much lower amounts throughout the samples and control (Figure 5.14). The abundances of all of the sterols throughout the different sampling points were analysed to see if the cholesterol and its degradation product are linked to the remains or are part of the background soil. The areas sampled from beneath the right shoulder (6Z), adjacent to the right arm (8Y) and beneath the skull (A9) display the highest sterol contributions (Figure 5.14). As similar relative abundances of each of the different sterols and stanols were concentrated within the same sampling points (8Y, 6Z and A9) it is likely that they are from the same source and likely to be of part of the background soil.

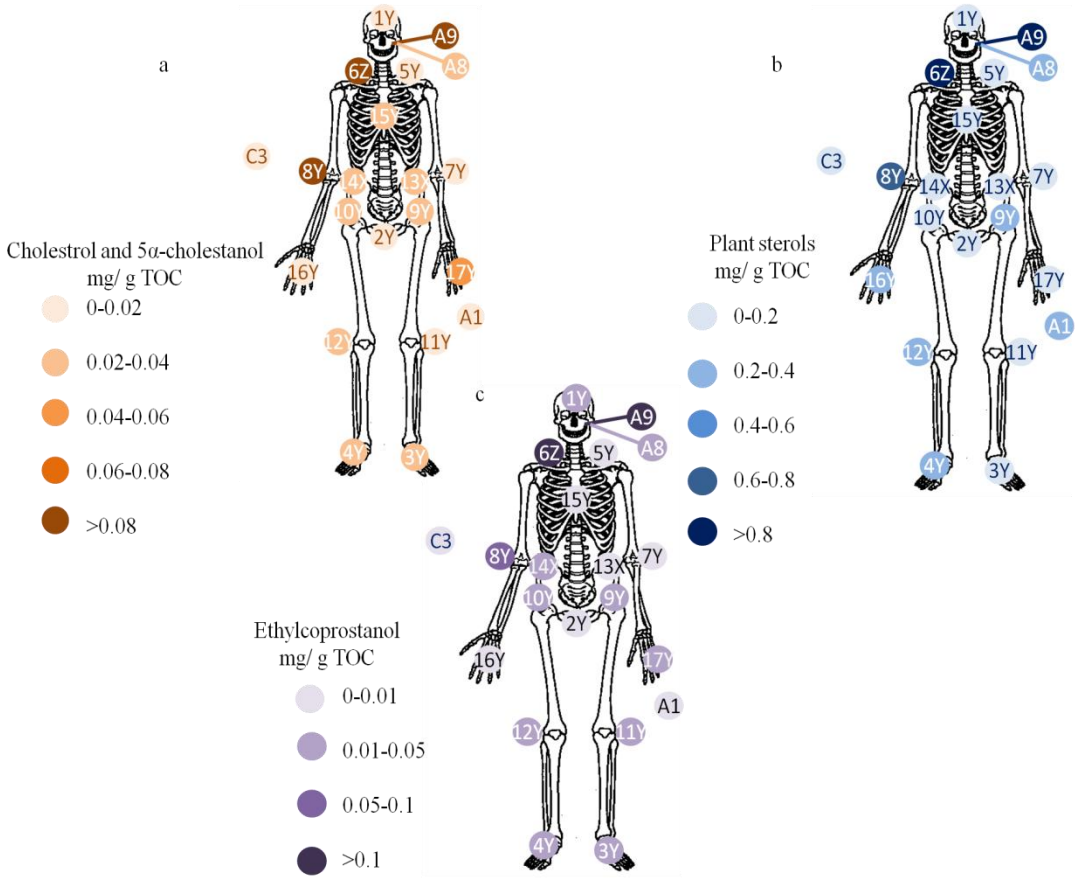

Figure 5.14: Distributions of sterols in grave RAA 7464; (a) distributions of cholesterol and its reduction product 5α-cholestanol (b) distributions of the total plant sterols and (c) distributions of 24-ethyl coprostanol. Originally in colour.

5.3.2.5 High polar fractions

The high polarity fractions for all samples contained a series of plant fatty acids ranging from (C22-C30) and having an even-over-odd predominance indicative of plant sources. All samples, excluding C3, contain a series of LMW saturated fatty acids ranging from C14-C18 accompanied by the unsaturated counterparts C16:1, C18:1 and C18:2. In addition, the samples from around the remains and the controls contain *iso*- and *antiso*- branched C15 components, indicating a bacterial input (Zelles, 1997). The C16 straight chain fatty acid had the greatest abundance with the C16:1 and C18 components being present at similar levels and the other components being minor constituents.

The LMW components are not present in the C3 sample suggesting that these components are associated with the interred remains. They may reflect contributions from both bacteria (due to the presence of the branched fatty acids) and degraded adipose tissue. The ratio of the LMW to HMW fatty acids across the sampling points indicates that the higher proportions of LMW fatty acids were present in the samples above the right hip (14) and the chest (15). The ratio compares the abundance of the fatty acids relating to plant (HMW) with fatty acids that may relate to bacterial or animal contribution. Therefore, higher proportions of fatty acids relating to bacteria and animal tissues are evident at the right hip (14X) and chest area (15Y).

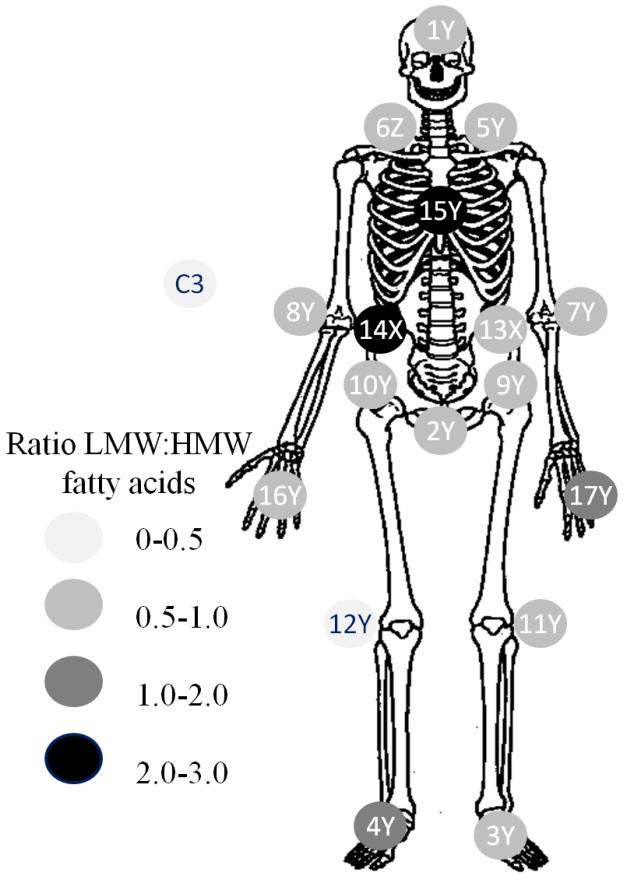

Figure 5.15: Ratio of LMW to HMW fatty acids in the samples collected from grave RAA 7464.

The samples from around the skeletal remains of RAA 7464 contained a series of diterpenoids including pimaric acid, abietic acid, didehydrobietic acid, dehydroabietic acid and 7-oxo-abietic acid, all analysed as their methyl esters, which are indicative of conifer resins (Colombini *et al.*, 2003). The abundance of the pine resins is elevated (Figure 5.16) in the sampling points from beneath the right shoulder (6Z), and beneath the skull (A9). Notably, the levels in the sample taken from the right arm were also quite high (0.3 mg/g TOC). By reference to the distributions of the sterols (Figure 5.14), similarities in the relationship between the distribution of plant sterols and the pine resins suggesting that the coffin wood could be the source of some of the plant sterols in the grave.

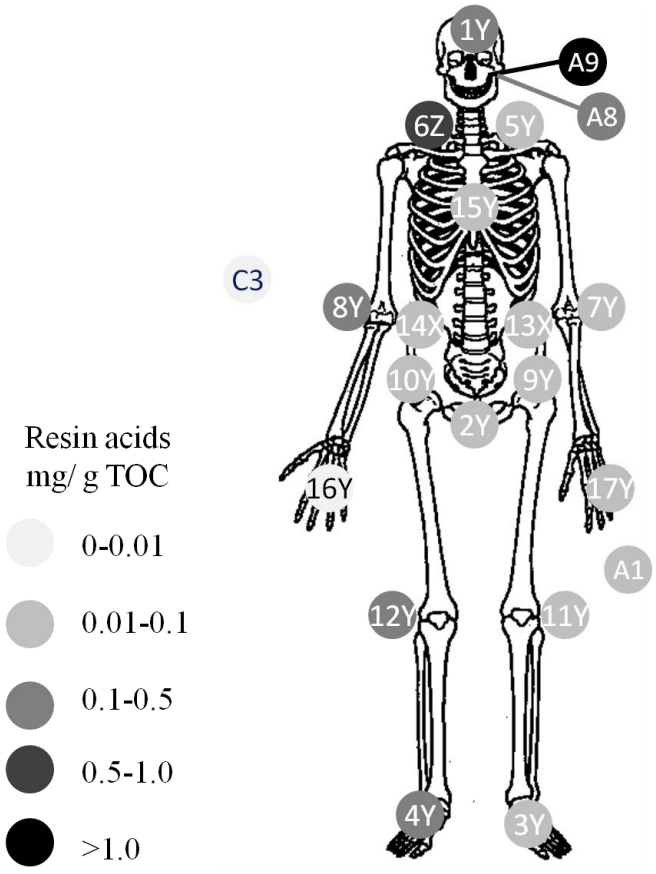

Figure 5.16: Distributions of pine resin acids around the skeletal remains of grave RAA 7464.

Py-GC analysis of the wood samples A2-A7 revealed them to be pine wood, consistent with the diterpenoid profiles from the soil extract analyses (Adam Pinder unpublished results). Unlike Iceland, pine wood is native to the Scandinavian countries, which could explain the large amounts of diterpenoids present in the soil; Sala samples contain ~0.1-1.0 mg/g TOC of diterpenoids whereas the diterpenoids in the Iceland samples were at trace levels. Abietic acid, present in high levels in fresh pine (Colombini *et al*., 2003), was not detected in the Iceland grave but was observed in the Sala samples, suggesting that in the former site they had degraded below the limit of detection. Therefore, there appears to be less extensive degradation of the conifer resin signature in the Sala samples than in the Iceland burial.

5.3.3 Conclusion

The study shows that signatures related to the remains are present within the soils. LMW fatty acids were present throughout the soil samples and were not observed in the control, suggesting that they are present due to the presence of the remains. These fatty acids have been present in other burial soil samples discussed in this thesis and are likely to derive from the hydrolysis of the acyl moieties of TAGs. The presence of branched fatty acids within the soils also indicates bacterial inputs.

Specific diterpenoid signatures relating to pine resin have also been found in sampling points from around the remains but not in the controls, consistent with the visible wood fragments within the grave. The results suggest that the remains were originally buried in a pine coffin. The pine resin signature was more abundant and signified a lower extent of degradation than that observed for the Icelandic burial. Hence, it is likely that the wood had degraded less in the soils from Sala than the soils from Iceland.

The results further illustrate that LMW fatty acids are likely to be present in the soil due to the presence of the burial and that they can reflect the degradation of adipose tissue or bacterial input. In addition, specific signatures relating to the coffin material can also be found within the soil from around the remains.

5.4 Thaon (TESP)

5.4.1 Site description and sampling

Thaon is a small town located in the Basse-Normandie region of north-west France. The excavation took place inside the church of St. Peter's (Figures 5.17 and 5.18) where several hundreds of bodies were found at different layers under the church.

Figure 5.17: Church of St. Peter's, location of the excavation in Thaon, France (photograph by Brendan Keely). Originally in colour.

