# The fate of human decomposition products in soils

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# Summary

During the decomposition of a human body, a variety of cadaveric lipids are introduced into the surrounding environment. The fate of cadaveric lipids in soils as well as the impact of soils on cadaver derived components is still poorly understood. Furthermore, the high diagnostic potential of lipids ($\Delta^5$-sterols, stanols, stanones, bile acids and fatty acids) and their long-lasting preservation in soils has been neglected in forensic research. Knowledge about the fate of human remains in soils can aid in selecting lipid biomarkers of human decomposition fluids and in understanding which soil abiotic and biotic factors regulate the decomposition of human remains in soils. For this purpose, a detailed assessment of the fate of cadaveric lipids was carried out. Soil samples were obtained from a case study where a body was found decomposing aboveground 11-18 days after deposition. Soil samples were taken 11-18 days after deposition and 358 days (one year) after removal of the body. The second soil sample set was obtained from putative mass graves (3 soil pits) from World War II, where 66 concentration camp prisoners were buried for 10 months (~late 1944-1945). Cadaveric lipids were determined in these soil samples after (I) testing suitable laboratory methods to determine (II) the concentration of $\Delta^5$-sterols, stanols, stanones, bile acids and fatty acids (III) to investigate the spatial distribution, transformation and degradation processes over time. The impact of soil biotic and abiotic parameters on decomposition was determined on adipocere from one individual. Minicontainers were used to assess decomposition of adipocere in undisturbed Calcisols and Arenosols.

Recovery surrogates of $\Delta^5$-sterols and stanols were between 78-97% ($R^2$=0.94-0.99) determined by standard addition on reference soil samples of the aboveground decomposition case study. 10-hydroxystearic acid, a typical fatty acid found in adipocere, was quantified with a precision of 97% by using effective carbon numbers (ECN) and relative standard response factors (SRF) by gas chromatography, because no analytical standard was available.

In the surface decomposition study several tissue and faecal steroids (cholesterol, 5α-cholestanol, coprostanol, epicoprostanol, 5β-stigmastanol, epi-5β-stigmastanol and 5α-stigmastanol) and fatty acids (myristic acid, palmitic acid, oleic acid, stearic acid and 10-hydroxystearic acid) were detected in higher concentrations in soil beneath the body than in the reference. Cholesterol (0.5-400 µg $g_{soil}^{-1}$) was the dominant steroid found beneath the thorax. Beneath the abdomen faecal steroids from the intestines predominated tissue steroids. Abundances of fatty acid salts and 10-hydroxystearic acid demonstrated potential anaerobic zones and adipocere formation beneath a decomposing body. After one year higher concentrations of fatty acids and steroids were still detected, but losses of total abundances were attributed to degradation and transformation processes. In the temporary mass graves faecal steroids and bile acids were indicative for human decomposition fluids in one of the three soil pits. Selected steroids (epicoprostanol, epi-5β-stigmastanol, isolithocholic acid) were unique for soil from the decomposition site. Cholesterol, 5α-cholestanol and 5α-cholestanone did not differ to the reference and were thus not suitable to indicate human decomposition fluids. Analysis of steroids revealed that at least one pit was likely used as mass grave, where human bodies were temporarily buried.

In the minicontainer experiment, a decrease in adipocere abundances was found over time. In the Arenosol, more adipocere was degraded which was attributed to the sandy texture and the high air capacity. The clayey texture and influence of groundwater in the Calcisol hampered a rapid adipocere degradation. The study also showed that the loamy Calcisol, where macrofauna species were found, had an impact on adipocere metabolisation.

In conclusion, combined analysis of $\Delta^5$-sterols, stanols, stanones, bile acids and fatty acids made it possible to track temporal pattern changes and processes involved in the fate of cadaveric lipids in soils. Selected lipids (steroids and bile acids) were suitable as indicators for decomposition fluids in soils, but the selection of lipids must always be adjusted to the specific forensic case. Furthermore, it has been shown that well aerated soils promote adipocere decomposition, which is important for decomposition rates at cemeteries to ensure a complete decomposition within the regular resting time.

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# Abbreviations

| | |
|---|---|
| 10-OH-$C_{18:0}$ | 10-hydroxystearic acid |
| 12-OH-$C_{18:0}$ | 12-hydroxystearic acid |
| AC | air capacity |
| ADDs | accumulated degree days |
| BD | bulk density |
| BSTFA | *N,O*-Bis(trimethylsilyl)trifluoroacetamide |
| $C_{14:0}$ | myristic acid |
| $C_{16:0}$ | palmitic acid |
| $C_{16:1\Delta9}$ | palmitoleic acid |
| $C_{17:0}$ | heptadecanoic acid |
| $C_{18:0}$ | stearic acid |
| $C_{18:1\Delta9}$ | oleic acid |
| $C_{18:2}$ | linoleic acid |
| $C_{21:0}$ | heneicosanoic acid |
| CDCA | chenodeoxycholic acid |
| CDI | cadaver decomposition island |
| CFE | chloroform fumigation extraction |
| DCA | deoxycholic acid |
| DCM | dichloromethane |
| ECN | effective carbon number |
| FA | fatty acid |
| FAME | fatty acid methyl ester |
| $FA_t$ | total fatty acids |
| FC | field capacity |
| GC/FID | gas chromatography/flame ionisation detection |
| GC/MS | gas chromatography/mass spectrometry |
| HDCA | hyodeoxycholic acid |
| ICP/OES | inductively coupled plasma optical emission spectrometer |
| IDCA | isodeoxycholic acid |
| ILCA | isolithocholic acid |
| IS | internal standard |
| LCA | lithocholic acid |
| LOD | limit of detection |

| | |
|---|---|
| LOQ | limit of quantification |
| MeOH | methanol |
| MW | molecular weight |
| NIST | National Institute of Standards and Technology |
| PMI | post-mortem interval |
| $PV_t$ | total pore volume |
| $R^2$ | regression coefficient |
| RSD | relative standard deviation |
| RT | retention time |
| SIM | selected ion monitoring |
| SPE | solid phase extraction |
| SRF | standard response factor |
| TMCS | trimethylchlorosilane |
| TLE | total lipid extract |
| TMS | trimethylsilyl |
| TOC | total organic carbon |
| wt | weight |
| WP | wilting point |

# Chapter 1

## Human taphonomic processes in relation to forensic pedology

## 1.1. Introduction

### *1.1.1. Cadaver decomposition processes*

Upon decomposition of a human body, a significant amount of degradation products are released into the surrounding environment (Carter and Tibbett 2008; Carter et al. 2007; Dent et al. 2004). Immediately after death (~4 min), the driving chemical processes of cadaver decomposition start with autolysis, the aerobic self-digestion of cells (Gill-King 1997). The following anaerobic stage putrefaction is characterised by invading internal (e.g. intestines, lung) and external (e.g. soil, skin) microorganisms, which continue to break down soft tissues (Powers 2010; Gill-King 1997; Evans 1963). During putrefaction and the subsequent stage of liquefaction a rapid loss of soft tissues occurs from peak insect maggot activity (Carter et al. 2007). In terrestrial ecosystems, the liquefied mass of degraded soft tissues leach into the surrounding soil and changes chemical, biological and physical soil properties (= grave soil; Carter and Tibbett 2008; Carter et al. 2007; Dent et al. 2004). A small area surrounding the cadaver is thus enriched with a wide diversity of easily degradable compounds and water which creates a hot spot of fertility (Parmenter and MacMahon 2009). This hot spot of fertility is commonly defined as the cadaver decomposition island (CDI). It represents a localised ecosystem with enhanced terrestrial biogeochemical cycling, heterogeneity and biodiversity (Cobaugh et al. 2015; Macdonald et al. 2014; Parmenter and MacMahon 2009; Carter et al. 2007; Towne 2000). Although cadavers represent about 1% of organic matter input into terrestrial ecosystems, they have been neglected in ecosystem research (Carter et al. 2007; Swift et al. 1979); the majority of research on cadavers was conducted in forensic entomology[1] to reconstruct the post-mortem interval (Marchenko 2001; Catts and Goff 1992; Schoenly et al. 1991). However, the fate of cadaveric products in soils as well as the impact of soils on cadaver derived components is still poorly understood. Decomposition products can be assessed beneath cadavers to identify which components derive from cadavers. In forensic investigations, knowledge about the fate of cadaveric products in soils may aid in the identification of decomposition fluids, the identification of the human origin of decomposition fluids and the subsequent estimation of decomposition rates of human remains.

For the characterisation of decomposition fluids, a variety of cadaver derived products have been found in soils. Forensic applications of cadaveric products in/on soils have mainly focused on the estimation of the post-mortem interval (PMI), the search for clandestine graves, the identification of temporary graves and the reconstruction of crime scenes which have been scavenged (Hunter et al. 2013; Bull et al. 2009; Carter et al. 2009; van Belle et al. 2009; Benninger et al. 2008; Vass et al. 2008; Vass et al. 2002; Vass et al. 1992). Inorganic components such as base cations (e.g. potassium, sodium, calcium, magnesium), nitrogen ($NO_3^-$, $NH_4^+$), phosphorous ($PO_4^-$) have been found in soil beneath decomposing cadavers (Stokes et al. 2013; Aitkenhead-Peterson et al. 2012; Benninger et al. 2008; Melis et al. 2007; Towne 2000). However, inorganic components in soils have various sources (e.g. parent material, plant litter, soil flora and fauna) and their occurrence is unspecific for the identification of human decomposition fluids.

[1] In forensic entomology the sequential arrival times and development stages of arthropods on cadavers is used to reconstruct the post-mortem interval (Swann et al. 2010a).

Organic decomposition products such as lipids could be more suitable indicators of human decomposition fluids in soils because they are relatively specific for their origin.

In a natural environment, cadaver decomposition mainly occurs on the soil surface (Melis et al. 2007; Towne 2000). Processes involved in belowground decomposition have been examined in a number of forensic studies (van Belle et al. 2009; Carter and Tibbett 2008; Carter et al. 2007; Fiedler et al. 2004; Hopkins et al. 2000; Mant 1987). It is known that environmental factors (e.g. temperature, moisture, acidity, soil texture) regulate the decomposition of cadavers in soil. There is still little known about which biotic and abiotic soil parameters regulate decomposition rates of buried cadavers. Information about the impact of soil parameters on decomposition could help in resolving the unexplained death of humans (Klemczak et al. 2015; Schotsmans et al. 2011) and in estimating the remediation of graves at cemeteries (Ferreira and Cunha 2013; Fiedler and Graw 2005; Fiedler and Graw 2003).

### *1.1.2. Cadaveric lipids in soils*

In forensic research cadaver derived lipids were extracted from soils and soil solutions to reconstruct the post-mortem interval (Vass et al. 2002; Vass et al. 1992) but using lipids as identifiers of human decomposition fluids was rarely investigated to date (Bull et al. 2009; Zimmermann et al. 2008). Suitable components for this purpose should be (I) preserved in soils over longer times (years to decades) and (II) be specific for their origin. In forensic investigations the observation periods of the presence of cadaveric lipids in soils were relatively short (days to months), whereas archaeological studies showed that cadaveric lipids are present in soils over longer times (up to thousands of years; von der Lühe et al. 2013; Shillito et al. 2011; Fiedler et al. 2009; Davies and Pollard 1988). In archaeology, lipid constituents including $\Delta^5$-sterols (e.g. cholesterol), stanols (e.g. coprostanol, 5α-cholestanol, epicoprostanol), stanones (e.g. 5β-cholestanone), bile acids (e.g. lithocholic acid, deoxycholic acid) and fatty acids (e.g. palmitic acid, stearic acid, oleic acid) were successfully used to identify tissues and degradation products in various environmental contexts (Shillito et al. 2011; Fiedler et al. 2009; Evershed and Connolly 1994; Evershed 1990; Gülaçar et al. 1990; Davies and Pollard 1988; Evershed and Connolly 1988). Furthermore, it is known that animal species (including humans) are distinguishable by analysing steroid ($\Delta^5$-sterols, stanols, stanones and bile acids) patterns in soils and sediments (Birk et al. 2011; Bull et al. 2003; Elhmmali et al. 2000; Bull et al. 1999a; Bull et al. 1999b; Leeming et al. 1997; Leeming et al. 1996; Knights et al. 1983). The high diagnostic potential of lipids ($\Delta^5$-sterols, stanols, stanones, bile acids and fatty acids) and their long-term preservation in soils has been neglected in forensic research. Up to now, there is little research conducted on the fate of cadaveric $\Delta^5$-sterols, stanols, stanones, bile acids and fatty acids in soils. The knowledge of the fate of human derived lipids in soils can aid in the selection of useful lipid biomarkers, which are indicative of human decomposition fluids. In forensic investigations, lipid biomarkers can then be applied to reconstruct crime scenes, to identify temporary graves or to support the search of clandestine graves.

Steroids belong to the class of lipids including $\Delta^5$-sterols, 5β-stanols, 5α-stanols, 5β-stanones, 5α-stanones and bile acids (Bull et al. 2002). Cholesterol is the common $\Delta^5$-sterol of higher animals and is also present in traces in plants, fungi and other eukaryotes and thus wide spread in soils (Christie and Han 2010; Weete et al. 2010; Mouritsen and

Zuckermann 2004). Excreted into the gut of the majority of higher animals, cholesterol is microbially reduced to coprostanol (Fig. 1.1), the major faecal 5β-stanol of human faeces (60% of total sterol content; Bull et al. 2002). Coprostanol is used as faecal biomarker in archaeological and environmental pollution research (Lauer et al. 2014; Bull et al. 2002; Leeming et al. 1996; Bethell et al. 1994; Leeming et al. 1994; Björkhem and Gustafsson 1971). In soils, cholesterol reduction yields 5α-cholestanol, the usual product of microbial biohydrogenation in the environment (Fig. 1.1). It is also present in minor amounts in animal tissues, faeces and plants (Bull et al. 2002; Noda et al. 1988; Hatcher and McGillivary 1979; Murtaugh and Bunch 1967). 5α-cholestanone and 5β-cholestanone are intermediate steroidal ketones of the environmental and intestinal cholesterol reduction to the respective products 5α-cholestanol and coprostanol (Grimalt et al. 1990; Gaskell and Eglinton 1975; Björkhem and Gustafsson 1971; Fig. 1.1). The occurrence of the epimer epicoprostanol in the environment has been attributed to microbial coprostanol conversion under anaerobic conditions (Bull et al. 2002; Quirk et al. 1980; Wardroper and Maxwell 1978) but it can also be found in traces in soils and faeces (Prost et al. (in prep); Birk et al. 2011; Fig. 1.1). The characteristic $\Delta^5$-sterol of plants is the higher molecular weight congener β-sitosterol (Bull et al. 2002). Its intestinal microbial reduction product is 5β-stigmastanol, a 5β-stanol commonly used as biomarker of ruminant faeces (Bull et al. 2002). 5α-stigmastanol is the respective environmental reduction product of β-sitosterol and widespread in soils and sediments (Bull et al. 2002). Plant $\Delta^5$-sterols and their associated 5β-stanols are present in human faeces as part of their diet (Leeming et al. 1996).

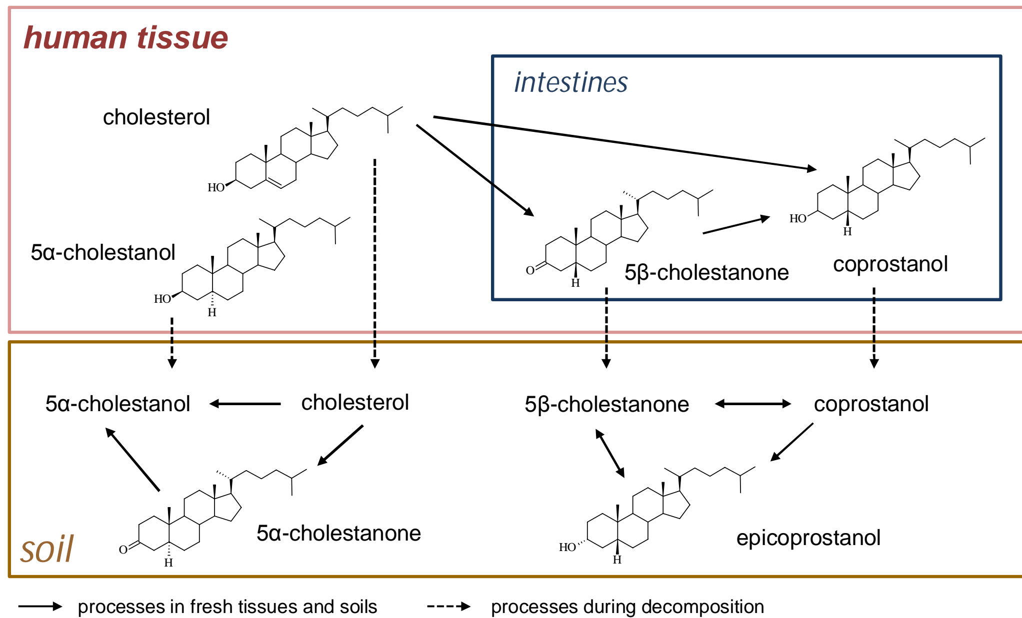


**Figure 1.1:** Major natural processes of cholesterol transformation in fresh human tissues, intestines and soils upon the decomposition of a human body. Modified after (Gérard 2014; Birk et al. 2011; Puglisi et al. 2003; Bull et al. 2002; Leeming et al. 1996).

Thus far there has been little research conducted on cadaveric derived $\Delta^5$-sterols, stanols and stanones and they have not been used in forensic case studies. Von der Lühe et al. (2013) demonstrated the presence of cholesterol, coprostanol and β-sitosterol beneath pig carcasses in shallow graves (~40 cm) 3 months post-burial but were not able to detect significant differences after six months probably because the extraction method (0.5 g soil, ultrasonic extraction, full scan with gas chromatography/mass spectrometry (GC/MS)) required optimisation (e.g. 10 g soil, Soxhlet extraction, selected ion monitoring (SIM) with GC/MS) for an increased sensitivity. Comparable research on cadaveric steroids in soft tissues preserved by mummification and bogs were conducted in an archaeological context (Evershed and Connolly 1994; Evershed et al. 1990; Gülaçar et al. 1990; Evershed and Connolly 1988). These studies demonstrated that $\Delta^5$-sterols, stanols and stanones conservation was promoted under moist and wet (bog bodies; Evershed 1990; Evershed and Connolly 1988) or under dry environmental conditions (mummies; Gülaçar et al. 1990). In soils, the presence of human derived cholesterol in an Anglo-Saxon grave (c. 400-700 AD) and cholesterol and coprostanol in a burial from Neolithic Çatalhöyük (c. 7400-6000 BC) in the periphery of skeletons indicated the long-term presence of animal derived $\Delta^5$-sterols and stanols in close proximity to decomposing bodies (Shillito et al. 2011; Davies and Pollard 1988).