The church of St Peter's was first established in the 11th century with a new church, the subject of the excavation was built in the 19th century. The grave discussed TESP 421 was one of seven graves that were excavated during the site visit in 2011. This grave was sampled in the final season of the church excavation and was located in the lowest level of graves. The soil at this level was mainly clay and had been waterlogged at the time of excavation, a common feature of these burials. The grave revealed preserved coffin panels within the burial and the skeletal remains were those of an adult lying supine with the hands crossed over the pelvis (Figure 5.19).

Figure 5.18: Inside St Peter's church during excavation (photograph by Brendan Keely). Originally in colour.

Figure 5.19: Grave TESP 421 (photograph by Brendan Keely). Originally in colour.

High intensity sampling was carried out on the remains: 15 samples were taken from around the body in accordance with the InterArChive sampling strategy. Samples were not obtained for the hands as they were difficult to locate within the

burial. Due to the nature of the excavation, with several layers of burials overlying this one, controls could not be taken (Figure 5.20; Table 4.)

Table 4: Samples collected from TESP 421 including sample numbers and the locations from where they were collected

Sample number	Sample position
1Y	Adjacent to the skull
1Z	Beneath the skull
2Z	Beneath the pelvis
3Z	Beneath the left foot
4Z	Beneath the right foot
5Y	Adjacent to the left shoulder
6Z	Beneath the right shoulder
7Z	Beneath the left arm
8Z	Beneath the right arm
9Z	Beneath the left pelvis
10Z	Beneath the right pelvis
11Z	Beneath the left knee
12Z	Beneath the right knee
13Y	Adjacent to the left hip
14Y	Adjacent to the right hip
15Y	Adjacent to the chest
15Z	Beneath the chest
A1	Below sacrum
A2*	Wood sample from chest
A3*	Wood sample from the chest
A4*	Wood samples from the legs
A5*	Wood sample from the knees
A6*	Wood sample from underneath the shoulders
A7*	Wood sample from underneath the shoulders
A8	Bone fragments from 2Z
A9	Bone fragments from 13Y
A10	Bone fragments from 14Y

*Samples were collected for Py-GC analysis and will not be fully discussed in this Chapter. Y relates to samples taken adjacent and Z relates to samples taken below the remains.

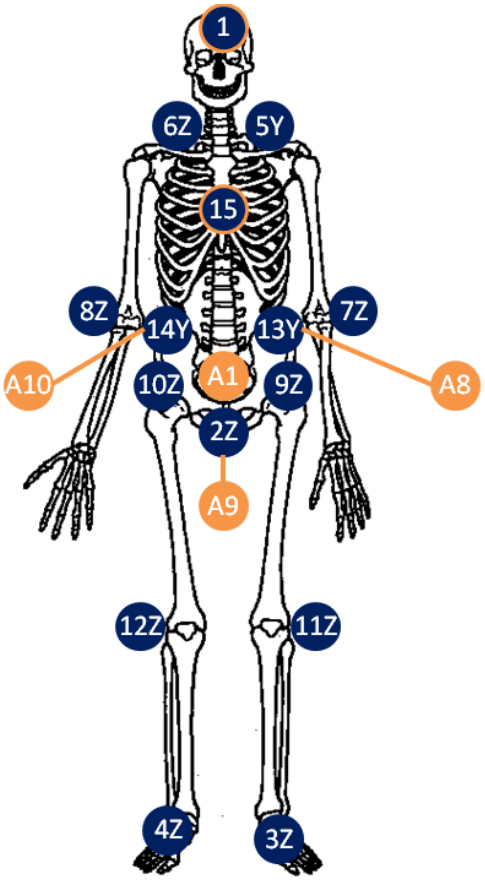

Figure 5.20: Samples taken from Thaon grave TESP 421, the control samples are labelled in green, the samples collected around the body in accordance with the InterArChive sampling strategy are labelled in blue and additional samples are labelled in orange. Originally in colour.

5.4.2 Results and Discussion

5.4.2.1 Bulk soil analysis

Elemental analysis of samples from grave TESP 421 shows that all of the samples have low levels of nitrogen (Figure 5.21; 0.15-0.6%). TOC levels are generally low compared with the total amount of carbon (Figure 5.21) suggesting that the soil contains proportionately more inorganic carbon. The sampling points from below the foot (4Z) showed a greater amount of total organic carbon than that seen in the other sampling points. It is possible that this reflects a contribution from fragments of coffin wood that may have been collected with this sample.

Notably the nitrogen contents in the bone fragments is higher than in the soil samples, which would be consistent with, and may indicate, the preservation of collagen-derived material in the bones.

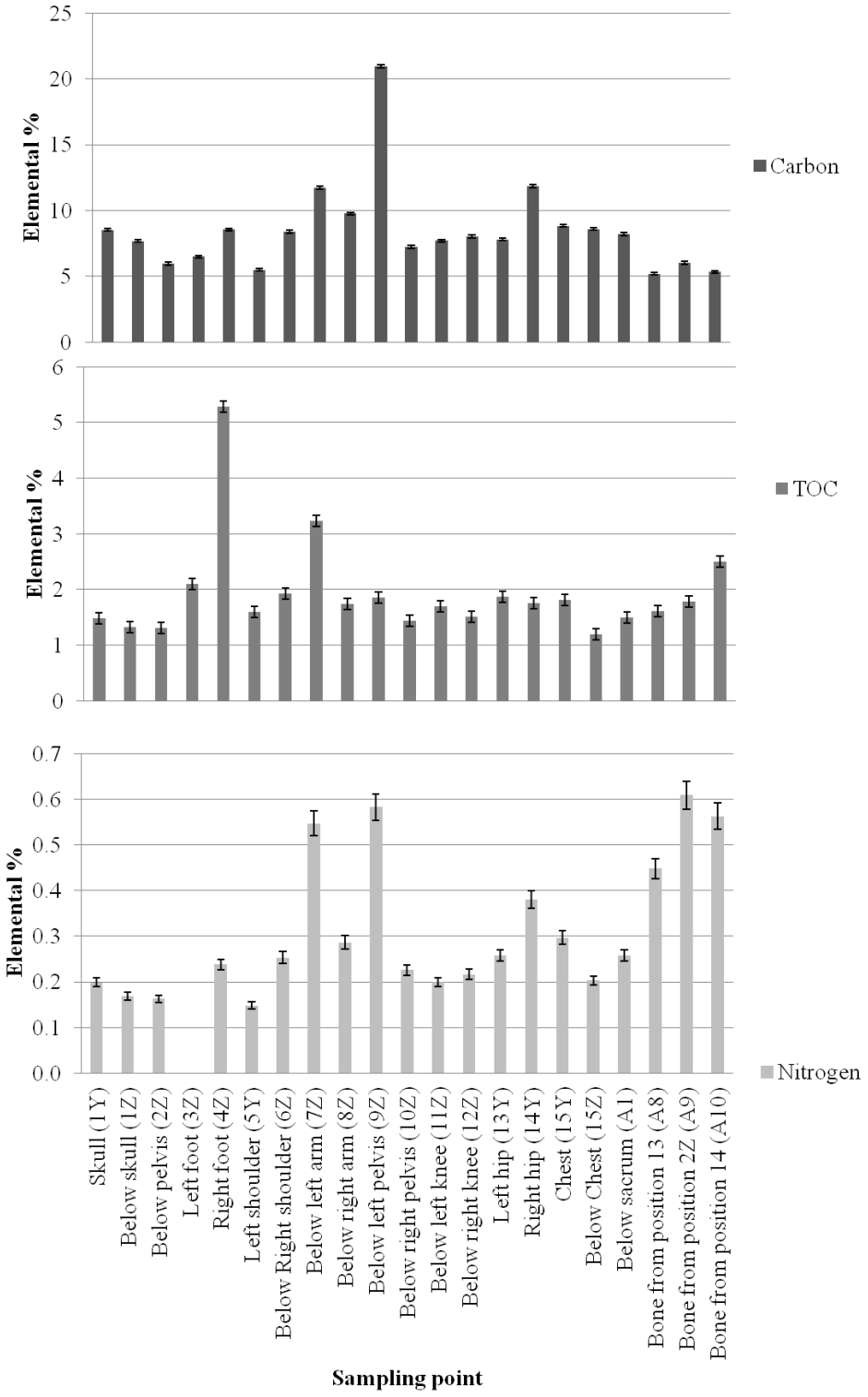

Figure 5.21: Bulk carbon, nitrogen and total organic carbon (TOC) elemental analyses of soil samples from TESP 421. Error bars represent ± 1 standard deviation calculated from replicates of standards (*n*=4).

All soil and bone samples contain a similar amount of extractable organic matter (15-70 mg/g TOC; Figure 5.22). The right foot (4Z) has the lowest amount of extractable organic matter even though it had the highest TOC content, which suggests that the sample mainly contained polymeric material, consistent with this sample having included some coffin wood. The sampling points with the highest amount of extractable organic matter (> 60 mg/g TOC) are from around the pelvic region, including pelvis (2Z), left and right pelvis (9Z and 10Z), right hip (14Y) and below sacrum (A1). Notably, samples taken from adjacent and below the remains (at the skull and the chest) contained similar amounts of extractable organic matter.

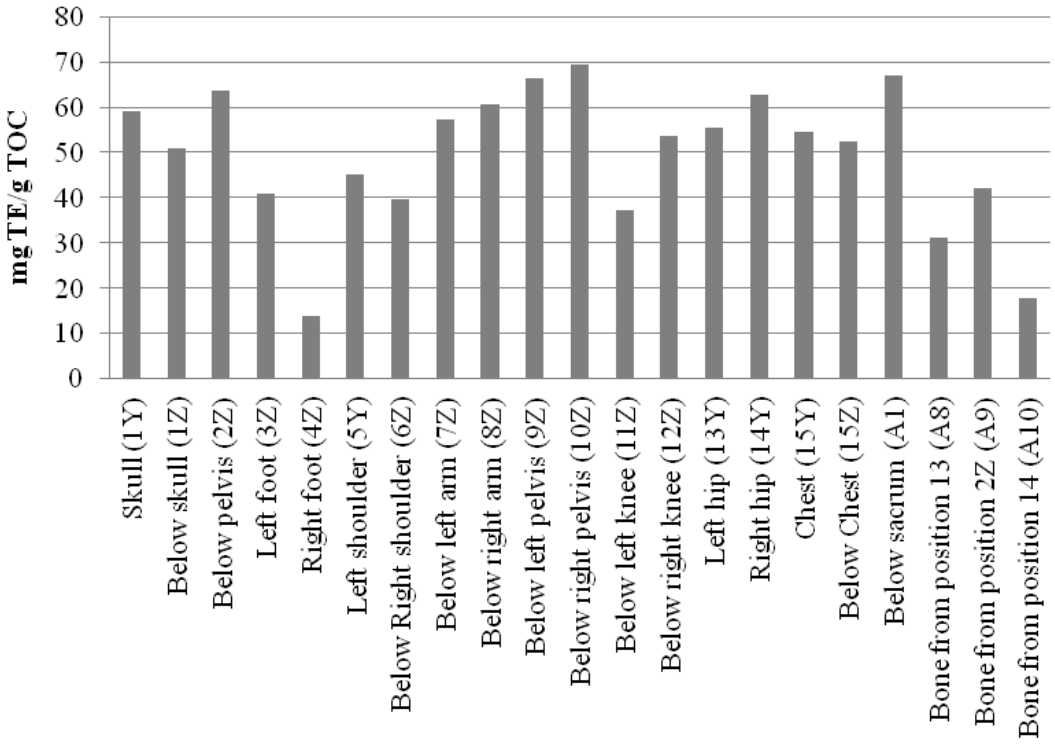

Figure 5.22: Solvent extractable organic matter in the soil samples from TESP 421 normalised to total organic content.