The presence of $\Delta^5$-sterols, stanols and stanones in soils thus demonstrated, that steroids are indicative of decomposition fluids and tissues but, as they were found 3 months post-mortem beneath pig cadavers (von der Lühe et al. 2013), it is still not known when steroids were introduced into the soil during the decomposition of a cadaver ≤ 3 months. To date, only cholesterol, coprostanol and β-sitosterol were determined beneath cadavers. However, a combination of other characteristic animal stanols (5α-cholestanol, epicoprostanol, 5β-stigmastanol and epi-5β-stigmastanol) and stanones (5α-cholestanone and 5β-cholestanone) strengthens the evidence of human decomposition fluids in soils. The presence of 5α-cholestanol, 5α-cholestanone, 5β-cholestanone, epicoprostanol, 5β-stigmastanol and epi-5β-stigmastanol in soil samples beneath decomposing cadavers was not investigated yet. Until the present, only surface samples were taken beneath decomposing cadavers, so the spatial distribution over soil depth and between different body positions (e.g. thorax, abdomen) is still unknown.

Bile acids belong to the group of steroids and are only produced by vertebrate organisms (Prost et al. (in prep) Hofmann and Hagey 2008; Bull et al. 2002; Haslewood 1967). Primary bile acids (e.g. cholic acid, chenodeoxycholic acid) are formed in the liver from cholesterol and are excreted into the intestinal tract via the bile (Gérard 2014; Fig. 1.2). In the intestines, microorganisms produce secondary bile acids (e.g. lithocholic acid (LCA), deoxycholic acid (DCA)) from primary bile acids (Gérard 2014; Stellwag and Hylemon 1979; Hayakawa 1973; Fig. 1.2). About 95% of bile acids are reabsorbed and returned to the liver by the enterohepatic circulation hence small proportions are excreted within faeces (Zwicker and Agellon 2013; Bull et al. 2002). The major bile acids in human faeces are lithocholic acid and deoxycholic acid with low abundances of isolithocholic acid (ILCA) and chenodeoxycholic acid (CDCA; Prost et al. (in prep)). Bile acids are a relatively novel biomarker class in detecting faecal sources in archaeological and environmental pollution research and have not been widely used to date (Bull et al. 2002). Because they are only produced by vertebrates, they are highly specific for the input of faecal material into soils. Bile

acid analysis in combination with stanols was recently used to distinguish between animal species (Prost et al. (in prep); Tyagi et al. 2007; Simpson et al. 1998; Leeming et al. 1997; Leeming et al. 1996; Gülaçar et al. 1990). Furthermore it has been shown that bile acids are more stable against microbial degradation than 5β-stanols (Elhmmali et al. 1997).

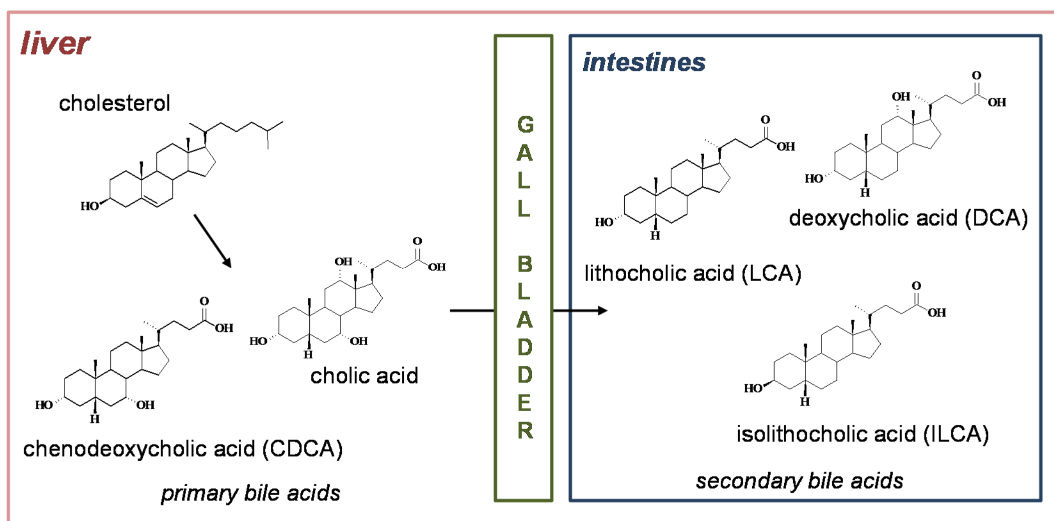


Figure 1.2: Example of primary bile acid formation from cholesterol in the liver and microbial transformation to secondary bile acids in the intestines of humans. Modified after (Bull et al. 2002).

As far as known, there is only one study that determined bile acids in a grave containing human skeletal remains. Shillito et al. (2011) found LCA in one and DCA in two of five burial samples at Neolithic Çatalhöyük (c. 7400-6000 BC). However, no reference sample was analysed in that study; it is therefore uncertain if LCA and DCA from the burials were from the decomposition of the bodies or naturally present (Shillito et al. 2011). It is unknown if bile acids are present in detectable amounts beneath decomposing human cadavers. Due to their high specificity for certain taxa (including humans) and their relative stability against microbial degradation, they might be highly worthwhile as markers of human decomposition fluids in soils.

As explained above, steroids have different origins in a human body and in the environment. In this work, (soft-) tissue steroids are defined as any steroids which are not from the digestive tract of a human body. Faecal steroids are understood as steroids which are produced in the intestines by microorganisms. A clear separation between tissue and faecal steroids is not always possible, because steroids are also ingested as part of the human diet and thus occur in faeces. The main occurrences of steroids in a human body and the fate of human derived steroids in the environment is summarised in Table 1.1.

Table 1.1: Occurrence of steroids in the human body and in the environment. Soft tissues are all tissues except contents of the digestive tract. Faeces derived steroids are ingested as part of the human diet and faecal steroids are microbially produced (e.g. coprostanol) and present (e.g. chenodeoxycholic acid) in the intestines of a human body. Steroids do also occur in the environment (e.g. plants, fungi) and human derived tissue, faecal and faecal derived steroids are further transformed in the environment. ? = unknown process, (x) = produced to a minor extent.

| steroid | occurrence | | | | References |
|---|---|---|---|---|---|
| | tissue | faeces derived | faecal | environment | |
| cholesterol | x | x | x | x | Prost et al. (in prep); Christie and Han 2010; Weete 2010; Behrman and Gopalan 2005; Mouritsen and Zuckermann 2004; Hartmann 1998; Grandmougin-Ferjani et al. 1999 |
| 5α-cholestanol | x | x | ? | x | Prost et al. (in prep); Piironen et al. 2000; Bull et al. 2002; Leeming et al. 1996; Noda 1988; Nishimura 1977; Gaskell and Eglinton 1975; Danielsson and Tchen 1968 |
| 5α-cholestanone | x | x | ? | x | Prost et al. (in prep); Bull et al. 2002; Gaskell and Eglinton 1975; Daniellson and Tchen 1968; |
| coprostanol | | | x | (x) | Prost et al. (in prep); Bull et al. 2002; Leeming et al. 1994; Gaskell and Eglinton 1975; Björkhem and Gustaffson 1971 |
| 5β-stigmastanol | | | x | (x) | Prost et al. (in prep); Gill et al. 2010; Bull et al. 2002 |
| 5β-cholestanone | | | x | x | Gérard 2014; Bull et al. 2002; Gaskell and Eglinton 1975; Björkhem and Gustaffson 1971 |
| epicoprostanol | | | x | x | Prost et al. (in prep); Leeming et al. 1996; Wardroper and Maxwell 1978; McCalley et al. 1981 ; Quirk et al. 1980 |
| epi-5β-stigmastanol | | | | x | McCalley et al. 1981 |
| β-sitosterol | | x | | x | Christie and Han 2010; Behrmann and Gopalan 2005; Hartmann 1988 |
| 5α-stigmastanol | | x | ? | x | Prost et al. (in prep); Bull et al. 2002; Piironen et al. 2000; Nishimura and Koyama 1976 |
| lithocholic acid (LCA) | x | | x | | Prost et al. (in prep.); Bull et al. 2002 |
| deoxycholic acid (DCA) | x | | x | | |
| chenodeoxycholic acid (CDCA) | x | | x | | |
| isolithocholic acid (ILCA) | x | | x | | |

Human adipose tissue consists by weight of 5-30% water, 2-3% proteins and 60-85% of lipids (fat) of which 90-99% are triacylglycerols (Dent et al. 2004). In fresh human adipose tissue the major fatty acid is the monounsaturated oleic acid ($C_{18:1\Delta9}$), followed by palmitic acid ($C_{16:0}$), linoleic acid ($C_{18:2}$), stearic acid ($C_{18:0}$), palmitoleic acid ($C_{16:1\Delta9}$) and myristic acid ($C_{14:0}$; Notter et al. 2009). Following death, triacylglycerols are enzymatically hydrolysed by intrinsic tissue lipases to glycerol and free fatty acids (Dent et al. 2004; Fig. 1.3). During advanced decomposition an extensive hydrolysis and hydrogenation yields increased abundances of saturated fatty acids (Dent et al. 2004; Evans 1963). Under aerobic conditions, fatty acids from tissues undergo oxidation to yield short-chain fatty acids, aldehydes and ketones by the activity of bacteria, fungi and atmospheric oxygen (Dent et al. 2004; Evans 1963; Fig. 1.3). Research focusing on human derived fatty acids under natural oxidative processes was neglected in the past. It was demonstrated, that fatty acids from various sources (e.g. plants, fungi, atmospheric deposition, animals) can have mean residence times in soils of several decades (Wiesenberg et al. 2010; Quénéa et al. 2006; Wiesenberg et al.

2004). In forensic studies, the presence of human derived volatile fatty acids in soils was demonstrated in short periods of 70 days and long-chain fatty acids were detected in decomposition fluids of pigs up to 60 days post-mortem (Swann et al. 2010b; Vass et al. 2002). The long-term (≥ 70 days) presence and pattern changes of relative abundances of fatty acids from decomposition fluids in soils under oxidative environmental conditions are still unknown.

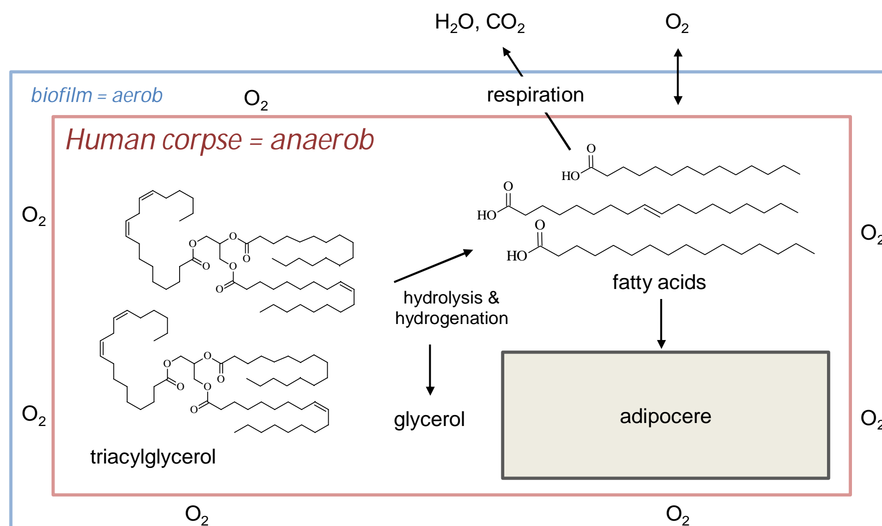


**Figure 1.3:** Simplified processes of triacylglycerol degradation in a human corpse after death under aerobic and anaerobic conditions involving fatty acids respiration and adipocere formation. The thin layer of water and microorganisms (= biofilm) accelerate fatty acid respiration. Modified after Schoenen and Schoenen (2013).

In forensic science the majority of the published literature concerning cadaveric derived fatty acids in soil environments is in association with adipocere formation, because it is relatively often formed in burials and causes difficulties in the reuse of graves in cemeteries (Fiedler et al. 2004; Fiedler and Graw 2003). In burials, moist and anaerobic conditions promote adipocere formation (Fiedler and Graw 2003). It comprises of saturated free fatty acids ($C_{16:0}$ > $C_{18:0}$ > $C_{14:0}$), $Na^{+}$-, $K^{+}$-, $Ca^{2+}$- and $Mg^{2+}$-fatty acid soaps, hydroxy- and oxo-fatty acids and polyhydroxy fatty acids (Schoenen and Schoenen 2013; Takatori 2001; Takatori 1996; Takatori et al. 1988; Takatori et al. 1987; Tomita 1984; Fig 1.3). When environmental conditions are constant, adipocere remains as stable degradation product over thousands of years, e.g. the Tyrolean Iceman ("Ötzi") is a prominent example for adipocere formation and preservation of soft tissues (Bereuter et al. 1997). The high stability of adipocere is attributed to the insolubility of fatty acid soaps and the high melting points of hydroxy- and oxo-fatty acids (Takatori 2001; Takatori et al. 1988). There is no mechanism of fatty acid fermentation under anaerobic conditions consequently fatty acids are preserved in a grave environment (Schoenen and Schoenen 2013). Fiedler et al. (2015) found black humic material surrounding adipocere carcasses in water-saturated graves and proposed a possible way of adipocere decomposition under poikiloaerobic conditions. Although the formation of adipocere has been well studied, knowledge of factors which regulate adipocere decomposition is still lacking. Fründ and Schoenen (2009) observed adipocere decomposition in laboratory and field

experiments. They found low decomposition rates (1.5 years) of adipocere buried in soil and presumed that adipocere decomposition is stimulated by soil flora and fauna. No study has yet provided a detailed assessment of the contribution of the micro-, meso- and macrofauna on the decomposition of adipocere in soils.

## 1.2. Aims of the study

The aim of the present work was to investigate if cadaveric lipids are present in soil comprising human decomposition fluids. The fate of the identified cadaveric lipids was then investigated over different periods of time. Furthermore, the impact of soil parameters on the decomposition of human remains was investigated. Knowledge about the fate of human decomposition products in soils aids the identification of diagnostic lipids for human decomposition fluids and in understanding which soil abiotic and biotic factors regulate the decomposition of human remains.

The main objectives of this study were

(I) to develop suitable laboratory methods which can then be applied to detect cadaveric lipids in soil samples containing human decomposition fluids by gas chromatography/flame ionisation detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS)

(II) to discover the presence of $\Delta^5$-sterols, stanols, stanones and bile acids in soil samples comprising decomposition fluids and test (I) their suitability as diagnostic markers of decomposition fluids and (II) to identify the human origin

(III) to investigate the fate of cadaveric lipids in soil, namely their (I) temporal presence (II) spatial distribution and (III) transformation and degradation processes of steroids and fatty acids examining factors which influence their presence

(IV) to explore the impacts of biotic and abiotic parameters on the decomposition of adipocere in a soil environment.

For the present work, soil samples from case studies were used, where human bodies were temporarily decomposing on the soil surface or in graves. Human adipocere was used to study degradation processes in soils. The knowledge obtained from the studies conducted is not only applicable in forensic investigations but can also be applied in archaeology and ecological sciences.

## 1.3. Outline of research

Analytical separation of lipid biomarkers is mainly divided into the total lipid extraction, saponification to achieve higher recoveries of lipids, separation of acidic and neutral lipids by liquid-liquid extraction, solid phase extraction to separate lipid classes and a subsequent methylation or silylation of functional groups for gas chromatographic (GC/FID) and gas chromatographic/mass spectrometric (GC/MS) analysis (Chapter 2). This chapter commences by testing preliminary laboratory methods suitable for analysing steroids in soil samples which received high amounts of human decomposition fluids. Methods tested (Chapter 2) were then adopted in the following Chapters 3-5.

Steroidal biomarkers were analysed in soil which had received high amounts of human decomposition fluids. The placement of a human body in an outdoor situation allowed the rapid degradation of soft tissues with the involvement of insect maggot activity and a subsequent development of a CDI. Soil was sampled 11-18 days after deposition of the body and 358 days after the body was removed. It was hypothesised, that steroids are released from the decaying body and are therefore present in high amounts in the soil 11-18 days after deposition and 358 days after removal of the body. It was further assumed that steroid composition patterns differ dependent on the release of the decomposition fluids, i.e. enhanced amounts of faecal steroids are measured beneath the abdomen (Chapter 3). Fatty acids analytics were applied on soil samples from the case study in Chapter 4. It was hypothesised that upon decomposition of a human body fatty acids are released from the body and leach into the soil beneath 11-18 days after the body was deposited. Fatty acids are still increased in the soil one year after the body was removed compared to reference soil samples taken in close proximity of the body (Chapter 4).

With the knowledge obtained from Chapter 2-4 a multi-lipid approach including $\Delta^5$-sterols, stanols, stanones and bile acids was applied to identify temporary graves from World War II (Chapter 5). This approach was performed in determination of the long-term presence (67 years) of temporary graves, where human bodies were previously buried for a period of 10 months. It was presumed, that steroids were indicative of decomposition fluids from the human bodies which were buried in the mass graves for a period of 10 months. It was suggested that amongst $\Delta^5$-sterols, stanols and stanones, bile acids aid in the identification of the human origin of decomposition fluids.

As adipocere is known to be a relatively stable human degradation product, the influence of soil biotic and abiotic parameters was tested on adipocere decomposition (Chapter 6). The access of soil flora and fauna provides information about which soil organisms are involved in the mass loss of adipocere over time in a forest ecosystem under temperate climatic conditions. It was hypothesised that (I) adipocere decomposition takes place along the organic matter necrophage food chain starting by microfauna/flora and increasing complexity to higher trophic levels; (II) adipocere decomposition is faster when meso- and macrofauna occurs in soil alongside microfauna, rather than when only microbes are present; (III) adipocere decomposition takes place at a higher rate in slightly alkaline soil with high levels of microbial biomass than in an acidic sandy soil with low levels of microbial biomass.

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# Chapter 2

## Analytical approaches in detecting steroids and fatty acids from body decomposition fluids in soil

## 2.1. Introduction

In this chapter two initial laboratory approaches are introduced to detect cadaveric lipids from decomposition fluids in soil samples. This chapter serves as preparation for the following Chapters 3-5, where methods described below were used. Chapter 2.2. describes a laboratory method for $\Delta^5$-sterol and stanol analysis tested on reference soil samples taken from the case study described in Chapter 3 and 4. The extraction of neutral steroids was also carried out on soil samples in Chapter 5. Chapter 2.3. introduces a method to estimate the concentration of 10-hydroxystearic acid by gas chromatography equipped with a flame ionisation detector (GC/FID), where no analytical standard was available. The estimation of 10-hydroxystearic acid concentrations was adapted in Chapter 4.

## 2.2. Steroid method test

In a first approach a new method to extract consistently high amounts of steroids from soil samples was tested. The test soil was reference material from a case study, where a body was found 11-18 days after deposition (detailed description of the case in Section 3.2.1.). A previously published method used on soil samples adapted from Dove and Mayes (2006) was modified to extract high amounts of steroids from soil (von der Lühe et al. 2013). Extraction of the total lipid extract (TLE) was carried out after a method previously published by Isobe et al. (2002). The combined method was tested by standard addition on matrix samples (TLE from reference soil; 0-0.5 cm depth) in order to quantify sample loss during saponification, solid phase extraction (SPE) and derivatisation to achieve high recovery surrogates of added steroid standards and to test the suitability of the method on soil found at the crime scene. The extraction and purification procedure is graphically summarised in Fig. 2.1. This newly developed method was then applied to soil samples, which were in contact with decomposition fluids (Chapter 4).

### *2.2.1. Reference soil samples from the case study*

Reference soil samples were taken 10 m away from a surface decomposition case study, where a partly burned female body was found in a suburban area. After removal of the vegetation and the litter cover, the soil was sampled at 0–0.5 cm depth starting at the mineral surface. Soil samples were freeze-dried for 24 h (Alpha 1-4 LSC, Christ, Osterode, Germany), sieved (≤ 2 mm) and milled (450 rpm, 5 min; PM 200, Retsch, Haan, Germany). Samples were stored in a desiccator with dried silica gel (105°C, 24 h) at room temperature (18°C) until analysis.

### *2.2.2. Laboratory equipment and materials*

To avoid contamination reusable glassware and plastic materials were rinsed five times with deionised water. All disposable and reusable glassware was heated at 450°C for 12 h. Plastic materials were rinsed twice in *n*-heptane prior use. The solvents methanol, dichloromethane (DCM), *n*-heptane, toluene, ethanol, ethyl acetate were purchased from Carl Roth (Karlsruhe, Germany) and were at least GC grade. Ultrapure water (18-MΩ grade; Barnstead

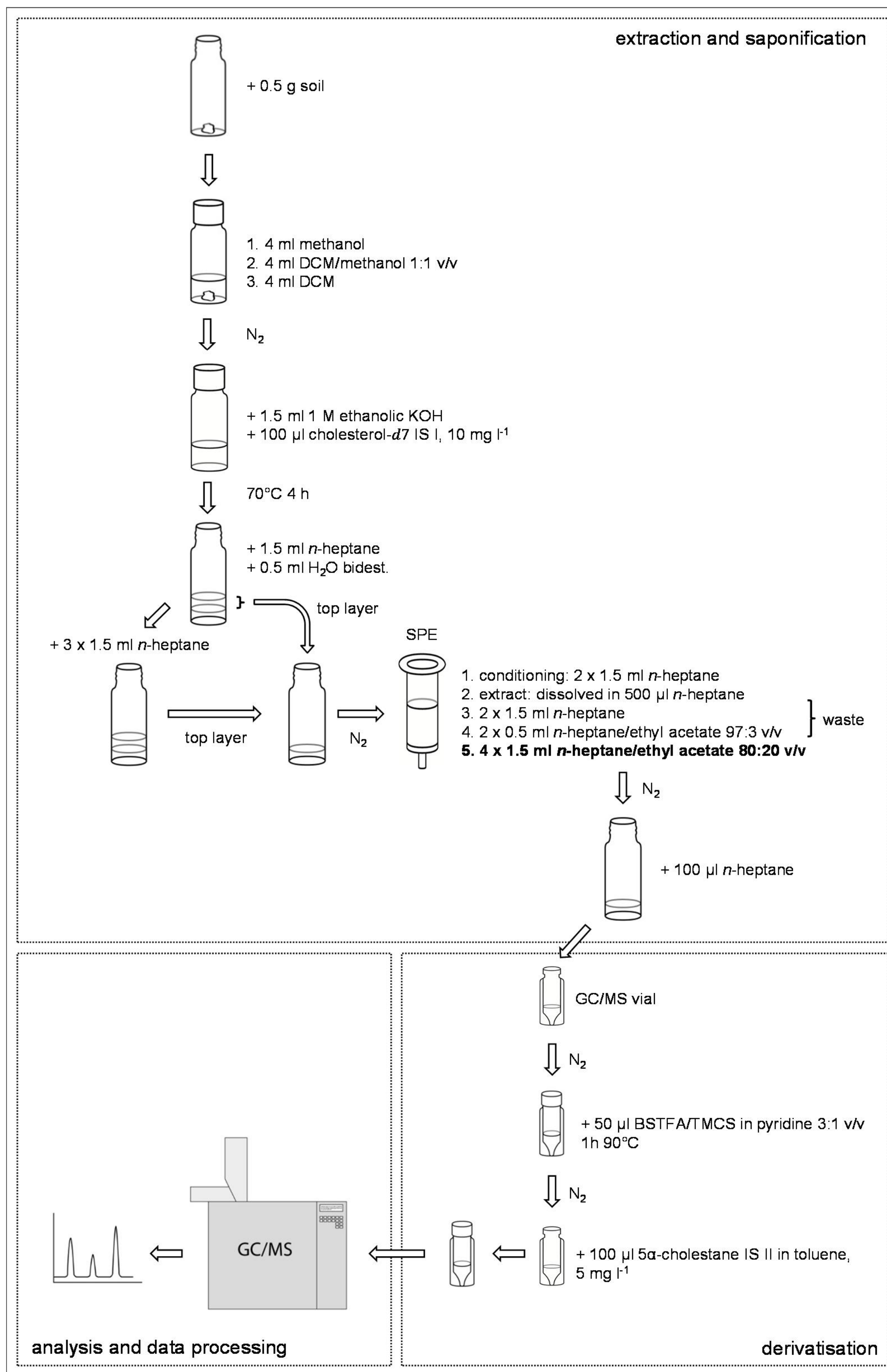


**Figure 2.1:** Steroid analytical procedure of soil samples adapted from von der Lühe et al. (2013), Dove and Mayes (2006) and Isobe et al. (2002). DCM = dichloromethane, IS = internal standard, GC/MS = gas-chromatograph/mass spectrometer, BSTFA+TMCS = *N,O*-Bis(trimethylsilyl)trifluoroacetamide+trimethylchlorosilane.

Nanopure, Thermo Scientific, Waltham, MA, USA) was used for the liquid-liquid extraction. The silica gel (60 Å, 0.063-0.200 mm; Merck, Darmstadt, Germany) used for SPE was heated at 380°C for 2 h to remove any organic contaminants, activated at 200°C for 5 h and subsequently stored in *n*-heptane until use (Isobe et al. 2002). The derivatisation mixture containing *N,O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS, 99:1, v/v) was purchased from Sigma Aldrich (Steinheim, Germany) and pyridine from Merck (Darmstadt, Germany). Nitrogen ($N_2$) was obtained in a purity of 99.998% (Westfalen, Münster, Germany).

### 2.2.3. Steroid standards

Steroid standard specifications are listed in Tab. 2.1. Single stock solutions of each steroid standard were prepared in a concentration of 1 g $l^{-1}$ in *n*-heptane. Two steroid mixed standard solutions in *n*-heptane were diluted from single stocks with a concentration of 20 mg $l^{-1}$ and 1 mg $l^{-1}$ containing coprostanol, epicoprostanol, cholesterol-25,26,26,26,27,27,27-d7 (internal standard I, IS I), cholesterol, 5α-cholestanol, 5β-stigmastanol, epi-5β-stigmastanol, β-sitosterol and 5α-stigmastanol (Tab. 2.1). All standard solutions were stored at -20°C. For quantification an external calibration was prepared from the 20 mg $l^{-1}$ stock solution with concentrations ranging from 100, 500, 1000, 1500, 2000, 3000 to 4000 ng $l^{-1}$. For low concentrations an external calibration from the 1 mg $l^{-1}$ stock solution ranging from 10, 20, 40, 80 and 100 ng $l^{-1}$ was prepared. Calibration curves had coefficients of determination ($R^2$) between 0.98 and 0.99.

**Table 2.1:** Steroid standards used for standard addition method test and external calibration.

| Substance (trivial name) | biomarker group | RT[d] min | ion fragments *m/z* | standard added ng |
|---|---|---|---|---|
| **5α-cholestane**[a] | IS II | 22.514 | 217.2; 357.4; 372.4 | |
| 5β-cholestan-3β-ol[a] (**coprostanol**) | 5β-stanol | 26.139 | 215.2; 257.2; 370.4 | 0-100-500-1000-2000 |
| 5β-cholestan-3α-ol[a] (**epicoprostanol**) | 5β-stanol | 26.700 | 215.2; 355.4; 370.4 | 0-50-100-500 |
| **cholesterol-d7**[b] | IS I | 27.999 | 336.4; 375.4; 465.5 | 0-500-1000-1500-2000 |
| cholest-5-en-3β-ol[a] (**cholesterol**) | $\Delta^5$-sterol | 28.165 | 329.3; 368.3; 458.4 | 0-500-1000-2000 |
| 5α-cholestan-3β-ol[a] (**5α-cholestanol**) | 5α-stanol | 28.445 | 215.2; 445.4; 460.4 | 0-100-500 |
| 5β-stigmastan-3β-ol[c] (**5β-stigmastanol**) | 5β-stanol | 30.705 | 215.2; 383.4; 398.4 | 0-50-100-500-1000 |
| 5β-stigmastan-3α-ol[c] (**epi-5β-stigmastanol**) | 5β-stanol | 31.386 | 215.2; 383.4; 398.4 | 0-100-200-500 |
| stigmast-5-en-3β-ol[a] (**β-sitosterol**) | $\Delta^5$-sterol | 33.349 | 357.3; 396.4; 486.5 | 0-1000-1500-2000 |
| 5α-stigmastan-3β-ol[a] (**5α-stigmastanol**) | 5α-stanol | 33.721 | 215.2; 473.4; 488.5 | 0-50-100-500 |

[a]Sigma Aldrich, Steinheim, Germany │ [b]Avanti, Alabaster, AL, USA │ [c]Chiron, Trondheim, Norway │ [d]retention time

### 2.2.4. Extraction of TLE

Reference soil samples (0.5 g) were weighed into glass centrifuge tubes. Extraction was carried out by ultrasonication (Elmasonic P60H, Singen, Germany) for 30 min at 30-35°C with three different solvents and solvent mixtures: 4 ml methanol (MeOH), 4 ml dichloromethane/methanol (1:1, v/v) and 4 ml dichloromethane (DCM). Between each

extraction, tubes were centrifuged (3000 rpm, 6 min; Megafuge 1.0, Heraeus Instruments, Hanau, Germany) and the supernatant was transferred to a glass vial. The TLE was then dried under a gentle stream of $N_2$.

### 2.2.5. Standard addition

For standard addition TLE of reference samples were spiked with the 20 mg $l^{-1}$ standard mixture within the range of steroids amounts expected in soil samples contaminated with decomposition fluids (Tab. 2.1).

### 2.2.6. Saponification

For saponification 1.5 ml of 1 M ethanolic KOH was added to the TLE and samples were heated at 70°C for 4 h on a dry-block heater (IVA, Düsseldorf, Germany).

### 2.2.7. Liquid-liquid extraction

To extract the neutral lipid fraction, saponified samples were cooled to 50-60°C and 1.5 ml *n*-heptane was added and vials were capped and vortexed. Ultrapure water (0.5 ml) was added and samples were capped and vortexed. The upper organic layer was carefully removed and transferred with a Pasteur pipette into a glass vial. This procedure was repeated by adding 3 x 1.5 ml *n*-heptane. The collected extracts were combined and dried under $N_2$.

### 2.2.8. SPE extraction

Empty 3 ml SPE glass columns were fitted with a PTFE frit and 0.75 ml silica gel in *n*-heptane (approximately 1 cm of bed volume). Columns were conditioned with 2 x 1.5 ml *n*-heptane. The neutral lipid extracts were dissolved in 500 µl *n*-heptane by warming at 50°C and transferred to the SPE columns. Hydrocarbons and other short-chain compounds were washed to waste by adding 2 x 1.5 ml *n*-heptane. Ketones and aldehydes were eluted to waste by adding 2 x 0.5 ml *n*-heptane/ethyl acetate (97:3, v/v). Steroids and alcohols were collected in glass vials by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (80:20, v/v) and eluates were dried under a gentle stream of $N_2$.

### 2.2.9. Silylation of steroids

The steroid extracts were silylated with 50 µl BSTFA+TMCS/pyridine (3:1, v/v) at 90°C for 1 h. After derivatisation the mixture was evaporated under $N_2$ and 100 µl of 5α-cholestane (5 mg $l^{-1}$ in toluene, IS II; Tab. 2.1) was added as injection IS II.

### 2.2.10. GC/MS conditions

Analysis was performed on an Agilent 6890 gas chromatograph coupled to a 5975B mass spectrometer ((GC/MS) Agilent Technologies, Santa Clara, CA, USA). A DB-5ms Ultra Inert (Agilent Technologies, Santa Clara, CA, USA) fused silica capillary column (30 m length x 250 µm internal diameter x 0.25 µm film thickness) was used with helium (99.9995%) as carrier gas at 1.1 ml $min^{-1}$ constant flow. The injection port was maintained at 250°C and an aliquot of 1 µl was injected in splitless mode. The initial oven temperature was held at 80°C for 1.5 min, then programmed at

12°C $min^{-1}$ to 265°C, at 0.8°C $min^{-1}$ to 280°C and at 10°C $min^{-1}$ to 300°C, and held for 12 min. The solvent delay was 20 min and electron ionisation was set at 70 eV. For quantification characteristic ions (Tab. 2.1) for each analyte were selected, and samples were run in selected ion monitoring mode (SIM). Quantification was carried out with the analyte/IS II ratios and the corresponding ratios of the external calibration. Recoveries of the standard addition test of each analyte were calculated by comparing the amount added to the reference TLE with the amount extracted from the reference TLE (Tab. 2.2).

**Table 2.2:** Results of the standard addition experiment; recovery (%) and regression coefficients ($R^2$) of linear regression analysis of added $\Delta^5$-sterols, 5β-stanols and 5α-stanols to the TLE of reference block samples.

| compound | recovery % | $R^2$ | LOD[a] ng $g_{soil}^{-1}$ | LOQ[b] ng $g_{soil}^{-1}$ |
|---|---|---|---|---|
| coprostanol | 87 | 0.99 | 0.2 | 0.7 |
| epicoprostanol | 97 | 0.99 | 0.2 | 0.8 |
| cholesterol-d7 | 82 | 0.99 | n/a | n/a |
| cholesterol | 78 | 0.98 | 0.4 | 1.4 |
| 5α-cholestanol | 84 | 0.99 | 0.5 | 1.7 |
| 5β-stigmastanol | 83 | 0.99 | 0.6 | 2.0 |
| epi-5β-stigmastanol | 90 | 0.99 | 0.4 | 1.3 |
| β-sitosterol | 84 | 0.94 | 0.9 | 2.8 |
| 5α-stigmastanol | 85 | 0.99 | 1.0 | 3.5 |

[a]limit of detection (signal-to-noise ratio S/N) = 3 │ [b]limit of quantification S/N = 10 │n/a not analysed

### *2.2.11. Summary*

A high yield of steroids (78-97%) added to reference soil TLEs was recovered with a high precision ($R^2$ 0.94-0.99) showing that the method is applicable to soil found at the crime scene (Tab. 2.2). The relative standard deviation (RSD %) of the 8 sterols examined through four replicate assays of a reference block sample was 2-11%. Low limits of detection (LOD) and limits of quantification (LOQ) were calculated as signal-to-noise ratio S/N=3 and 10, respectively (Tab. 2.2). Using GC/MS and selected characteristic ions for quantification with low detection and quantification limits is a sensitive method that also allows the determination of trace amounts such as epicoprostanol (LOD at 0.8 ng $g_{soil}^{-1}$; Tab. 2.2). For instance, Birk et al. (2012) found detection limits of epicoprostanol at 1.4 ng $g_{soil}^{-1}$ and Isobe et al. (2002) at 0.1 ng $g_{sediment}^{-1}$. This highlights the sensitivity of GC/MS analysis of steroids extracted from soil and sediment samples. An ultrasonication extraction method with three successive solvents (MeOH, MeOH/DCM and DCM) introduced by Isobe et al. (2002) was applicable for cadaver decomposition island (CDI) and reference samples. It showed low standard errors between replicates and acceptable RSD of individual steroids of one test sample (n=4, 2-11%). Saponification in an alkaline solution was suggested by other researchers to yield amongst free steroids also the esterfied forms and was therefore applied here (Birk et al. 2012; Dove and Mayes 2006; Grimalt et al. 1990; Dutka and El-Shaarawi 1975). For future analysis of stanones as additional lipid fraction, concentration of the KOH, the saponification temperatures and the duration of saponification has to be modified because stanones are known to be sensitive to rigorous saponification (Birk et al. 2012; Isobe et al. 2002). For additional valuable

information, a further fractionation of the TLE into various lipid fractions is recommended as steroids, fatty acids and bile acids could be extracted from one soil sample (Birk et al. 2012). Application of the above tested method to soil samples contaminated with decomposition fluids showed that standard errors between replicate analysis were comparably low (below 10% deviation from mean) following that the extraction and purification method can be applicable on soil samples containing decomposition fluids (Chapter 3). The standard addition experiment worked well for all steroids in the range of the standard amounts added. However, in a few cases selected TLEs of cholesterol exceeded amounts of the added cholesterol (Chapter 3). It has been shown that the method established for the extraction and purification of CDI samples is simple and sensitive and can deal with a large set of samples.

## 2.3. Quantification of 10-hydroxystearic acid without analytical standard

Fatty acids have been recently used as decomposition markers in soil and related materials in forensic sciences, especially in the identification of adipocere in soils and tissues (Bull et al. 2009; Zimmermann et al. 2008; Forbes et al. 2002). Amongst the typical saturated fatty acids, which can be detected in adipocere, oxo- and hydroxy-fatty acids, mainly 10-hydroxystearic acids (10-OH-$C_{18:0}$) have been identified to be present in adipocere (Schoenen and Schoenen 2013; Takatori 2001; Takatori 1996; Takatori et al. 1988; Takatori and Yamaoka 1977).

During routine laboratory procedure total lipid extracts from soil samples were obtained from a case study where a body was discovered decomposing aboveground 11-18 days since deposition (detailed case study description Chapter 3 and 4). Additionally, further soil samples were taken one year (358 days) after the body was removed to track the preservation of the human derived lipids in soils. The dried TLE samples from soil taken one year after the body was removed were a yellow-whitish firm-crumbly substance, comparable to the consistency of adipocere (Fig. 2.2). Therefore, it was decided to run a fatty acid analysis to identify presumptive adipocere in the soil extracts. An unidentified peak was found in the fatty acid chromatograms, run by GC/FID, and it was presumed that this component is 10-hydroxystearic acid. Tests with changing derivatisation methods and the analysis of mass spectrums by GC/MS confirmed the presence of 10-hydroxystearic acid in the soil samples. As there was no analytical standard of 10-hydroxystearic acid available, an alternative method was developed to estimate concentrations of 10-hydroxystearic acid in the soil samples.

**Figure 2.2:** Total lipid extracts of soil from a case study, where a body was found decomposing aboveground for 11-18 days. (a) lipid extract from soil taken at 11-18 days after the body was removed (b) lipid extract from soil, one year (358 days) after the body was removed.