5.4.2.2 Total extracts

GC analysis of the total extracts reveals them to exhibit two distinct profiles, with the soil samples having a different profile to the bone samples. All soil samples have total extracts that display two distinct compound groups in the chromatogram, LMW components (< 30 min) and HMW components (> 35 min; Figure 5.23a). The samples of bone fragments contained some components similar to the soil extracts and also contained two large peaks around 46 min (Figure 5.23b), identified as being cholesterol and 5α-cholestanol. These have been reported to be the major lipid components in modern and archaeological bone (e.g. 4th -6th century human tibia Evershed *et al.*, 1995). Investigation of the chromatographic fractions was performed to reveal further differences between the individual sampling points.

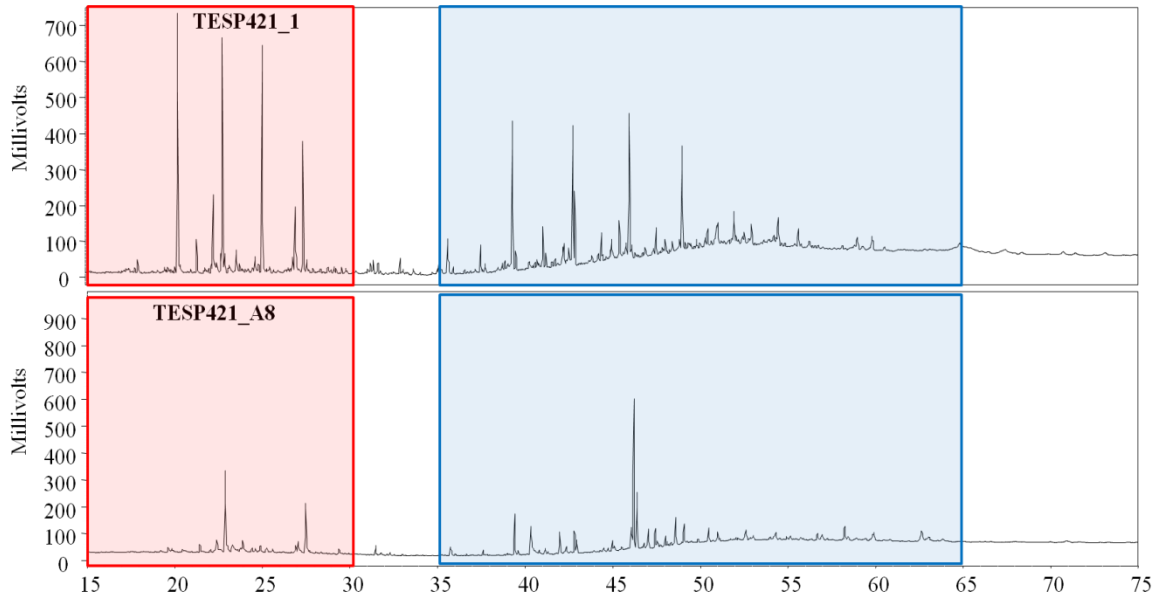

Figure 5.23: Partial GC-FID chromatogram from a) the skull (1), which represents the typical profile from all soils samples collected and b) from the bone sample (A8), which typifies the bone samples collected. The area highlighted in red shows the LMW components and the area highlighted in blue represents the HMW components. Originally in colour.

5.4.2.3 Hydrocarbon fractions

The hydrocarbon fractions from the bone fragments (A8-A10) contained no detectable components. All other sampling points exhibit a unimodal distribution of plant-derived *n*-alkanes ranging from C23-C33 (Eglinton and Hamilton, 1967; Jambu *et al.*, 1991). In addition, diploptene was present in low relative abundance in the samples from around the remains but was absent from the bone samples (Figure 5.24). Diploptene was also found in samples from a grave from Edinburgh (Chapter 4) and it was suggested that it may have been sourced from soil microbes involved in the degradation of the bone or the tissues associated with the bone (Evershed *et al*., 1995). Notably, however, diploptene was not present in the samples of bone analysed here, so it is likely that diploptene is related to the soil bacteria and not to bone degradation within this burial though it could still be present due to the degradation of the remains.

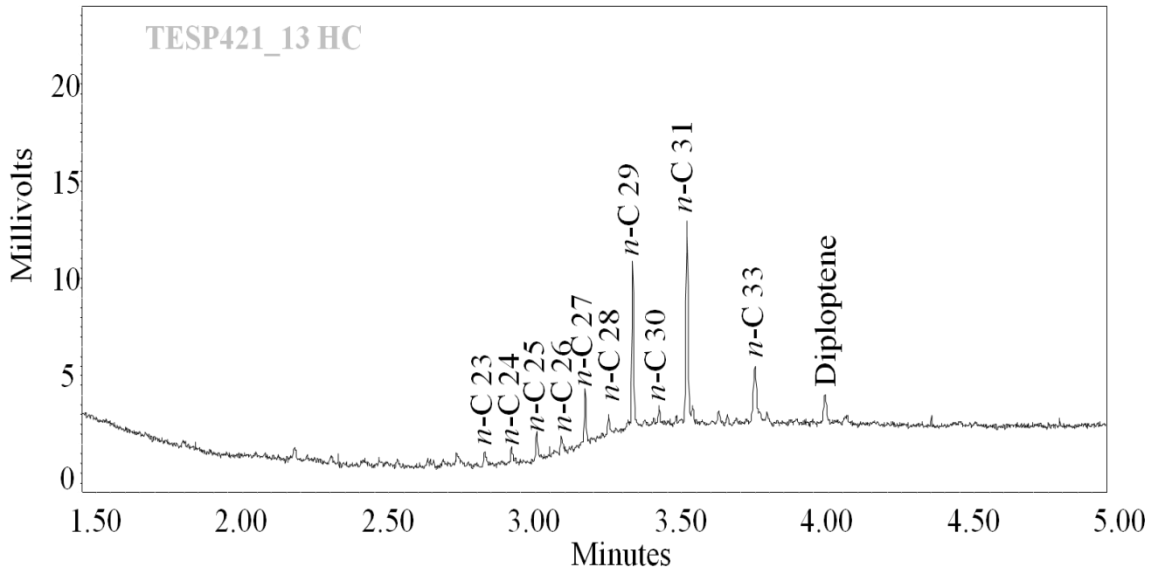

Figure 5.24: Partial GC-FID chromatogram from the hydrocarbon fraction of the left hip (13), which typifies the hydrocarbon profile of all the soil samples.

5.4.2.4 Aromatic fractions

The GC chromatograms of the aromatic fractions from bone fragments (A8-A10) contain a small cluster of six peaks eluting between 3.25-3.5 min (Figure 5.25).

GC-MS of the aromatic fractions from A10 revealed the peaks to correspond to cholestene, cholestadiene and cholestatriene components.

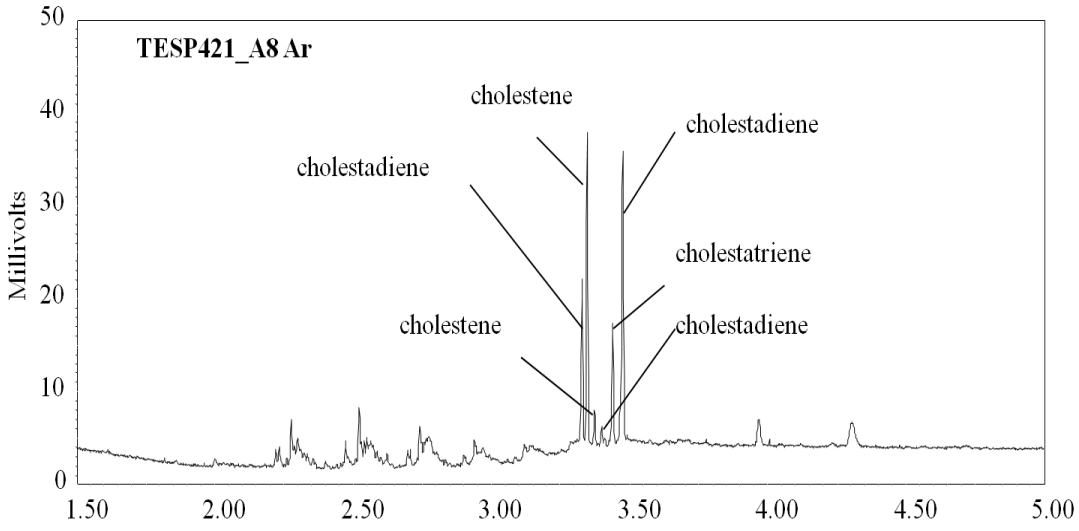

Figure 5.25: Partial GC-FID chromatogram of the aromatic fraction from sample containing bone A8.

Steroidal components have previously been found in recent sediments as degradation intermediates for the transformation of sterols to steranes (Bortolomeazzi *et al.*, 2000; Dastillung and Albrecht, 1977). A pathway from sterols to sterenes, via 5α and 5β stanols, and their subsequent digenetic transformation to sterenes has been suggested for the early digenesis of sterols (van Kaam-Peters *et al.*, 1998; Killops and Killops, 2005). Accordingly the cholestenes occurring in the bone and soil samples discussed here most likely represent early digenetic products of cholesterol derived from the bone and/or associated body tissues.

The presence or absence of steroidal hydrocarbons in the lipid analysis of bone was not discussed by Evershed *et al.* (1995). Cholestatriene and cholestadiene components have been found in bandage/resin/tissue samples from the Khnum Nakht mummy (Buckley *et al.*, 1999) indicating that they can form over archaeological timescales. As there is very little contribution from other

components in the extracts of the bone fragments it is most likely that these steroidal components are from the cholesterol in the bone.

In addition to their presence in the bone samples, the steroidal hydrocarbon components are also present in low levels within the soil samples (Figure 5.27). The bone samples seem to contain very few components that relate to the soil organic matter, as their total extract profiles are very different from the soil samples, which suggests that the steroidal components are directly related to the bone. The presence of sterenes and steranes in the soil samples may be due to their leaching from the degrading bone into the soil surrounding the remains or that there are very small fragments of bone are present within the soil.

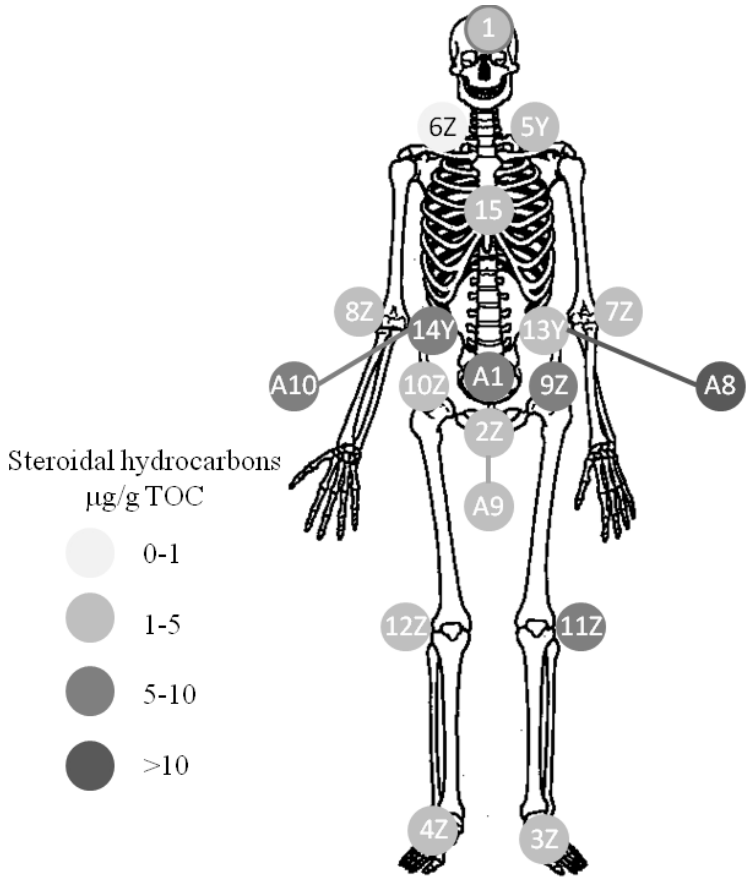

Figure 5.26: Distribution of steroidal hydrocarbons in samples across the remains.

The soil samples also contain a series of *n*-alkanals ranging from C15-C17 with C16:0 and C18:0 being the dominant components (Figure 5.27).

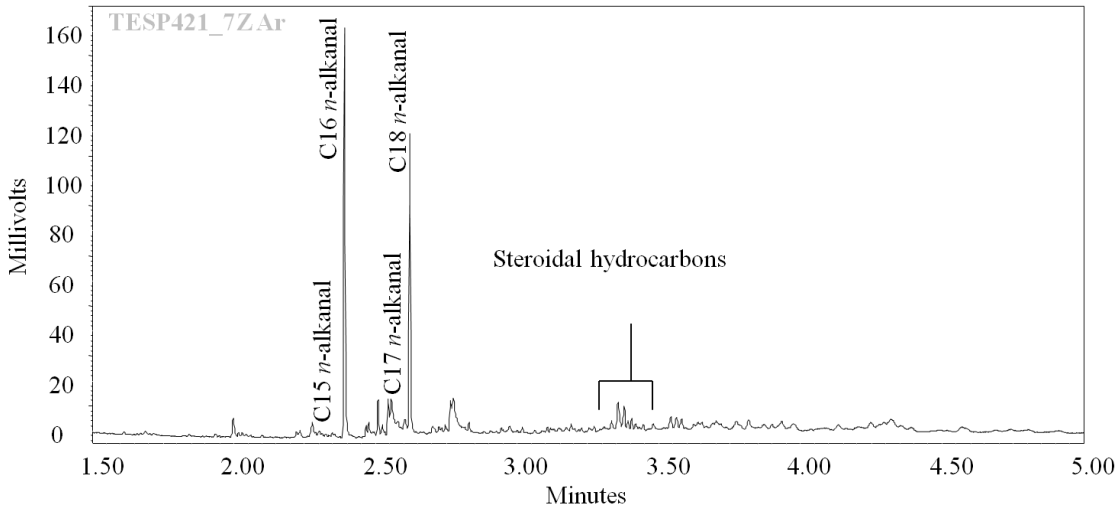

Figure 5.27: Partial GC-FID chromatogram of the aromatic fraction of the soil extract from sampling point 7Z, which represent the profile of all the soil extracts taken from TESP 421.

The LMW *n*-alkanals have previously been observed in the grave from Mechelen (Chapter 4) and from other sites analysed; Thessaloniki T182 (Chapter 3), the analysis of the Heslington brain, Syningthwaite G1, TESP 417, Hungate SK53700 and MESW SK27 (unpublished results) and are believed to represent reduction products of C16 and C18 fatty acids (Naccarato et al., 1972; Day, 1978). In these particular graves the location of the LMW *n*-alkanals was restricted to areas around the skull, including the shoulders. The analysis of tissue from the Heslington brain revealed LMW *n*-alkanals, inferring a direct source from brain tissue. Within the TESP 421 grave, however, the *n*-alkanals present throughout the sampling points (Figure 5.27) and are present in highest abundance in the sampling points from beneath the lower pelvis (2Z, 10Z, A9), the left shoulder (5Z), the right arm (8Z) and the left knee (11Z). The distribution suggests that the origin of the *n*-alkanals is not restricted to brain tissue and that they could be formed by the reduction of fatty acid components of the body tissues (e.g. TAGs in adipose tissue). Operation of this reduction pathway from fatty acids to *n*-alkanals, and the preservation of *n*-alkanals, which are reactive species, is most likely to indicate anaerobic/reducing conditions in the grave.

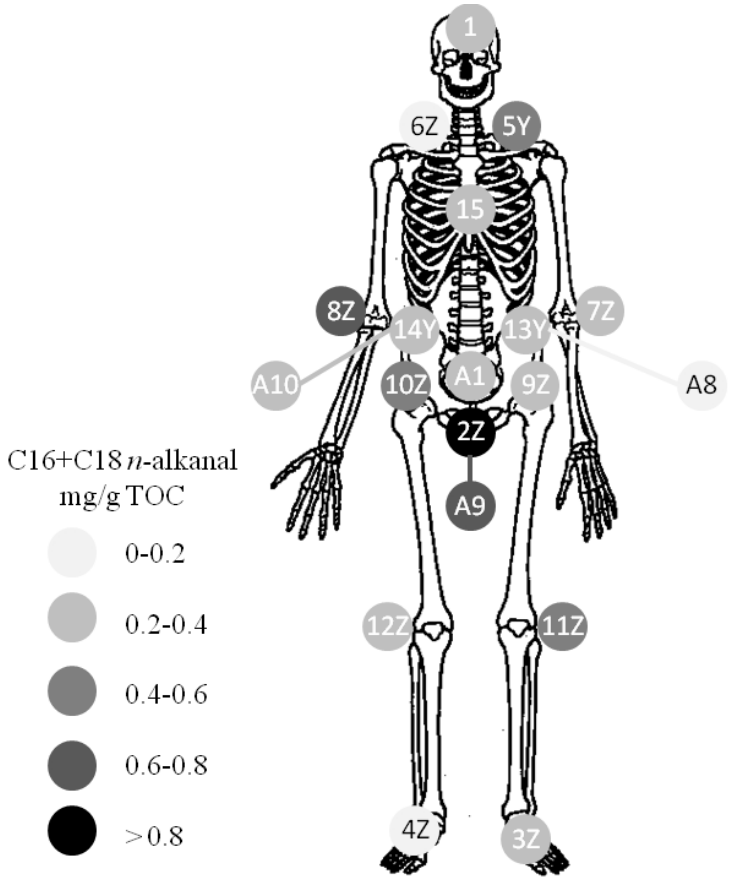

Figure 5.28: Distribution of LMW *n*-alkanals around the remains of TESP 421.

Unlike the burial from Mechelen, no LMW *n*-alkanes or C16 and C18 *n*-alkanols (that could relate to the further reduction of *n*-alkanals and fatty acids) were present within the Thaon samples. The presence of LMW *n*-alkanes indicates further degradation of the *n*-alkanals in the samples from Mechelen suggesting degradation to be more advanced than for Thaon. This would also explain why the *n*-alkanals are found within all Thaon samples whereas in Mechelen only the shoulder and the right hand contained *n*-alkanals.

5.4.2.5 Medium polar fractions

The GC chromatograms of the medium polarity fractions from all soil samples contain a series of plant *n*-alkanols (C22-C30) with an even over odd predominance (Eglinton and Hamilton, 1967) and a maximum at C26, typical of a grassland distribution (van Bergen *et al.*, 1997). The presence of the plant sterols, sitosterol and stigmasterol and pentacyclic terpenoid β-amyrin are also consistent with grassland vegetation (van Bergen *et al.*, 1997).

Cholesterol was present in the samples from TESP 421 along with 5α-cholestanol, the major reduction product of cholesterol in the environment (Bethell *et al.*, 1994). Coprostanol, the major reduction product of cholesterol in the gut of most higher animals (Bull *et al.*, 1999a; Leeming *et al.*, 1996) and epicoprostanol, which has been suggested as a further transformation product of coprostanol in the environment (Bull *et al.*, 2002) were also present. The presence of the ketone intermediate in the reduction of cholesterol to stanols, cholestanone (Bull *et al.*, 2002), further suggests reducing conditions in the burial environment.

To understand the significance of the presence of coprostanol, Bull *et al*., (2002) describe a ratio for the determination of faecal contamination. The ratio is based on the equation proposed by Grimalt *et al*. (1990; Equation 1) with values ≥ 0.7 being suggestive of faecal contamination.

$$\frac{coprostanol + epicoprostanol}{coprostanol + epicoprostanol + 5\alpha - cholestanol}$$

Equation 1: Ratio for the determination of faecal contamination in soils incorporating epicoprostanol.

The bone samples all displayed ratios lower than 0.7 (~0.67), whereas all the soil samples displayed ratios of around 0.9. The levels of coprostanol in all of the

sampling points were quite high by comparison with the grave analysed from Mechelen (G422; Chapter 4) where coprostanol and epicoprostanol was localised under the left hip. The significant levels of coprostanol in TESP 421 and lack of localisation to the pelvic region, as was the case with the Mechelen burial (Chapter 4), may suggest either better preservation or additional sources of coprostanol in Thaon. Coprostanol has been found in the muscle tissue of bog bodies (Evershed and Connolly, 1994) and mummies (Gulaçar *et al*., 1990) and it was suggested that its presence was due to migration and activity of gut microbiota. The formation of coprostanol outside the gut has not been reported in the literature and the presence of coprostanol in soils and sediment is usually attributed to sewerage or faecal pollution (Bull *et al*., 1999a). The conditions in the gut, such as pH and temperature, are factors that have not been investigated in relation to the formation of coprostanol although it is likely they do play a role in its formation. Therefore it is not known if the microbes responsible for the transformations would survive and be sufficiently active after death: the conditions in the thorax (where samples were taken from the mummy; Gulaçar *et al*., 1990) would not replicate those in the intestinal tract, for example body temperature decreases significantly after death.

During the process of mummification organs are often removed. Gulaçar (1990) does not mention if the organs had been removed during the mummification process. Furthermore, it is also conceivable that the gut may have ruptured during the mummification, allowing its contents to enter the thoracic area. Similarly, if organs were left within the body, they may have degraded, allowing the gut contents to move within the body cavity. The samples analysed from the Nubian mummy where coprostanol was observed (Gulaçar *et al*., 1990) showed much higher levels of degradation compared with the Pharoanoic mummies analysed by Buckley *et al*. (1999) (where coprostanol was not observed in the tissue) which could suggest a lower level of skill/care during the mummification process. Hence, the greater extent of preservation of the Pharoanoic mummies and the lack of coprostanol could be related to the nature and success of the preparation process (Buckley *et al*., 1994).

The skin sample that was analysed from the bog body (Evershed and Connolly, 1994) was taken above the abdomen hence it is conceivable that peri- or post-mortem disruption of the integrity of the abdominal area may have released gut contents, contaminating the skin. But as discussed previously, it has not been established if the conditions in a bog would provide suitable conditions for the gut flora to be viable and for the transformation of cholesterol to coprostanol. Although the authors dismissed the introduction of coprostanol through contamination due to the lack of phytosterols and their 5β-stanol degradation products, no data on other faecal signatures from the body were presented, hence the presence of those sterols was not established.

If gut flora was responsible for the degradation of the remains to such an extent it would be likely that other burials analysed in this study would contain significant amounts of coprostanol around the gut region where the gut bacteria would originate, which has not been observed. As the presence of cholesterol is widespread across the body it would be likely that coprostanol would be present in samples throughout the remains and that 5α-cholestanol (cholesterol reduction product in the environment) would be observed in samples where the gut flora may not have necessarily reached. Patterns such as this have not been observed in any of the burials previously analysed

Clearly, the conditions in the gut region are quite specific and conditions favourable to the activity of the gut microflora may not exist within the grave. It is, therefore, possible that the presence of coprostanol is due to contamination from the gut region and better conditions for its preservation, and/or from material derived from other remains at the site.