### 2.3.1. *Laboratory equipment*

Reusable glassware was washed five times with deionised water and subsequently heated at 450°C for 24 h to avoid contamination. Disposable glassware was heated at 450°C for 24 h prior use. Plastic materials were washed five times with deionised water and twice with *n*-heptane. The solvents *n*-heptane, ethanol, ethyl acetate, toluene, methanol and DCM were purchased from Roth (Karlsruhe, Germany) and were at least in GC grade. HCl (37%), $H_2SO_4$ (98%), KOH and $K_2CO_3$ were purchased from Roth (Karlsruhe, Germany). For the liquid-liquid extraction 18-MΩ grade $H_2O$ (Barnstead Nanopure, Thermo Scientific, Waltham, MA, USA) was used. The silica gel (60 Å, 0.063-0.200 mm; Merck, Darmstadt, Germany) used for SPE was heated at 380°C for 2 h and activated at 200°C for 5 h and subsequently stored in *n*-heptane. Analytical standards of fatty acid methyl esters and fatty acids were obtained from Sigma Aldrich (Steinheim, Germany) with a purity of ≥ 98%. The derivatisation agent *N,O*-Bis(trimethylsilyl)-trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS, 99:1, v/v) was purchased from Sigma Aldrich (Steinheim, Germany) and pyridine from Merck (Darmstadt, Germany). Nitrogen ($N_2$; 99.998% purity, Westfalen, Münster, Germany) was used to dry the extracts.

### 2.3.2. *Fatty acid standards*

Stock solutions of heptadecanoic acid ($C_{17:0}$), methyl heneicosanoate ($C_{21:0}$), methyl 12-hydroxystearate (12-OH-$C_{18:0}$) were prepared with a concentration of 1 g $l^{-1}$. From these stock standards, diluted standards were prepared: heptadecanoic acid in 100 mg $l^{-1}$, methyl heneicosanoate in 10 mg $l^{-1}$ and methyl 10-hydroxystearate in 100 mg $l^{-1}$.

### 2.3.3. *Extraction and purification of fatty acids*

Soil samples were taken from a forensic case study site where a female body was placed in a thicket of bushes and trees in a suburban area (Chapter 3 and 4). Soil samples containing decomposition fluids were freeze-dried for 24 h (Alpha 1-4 LSC, Christ, Osterode, Germany), sieved at ≤ 2 mm and finely ground in a ball mill (450 rpm, 5 min; PM 200, Retsch, Haan, Germany). Samples were stored in a desiccator at room temperature (18°C) until analysis.

For extraction, an aliquot of 0.5 g was consecutively extracted with 4 ml each of methanol, DCM/methanol (1:1, v/v) and DCM for 30 min in an ultrasonic bath at 30-35°C. Soil samples were centrifuged (3000 rpm, 6 min; Megafuge 1.0, Heraeus Instruments, Hanau, Germany) and the lipid extract was transferred to a new glass vial. Lipid extracts were combined and dried under a gentle stream of nitrogen.

Heptadecanoic acid ($C_{17:0}$, IS I, 200 µl, 100 mg $l^{-1}$ in *n*-heptane) was added to the lipid extracts as an internal standard. To yield free and esterfied fatty acids, saponification was carried out by adding 1.5 ml 1 M ethanolic KOH and heating at 70°C for 4 h. Neutral lipids, such as steroids and alcohols were removed with liquid-liquid extraction by adding 0.5 ml distilled water and 4 x 1.5 ml *n*-heptane. The aqueous residual solution was acidified with 0.4 ml 5.8 M HCl. The acid fraction containing fatty acids were extracted by liquid-liquid extraction with 4 x 1.5 ml *n*-heptane after warming to 60°C. After extracts were dried under nitrogen a SPE was carried out, to purify extracts from any other acid fraction such as bile acids. SPE was prepared by adding 0.75 ml silica gel in *n*-heptane into 3 ml glass SPE

columns and preconditioning with 2 x 1.5 ml *n*-heptane. Extracts were redissolved in 100 µl *n*-heptane and transferred to the SPE columns. Columns were washed with 3 x 1 ml *n*-heptane/ethyl acetate (98:2, v/v) and eluates containing fatty acids were collected in glass vials by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (70:30, v/v). Eluates were dried under a gentle stream of nitrogen.

To convert fatty acids to their methyl esters, 0.5 ml toluene and 1 ml acidified methanol (2% $H_2SO_4$) were added to the dried extract and heated at 50°C overnight (~14 h). Fatty acid methyl esters (FAME) were extracted with 1 ml 0.5 M $K_2CO_3$ solution and 3 x 1 ml *n*-heptane by liquid-liquid extraction. Extracts were dried under nitrogen and 100 µl methyl heneicosanoate was added as second internal standard ($C_{21:0}$, IS II, 10 mg $l^{-1}$ in *n*-heptane).

As 10-OH-$C_{18:0}$ was presumably present in most of the soil samples containing decomposition fluids an aliquot was further derivatised by adding 50 µl of BSTFA+TMCS/pyridine (3:1, v/v) to replace the free hydroxyl group at the $C_{10}$ position with a trimethylsilyl group. The aliquot was derivatised for 45 minutes at 90°C on a dry block heater. The solvent was evaporated under a stream of $N_2$ and 100 µl methyl heneicosanoate was added as the second internal standard. The analytical scheme is shown in Fig 2.3.

### *2.3.4. Instrumentation*

Analysis of FAME and silylated FAME was performed on an Agilent 7890B gas chromatograph equipped with a flame ionisation detector (GC/FID, Agilent Technologies, Santa Clara, CA, USA) and a fused-silica capillary column (DB 23, 30 m x 250 µm x 0.25 µm). Helium was used as a carrier gas with constant pressure at 100 kPa. The injection port was maintained at 240°C and a sample volume of 1 µl was injected in splitless mode. The initial temperature was 50°C (2 min isothermal). The temperature rate was set from 7°C $min^{-1}$ to 240°C (10 min isothermal). The detector temperature was 300°C, $H_2$ flow was 30 ml $min^{-1}$, air flow was 400 ml $min^{-1}$ and make up gas (Helium) was set at 25 ml $min^{-1}$.

Further analysis of 10-OH-$C_{18:0}$ was carried out by GC/MS with an Agilent 6890 N gas chromatograph equipped with a 5975 B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). A DB-5ms Ultra Inert (Agilent Technologies, Santa Clara, CA, USA) fused silica capillary column (30 m x 250 µm x 0.25 µm) was used with helium (99.9995%) as the carrier gas at 1.1 ml $min^{-1}$ constant flow. The injection port was maintained at 250°C under 84 kPa and an aliquot of 2 µl was injected in split mode (10:1 split ratio, 12.5 ml $min^{-1}$ split flow). The initial oven temperature was held at 80°C for 2 min, then programmed at 10°C $min^{-1}$ to 290°C and held for 5 min. The solvent delay was 5 min and electron ionisation was set at 70 eV in scan mode.

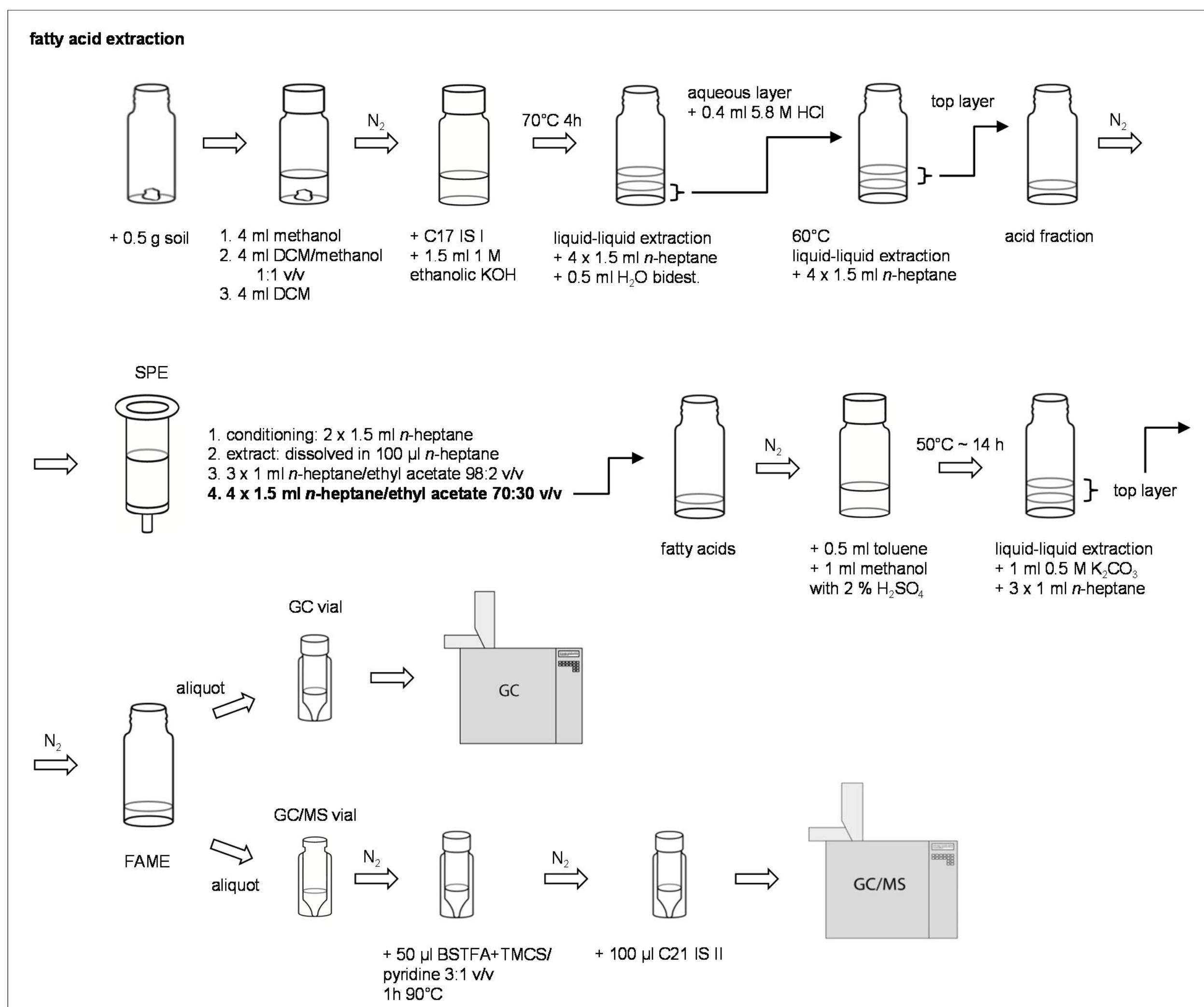


**Figure 2.3:** Analytical scheme of fatty acid extraction from soil.

## *2.3.5. Identification of 10-hydroxystearic acid*

During analysis of fatty acid extracts of soil from the surface decomposition case study (Chapter 3 and 4), a dominant unidentified peak was determined in the chromatogram, which showed obvious peak tailing assuming a free hydroxy group as cause (Fig 2.4a). The sample was rerun on GC/MS and identified as the methylated derivate of 10-OH-$C_{18:0}$ with characteristic molecular fragments at *m/z* 169 and 201 (Fig 2.5). The high mass range *m/z* 264 is dominant and attributed to M-50 (= 314-[32+18]) due to the loss of methanol and water (Takatori 2001; Takatori and Yamaoka 1977). As there was no analytical standard of 10-OH-$C_{18:0}$ available, the mass spectrum (Fig. 2.5) was compared to literature findings where it is characterised as a typical constituent of adipocere and the (Christie 2015; Takatori 2001; Takatori and Yamaoka 1977; Appendix 1, Fig. SI 1.1 and Fig. SI 1.2). The typical shift of trimethylsilyl (TMS) methyl 10-OH-$C_{18:0}$ to the left side of the chromatogram is attributed to the hydroxy group (Fig 2.4b) and a further index of 10-OH-$C_{18:0}$ (Takatori 2001; Takatori and Yamaoka 1977).

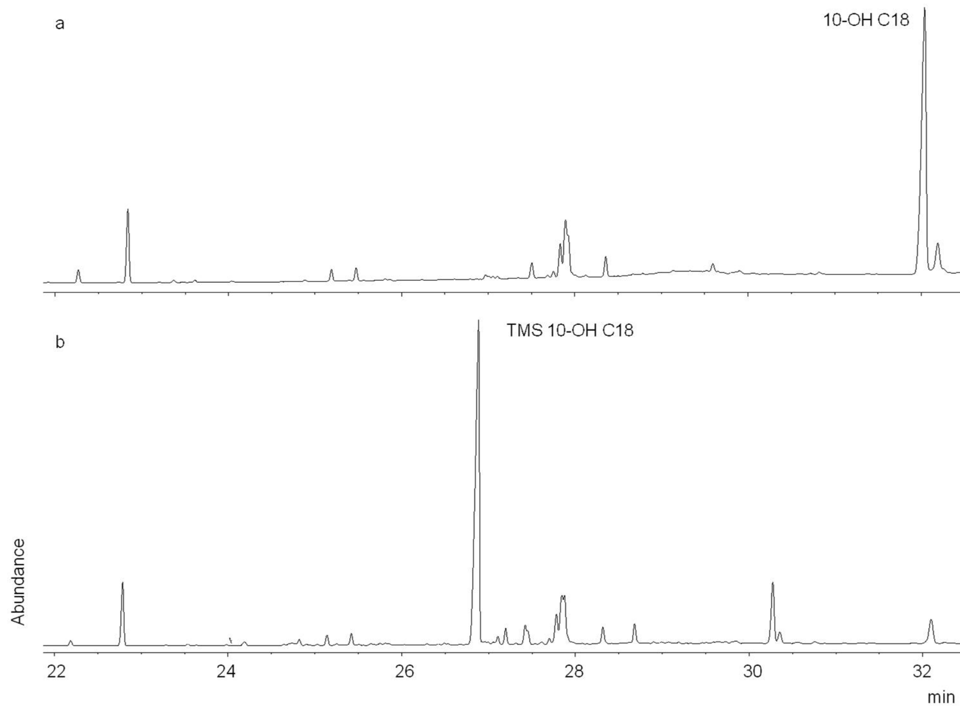


**Figure 2.4:** (a) Chromatogram of methylated fatty acids with methyl-10-hydroxystearate eluting at 32.039 min from the case study soil sample (one year after removal of the body; 1-1.5 cm soil depth). (b) TMS methyl 10-hydroxy stearate eluting at 26.885 min shows the typical shift to the left side of the chromatogram with the additional trimethylsilylation of the free hydroxy group.

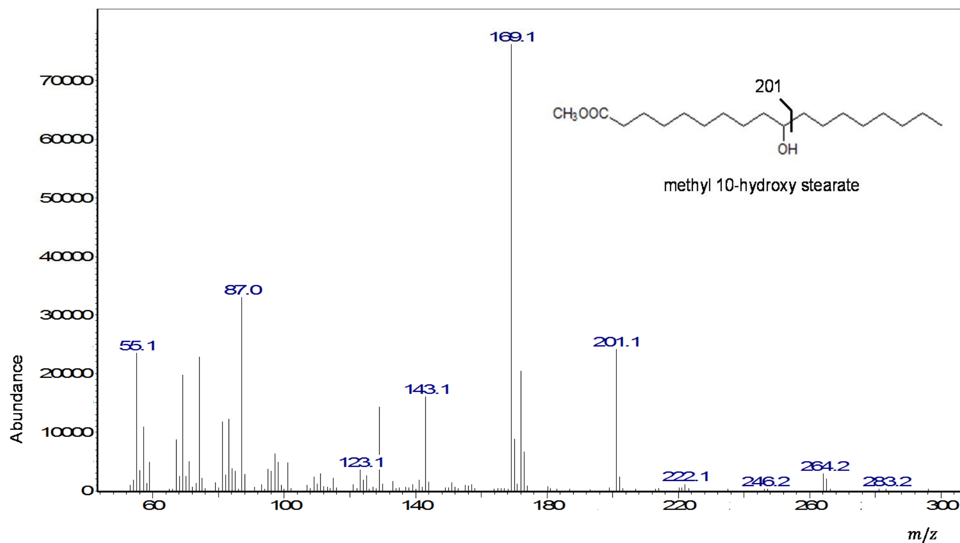


**Figure 2.5:** Mass spectrum of methyl 10-hydroxystearate (retention time 17.08 min) in one of the case study soil samples (one year after removal of the body; 1-1.5 cm soil depth).

### 2.3.6. *Estimation of the concentration of 10-hydroxystearic acid*

As a flame ionisation detector is a mass-sensitive detector, a linear response was expected and therefore internal standardisation was used for the estimation of the concentration of 10-hydroxystearic acid (Grob and Barry 2004). Concentrations of fatty acids with internal standardisation were normally calculated as follows: a mean standard response factor ($SFR_{FA}$) of all analysed external standards of the sample set was determined with known weight-% related to the quantification reference $C_{17:0}$ (equation I), where $SRF_{FA}$ is the standard response factor of the fatty acid, peak $area_{FA}$ is the peak area of the fatty acid, peak $area_{C17-IS}$ is the peak area of the internal standard $C_{17:0}$ and $wt\%_{FA}$ is the weight-% of the fatty acid related to the weight of $C_{17:0}$ in mg in the external standard solution (Dove and Mayes 2006; Grob and Barry 2004). Concentration of fatty acids in the case study soil samples were calculated in respect of the added internal standard $C_{17:0}$ with a known concentration (equation II) where FA (mg $g^{-1}$) is the concentration of the analysed fatty acid in mg $g^{-1}$, $conc_{C17-IS}$ is the concentration of $C_{17:0}$ added at the beginning of the extraction in mg $g^{-1}$, $wt_{C17-IS}$ is the weight of the added $C_{17:0}$ internal standard solution in g and $wt_{soil}$ is the weight of the soil sample in g (Dove and Mayes 2006; Grob and Barry 2004).

(equation I) $$SRF_{FA} = \frac{peak\ area_{FA} / peak\ area_{C17\ IS}}{wt\%_{FA}}$$

(equation II) $$FA\ (mg\ g^{-1}) = \frac{\frac{peak\ area_{FA}}{peak\ area_{C17\ IS}} * conc_{C17\ IS} * wt_{C17\ IS}}{wt_{soil}} \Big/ SRF_{FA}$$

As there was no analytical standard of 10-OH-$C_{18:0}$ available, it was not possible to calculate a SRF for 10-OH-$C_{18:0}$. During laboratory analysis, it was determined that TMS 10-OH-$C_{18:0}$ was more reproducible than estimations of concentrations of methylated 10-OH-$C_{18:0}$, which yielded high deviation between replicates. It was therefore decided to estimate the concentration of 10-OH-$C_{18:0}$ with its TMS derivate. The concentration was estimated by calculating the effective carbon number (ECN) introduced by Sternberg et al. (1962). The ECN is a number, which considers the mass-selectivity of a FID-detector. The FID responses to the number of carbon atoms entering the detector and it decreases in the presence of certain heteroatoms (e.g. O, S, halogens) in functional groups of a molecule (Grob and Barry 2004). With tabulated ECN increments of various types of atoms and groups an ECN of an unknown compound can be calculated (Grob and Barry 2004). According to Scanlon and Willis (1985) the ECN is calculated by using equation III where $MW_{TMS-10-OH-C18}$ is the molecular weight of TMS methyl 10-hydroxystearate (TMS 10-OH-$C_{18:0}$), $ECN_{C17-IS}$ is the calculated ECN of methyl heptadecaonate ($C_{17:0}$ IS I) according to the listed ECN increments shown in Table 2.3, $MW_{C17-IS}$ is the molecular weight of methyl heptadecaonate and $ECN_{TMS-10-OH-C18}$ is the ECN of the TMS 10-OH-$C_{18:0}$.