Analysis of a First World War mass grave from Fromelles, France also detected high levels of coprostanol (Scott Hicks unpublished results) at all of the sampling points other than the pelvis. Both of the sites (Fromelles and Thaon) had been prone to waterlogging, with both burials containing significant levels of water during their excavation. In addition, both sites contained many graves. The bog body analysed by Evershed and Connolly (1994) is also likely to have been under

conditions where significant water was present thus the water logging within the burials may have allowed movement of these steroidal components around the grave. The grave TESP 421 was also on the bottom layer of a multiple burial site and it is conceivable, though perhaps unlikely, that the coprostanol may represent contamination from graves above.

5.4.2.6 High polar fractions

The GC chromatograms of the high polarity fractions displayed a bimodal distribution comprising HMW fatty acids (C24-C30) typical of higher plant waxes (Eglinton and Hamilton, 1967) and LMW fatty acids C14-C18 with a maximum at C16:0 and C18:0 (accompanied by their unsaturated components C16:1, C18:2 and C18:1) sources including animals plants and fungi. In addition, *iso*- and *anteiso*- branched components (C15 and C17), which represent a bacterial input (Zelles, 1997), were particularly abundant in the samples. A ratio of LMW:HMW fatty acids was calculated (Figure 5.29). The samples that had a higher LMW:HMW fatty acid ratio were located around the central part of the body suggesting that there is more input from the fatty acids relating to bacteria and degradation of adipose tissue in those particular sampling locations.

Due to the nature of the burial no controls could be taken and so it cannot be determined if the signatures are also present in the background soil. The identification of same LMW fatty acid components in many of the other burials examined (including Sala discussed above) and the increased abundance levels around the remains in those cases indicates that the signatures from LMW fatty acids relate to the human remains rather than the natural soil organic matter.

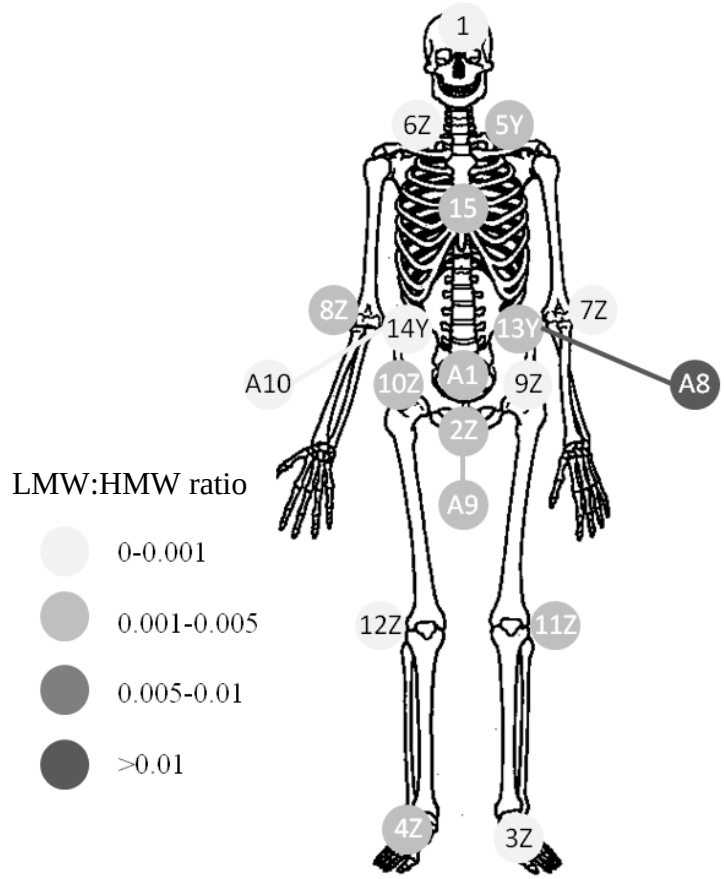

Figure 5.29: Distribution of LMW:HMW ratio distributions across the different sampling points.

5.4.3 Conclusion

The samples from Thaon show the presence of fatty acid signatures relating to the remains as has been observed for several of the other graves analysed previously. The aromatic fraction from the soil samples from around the body display low levels of steranes and sterenes, representing the degradation products of cholesterol. These sterenes and steranes are present in high abundance in the bone samples that have been analysed suggesting that the steranes and sterenes present in the soil result from leaching of steroidal components from the bone. Hence, cholesterol-derived sterene and steranes present in soil could provide evidence of degraded bone within a burial.

The soil samples also contain significant levels of coprostanol, which is related to the presence of faecal matter, in all of the soil samples associated with the remains and in higher abundance than has been observed in the grave from Mechelen. The levels of coprostanol are likely to derive from the remains though a source of contamination from other nearby burials on the site cannot be firmly excluded. Water movement through the site may have aided the dispersal of sterols within the grave at this site.

LMW *n*-alkanals, suggesting a reductive microbial transformation pathway for fatty acids relating to the remains, were observed in all of the soil samples; these components have previously been observed in shoulder (5 and 6) and right hand (16) samples of a grave from the Mechelen site (Chapter 4). Certainly, waterlogged conditions such as were observed for the site would promote an oxygen limited and reducing environment. Unlike the Mechelen grave, however, no *n*-alkanes or *n*-alkanols, which are also form from the microbial reduction of fatty acids, were observed for the grave from Thaon. The absence of *n*-alkanes suggest that degradation was at an earlier stage than for the Mechelen grave, this was also reflected in the Mechelen grave as the earlier degradation products of *n*-alkanals were only observed in three samples.

5.5 Conclusions

Analysis of the burials described in this Chapter further indicates the relationship between the presence of interred human remains and elevated levels of LMW fatty acids within the burial soils, and that this most likely relates to the degradation of adipose tissue and bacterial activity around the remains. The LMW fatty acids were present in all soil samples analysed from Thaon and in all soil samples except the control taken from the Sala burial site. In addition the graves both from Sala and Thaon displayed branched fatty acids relating to a bacterial input, suggesting that signatures of bacterial degraders are evident in the soils from sampling positions around the remains. Samples taken from Iceland,

however, did not contain any signatures that could be related to the presence of interred human remains and were dominated by signatures reflecting the overlying vegetation. This most likely reflects the loss of the organic signatures through soil erosion which is common in the soil that is found at the burial site, or simply to efficient degradation of organic matter.

Coprostanol, which is known to indicate human faecal input, was found in all soil samples taken from around the skeletal remains from Thaon, whereas previously it had only been detected in soils from the pelvic region (Chapter 4). The presence of coprostanol in all of the samples from Thaon is likely to be due to the dispersal of the signatures throughout the coffin (which was still largely intact) aided by water transportation, water logging being prominent at the level of the grave within the burial site.

Bone samples from Thaon also displayed the presence of components relating to the degradation of cholesterol in bone. The bone samples contained a number of sterene and steranes, which have also been observed in the tissues of mummies and were related to the degradation of cholesterol. The sterenes and steranes were also present in lower amounts in the soil suggesting that they have leached into the soil from the bone and that these components could relate to degraded bone.

Although Sala contained fatty acid components that may have related to degraded adipose tissue it does not contain any evidence for products of fatty acid degradation. The soil samples analysed from Thaon, however, contain C16 and C18 *n*-alkanals which most likely represent microbial degradation products of fatty acids. These components were present in all of the samples whereas when previously observed for the Edinburgh burial they were limited to only three samples. Extracts from the latter burial, however, also contained C16 and C18 *n*-alkanes, possibly representing a further stage in the progressive reduction of fatty acids from the body tissues. This suggests that while there were reducing conditions present within the Thaon grave, degradation of the fatty acids relating to the remains is at an earlier stage than in the Edinburgh burial. The steranes and

sterenes present in the Thaon samples are also formed under anoxic conditions, thus, water logging of the grave may have provided these conditions.

All of the burial sites discussed in this Chapter contained evidence of wood relating to a coffin. Specific signatures relating to wood were observed in the extracts from the burials from Iceland and Sala. Samples from both sites contained a number of diterpenoids including abietic acid, dehydroabietic acid, didehydrobietic acid and pimaric acid, all of which are related to pine resins. The abundances and distributions of the diterpenoids indicate a greater extent of degradation of the resin signatures for the Icelandic grave compared to the wood in the Sala burial. The presence of the specific signatures reveals information regarding the materials used to construct the coffin, which may not have been accessible through visual or microscopic analysis of the surviving wood fragments. These findings are particularly interesting in case of the Icelandic burial as pine trees are not native to Iceland. This means the pine wood used for the coffin would either have to be imported through trade from neighbouring countries arrived on the shores as driftwood and been collected. Thus not only do these specific signatures relate to materials used for coffin manufacture but may have implications for the significance of using this, presumably valuable, commodity to bury the dead.

The study further provides evidence for chemical signatures which can be linked to the presence of interred human remains including; degraded adipose tissue (LMW fatty acids and *n*-alkanals), degraded bone (steranes and sterenes), the presence of faecal material (coprostanol) and bacteria (LMW and branched fatty acids). In addition to these components *n*-alkanals, steranes and sterenes infer anoxic conditions within the burial environment (e.g. Thaon) and operation of reductive transformation pathways for the degradation of organic matter from the remains. There are, however, cases where components relating to the remains are not present within a burial and this could be due to the soil type. In the case of the Icelandic burial site the soil was prone to erosion. In addition this chapter has

revealed the presence of signatures related to materials included with the corpse (e.g. coffin wood).

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1. Conclusions

The overall aim of the thesis was to develop and test an approach for obtaining organic signatures from burial soils that inform an understanding of the burial environment, encompassing aspects relating to the individual and/or culture in which they lived. This involved several key areas;

- Development of an analytical strategy.
- Determination of a range of GC amenable compound classes typically associated with grave soils and assessment of their origins and possible degradation pathways.
- Evaluation of the variability of the organic signatures throughout the grave.
- Development of an understanding of the potential for migration of lipids within burial soils.
- Identification of specific signatures associated with the buried human remains and assessment of their potential to reveal features of the burial.

6.1.1. Analytical strategy

The method that was developed has enabled high numbers of samples to be analysed in a systematic and semi-automated manner. A systematic approach ensures that all of the samples were prepared and analysed under the same conditions, allowing for meaningful comparison of samples within a particular burial and between different burial sites. Following the recognition of contamination within early sample sets further development enabled reduction of sample contamination (from Whirl-Pak bags) during sample collection and storage and extraction (from plastics within the ASE system). Thus, wrapping soils in furnace cleaned aluminium foil prior to storage in Whirl-Pak bags prevents contact of the soil with the bag and was found to be sufficient to eliminate the contamination from this source of plasticisers.

Performing blank extractions on all ASE cells prior to sample extraction and limiting the extraction temperature to 100°C reduced contamination from plasticisers that had been identified and linked to the cell rings.

The GC and fast GC methods developed both allow analysis of a wide range of components encountered during the study, enabling the detection of components relating to the dominant extractable organic matter sources within the burials. The fast GC was used as a screening process throughout the study to allow for rapid assessment of key differences between the sampling locations, while the GC coupled with MS allowed for detection and identification of specific components.

6.1.2. GC amenable components

6.1.2.1. Lipids associated with the remains and their origins

Notable components relating to human remains that have been discussed in the literature include fatty acid and steroidal components. Both compound classes have been observed frequently in grave soil samples collected during this study. In particular, straight chain fatty acids in the range C14-C18 (including saturated and unsaturated components) were observed in many of the grave soils collected from the burial sites discussed in this study. Components in this carbon number range are known to have many sources including plants (Eglinton and Hamilton, 1967), bacteria (Zelles, 1997), fungi (Ruess *et al*., 2002) and degraded adipose tissue (Gulaçar *et al*., 1990; Forbes *et al*., 2005). Each source can be identified through the abundance of particular components; bacteria are dominated by the C16:1 and C16:0 components, fungi are dominated by C18:2 and C18:0 and degraded adipose tissue by the C16:0 and C18:0 components. Plants are dominated by HMW fatty acids, however they can contain small abundance of LMW n-alkanes mainly C16: and C18:2.