(equation III) $$SRF_{TMS\ \mathbf{10}-OH\ C\mathbf{18}} = \frac{MW_{TMS\ \mathbf{10}-OH\ C\mathbf{18}} * ECN_{C\mathbf{17}\ IS}}{MW_{C\mathbf{17}\ IS} * ECN_{TMS\ \mathbf{10}-OH\ C\mathbf{18}}}$$

The theoretical $SRF_{TMS-10-OH-C18}$ was between 1.14-1.15 (mean=1.145; Tab. 2.3). To test if the calculated $SRF_{TMS-10-OH-C18}$ is a decent estimate of the real SRF of TMS 10-OH-$C_{18:0}$, the analytical SRF of TMS methyl 12-hydroxystearate (TMS 12-OH-$C_{18:0}$) was determined, as it has the same molecular weight as TMS 10-OH-$C_{18:0}$ and therefore a comparable response on a flame ionisation detector. The analytical $SRF_{TMS-12-OH-C18}$ was 1.11, which is 97% of the theoretical $SRF_{10-OH-C18}$.

**Table 2.3:** Effective carbon number (ECN) increments and their contribution to the ECN of TMS methyl 10-hydroxystearate (TMS 10-OH-$C_{18:0}$); MW = molecular weight g $mol^{-1}$; $C_{17:0}$ IS = methyl heptadecaonate, fatty acid internal standard; HC-O-TMS = trimethylsilyl derivate of the 10-hydroxy group; $ECN_{cal}$ = calculated ECN.

| component | TMS 10-OH-$C_{18:0}$ | $C_{17:0}$ IS |
|---|---|---|
| **formula** | $C_{22}O_3SiH_{46}$ | $C_{18}O_2H_{36}$ |
| **MW** | 402.68374 | 284.47724 |
| **ECN increments** | | |
| aliphatic | 17 x 1.0⁺ | 17 x 1.0⁺ |
| carbonyl | 1 x 0.0⁺ | 1 x 0.0⁺ |
| ether | 1 x -1.0⁺ | 1 x -1.0⁺ |
| HC-O-TMS (alcohol) | 1 x 3.69-3.78* | - |
| **$ECN_{cal}$** | **19.69-19.78** | **16.0** |
| **SRF** | **1.14-1.15** | |

⁺(Grob and Barry 2004); *(Scanlon and Willis 1985)

### 2.3.7. Summary

Using the ECN factor to calculate a theoretical SRF for GC/FID analysis of a compound, where no analytical standard is available worked well for 10-OH-$C_{18:0}$; a compound found in high abundances in soil samples contaminated with body decomposition fluids. It was possible to identify 10-OH-$C_{18:0}$ with its characteristic molecular fragments with a combination of derivatisation methods, generating mass spectrums with GC/MS and comparison with data from the literature and the National Institute of Standards and Technology (NIST) mass spectral library. It was further shown, that 12-OH-$C_{18:0}$ with the same molecular weight and ECN factor showed a comparable response to the theoretical SRF of 10-OH-$C_{18:0}$. This method was used for 10-OH-$C_{18:0}$ concentration estimations to fit the analytical challenge in Chapter 4, where unexpected high abundances of 10-OH-$C_{18:0}$ were found in soil samples from an aboveground body decomposition case study.

## 2.4. References

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# Chapter 3

## Vertical and temporal distribution of steroids from human decomposition fluids in soils – a case study

# Abstract

In forensic pedology, steroids can be used as long-term markers of human decomposition fluids in soils as they are detectable for several years hence. The objective of this study was to investigate if steroids are detectable in soil beneath a decomposing human body which was only temporarily deposited and removed before the degradation of the soft tissues had been completed. The distribution of steroids was analysed at two time periods: 11-18 days after deposition and one year (358 days) after removal of the body. A range of tissue and faecal steroids were extracted from soil samples and analysed by gas chromatography/mass spectrometry (GC/MS). After 11-18 days, high concentrations of steroids were found close to the soil surface. Faecal steroids were dominant beneath the abdominal region of the body, while a mixture of tissue and faecal steroids were found beneath the thorax. After one year, steroids were still detected, but initial transformation and degradation processes were observed. In conclusion, even after a short period of deposition, steroids were indicative of human decomposition fluids in soil and were vertically distributed and present up to one year after the removal of the body. This study provides a valuable insight into steroid depth distributions and changes of the human derived steroidal fingerprint in soils over time.

## 3.1. Introduction

During the decay of a human body, decomposition fluids containing organic degradation products (e.g. adipose tissue constituents, organic acids, organic nitrogen; Vass et al. 1992) of the soft tissues leach into the surrounding environment, mainly during the bloat and active stage of decomposition (Carter et al. 2007). With advancing decomposition, a cadaver decomposition island (CDI) develops, observable as a dark stain surrounding the body (Carter et al. 2007). The diversity of the nutrient rich fluids stimulate the soil microbial activity, hence a CDI represents an ecosystem of enhanced biogeochemical cycling (Cobaugh et al. 2015; Carter et al. 2007).

It has been recently demonstrated that a range of tissue and faecal steroids are present in soil surrounding a decomposing carcass (von der Lühe et al. 2013; Davies and Pollard 1988). In an experimental approach, von der Lühe et al. (2013) detected decomposition fluids from pig carcasses in soil samples. They found increased cholesterol, coprostanol and β-sitosterol contents beneath the carcasses three months post-mortem compared to reference soil. In an Anglo-Saxon grave (c. 400-700 AD), Davies and Pollard (1988) detected three fold increases in cholesterol levels in soil beneath a skeleton compared to a reference sample. These studies demonstrated that once steroids are introduced into soil they are indicative of decomposition fluids over short e.g. 3 months (von der Lühe et al. 2013) and long periods of time e.g. 1300-1700 years (Davies and Pollard 1988). So far, there have been few investigations conducted in relation to forensic pedology, but faecal steroids have been widely used in archaeological investigations (Birk et al. 2011; Shillito et al. 2011; Bull et al. 2001; Simpson et al. 1998; Bethell et al. 1994). Knowledge about the presence of cadaveric lipids in soils is applicable in forensic investigations, e.g. to identify temporary graves. In temporary graves, body decomposition is often incomplete, it is hence unknown if cadaveric steroids are present after a short period (≤ 3 months) of decomposition.

Archaeological studies showed that steroids are indicative of their original biogenic source and retain their information for a certain period of time depending on the physicochemical conditions of the depositional environment (Peters and Moldowan 1993). Whereas cholesterol is the main $\Delta^5$-sterol in eukaryotic cells, β-sitosterol is the main $\Delta^5$-sterol in plants (Christie and Han 2010; Mouritsen and Zuckermann 2004). Cholesterol and β-sitosterol are microbially reduced in the intestinal tract of the majority of higher animals to the respective 5β-stanols coprostanol and 5β-stigmastanol (Hatcher and McGillivary 1979; Murtaugh and Bunch 1967), with coprostanol being the major 5β-stanol found in human faeces (Bull et al. 2002; Leeming et al. 1994). Once introduced into the environment, coprostanol and 5β-stigmastanol undergo further microbial conversion to epicoprostanol and epi-5β-stigmastanol (McCalley et al. 1981; Quirk et al. 1980; Wardroper and Maxwell 1978), whereas cholesterol and β-sitosterol are mainly transformed to 5α-cholestanol and 5α-stigmastanol (Bull et al. 2002). Cholesterol, 5α-cholestanol, epicoprostanol, 5β-stigmastanol, 5α-stigmastanol and β-sitosterol are also present in human faeces (Prost et al. (in prep, a); Leeming et al. 1996). Therefore, several tissue (cholesterol, 5α-cholestanol) and faecal as well as faecal derived (cholesterol, 5α-cholestanol, coprostanol, epicoprostanol, 5β-stigmastanol, β-sitosterol and 5α-stigmastanol) steroids can be potential identifiers of human decomposition products in soils.

In the past steroids were rarely used as indicators for human decomposition fluids in a forensic context. They provide a high potential as indicators of human decomposition fluids, due to their known long-term presence and their source specificity. The preservation of cadaver derived steroids in soil samples might help in identifying temporary graves or to locate burials with minimal destruction of a crime scene. Furthermore, the high specificity of $\Delta^5$-sterols and their associated stanols might aid the identification of decomposition fluids of human origin in soil samples. Their presence beneath decomposing bodies and pig carcasses was demonstrated in earlier studies (von der Lühe et al. 2013; Shillito et al. 2011; Davies and Pollard 1988), but the soil associated preservation and change in patterns of body derived steroids is largely unknown.

This study was conducted to investigate the presence of $\Delta^5$-sterols and stanols from human decomposition fluids, their temporal changes and their distribution in soil over two time periods. Soil samples beneath a human body decomposing for a short period (11-18 days) were analysed for the presence of $\Delta^5$-sterols as well as 5β- and 5α-stanols. After removal of the body and a period of one year, another soil sample was taken from the CDI in order to determine the influence of soil processes on human derived steroids over time. We hypothesised that (I) steroids are released from the decaying body and are therefore present in high amounts in the soil 11-18 days after deposition with the largest accumulation close to the soil surface compared to reference soil receiving no impact of a decaying human body; (II) steroid composition pattern differs dependent on the release of the decomposition fluids, i.e. enhanced amounts of faecal steroids are assessed beneath the abdomen; (III) steroids are detectable in accelerated amounts in CDI samples compared to reference soil one year after the body had been removed.

## 3.2. Material and Methods

### 3.2.1. *Case study*

Soil samples were taken from a forensic case study site where a female body was placed in a thicket of bushes and trees in a suburban area. The naked body was lying on its back on the soil surface with parts of the shoulder and the head areas burned with scavenging observed. The stage of decomposition was advanced and soft tissues except the skin were liquefied and settled by insect larvae. The time since deposition was estimated between 11 and 18 days by the development stages of insect larvae (*Phormia regina*). The body was found during summer time; temperatures between the deposition (18 days) and the recovery were reconstructed with measured data from a weather station 10 km away from the crime scene and ranged between 13-23°C. Precipitation at the weather station was 20 mm during the period of 18 days.

### 3.2.2. *Soil type*

In immediate vicinity of the crime scene (10 m away) a soil profile was dug in order to characterise local soil properties. Disturbed topsoil samples (≤ 20 cm) were taken for chemical analysis (Tab. 3.1). Undisturbed soil samples were taken with soil cores (100 $cm^3$) to estimate aeration and leaching conditions of the soil found at the crime scene (Tab. 3.1). The soil type at the study site was classified as Fluvic Cambisol Prototechnic (Schad 2008) and its chemical and physical characteristics are summarised in Table 3.1. The soil contained several artefacts such as ceramic and brick fragments (≥ 5%, ≤ 100 cm) indicating a former anthropogenic impact. The topsoil of the Cambisol showed a homogenous sandy texture (up to 80% sand) and a neutral pH at ~7 (Tab. 3.1). Due to the sandy texture, the Cambisol has a high air capacity (34-38 vol.%), which dominates the total pore volume (Tab. 3.1). Consequently the topsoil of the Cambisol, characteristic of soil at the crime scene, is well aerated and is freely draining (Tab. 3.1).

**Table 3.1:** Soil chemical and physical parameters (mean of n=2 laboratory replicates ± standard error) of the Cambisol topsoil from the study site.

| depth | sand[a] | silt[a] | clay[a] | $CaCO_3$[b] | pH[c] | TOC[d] | BD[e] | AC[f] | $PV_t$[g] |
|---|---|---|---|---|---|---|---|---|---|
| cm | % | | | % | | g kg$^{-1}$ | g cm$^{-3}$ | vol.% | |
| -6 | 80±0.1 | 9±0.1 | 11±0 | n.d. | 6.8 | 15 | 1.2 | 34.4 | 53.1 |
| -10 | 79±0.1 | 9±0.1 | 12±0.2 | 0.6±0 | 7.0 | 12 | 1.3 | 36.5 | 52.4 |
| -14 | 80±0.1 | 9±0 | 11±0.1 | 0.6±0 | 7.1 | 11 | 1.3 | 38.0 | 52.5 |
| -20 | 80±0.1 | 9±0 | 11±0.1 | 1.0±0 | 7.1 | 9 | 1.4 | 34.6 | 49.0 |

n.d. = not detected │ [a]sand 2000-63 µm; silt 63-2 µm; clay ≤2 µm (DIN ISO 11277) │[b]carbonate content (Norm DIN ISO 10693) │[c]pH in $CaCl_2$ (DIN ISO 10390) │ [d]total organic carbon analysed by dry combustion (Vario EL Cube, Elementar, Hanau, Germany) after destruction of carbonates by acid fumigation (Harris et al. 2001) │ [e]bulk density (DIN ISO 11272) │[f]air capacity = coarse pore volume, equivalent pore size > 50 µm; [g]total pore volume (DIN ISO 11274)

### *3.2.3. Soil samples*

After discovery, the body was removed and soil blocks (~20 cm length x 20 cm width) were taken with a clean spade directly beneath the body ($B_T$ = beneath thorax with 9 cm depth, $B_A$ = beneath abdomen with 13.5 cm depth) and one reference block ($B_{Ref}$ = reference block with 8.5 cm depth) was sampled 10 m apart from the CDI. Approximately one year (358 days) after the body removal another block ($B_{1ya}$) was taken from the CDI beneath the thorax. Organic litter and the first 5 mm soil were removed from the block surfaces to level the block. Afterwards, soil blocks were separated into 5 mm-interval samples. Soil samples were freeze-dried for 24 h, sieved (≤ 2 mm) and finely ground in a ball mill (450 rpm, 5 min). Samples were stored in a desiccator at room temperature until analysis.

### *3.2.4. Total organic carbon analysis of block samples*

Total organic carbon (TOC) was analysed on block samples by dry combustion (Vario EL Cube; Elementar, Hanau, Germany) after destruction of carbonates by acid fumigation (Harris et al. 2011).

### *3.2.5. Laboratory procedure*

To avoid contamination, reusable glass and plastic materials was rinsed five times with deionised water. All disposable glass and reusable glassware was heated at 450°C for 12 h in a muffle furnace. The solvents were purchased from Carl Roth (Karlsruhe, Germany) and were at least GC grade. The silica gel (60 Å, 0.063-0.200 mm particle size; Merck, Darmstadt, Germany) for solid phase extraction (SPE) was heated at 380°C for 2 h to remove any organic contaminants and activated at 200°C for 5 h and subsequently stored in *n*-heptane until use (Isobe et al. 2002). The derivatisation mixture containing *N,O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS, 99:1, v/v) was obtained from Sigma Aldrich (Steinheim, Germany) and pyridine from Merck (Darmstadt, Germany). Nitrogen ($N_2$) was purchased in a purity of 99.998% (Westfalen, Minster, Germany).

Steroids analysed in this study are listed with trivial names in Table 3.2. Extraction of the total lipid extract (TLE) was carried out after a method previously published by Isobe et al. (Isobe et al. 2002). A method used on soil samples, which was adapted from Dove and Mayes (2006) was modified for further sample purification (von der Lühe et al. 2013).

Duplicate soil samples (0.5 g) were ultrasonically extracted for 30 min with 4 ml each of methanol, dichloromethane/methanol (1:1, v/v) and dichloromethane, consecutively. After sonication, the suspension was centrifuged (3000 rpm, 6 min) and the supernatant was transferred to a 4 ml vial and dried under a gentle stream of $N_2$. Cholesterol-25,26,26,26,27,27,27-d7 (cholesterol-d7) was added (100 µl, 10 mg $l^{-1}$) as recovery internal standard (IS I; Tab. 3.2). The TLE was saponified with 1.5 ml of 1 M ethanolic KOH at 70°C for 4 h. The neutral lipids were obtained by a repeated liquid-liquid extraction with 4 x 1.5 ml *n*-heptane and 0.5 ml distilled water. The collected extracts were dried under $N_2$. A solid phase extraction (SPE) was carried out with 0.75 ml silica gel (1 cm bed volume) in 3 ml SPE glass columns. Columns were conditioned with 2 x 1.5 ml *n*-heptane. Hydrocarbons and other short-chain

**Table 3.2:** Tissue and faecal steroids used to characterise steroids from a human cadaver decomposition island (CDI); names in bold are used in the text. Steroid standards used for standard addition method test for soil found at the crime scene and for external calibration. Results of the standard addition experiment; recovery (%) and regression coefficients ($R^2$) of the linear regression analysis, limit of detection (LOD; signal to noise ratio (S/N) = 3) and limit of quantification (LOQ; S/N = 10) of added $\Delta^5$-sterols, 5 β-stanols and 5 α-stanols to the total lipid extract (TLE) of reference block samples.

| substance (trivial name) | biomarker group | RT[d] | ion fragments | standard added | recovery | $R^2$ | LOD | LOQ |
|---|---|---|---|---|---|---|---|---|
| | | min | *m/z* | ng | % | | ng $g_{soil}^{-1}$ | ng $g_{soil}^{-1}$ |
| **5α-cholestane**[a] | IS II | 22.514 | 217; 357; 372 | | | | | |
| 5β-cholestan-3β-ol[a] (**coprostanol**) | 5β-stanol | 26.139 | 215; 257; 370 | 0-100-500-1000-2000 | 87 | 0.99 | 0.2 | 0.7 |
| 5β-cholestan-3α-ol[a] (**epicoprostanol**) | 5β-stanol | 26.700 | 215; 355; 370 | 0-50-100-500 | 97 | 0.99 | 0.2 | 0.8 |
| **cholesterol-d7**[b] | IS I | 27.999 | 336; 375; 465 | 0-500-1000-1500-2000 | 82 | 0.99 | n/a | n/a |
| cholest-5-en-3β-ol[a] (**cholesterol**) | $\Delta^5$-sterol | 28.165 | 329; 368; 458 | 0-500-1000-2000 | 78 | 0.98 | 0.4 | 1.4 |
| 5α-cholestan-3β-ol[a] (**5α-cholestanol**) | 5α-stanol | 28.445 | 215; 445; 460 | 0-100-500 | 84 | 0.99 | 0.5 | 1.7 |
| 5β-stigmastan-3β-ol[c] (**5β-stigmastanol**) | 5β-stanol | 30.705 | 215; 383; 398 | 0-50-100-500-1000 | 83 | 0.99 | 0.6 | 2.0 |
| 5β-stigmastan-3α-ol[c] (**epi-5β-stigmastanol**) | 5β-stanol | 31.386 | 215; 383; 398 | 0-100-200-500 | 90 | 0.99 | 0.4 | 1.3 |
| stigmast-5-en-3β-ol[a] (**β-sitosterol**) | $\Delta^5$-sterol | 33.349 | 357; 396; 486 | 0-1000-1500-2000 | 84 | 0.94 | 0.9 | 2.8 |
| 5α-stigmastan-3β-ol[a] (**5α-stigmastanol**) | 5α-stanol | 33.721 | 215; 473; 488 | 0-50-100-500 | 85 | 0.99 | 1.0 | 3.5 |

Purchased at [a]Sigma Aldrich (Steinheim, Germany) │ [b]Avanti (Alabaster, AL, USA) │ [c]Chiron (Trondheim, Norway) │ [d]retention time

compounds were washed to waste by adding 2 x 1.5 ml *n*-heptane. Ketones and aldehydes were eluted to waste with 2 x 0.5 ml *n*-heptane/ethyl acetate (97:3, v/v). Steroids and alcohols were collected in glass vials by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (80:20, v/v) and eluates were dried under $N_2$. The steroid extracts were silylated with a mixture containing BSTFA+TMCS/pyridine (3:1, v/v; 50 µl) at 90°C for 1 h. After derivatisation, the mixture was evaporated and 100 µl of 5α-cholestane (5 mg $l^{-1}$ in toluene, IS II; Tab. 3.2) was added.

### *3.2.6. GC/MS analysis and quantification*

Analysis was performed using an Agilent 6890 gas chromatograph coupled to a 5975B mass spectrometer (GC/MS; Agilent Technologies, Santa Clara, CA, USA). A DB-5ms Ultra Inert fused silica capillary column (30 m length x 250 µm internal diameter x 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used with helium (99.9995%) as carrier gas at 1.1 ml $min^{-1}$ constant flow. The injection port was maintained at 250°C and 1 µl was injected in splitless mode. The initial oven temperature was held at 80°C for 1.5 min, then programmed at 12°C $min^{-1}$ to 265°C, at 0.8°C $min^{-1}$ to 280°C and at 10°C $min^{-1}$ to 300°C, and held for 12 min. The solvent delay was 20 min and electron ionisation was set at 70 eV. For quantification characteristic ions (Tab. 3.2) for each analyte were selected, and samples were run in selected ion monitoring mode (SIM). Quantification was carried out with the analyte/IS II ratios and the corresponding ratios of the external calibration. Concentrations in µg $g_{freezed\ dried\ soil}^{-1}$ (in all following sections µg $g_{soil}^{-1}$) were corrected by the recovery of IS I and summarised with standard error of duplicate analysis. Recovery surrogates of IS I of all analysed block samples averaged at 93±15%. A typical chromatogram of the steroid analysis by GC/MS is shown in Figure 3.1.

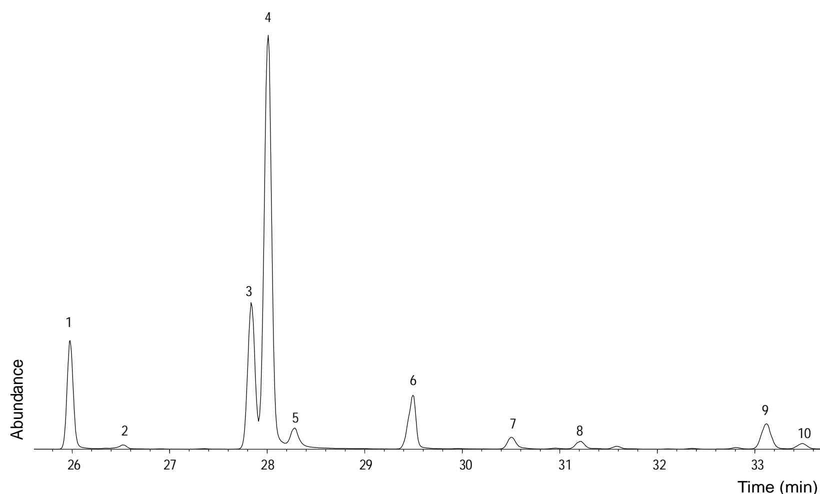



**Figure 3.1:** Partial chromatogram of the steroid extract from the soil beneath the corpse one year after its removal ($B_{1ya}$; 4.5-5 cm soil depth). 1 = coprostanol, 2 = epicoprostanol, 3 = cholesterol-d7, 4 = cholesterol, 5 = 5α-cholestanol, 6 = unidentified, 7 = 5β-stigmastanol, 8 = epi-5β-stigmastanol, 9 = β-sitosterol, 10 = 5α-stigmastanol.

### *3.2.7. Method test*

The steroid purification method was tested by standard addition on soil TLEs from one reference soil sample (0-0.5 cm depth). Recovery rates of analytes ranged between 78-97% and limits of detection varied (LOD; S/N=3) between 0.2 ng $g_{soil}^{-1}$ and 1.0 ng $g_{soil}^{-1}$ (Tab. 3.2; detailed description in Section 2.2.).

## 3.3. Results

Directly after the removal of the body (11-18 days) TOC and steroid concentrations (in µg $kg_{soil}^{-1}$; data normalised to TOC found in Appendix 2, Figure SI 2.1-2.4) were higher in soil beneath the body to a depth of 9-13.5 cm compared to TOC and steroid concentrations in reference samples (Fig. 3.2). The largest TOC and steroid concentrations were found beneath the thorax ($B_T$; Fig 3.3-Fig. 3.6). Various tissue (cholesterol, 5α-cholestanol) and faeces derived (coprostanol, epicoprostanol, 5β-stigmastanol) steroids were found in soil beneath both body positions (Fig 3.3-Fig. 3.6). Cholesterol was the most abundant steroid in $B_T$, comprising 650 times higher contents in 0.5-1 cm soil depth compared to the reference soil (Fig. 3.3). The plant $\Delta^5$-sterol β-sitosterol was slightly increased in some depth samples beneath the body compared to the reference, but most of β-sitosterol found beneath the body was in the same order as the reference (Fig. 3.6). Steroids which were found in traces beneath the thorax (epicoprostanol, epi-5β-stigmastanol and 5α-stigmastanol) and the abdomen (5α-cholestanol, epicoprostanol, epi-5β-stigmastanol and 5α-stigmastanol) did not differ to the reference when related to TOC contents (Appendix 2, Figure SI 2.1-2.4).

Soil blocks taken beneath the thorax ($B_T$) and the abdomen ($B_A$) differed in steroid concentrations and distributions. Beneath the thorax, increased tissue steroids were found and faecal steroids were comparably lower in abundance (Fig 3.3-Fig. 3.6). In contrast, beneath the abdomen the faecal stanols coprostanol (Fig. 3.4) and 5β-stigmastanol (Fig. 3.5) predominated the tissue steroids cholesterol and 5α-cholestanol (Fig. 3.3).

A sharp decrease of steroids was observed in the vertical steroid distribution beneath the thorax (11-18 days), where most of the steroids accumulated predominantly ≤ 2 cm (Fig. 3.3-Fig. 3.6). However, β-sitosterol showed an increase ≥ 2 cm in concentrations beneath the thorax (Fig. 3.6). In contrast, steroids in soil under the abdomen were more distributed into lower depths, but likewise to the thorax, highest amounts of steroids in $B_A$ were detected close to the soil surface (Fig. 3.3-Fig. 3.6).

One year after the removal of the body, steroids were still detectable in higher concentrations to a depth of 17 cm compared to the reference soil. 5α-cholestanol (Fig. 3.3) and 5α-stigmastanol (Fig. 3.6) had the largest contents of all steroids in a soil depth ≤ 6 cm, whereas cholesterol was the dominant steroid in larger depths (Fig. 3.3). The patterns of epicoprostanol, 5α-cholestanol, 5β-stigmastanol and 5α-stigmastanol (Fig 3.3-Fig. 3.6) displayed a minor increase of steroids in a soil depth between 11.5 and 14 cm. During block preparation it was visually observed, that these depths (≥ 6 cm) were darker in soil colour and higher in moisture. Epicoprostanol, epi-5β-stigmastanol and β-sitosterol concentrations only differed to the reference when related to soil (µg $g_{soil}^{-1}$), but not to TOC contents (Appendix 2, Figure SI 2.2-2.4).

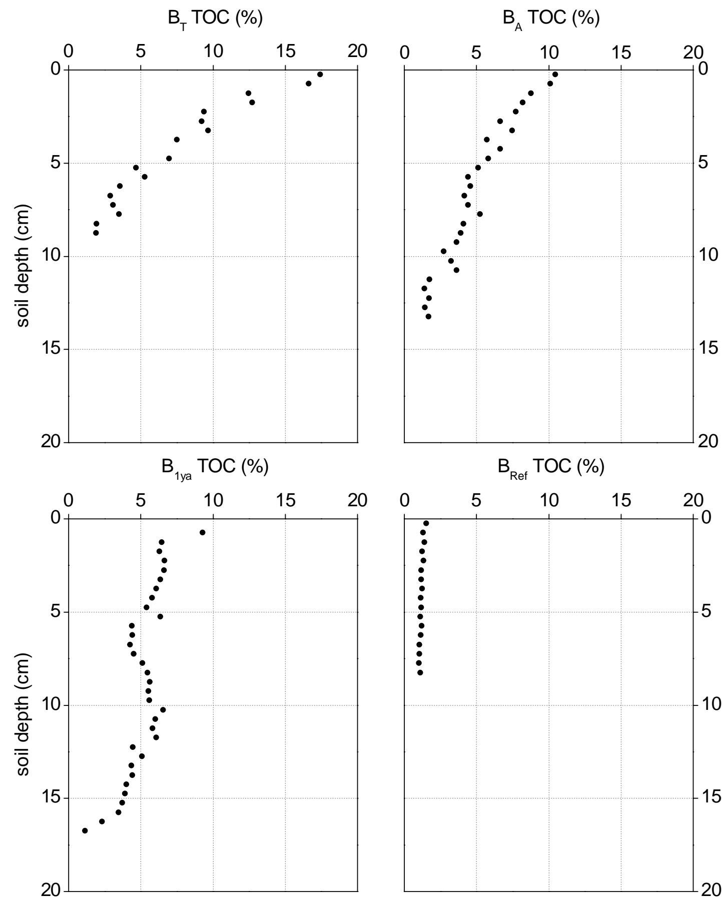



**Figure 3.2:** Depth distribution of total organic carbon (TOC) in soil beneath a cadaver decomposition island (CDI) where a human body was decomposing for 11-18 days; $B_T$ = soil beneath thorax; $B_A$ = soil beneath abdomen; $B_{1ya}$ = soil taken 358 days after body removal beneath thorax; $B_{Ref}$ = reference soil (distance to the body 10 m).

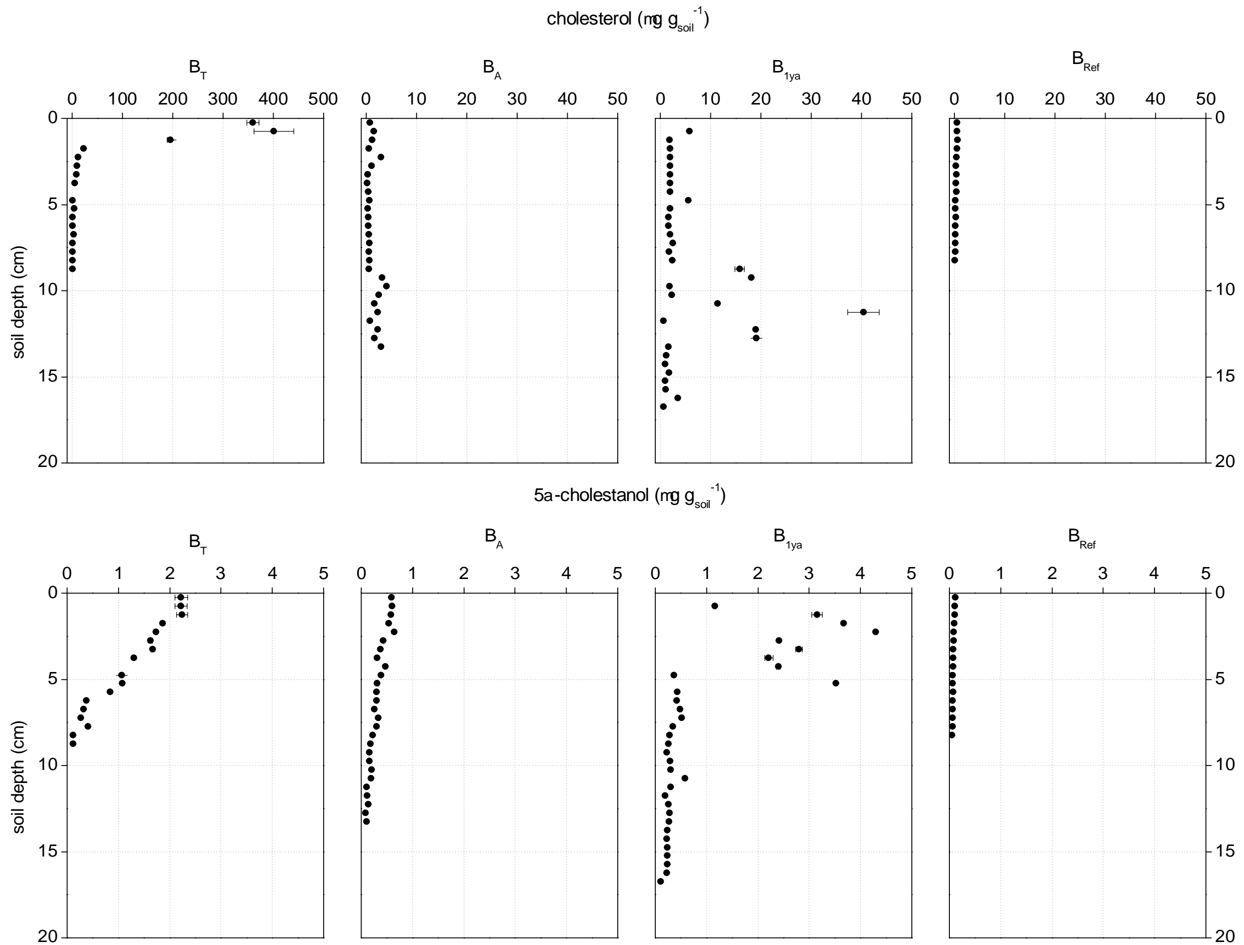



**Figure 3.3:** Depth distributions of cholesterol and 5α-cholestanol (µg $g_{soil}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

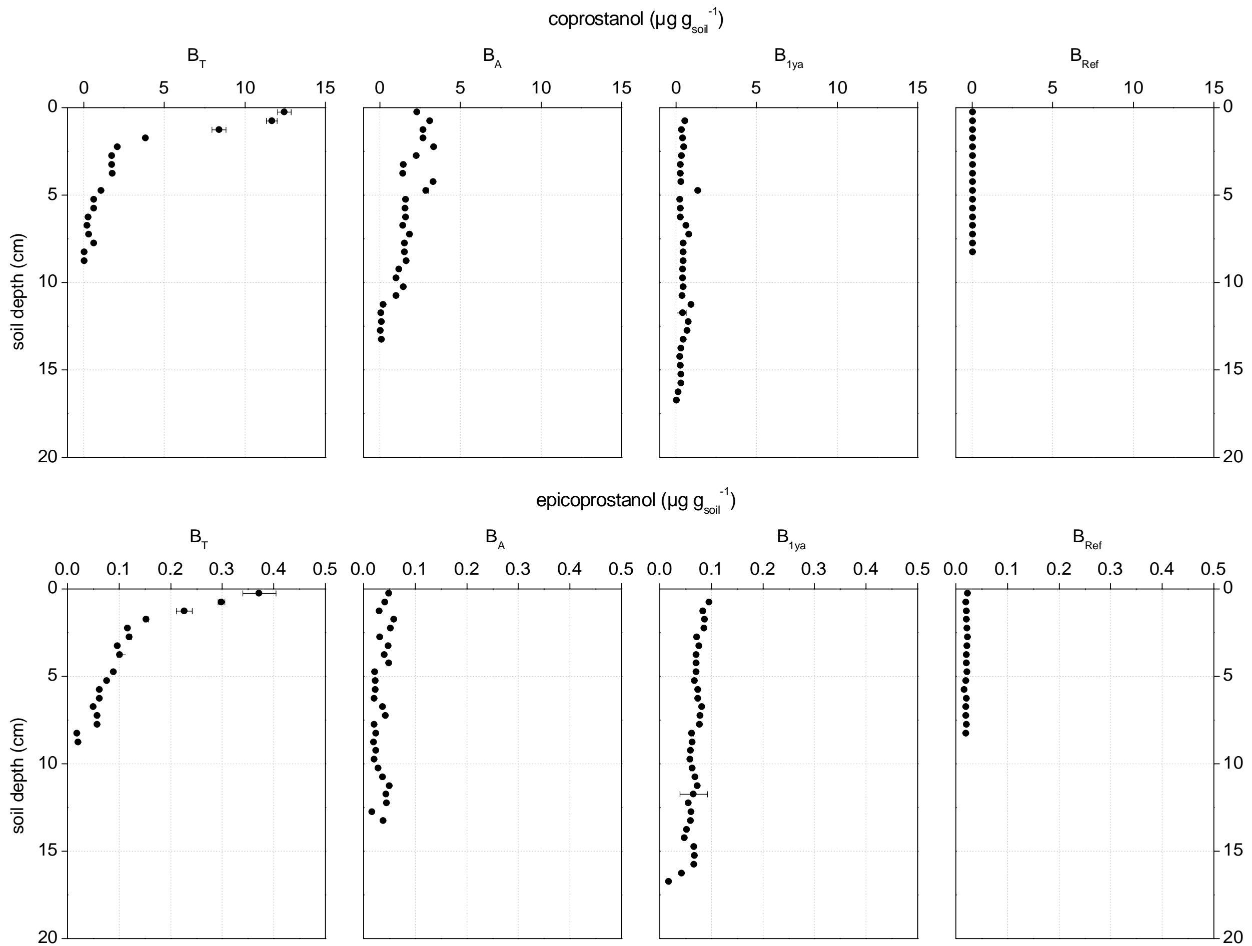



**Figure 3.4:** Depth distributions of coprostanol and epicoprostanol (µg $g_{soil}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