Grave soils from Basly, Syningthwaite, Thaon, Iceland and Sala contained low levels of the C18:2 fatty acid whereas this component was absent from the other

burial soils. The presence of low levels of C18:2 fatty acid in some environments but not in others suggests that some fatty acids have contributions from sources other than the human remains, potentially plant or fungal inputs, but that their contributions are not dominant. The distributions of fatty acids in the grave soils have been compared to those obtained from tissues of mummies (Gulaçar *et al*., 1990; Buckley *et al*., 2006), bog bodies (Evershed and Connolly, 1998) and adipocere (Forbes *et al*., 2005). The significant similarities in distribution suggest that the signatures result from the hydrolysis of the TAGs of the adipose tissue from the remains. Notably, however, the levels of the C16:1 fatty acid in most soil samples suggests a significant bacterial contribution. Accordingly, the fatty acids present in the samples from around the remains are not likely to be exclusive to the degraded adipose tissue. Signatures from LMW fatty acids have been present in the control samples (with the exception of the Sala burial), further indicating that the fatty acids also derive, in part, from the soil background organic matter. The elevated levels of LMW fatty acids in close proximity to the remains, in particular in samples collected from the gut area in graves from Mechelen, Edinburgh and Sala reveals the direct influence of the remains. The gut region of the body is an area where there are high amounts of fatty tissue and additional bacterial input from the gut flora. Thus, the fatty acid signatures are likely to derive mainly from the degraded remains. Although the LMW fatty acid signatures cannot explicitly indicate the presence of degraded remains the fatty acid composition, abundance and variability among the anatomical positions sampled and the controls may infer the presence of degraded remains.

Despite the significant input of bacterial fatty acids to the grave soils collected around the remains, bacterial *iso- and anteiso-* branched fatty acid components, which have been identified as abundant components in degraded human tissues (Gulaçar *et al*., 1990; Buckley *et al*., 1999), were absent from all of the burial soils except those from Sala and Thaon. Notably, however, Zelles (1997) extracted and analysed fatty acids from the cell membranes of several types of bacteria and showed that several gram negative bacterial species did not contain any *iso- and anteiso-* branched fatty acid components. Hence, specific

components of the fatty acid signatures (in particular C16:1) may indicate bacterial activity related to the degradation of the remains , but by bacteria present in the soils that differ from the dominant species that affected the mummies (Gulaçar *et al*., 1990; Buckley *et al*., 1999).

Further bacterial markers present in the soil include LMW *n*-alkanes and diploptene. While diploptene has been observed in the analysis of archaeological bone, LMW *n*-alkanes have not previously been observed in degraded human tissue. Diploptene was present in soils from Edinburgh and Thaon and it was originally thought that it could originate from bacteria that were responsible for the degradation of human bone (Evershed *et al*., 1995). Further analysis of bone fragments from Thaon did not reveal the presence of diploptene, even at trace levels, implying that microbial degradation was not focused on the bone material. For that reason, the presence of diploptene cannot suggest exclusive microbial attack on bone as the soil microbes could also be responsible for the degradation of human tissue. The presence of diploptene observed on the bone in the study by Evershed *et al*., (1995) could indicate the presence of microbial degraders that are not limited to microbial degradation of bone.

The LMW *n*-alkane components observed in several of the burials in this study (Heslington East, Thessoloniki, Syningthwaite, Mechelen and Edinburgh) have not been observed previously either in degraded human tissue or associated with human activity. It is known that LMW *n*-alkanes are normally present in soils as products of bacterial inputs (Ladygina *et al*., 2006) and they can be formed through the microbial reduction of fatty acids (Naccarato *et al*. 1972; Day, 1978). In all of the burial sites the LMW *n*-alkane components were either not observed within the control samples or their abundance increased in samples that were taken from around the remains. Thus, the *n*-alkanes are likely to originate from the remains and could be present due to a direct bacterial input or from the microbial reduction of fatty acids.

Several steroidal components, including cholesterol, coprostanol, epicoprostanol and a number of phytosterols, were observed in the grave soils taken from around the remains. The main steroids discussed in the literature in relation to human input in soils (Bull *et al*., 1999) and degraded human tissue (Gulaçar *et al*., 1990; Evershed and Connolly, 1988) were cholesterol and coprostanol. In most of the burial soils examined here cholesterol was present as a component of the background soil organic matter. In the case of samples collected in Mechelen, the abundance of cholesterol was greater below the remains than adjacent to or above them. The higher abundance levels below the remains are likely to represent a cholesterol input from the degraded remains, though it is difficult to distinguish between the cholesterol inputs in the soil and any coming directly from the remains.

Unlike cholesterol, coprostanol was only observed in two of the burials, although the abundance and location within the two burials was very different. Coprostanol was observed in low quantities at the right side of the pelvis in the Mechelen burial whereas in the Thaon burial it was observed throughout the sampling points. Due to its specific location the coprostanol in Mechelen is likely to derive directly from the remains whereas the coprostanol in Thaon may be present due to contamination from the gut region of the remains in that burial or possibly from other burials within the site. Burials where coprostanol is exclusively present in the abdominal area (and most likely derive from the gut region) are more likely to reveal information about the remains (last meal) than burials that contain levels of coprostanol throughout the sampling points. Nevertheless, the presence of coprostanol in the grave soil still infers that degraded remains were present. Analysis of a grave from Fromelle, a WW1 multiple burial site, revealed that, like the grave from Thaon, coprostanol was present in all sampling points (Scott Hicks unpublished results). Both sites were waterlogged and both sites contained multiple burials. It could, therefore, be inferred that the presence of coprostanol is related to the degraded remains and that its presence throughout the burial could be due to the water logging.

6.1.2.2. Degradation pathways inferred from the analysis of grave soils

The occurrence of LMW *n*-alkanes signatures in several of the burial sites supports the interpretation that they originate from the remains and that there are two possible sources; (i) a bacterial input (Ladygina *et al*., 2006) and (ii) microbial reduction of fatty acids (Naccarato *et al*. 1972; Day, 1978) relating to human adipose tissue. The latter would suggest that the degradation of human tissue within the burials involved microbial reduction, whereas previous discussion of human tissue degradation in mummies (Gulaçar *et al*., 1990; Buckley *et al*, 1999) and the formation of adipocere (Adachi *et al*., 1997; Forbes *et al*., 2005) have highlighted the involvement of oxidative transformation pathways. Oxidative pathways were considered due to the presence of hydroxy fatty acids and di-carboxylic acids, both of which are formed through oxidation reactions of unsaturated fatty acids. Notably, neither of the oxidation products was observed in the grave soils, inferring that oxidative transformation pathways centred on the unsaturated fatty acids were not prevalent in the environments examined.

Further suggestions of microbial reduction can be inferred from the presence of the C16 and C18 *n*-alkanals that were observed in the Mechelen and Thaon burials, with the Mechelen burial also containing C16 and C18 *n*-alkanols. Like the *n*-alkanes, the *n*-alkanals and the *n*-alkanols can also be formed through microbial reduction of fatty acids but are earlier stage degradation products (Figure 6.1). The burial samples from Mechelen contained a series of LMW *n*-alkanes (C14-C18), whereas no LMW *n*-alkanes or *n*-alkanols were observed in Thaon, suggesting that the organic matter in the latter was at an earlier stage of degradation than in Mechelen burial. Other sites in the study that contained LMW *n*-alkane signatures but not *n*-alkanals and *n*-alkanols included Syningthwaite, Thessoloniki and Edinburgh suggesting that the organic matter in these grave soils has undergone more extensive microbial reduction than that in the Mechelen and Thaon burials.

In addition to the *n*-alkanes, *n*-alkanals and *n*-alkanols, the cholesterol reduction products coprostanol, epicoprostanol, steranes and sterenes have been observed in the grave soils from Mechelen and Thaon. As coprostanol is formed in the gut region of higher animals rather than the soil it does not provide evidence for reducing conditions in the soil. Coprostanol can further undergo transformation to epicoprostanol in anaerobic environments (Bull *et al*., 1999). The presence of epicoprostanol together with coprostanol in the pelvic area in Mechelen provides further evidence that anaerobic conditions existed in the grave soil.

Additional reduction products of cholesterol detected in the analysis of bone samples collected from Thaon include a group of steranes and sterenes relating to the bones. These sterene and steranes are known to form from cholesterol (a natural component in bone) under anaerobic conditions in the water column and surface sediments of aquatic environments and it is likely that the same conditions were important to the origins of the sterenes and steranes in Thaon, where waterlogging was apparent and known to have been persistent within the burial horizon.

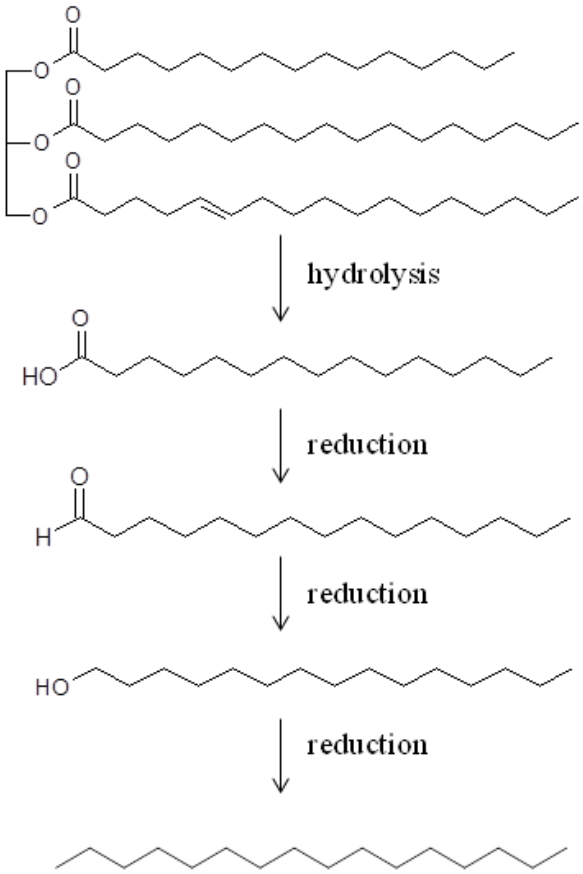

Figure 6.1: Putative pathway for the transformation of TAGs to *n*-alkanes, modified from Naccarato *et al*. (1972) where the reduction of tracer fatty acids to alcohols in the presence of a cell-free preparation of E coli and Day, (1978), where the biosynthesis of *n*-alkanals and alkanols by the bacterium *Clostridium butyricum* are discussed.

Based on the presence of *n*-alkanes and *n*-alkanals inferring reducing conditions within the burial environment, it can be interpreted that several of the grave sites in this study: Heslington East, Thessoloniki, Syningthwaite, Mechelen, Edinburgh and Thaon experienced anaerobic conditions. As Thaon was water logged it could be expected that the burial experienced reducing conditions. Similarly, the fluctuating groundwater at Thessoloniki may have induced at least periods of anaerobic conditions as suggested (Fiedler *et al*., 2009). It is not evident that any of the other graves experienced waterlogging or fluctuating groundwater levels.

Accordingly, other factors that may induce anaerobic conditions in the burial soils should also be considered. Among the burials that displayed evidence of reducing conditions only that from Thaon showed evidence for the presence of a coffin. Forbes *et al*. (2005) suggested that coffins can created an aerobic environment and experimental; studies showed that pig adipose tissue degraded much more rapidly in a coffin environment. Burials from Iceland and Sala, both of which showed evidence of a coffin, did not contain C16 or C18 *n*-alkanals, *n*-alkanols or LMW *n*-alkanes. Conversely, graves that have not shown evidence of a coffin being present (Heslington East, Thessoloniki, Syningthwaite, Mechelen and Edinburgh) have revealed the presence of reduction products within the grave soils. Hence, it can be inferred that microbial reduction can affect the organic remains in burials where a coffin is not present though it is not conclusive that coffins were absent and further investigations are required to confirm these findings.