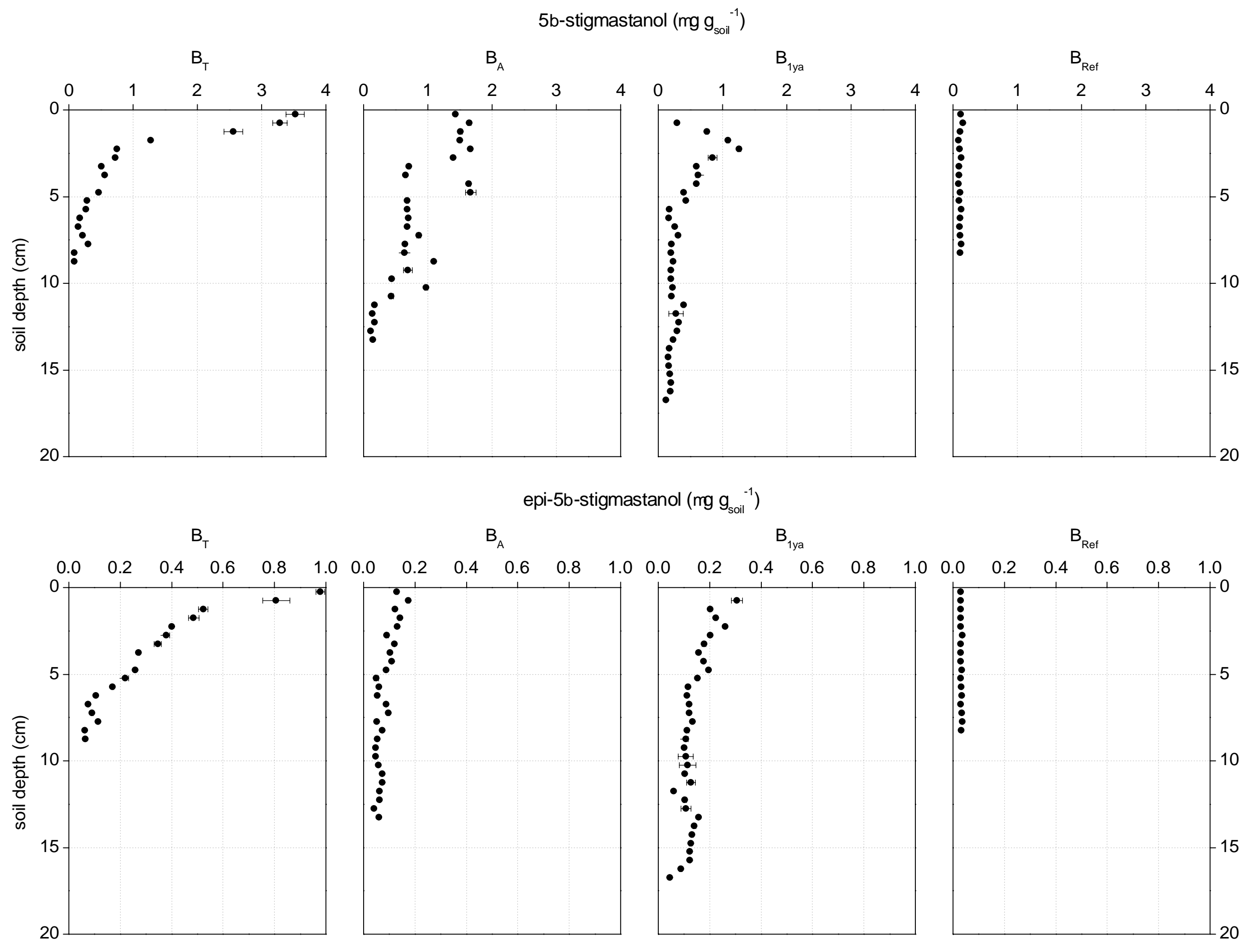



**Figure 3.5:** Depth distributions of 5β-stigmastanol and epi-5β-stigmastanol (µg $g_{soil}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

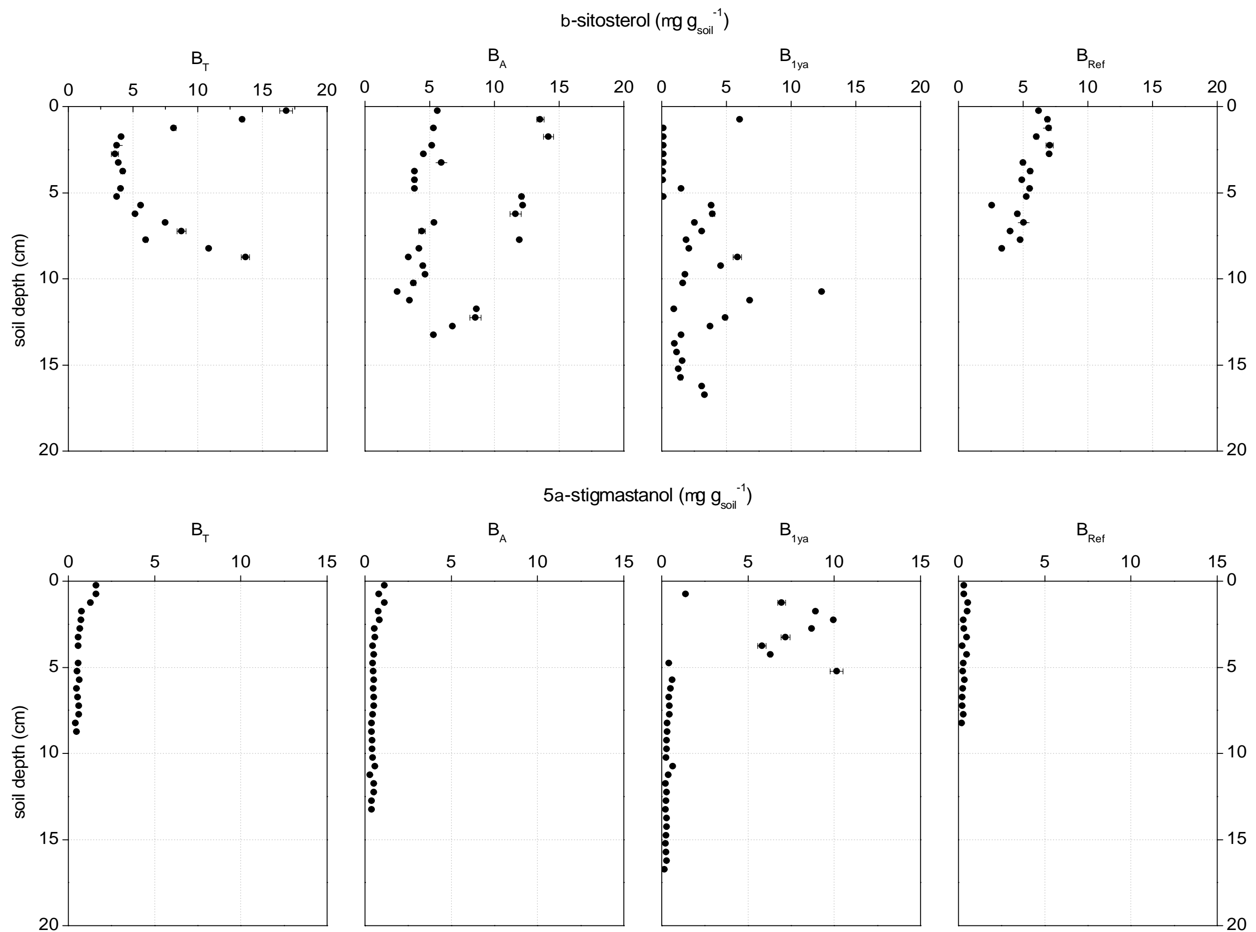


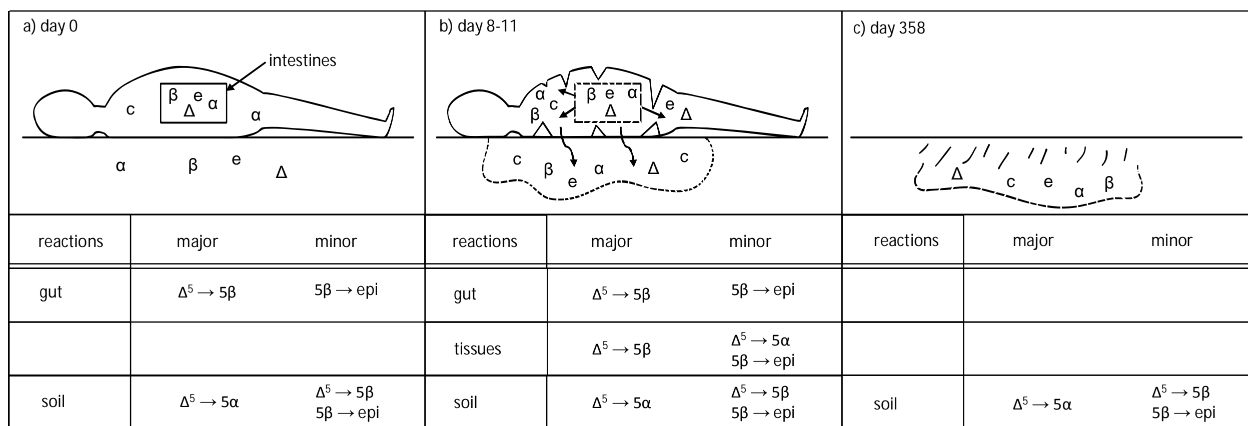

**Figure 3.6:** Depth distributions of β-sitosterol and 5α-stigmastanol (µg $g_{soil}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

## 3.4. Discussion

Directly after the removal of a body that had been decomposing for 11-18 days, the presence of decomposition fluids in soil was indicated by enhanced TOC and steroid contents (Fig. 3.2-Fig. 3.6). Previous studies were able to detect cadaver derived organic decomposition products (e.g. fatty acids, volatile organic compounds, amino acids) which leached into soil beneath animal and human carcasses (Swann et al. 2010; Vass et al. 2004; Vass et al. 2002). In this study, various faecal and faecal derived (cholesterol, 5α-cholestanol, coprostanol, epicoprostanol, 5β-stigmastanol, β-sitosterol, 5α-stigmastanol) and tissue steroids (cholesterol, 5α-cholestanol) were found in the soil beneath the body and a variety of different transformation and translocation processes are attributed to their presence.

Cholesterol was the most abundant steroid and is therefore a viable indicator of decomposition fluids in soils. Increased cholesterol contents were also found beneath pig carcasses three months after burial as well as in an Anglo-Saxon grave compared to reference soil (von der Lühe et al. 2013; Davies and Pollard 1988; Newman and Parkin 1986). 5α-cholestanol is also present in soft tissues and faeces (Leeming et al. 1996; Hatcher and McGillivary 1979; Murtaugh and Bunch 1967). Higher abundances of 5α-cholestanol in soil samples beneath the thorax and abdomen are therefore attributed to derive from the decomposition of the bodies soft tissues and from faecal material.

Coprostanol is the dominant steroid in human faeces (Leeming et al. 1996; Hatcher and McGillivary 1979; Murtaugh and Bunch 1967) and its presence in $B_A$ and in $B_T$ can be explained by the input of faecal material deriving from the gut content of the decomposing body. All the other steroids (except epi-5β-stigmastanol) analysed in this study are also present in human faeces, their increased concentrations in $B_T$ and $B_A$ compared to concentrations found in the reference block ($B_{Ref}$), can be explained similarly (Leeming et al. 1996; Hatcher and McGillivary 1979; Murtaugh and Bunch 1967). Faecal and tissue steroids were found in all soil blocks beneath the decaying body (Fig. 3.7). They have been likely mixed with tissue steroids during the decomposition process where microorganisms migrate from the intestines into the surrounding tissues and further mediate the breakdown of soft tissues (Carter and Tibbett 2008; Evans 1963). Tissues soften during the decomposition process, which results in a liquefied mass mixed with contents from the intestines (Evans 1963) and a leachate containing both tissue and faecal steroids was likely introduced into soil beneath. However, coprostanol and 5β-stigmastanol do not exclusively derive from faeces, but can also be produced during degradation and transformation processes in the corpse (Fig. 3.7). The translocation and transformation of steroids inside human bodies has been previously discussed in an archaeological context in mummified tissues and bog bodies (Evershed and Connolly 1994; Gülaçar et al. 1990). Evershed and Connolly (1994) characterised the steroidal content of tissues from a bog body and found coprostanol in skin tissues assuming that it was formed by intestinal microorganisms migrating into the skin after death. Gülaçar et al. (1990) detected coprostanol and traces of 5α-cholestanol in thorax concretions in a 4000-year-old Nubian mummy and assumed that intestinal contents were displaced in the thorax.




| reactions | major | minor | reactions | major | minor | reactions | major | minor |
|---|---|---|---|---|---|---|---|---|
| gut | $\Delta^5 \rightarrow 5\beta$ | $5\beta \rightarrow$ epi | gut | $\Delta^5 \rightarrow 5\beta$ | $5\beta \rightarrow$ epi | | | |
| | | | tissues | $\Delta^5 \rightarrow 5\beta$ | $\Delta^5 \rightarrow 5\alpha$<br>$5\beta \rightarrow$ epi | | | |
| soil | $\Delta^5 \rightarrow 5\alpha$ | $\Delta^5 \rightarrow 5\beta$<br>$5\beta \rightarrow$ epi | soil | $\Delta^5 \rightarrow 5\alpha$ | $\Delta^5 \rightarrow 5\beta$<br>$5\beta \rightarrow$ epi | soil | $\Delta^5 \rightarrow 5\alpha$ | $\Delta^5 \rightarrow 5\beta$<br>$5\beta \rightarrow$ epi |

$\Delta$, $\Delta^5$ = $\Delta^5$-sterols | $\beta$, $5\beta$ = $5\beta$-stanols | $\alpha$, $5\alpha$ = $5\alpha$-stanols | c = cholesterol | e, epi = epi-5β-stanols

Figure 3.7: Possible transformation processes of steroids from an aboveground body decomposition study at (a) day zero, deposition of the body to an outdoor environment, (b) 11-18 days after deposition and (c) 358 days after removal of the body.

Further processes commence as human associated microbes are introduced into the soil beneath the body and presumably continue to convert tissue and faecal steroids. Cobaugh et al. (2015) observed the migration of human associated bacteria in soil beneath decomposing human bodies during the active and advanced stage of decomposition. Abundances of human associated bacteria declined below the detection limit after the remains of the cadavers were removed (Cobaugh et al. 2015). Therefore, it is assumed that in this study certain intestinal microbial taxa responsible for the formation of $\Delta^5$-sterols to the corresponding 5β-stanols survived in the soil beneath the body during the presence of the body and continued to form 5β-stanols.

Cholesterol, 5α-cholestanol, coprostanol and 5β-stigmastanol clearly dominated the steroid patterns after 11-18 days. Only traces of epicoprostanol, epi-5β-stigmastanol, 5α-stigmastanol were found beneath the thorax and 5α-cholestanol, epicoprostanol, epi-5β-stigmastanol and 5α-stigmastanol beneath the abdomen. Differences between CDI and reference samples related to TOC were not apparent, which suggest that other organic decomposition products dominated the total TOC contents beneath the body.

Soil blocks taken beneath the thorax and the abdomen differed mainly in their total contents. Other than expected largest concentrations of faecal and tissue steroids were found beneath the thorax. Concentrations of faecal steroids beneath the abdomen were lower than beneath the thorax. But soil beneath the abdomen exhibited a dominance of faecal steroids (coprostanol, 5β-stigmastanol) attributed to the position of the intestinal tract (Fig. 3.4, Fig. 3.5). It can thus be concluded that larger amounts of decomposition fluids were introduced into the soil under the thorax released through the burned parts close to the shoulder. Hence, for further forensic investigations on steroids it should be considered that steroid patterns and contents can differ beneath body parts.

Although lipids are hydrophobic and associated with organic and mineral particles in soils (Bull et al. 2002; Schnitzer and Schulten 1989; Stevenson 1966), a property that usually inhibits a transfer along the soil profile, they were found

to be vertically distributed in this study. This observation may be explained by the fact that the soil had a high air capacity, with sand as the dominating particle size (Tab. 3.1). Hence, fluids may have freely drained through the soil macropores. Additionally, preferential flow of decomposition fluids was observed during block preparation of $B_A$ which presumably caused the infiltration to a depth of 7-11 cm. After one year ($B_{1ya}$), steroids were more distributed into depth. This steroid distribution might be explained by bioturbation, which was previously proposed by Evershed et al. (1997) and Bethell et al. (1994).

The $\Delta^5$-sterol β-sitosterol indicative for plant material, can also be found in faecal material as part of the diet (Leeming et al. 1996; Eneroth et al. 1964). In all soil block samples it was diffusely distributed along the examined soil profile (Fig. 3.6). Compared to reference soil, three times enhanced β-sitosterol contents in $B_T$ may also indicate the input of β-sitosterol from gut content, as comparable results could also be observed for β-sitosterol concentrations beneath buried pig carcasses (von der Lühe et al. 2013). An increase of β-sitosterol in the reference block ≥ 5 cm is presumably caused by plant-derived organic material in this block, e.g. roots. However, low β-sitosterol contents compared to the reference were found beneath the body, when β-sitosterol was related to TOC (Appendix 2, Figure SI 2.4). It is a dominant steroid in the natural background, but during decomposition other organic decomposition products lowered the total abundance of β-sitosterol related to TOC (Appendix 2, Figure SI 2.4).

After one year steroids were still present in larger amounts compared to the reference soil showing under the environmental conditions in this study their preservation over a period of one year. The presence of steroids observed for faecal biomarkers introduced into soils was several thousands of years (Birk et al. 2011; Bull et al. 1998; Bethell et al. 1994) and for cholesterol that had derived from decaying carcasses hundreds to thousands of years (2.6 $\mu g\ g^{-1}$ of cholesterol; Shillito et al. 2011). Comparing steroid concentrations directly after removal of the corpse and one year later, a dilution effect could be observed due to translocation, degradation and transformation of steroids. In a natural aerobic environment $\Delta^5$-sterols undergo microbial hydrogenation to 5α-stanols, which are thermodynamically more stable than their precursors (Leeming et al. 1996; Mackenzie et al. 1982; van Grass et al. 1982; Fig. 3.7). It was thus concluded that at the soil surface cholesterol and β-sitosterol were microbially transformed to their corresponding 5α-stanols over time (5α-cholestanol and 5α-stigmastanol, respectively; Fig. 3.3, Fig. 3.6, Fig. 3.7). With increasing soil depth 5α-stanol contents decreased while $\Delta^5$-sterol contents increased ($B_{1ya}$; Fig. 3.3-Fig. 3.6). It seems that the transformation process began at the soil surface and continued to depth, where cholesterol and β-sitosterol are still abundant in higher concentrations. Additionally, a slight increase of the other steroids indicates a better preservation in depths below 11.5 cm (Fig. 3.3-Fig. 3.6). As the soil surface environment beneath the body may have been anaerobic directly after the removal of the body (Cobaugh et al. 2015), it should have become more aerobic in the course of time, so that aerobic microbial transformation and degradation processes restarted at the soil surface. Losses of 5β-stanols are attributed to microbial degradation under aerobic conditions (Bull et al. 2002; Elhmmali et al. 1997; Bartlett 1987). This process might have occurred in the patterns of coprostanol in the first 5 cm and for 5β-stigmastanol in the first 2 cm of soil depth ($B_{1ya}$; Fig. 3.3-Fig. 3.6, Fig. 3.7). Epicoprostanol and epi-5β-stigmastanol were still enhanced in the uppermost soil layers but contents dropped to a level comparable to the reference soil

presumably indicating a decomposition of these compounds over time. Epi-5β-stigmastanol was found during compost formation (Prost et al. (in prep, b)) and in aged sewage sludge (McCalley et al. 1981), presumably formed from 5β-stigmastanol. Epicoprostanol and epi-5β-stigmastanol epimerisation was attributed to anaerobic processes in the past (Bull et al. 2002; McCalley et al. 1981), which presumably occurred during the presence of the body (11-18 days). However, low abundances of epicoprostanol and epi-5β-stigmastanol in soil of the CDI let suggest, that epimerisation of coprostanol and 5β-stigmastanol was apparent but compared to 5α-stanols not a dominant process in the soil.

Tissue, faecal derived and faecal steroids were found beneath a decomposing body after a depositional period of 11-18 days (Fig. 3.7b). Upon decomposition it was assumed, that intestinal microorganisms continued to transform $\Delta^5$-sterols to stanols from intestinal material and soft tissues (Fig. 3.7b). It was predicted that steroids released from a decomposing body undergo different transformation and degradation processes inside the body and in soils (Fig. 3.7b). During the presence of the body intestinal microorganisms might have survived in the soil to continue formation of 5β-stanols from $\Delta^5$-sterols (Fig. 3.7b). In soil, cadaver derived steroids were presumably further degraded and transformed to 5α-stanols rather than epi-5β-stanols during the presence of the body and during one year after the removal of the body (Fig. 3.7c).