6.1.3. Variability of molecular signatures with anatomical position

The signatures relating to degraded remains have mainly been prominent in samples collected from the abdominal area as seen through the abundances of LMW *n*-alkanes and fatty acids observed in samples collected from Heslington East, Edinburgh, Mechelen, and Sala. Nevertheless other sampling points should not be discounted as specific signatures and an increase in abundance of organic materials have been observed from the shoulder (Mechelen), the knee (Edinburgh) and the feet (Heslington East). At this stage in the InterArChive project removing any of the sampling points from the high intensity sampling strategy would not be advised as all of the points have the potential to reveal information about the grave. Not only do several of the sampling points reveal specific signatures relating to the burial but they also ensure that the variation of components across the burial can be examined closely, allowing for better assessment of the origins of the lipid components. Conversely, the addition of sampling points at different anatomical positions does not appear to be necessary as many of the additional samples collected from the graves did not enhance the information from the burial.

The additional samples taken from the Mechelen burial did provide further insight into how the signatures vary within the burial; the collection of samples in accordance with the high intensity sampling strategy but with recovery of discreet sample from adjacent to and below the remains was carried out. The analysis of these samples concluded that *n*-alkanes, fatty acids and cholesterol relating to the remains were present in higher abundance in samples collected from below the remains. In addition, coprostanol was located exclusively in the right hip sample that was collected from below the remains.

The results from high intensity sampling of the grave soils indicate that the sampling strategy provides a good range of information about the burial. Furthermore, it is evident from the analysis of the Mechelen burial that the signatures relating to the burial are better preserved in samples collected below the remains and it is recommended that, where possible, samples should be collected below the remains and that the exact sampling position (above, adjacent or below the remains) should be reported.

6.1.4. Transportation of lipid signatures

Several of the burials have shown evidence that lipids have been transported throughout and around the grave. These observations have compared to soil erosion experiments where agricultural organic matter was transported down hillslope. The main processes for the transportation of lipids within the grave seem to be related to the burial environment and the presence of water. Water plays a vital role in soil erosion, transport and sorting processes, processes critical to the development of soils (Oleschko et al., 2004). It is also critically important in the processes of transportation and re-deposition that affect lipid components owing to the adsorption of lipids mainly to clay particles (Zhang *et al*., 2009; Wang *et al.*, 2014). Samples collected from Heslington East showed the highest lipid contents in the abdominal region as well as at the feet. The high levels of lipids around the abdomen are likely to be due to the high levels of adipose tissue

and bacteria associated with that anatomical region. Such an explanation cannot account for the high levels at the feet. Consideration of the position and elevation of the grave revealed that it was at a similar elevation to a natural spring and on a slight slope inclined towards the feet. Hence, the high levels of lipids at the feet are likely be due to the transportation of small soil particles (mainly clays) from other parts of the grave and their accumulation at the foot area. This interpretation is a similar to that accounting for observations in soil erosion experiments (Zhang *et al*., 2006; Wang *et al*., 2009), where soil particles were transported from the top of a slope and re-deposited at the bottom of the slope. Although the experiments by Zhang *et al*. (2006) and Wang *et al*. (2009) did not contain any obstructions (such as skeletal remains), it is conceivable that small soil particles could also be transported within a grave.

The burials from Thessoloniki and Thaon displayed similar abundance of *n*-alkanols, fatty acids and sterols throughout the sampling positions. It was evident that both burials had significant levels of water movement with Thessoloniki being subjected to groundwater fluctuation and Thaon being subjected to waterlogging. As the levels of components were similar throughout the samples it was suggested that the lipids may have been transported within the graves and distributed equally throughout the different areas of the remains rather than being associated with one particular area (as seen in Heslington East). Notably another burial from the Thessoloniki site (Tϕ178) showed distinct differences in fatty acid signatures in samples taken from around the remains, suggesting that the burial had not been subject to conditions that would have transported the lipids to other areas of the burial. Distinctive features of the Tϕ178 burial included a tiled floor which could have prevented fluctuating ground water from entering the burial and transported fine particles and associated lipids and a greater elevation (by c. 1 m) than the Early Hellenistic grave examined in this study.

Some of the graves examined in the study did not reveal the presence of lipids relating to the remains. These include Basly and Iceland: Basly had very low levels of organic matter and the Iceland grave soils contained high levels of lipids

relating to the background soil organic matter. Both sites exhibited features that would allow for erosion of soil to occur; the graves at Basly were cut into well-draining limestone rock and the soil in the grave appeared to be associated mainly with erosional deposition from the surrounding area. The Iceland site featured an andosol, which can be very prone to erosion. In both cases, the samples from around the remains featured lipids that were part of the soil background organic matter. It is likely, therefore, that the lipids originated from the soil and had been transported via water movement through the graves. This would account for the low level of variation observed in lipid signatures between the samples taken around the body and the controls taken from the upper soil layers.

6.1.5. Specific molecular signatures associated with archaeological human burials

The analysis of the grave soil samples has revealed several components that have maximised information about the remains, the nature of the burial and materials used within the burial. These signatures have been specific to the anatomical sampling points and have not been observed in the controls, allowing them to be identified as being present due to the burial and not part of the background organic matter.

Coprostanol has been discussed previously as a component relating to the remains however; its presence in the right hip samples (14) from the Mechelen burial has the potential to provide evidence of the nature of the last meal eaten by the individual who was buried. Coprostanol has previously been considered to provide evidence of an omnivore and carnivore diet (Leeming *et al*., 1996) and has been found in 2000 year old coprolites (Lin *et al*., 1978). Its location in the pelvic area suggests that it derived from the gut of the individual and therefore has the potential to reveal information about the last meal. The hip sample also contained the plant-derived marker 5β-stanol 24-ethyl coprostanol, which had not been observed in the other samples or the controls. The presence of 24-ethyl coprostanol could further infer information about the last meal. As both

coprostanol and 24-ethyl coprostanol are both present within the hip sample it is likely that the last meal included a meat and plant matter (Leeming *et al*., 1996).

Coprostanol was also observed in samples from Thaon although, unlike in the Mechelen samples, coprostanol was present throughout the grave and was not limited to a particular sampling area. As coprostanol was present throughout the remains it is likely that coprostanol from the gut region was distributed amongst the burial due to the water that was present. Therefore the coprostanol present in the Thaon burial could infer information about diet but further investigation to the source of the coprostanol would be required.

Specific signatures identified do not relate just to the remains, they have the potential to reveal information about the nature of the burial. Hopenone *b* is one component that was observed in the medium polar fractions of several samples collected from the Edinburgh burial. It is known to occur in a particular plant - Cypress Spurge or grave yard weed (Starratt, 1969). This type of plant was not known to be used for decorative or ritual purposes; hence the presence of its signature in the grave probably implies an origin from the burial soil above the coffin. The presence of the signature of Cypress Spurge clearly infers that the particular plant was present around the time of burial. Furthermore, it indicates that, although plant material is common in the background soil, signatures that relate to particular species can be identified in grave soils. Such information can augment understanding of the nature of the burial environment or the use of plants as funerary ornaments.

Specific signatures relating to wood were observed in the extracts from the burials from Iceland and Sala. Samples from both sites contained a range of diterpenoids including abietic acid, dehydroabietic acid, didehydrobietic acid and pimaric acid, all of which are related to pine resins. Pyrolysis GC analysis of small fragments of wood that were found in the graves revealed lignin derivatives consistent with gymnosperm wood (e.g. pine) supporting the findings from the lipid analysis (Pickering *et al*., 2011). The presence of the specific signatures confirms the presence of a coffin and reveals information on the materials used in its

construction, which may not have been accessible through visual or microscopic analysis of the small and infrequent surviving wood fragments. Although both burials contained evidence of a coffin through the presence of wood fragments the samples that were analysed are unlikely to contain detectable wood fragments as these would have been removed by sieving in during the sample preparation. It is likely, therefore, that the resin acids from degraded wood fragments are present in the soils or within microscopic particles of wood. It is not clear if resin acids would be observed in soils that do not contain wood fragments and other burials will have to be analysed to confirm this.

The presence of pine resins is particularly interesting in the case of the Icelandic burial as pine trees are not native to Iceland. This indicates that the pine wood used for the coffin would either have been imported through trade with neighbouring countries or collected on the shores after its arrival as driftwood. Thus, not only do these specific signatures relate to materials used for coffin manufacture, they may also have implications for the significance of using this, presumably valuable, commodity to bury the dead.

6.2. Overall conclusion

The studies have shown that organic biomarkers relating to interred remains can be identified in grave soils and include wide variety of lipids that have been previously observed in archaeological materials; fatty acids, sterols, resin acids as well as components that have not been previously observed; *n*-alkanals, *n*-alkanes. Some of the components that were identified do not provide unambiguous evidence for the presence of the remains hence the locations and abundances of these signatures within the grave need to be taken into account. It is apparent that not all burials contain components relating to the remains and this may be consequent on factors including erosion of the soil, where organic material is lost to lower levels in the soil or re-deposited elsewhere in the grave.

Furthermore there is evidence to suggest that the molecular signatures can be subjected to microbial reduction, a transformation pathway that has not previously been described explicitly in relation to the degradation of human remains. Notably, evidence of oxidative transformation pathways was not observed in this study. The causes of the reducing conditions in the burial soils have not been clearly established, though the evidence suggests that even burials that were not within a coffin have been subject to a reductive degradation. Further studies would be needed to clarify this.

The study has also demonstrated that analysis of soils can reveal specific signatures that are exclusive to a limited number of sampling positions around the remains. These have provided information on the individual (last meal), style of burial (coffin material, plant materials) and the culture (sources of coffin materials in Iceland). Molecular signatures may relate to contamination and/or background material associated with the soil. It is, therefore, necessary that the location of the signatures in relation to the skeletal anatomy is analysed thoroughly.

Analysis of burials soils has wider implications in the archaeological world as there is potential to provide information about a particular burial without the presence of skeleton or physical remains. As it is the soil that is being analysed, highly sensitive but destructive techniques such as GC-MS can be used in the analysis of samples without compromising any artefacts that may have been collected.

6.3. Future work

To ensure recovery of the maximum information relating to the burial, future sampling of grave soils should include all of the samples denoted in the InterArChive high intensity sampling strategy. All samples have the potential to reveal information about the burial. Where possible, however, samples should be collected from below the remains rather than adjacent to them.

The sterol components have been mainly observed in the medium polar fractions, which are regularly dominated by plant *n*-alkanols and other components, making it difficult to observe sterols and their degradation products. Given that the steroidal components have provided good insight into the burial, their isolation in greater purity would allow them to be studied individually and in more detail. Further analysis should be targeted towards establishing the origins of cholesterol in order to determine exactly how much cholesterol derives directly from the remains and how much derives from the soil background organic matter. Such insights would be aided by compound-specific stable carbon isotope analysis and, for cholesterol and its derivatives that can be linked directly to the remains (e.g. the sterenes and steranes associated with bone) this analysis could provide insights into the diet of the individual, as it has been demonstrated previously for cholesterol analysed from bone (Stott *et al*., 1999).

Stable carbon isotope analysis could also be used to assess the sources of the fatty acids from around the remains. This would provide further evidence as to which fatty acids arise from the background soil and which arise from the degraded adipose tissues and could enable the assessment of the proportions of each in cases of mixed inputs.

Studies on the distribution of soil particles in the graves could also provide further evidence of how the nature of a burial could affect soil erosion. Although erosion experiments have been carried out in the literature, this does not include burial environments in which skeletal remains could provide barriers where soil particles could accumulate.

Evidence of a reductive degradation pathway has been observed in several of the soils. The causes of the reducing conditions in the burial could not be clarified and this should be a target for future studies. The InterArChive project encompassed a series of experimental burials of piglets both in coffins and directly into the soil. Analysis of samples collected from these burials should greatly assist in determining if the remains in coffined and non-coffined burials undergo different transformation pathways.

CHAPTER 7. EXPERIMENTAL

7.1. General procedures

The final chapter details the experimental procedures used throughout the project. All solvents used were HPLC grade and all standards and reagents were of the highest purity possible.

7.2. Glassware

Prior to use, all glassware was baked at 450°C for 6 h to remove organic contaminants using a Pyro-Clean oven (Barnstead/Thermolyne Pyro-Clean Trace).

7.3. Sample collections

To gain a comprehensive overview of the organic matter contained within a particular grave site, soils were collected from 17 sample points around the body (Figure 7.1; a). For each location samples may be taken from above, adjacent to or below the skeletal remains and are labelled as x, y and z respectively. In addition, three control samples (Figure 7.1; b) were collected in order to determine the background organic matter content of the soil: C1, non-grave soil from the site; C2, soil from the upper grave fill; C3, soil from the lower grave fill. In some of the graves studied additional samples were taken if they were deemed of interest e.g. grave goods and coffin wood.

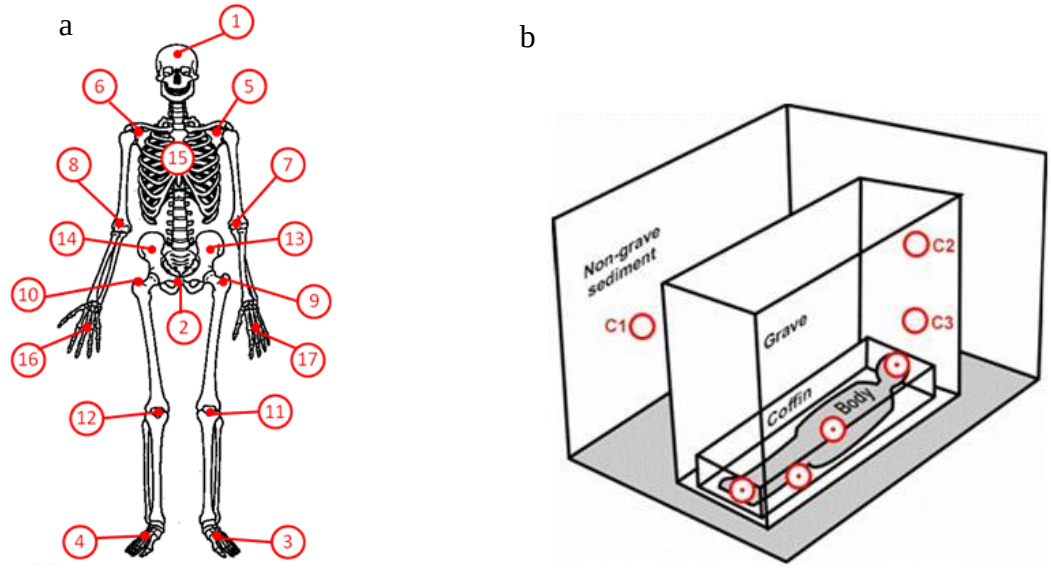

Figure 7.1: a) The positions of the 17 sampling points taken from around the remains, b) the areas where controls are collected from around the grave and grave site. Originally in colour.

7.4. Drying grinding and sieving

Frozen soil samples were spread out in glass petri dishes, introduced to the drying chamber of the freeze dryer (Thermo Heto PowerDry PL3000) and dried between 0.5-6 h (depending on the water content). Dried bulk soil was homogenised using a pestle and mortar and passed through a stack of sieves, of mesh sizes 1 mm, 400 µm, 200 µm, agitated using a sieve shaker (5 min; Endecottes Octagon Digital). Each individual fraction was stored separately according to particle size in a moisture proof container at -20°C until use. All further analyses were performed on the sub 200 µm fraction.

7.5. Elemental analysis

Non-extracted samples of grave soil were further dried using an SLS oven (60 °C). Elemental analysis was performed using a Thermo Flash EA 2000 elemental analyser fitted with a MAS 200 autosampler.

7.5.1. Carbon, hydrogen, nitrogen and sulfur (CHNS)

A set of standards and samples were weighed accurately (6 figure balance) into tin foil capsules (8 x 5 mm), folded into a compact package to exclude air and loaded into the autosampler. The sequence for each analysis (Table 7.1) included a bypass (i.e. an unknown amount of standard used to check that the instrument was working), a blank, two known amounts of a standard (~3 mg; to calibrate the instrument), a known mass of a separate standard (~3 mg; to check the calibration) and the soil samples (10-15 mg). Each sequence was finalised by repeat analysis of the non-calibrating standard to check for measurement drift.

Table 7.1: Elemental analysis sequence

Sample Run	**Purpose**	**Sample type**	**Approx weight required (mg)**
Methionine	Check instrument	Bypass	-
Empty Capsule	Calibration	Blank	-
Methionine	Calibration	Standard	2-3
Methionine	Calibration	Standard	2-3
Sulfanilimide	Check calibration	Unknown	2-3
Sample set (n)	Determine elemental comp	Unknown	10-15
Sulfanilimide	Check drift	Unknown	2-3

7.5.2. Total organic carbon (TOC)

A set of grave soil samples and standards were weighed accurately (on a 6 figure balance) into silver foil capsules (8 x 5 mm). Samples were placed into a cool heating block and 2 drops of aqueous HCl (18.5% w/v) were added to destroy inorganic carbonate. The heating block was placed onto a preheated hotplate (80^{o}C)

for 6 min to complete the reaction and remove excess HCl. Samples were allowed to cool before the capsule was folded for analysis. The analytical sequence was identical to that used for CHNS.

7.6. Extraction of grave soils

Grave soil (3-5 g) samples were accurately weighed into 5 ml ASE cells and extracted three times with DCM:MeOH (9:1 v/v) for 5 min at 100°C using an ASE 350 accelerated solvent extractor. The rinse volume was set at 50% and a nitrogen purge of 60 s was employed. Prior to extraction all the ASE cells that were used were pre-extracted using the same conditions in order to ensure the cells were clean. The solvent was removed using a rotary vacuum concentrator (Christ RVC; 25°C; 1500 rpm; 40 min), with the resulting residues dissolved in DCM (3 x 0.5 mL) and transferred to pre-weighed vials. The samples were subsequently reduced to dryness using the RVC and the weight of total extract recorded. Total extracts were then split, half for GC analysis and half for fractionation.

7.7. Sample fractionation and derivatisation

7.7.1. Fractionation

Fractionation was performed using miniature glass columns containing a sinter above the outlet which was protected by an overlying plug of DCM washed cotton wool, added to reduce the potential for blockage of the sinter. Silica gel (60 G) was loaded and tap packed into the column (approx. 15 mm column length). A small amount of silica gel (0.05 g) was placed into a small vial and the total extract added as a solution in DCM (250 µL). The top of the vial was plugged with DCM washed wool and placed under vacuum (Christ RVC; 25°C; 1500 rpm; 6 min) to remove solvent, leaving the sample impregnated on the silica. The column was washed with DCM:MeOH, 1:1(6 ml), DCM (3 ml) then equilibrated with hexane (3 bed volumes; under light vacuum) and the silica containing the test sample was loaded onto the column. Four eluent solvent compositions (hexane, hexane:toluene 1:1 (v:v), hexane:ethyl acetate 4:1 (v:v) and DCM:methanol 1:1 (v:v); 3 bed volumes) were

sequentially passed through the column under gravity to yield, in turn, hydrocarbon, low polar, mid polar and polar fractions.

7.7.2. GC sample preparation

Each fraction eluted from the pipette column was dried by centrifugation under vacuum (Christ RVC; 25°C; 1500 rpm; 20 min). The polar fraction was methylated by dissolution in a small amount of DCM:MeOH 2:1 (v:v) followed by the addition of trimethylsilyl diazomethane (2M in hexanes: 20µl). A 30 min period was allowed for reaction, before reduction to dryness under a gentle stream of nitrogen. The polar and mid polar fractions were silylated by adding bis (trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosilane (TMCS); 100 µL) and pyridine (4-6 drops), before being heated at 70°C for 90 min. Both fractions were reduced to dryness under a gentle stream of nitrogen and were then reconstituted in DCM (200 µL) for analysis. Hydrocarbon and low polar fractions were dissolved in DCM (100 µL) without further treatment. All samples were transferred to auto-sampler vials in preparation for gas chromatography (GC) analysis.

7.8. GC analysis

7.8.1. GC-FID

Analyses were performed on a Thermo Trace GC Ultra equipped with a Thermo Triplus autosampler and a FID detector. A DB5 column, (0.32 mm I.D x 60 m; 1.0 µm film thickness; A.J. and W Scientific) was employed, with the carrier gas (He) flow set to 2.0 ml min^{-1}. The injector was set to a temperature of 280°C and operated in the splitless mode. The detector temperature was set to 330°C. The column temperature was held at 70°C for 1 min before being ramped to 130°C at a rate of 20°C min^{-1} and then ramped to 320°C at 4°C min^{-1} and held for 40 min.

7.8.2. Fast GC

Fractionated samples were analysed using fast GC analysis performed on a Thermo Ultrafast GC equipped with a Thermo Triplus autosampler, FID detector, and fitted with a UFM column (0.1 mm I.D x 2.5 m; 0.4 µm film thickness; Thermo). The carrier gas (He) flow was set to 0.5 ml min^{-1}, the split injector temperature to 280°C with a split ratio of 1:100 and the detector temperature to 330°C. The column temperature was held at 50°C for 0.1 min before being ramped to 330°C at a rate of 90°C min^{-1} and held at this temperature for a further 4 min.

7.8.3. GC-MS

GC-MS was performed on selected fractions using an Agilent 7860A gas chromatograph equipped with a 7683B Series autosampler coupled to a Waters GCT Premier Micromass time of flight mass spectrometer. Separation was achieved on a fused silica capillary column (Zebron, ZB-5HT, 30 m x 0.25 mm i.d., 0.25 um film thickness). Samples were prepared in dichloromethane for analysis and injected onto the column via a split injection port (280°C, split flow 1:5). The oven temperature was programmed from an initial temperature of 70°C to 130°C at a rate of 20°C min^{-1} and then to 320°C at a rate of 4°C $min^{-1}$where it was held for 40 min. Helium was used as the carrier gas at a flow of 1 ml min^{-1}. Mass spectra were acquired using an electron impact energy of 70 eV. Compounds were identified from interpretation of their mass spectra and by comparison with library spectra (NIST 08) where available.

List of abbreviations

°C	Degrees Celsius
ASE	Accelerated solvent extraction
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DCM	Dichloromethane
EA	Elemental analysis
EI	Electron ionisation
FAME	Fatty acid methyl ester
FASE	Fatty acid silyl ester
FID	Flame ionisation detector
GC	Gas chromatography
HMW	High molecular weight
ID	Internal diameter
IR	Infra red
LC	Liquid chromatography
LMW	Low molecular weight
$[M]^+$	Molecular ion
MAG	Monoacylglycerol
Me	Methyl ester
MeOH	Methanol
MS	Mass Spectrometry
m/z	Mass to charge ratio

PCR	Polymerase chain reaction
SEF	Speed enhancement factor
SPE	Solid phase extraction
SOM	Soil organic matter
TAGs	Triacylglycerols
TE	Total extract
TMCS	trimethylchlorosilane
TMS	Trimethylsilyl
TOC	Total organic carbon
(v/v)	volume/volume

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