## 3.5. Conclusions

In this study, steroids were indicative of decomposition fluids in soil beneath a decomposing body 11-18 days after deposition. It has been demonstrated that faecal derived as well as tissue steroids are present in soil beneath a body even after a short depositional term of 11-18 days. Beneath the abdomen a predominance of faeces over tissue derived steroids were found, which was linked to the position of the intestinal tract. Highest concentrations of total steroids were detected beneath the thorax which was presumably due to leaching of decomposition fluids through the burned parts close to the shoulder. Indicative biomarkers for human derived decomposition products in this study were cholesterol, 5α-cholestanol and also coprostanol as most important human faecal steroids. Their presence over one year showed that decomposition fluids can be identified in the soil over longer periods, even when the body was removed before completing soft tissue decomposition. However, after one year microbial transformation of $\Delta^5$-sterols (cholesterol, β-sitosterol) to 5α-stanols (5α-cholestanol, 5α-stigmastanol) was observed close to the soil surface (≤ 6 cm). Furthermore, degradation and bioturbation showed initial in-situ changes of human derived steroids in soils after one year. Future research should focus on combined lipid biomarker analysis (e.g. steroids, bile acids, fatty acids) which strengthens the evidence of decomposition fluids in soils and might be helpful to identify the human origin of decomposition fluids. In archaeological sciences ratios between steroids were successfully applied to identify faecal sources (Prost et al. (in prep, a); Birk et al. 2011; Bull et al. 2002). The development of steroid ratios to identify mixtures of tissue and faecal materials can then help to ascertain the origin of steroids from a decomposing cadaver.

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# Chapter 4

# Temporal fatty acid patterns from human decomposition fluids in soils

## Abstract

Fatty acid analyses of materials in association with decomposing human bodies in soil environments have been widely used to understand adipocere formation or to reconstruct the post-mortem interval. Studies conducted under aerobic environmental conditions have covered relatively short observation periods up to 70 days. Longer periods of human derived fatty acids concentrations and pattern changes have, thus far not been investigated. The aim of the present study is to determine changes in concentrations and relative abundances of human derived fatty acids in soil over a period of one year. In a real case example, a body was deposited for 11-18 days on a soil surface before its removal. Soil was sampled after removal of the body (11-18 days) and additional soil samples were taken after one year. Total fatty acid concentrations ranged from 5 to 330 mg $g_{soil}^{-1}$ in soil taken after 11-18 days. Human derived fatty acids were still detectable in high abundances after one year (10-41 mg $g_{soil}^{-1}$). Patterns of fatty acids revealed that an extensive hydrogenation to saturated fatty acids occurred 11-18 days after deposition. Furthermore, 10-hydroxystearic acid and fatty acid salts, characteristic indicators for adipocere, were abundant in all soil samples beneath the body (11-18 days; one year). After one year (358 days) a decline in myristic acid and 10-hydroxystearic acid relative abundances were detected. In conclusion fatty acids are an important indicator of human decomposition fluids in soils and were still detectable after one year even when the body was removed after a short depositional term. Thus, in-situ processes - such as the formation of adipocere - promoted the preservation of human derived fatty acids over a period of one year.

## 4.1. Introduction

Fatty acids are constituents of the organic matter in soils. Originating from various sources (e.g. plants, fungi, atmospheric deposition, animals), they are ubiquitously distributed in soils (Bull et al. 2000). It is commonly known that once introduced into soils, fatty acids can have mean residence times of several decades (Wiesenberg et al. 2010; Quénéa et al. 2006; Wiesenberg et al. 2004). Due to their chemical properties they were used as diagnostic markers for source identification and turnover rate determinations (Wiesenberg et al. 2004). Patterns and abundances of fatty acids in soils can be utilised in forensic science where fatty acids are released into the surrounding environment from the decomposition of human corpses.

In forensic sciences, fatty acids have been widely used as indicators to understand human decomposition processes in soil environments. They were determined in association with the formation and identification of adipocere (Algarra et al. 2010; Notter et al. 2009; Forbes et al. 2003), to estimate the post-mortem interval (Vass et al. 2008; Vass et al. 1992) or to identify temporary graves (Bull et al. 2009).

Bull et al. (2009) found high abundances of fatty acids (up to 4200 µg $g_{soil}^{-1}$ of palmitic and stearic acid) in soil from a temporary grave compared to reference soil samples (20 µg $g_{soil}^{-1}$ of palmitic acid and stearic acid). Most of the fatty acids from the temporary grave were related to adipocere, a white firm substance, which is known to form during incomplete decomposition from adipose tissues, predominantly associated with anaerobic environments (Schoenen and Schoenen 2013; Ubelaker and Zarenko 2011; Bull et al. 2009). Zimmermann et al. (2008) observed fatty acids on a human-shaped stain on a concrete floor, presumably derived from a body which had been removed 30 years previously. It was assumed that the formation of fatty acids salts with $Ca^{2+}$ from the concrete and the dry conditions promoted the conservation of the stain (Zimmermann et al. 2008). The majority of studies engage fatty acid analysis in relation to adipocere formation (Forbes et al. 2005; Vane and Trick 2005; Forbes et al. 2002; Makristathis 2002; Takatori 2001). Once adipocere is formed it is relatively stable against degradation and was even found on archaeological specimens (Fiedler et al. 2009; Makristathis 2002). But the knowledge about the presence of human derived fatty acids in soils under natural aerobic decomposition conditions is rare (Swann et al. 2010a).

Under natural aerobic decomposition conditions, previous studies determined changes in fatty acid patterns over time to reconstruct the post-mortem interval (Swann et al. 2010a; Swann et al. 2010b; Vass et al. 2002; Vass et al. 1992). These studies observed relatively short observation periods up to 60 days for long-chain fatty acids in decomposition fluids (Swann et al. 2010b), and 70 days for human volatile fatty acids in soil solutions (Vass et al. 1992) during the continuous presence of decomposing bodies. Thus, fatty acids deriving from a decomposing body might be preserved in soils over longer time periods (≥ 70 days), even when the body is removed after a short depositional term (Bull et al. 2009).

It is uncertain how human fatty acids change in abundance and distributions over longer terms, especially when the body was removed before completing soft tissue decomposition. The knowledge about the long-term presence of fatty

acids (≥ 70 days) and their change in patterns over time is useful in forensic investigations to identify soil enriched with decomposition fluids even when a body was temporarily present. Additionally, the knowledge of preservation mechanisms of fatty acids in soils is useful in soil science for a better understanding of the fate and stability of these compounds in soils.

Therefore, a study was carried out on soil from a forensic case study. The aim of the study was to identify human fatty acids in soil samples where a body was temporarily deposited aboveground for 11-18 days. It was hypothesised that upon decomposition of the human body, (I) fatty acids are released from the body and leach into the soil beneath 11-18 days after the body was deposited, (II) fatty acids are still increased in the soil one year after the body was removed compared to reference soil samples taken in close proximity of the body.

Fatty acids were analysed in order to determine the presence and changes of concentrations and relative abundances of myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1\Delta9}$) and linoleic ($C_{18:2}$) acid over time, the common fatty acids associated to human tissues (Notter et al. 2009). In a preliminary examination of the lipid extracts, it was presumed that adipocere was formed in the soil. In a further approach the analysis of fatty acid salts and 10-hydroxystearic acid (10-OH-$C_{18:0}$) was included in this study, as these components are known to comprise adipocere (Forbes et al. 2005; Takatori 2001; Gill-King 1997).

## 4.2. Material and Methods

### 4.2.1. *Case study*

Soil samples were taken from a forensic case study where a female body had been placed in a thicket of bushes and trees in a suburban area. The naked body was lying on its back on the soil surface, with areas of the shoulder and the head having been burned and minor scavenging was observed. The stage of decomposition was advanced and soft tissues except the skin were liquefied and colonised by insect larvae. Time since deposition was estimated to be between 11 and 18 days by the development stages of insect larvae (*Phormia regina*). The body was found in summer time (June) and temperatures at the crime scene ranged from 13-23°C during the depositional time frame. Precipitation at the weather station (10 km from the crime scene) was 20 mm during the period of 18 days of the suspected depositional time.

### 4.2.2. *Soil type*

The soil type found at the study site was classified as Fluvic Cambisol Prototechnic (Schad 2008) and its chemical and physical characteristics are summarised in Tab. 4.1. The soil contained several artefacts such as ceramic and brick fragments (≥ 5%, ≤ 100 cm) indicating there had been a former anthropogenic impact. The topsoil of the Cambisol had a homogenous sandy texture (up to 80% sand) and a neutral pH at ~7 (Tab. 4.1). Due to the high abundance of sand, the Cambisol has a high air capacity of 34-38 vol.% (Tab. 4.1).

### 4.2.3. *Soil samples*

After removal of the body, soil blocks (~20 cm length x 20 cm width) were taken with a clean spade directly beneath the body ($B_T$ = beneath thorax, $B_A$ = beneath abdomen) and one reference block ($B_{Ref}$ = reference block) was sampled 10 m away from the body (Fig. 4.1). One year after (358 days) the body was removed another block ($B_{1ya}$) was taken from the cadaver decomposition island (CDI) where the thorax was located (Fig. 4.1). After sampling, the organic litter and the first 0 to 5 mm of soil were removed from the block surfaces and the soil blocks were separated into 5 mm-intervals to a depth of 5 cm for each block. Soil samples were freeze-dried for 24 h, sieved (≤ 2 mm), finely ground in a ball mill (450 rpm, 5 min) and subsequently homogenised with an agate mortar. Samples were stored in a desiccator at room temperature (18°C) until analysis.

### 4.2.4. *Laboratory equipment*

In order to avoid contamination reusable glassware was rinsed five times with deionised water and was subsequently heated with disposable glassware at 450°C for 24 h in a muffle furnace. Plastic materials were rinsed twice with *n*-heptane. Solvents were purchased from Carl Roth (Karlsruhe, Germany) and were GC grade. The silica gel (60 Å, 0.063-0.200 mm particle size; Merck, Darmstadt, Germany) for solid phase extraction (SPE) was heated at 380°C for 2 h to remove any organic contaminants, then activated at 200°C for 5 h and stored in *n*-heptane until use.

**Table 4.1:** Soil chemical and physical parameters (mean of n=2 laboratory replicates ± standard error) of the reference topsoil from the study site.

| depth | sand[a] | silt[a] | clay[a] | $CaCO_3$[b] | pH[c] | TOC[d] | BD[e] | AC[f] | $PV_t$[g] |
|---|---|---|---|---|---|---|---|---|---|
| cm | | % | | % | | g kg$^{-1}$ | g cm$^{-3}$ | vol.% | |
| -6 | 80±0.1 | 9±0.1 | 11±0 | n.d. | 6.8 | 15 | 1.2 | 34.4 | 53.1 |
| -10 | 79±0.1 | 9±0.1 | 12±0.2 | 0.6±0 | 7.0 | 12 | 1.3 | 36.5 | 52.4 |
| -14 | 80±0.1 | 9±0 | 11±0.1 | 0.6±0 | 7.1 | 11 | 1.3 | 38.0 | 52.5 |
| -20 | 80±0.1 | 9±0 | 11±0.1 | 1.0±0 | 7.1 | 9 | 1.4 | 34.6 | 49.0 |

n.d. = not detected │[a]sand 2000-63 µm; silt 63-2 µm; clay ≤ 2 µm (DIN ISO 11277) │[b]carbonate content (DIN ISO 10693)b│ [c]pH in $CaCl_2$ (DIN ISO 10390) │[d]total organic carbon analysed by dry combustion (Vario EL Cube, Elementar, Hanau, Germany) after destruction of carbonates by acid fumigation (Harris et al. 2001) │[e]bulk density (DIN ISO 11272) │[f]air capacity = coarse pore volume, equivalent pore size > 50 µm; [g]total pore volume (DIN ISO 11274)

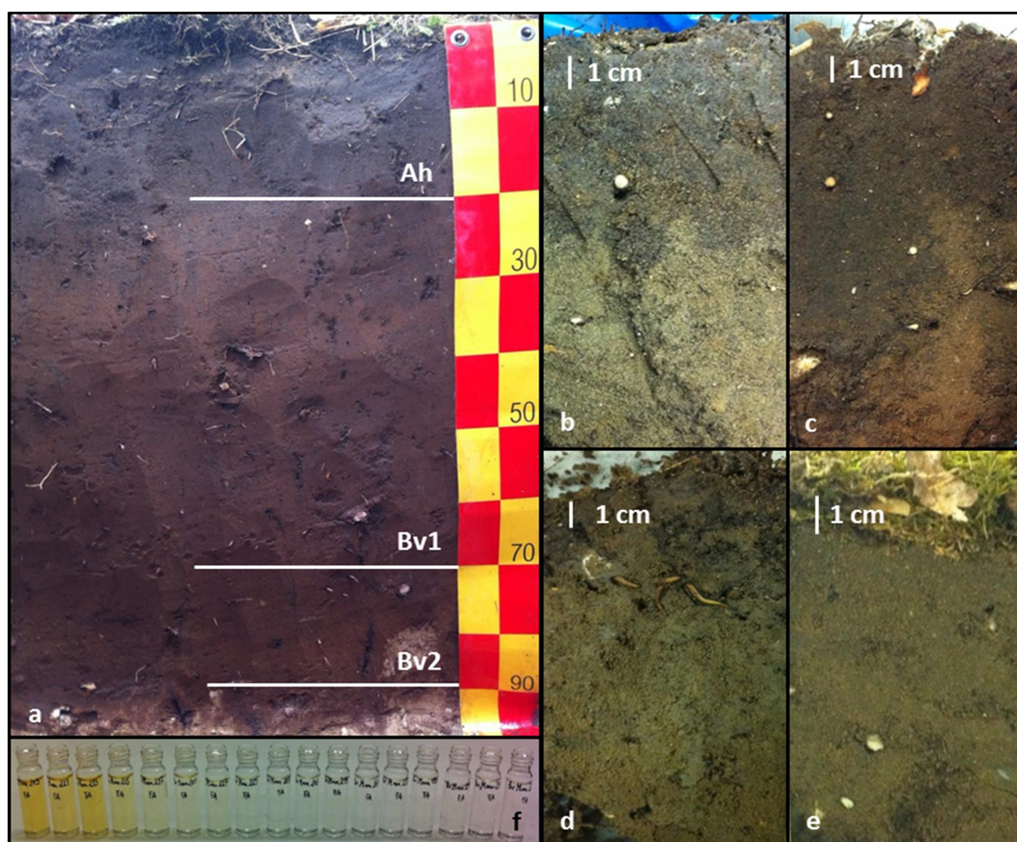


**Figure 4.1:** Reference soil profile and soil blocks of the case study; (a) reference soil profile, Ah horizon with accumulation of organic matter, Bv1 and Bv2 with a sandy soil texture (b) soil block $B_T$ taken 11-18 days after deposition of the body beneath the thorax; (c) soil block $B_A$ taken 11-18 days after deposition of the body beneath the abdomen; (d) soil block $B_{1ya}$ taken 358 days after the body was removed beneath thorax; (e) reference soil block taken 11-18 days after body deposition approximately 10 m away from the body; (f) lipid extract obtained after liquid-liquid extraction of soil samples from $B_T$; from left to right increasing soil depth showing enrichment of organic compounds in the uppermost intercepts; photos: B. von der Lühe 2013, 2014.

The derivatising agent *N,O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS, 99:1, v/v) was obtained from Sigma Aldrich (Steinheim, Germany) and pyridine from Merck (Darmstadt, Germany). Nitrogen ($N_2$) in a purity of 99.998% was used (Westfalen, Münster, Germany).

### *4.2.5. Extraction and purification of fatty acids*

Total lipids (TLE) containing fatty acids were consecutively extracted from a duplicate soil sample (0.5 g) with 4 ml each of methanol, dichloromethane/methanol (1:1, v/v) and dichloromethane (DCM) in an ultrasonic bath (30 min, 30-35°C). After centrifugation (3000 rpm, 6 min) lipid extracts were transferred to the same glass vial and dried under nitrogen.

Heptadecanoic acid ($C_{17:0}$, internal standard I (IS I), 200 µl, 100 mg $l^{-1}$) was added to the lipid extracts as an internal standard. To yield free and esterfied fatty acids, saponification was carried out with a 1 M ethanolic KOH solution (1.5 ml, 70°C, 4 h). Neutral lipids were discarded by adding 0.5 ml ultrapure $H_2O$ and 4 x 1.5 ml *n*-heptane. To obtain the acidic lipid fraction 0.4 ml 5.8 M HCl solution was added to the aqueous residual. Acidic lipids containing fatty acids were extracted with 4 x 1.5 ml *n*-heptane after warming to 60°C. After the extract was dried under nitrogen a solid phase extraction (SPE) was carried out to obtain the fatty acid fraction. Glass SPE columns (3 ml) were packed with 0.75 ml silica gel and subsequently preconditioned with 2 x 1.5 ml *n*-heptane. The extracts were redissolved in 100 µl *n*-heptane and transferred to the SPE columns. Columns were washed with 3 x 1 ml *n*-heptane/ethyl acetate (98:2, v/v) and fatty acids were eluted to glass vials by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (70:30, v/v).

Fatty acids were derivatised to their methyl esters with 0.5 ml toluene and 1 ml acidified methanol (2% $H_2SO_4$; 50°C, ~14 h). Fatty acid methyl esters (FAME) were extracted by adding 1 ml 0.5 M $K_2CO_3$ solution and 3 x 1 ml *n*-heptane. After drying ($N_2$), *n*-heptane was added and two aliquots from each sample were transferred into two GC/MS vials. The first aliquot was analysed by gas chromatography equipped with a flame ionisation detector (GC/FID) to quantify myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1\Delta9}$) and linoleic acid ($C_{18:2}$).

As 10-hydroxystearic acid (10-OH-$C_{18:0}$) was present in the soil samples containing decomposition fluids the second FAME aliquot was further derivatised by adding 50 µl of BSTFA+TMCS/pyridine (3:1, v/v; 90°C, 1 h) to form trimethylsilyl esters of hydroxylated FAME. The solvent was evaporated under a stream of $N_2$ and 100 µl *n*-heptane was added for analysis. The identification of 10-OH-$C_{18:0}$ was carried out by gas chromatography/mass spectrometry (GC/MS). All trimethylsilylated FAME extracts were analysed with GC/FID to obtain a proximate estimation of the 10-OH-$C_{18:0}$ concentration.

### *4.2.6. Instrument analysis*

Analysis of FAME samples was performed on an Agilent 7890B gas chromatograph equipped with a flame ionisation detector (Agilent Technologies, Santa Clara, CA, USA). A fused-silica capillary column (DB 23; Agilent Technologies, Santa Clara, CA, USA) with the dimensions of 30 m x 250 µm x 0.25 µm was used. The carrier gas was helium

(99.9999%) maintained at constant pressure (100 kPa). The injection port was set at 240°C and a sample volume of 1 µl was injected in splitless mode. The oven temperature was programmed from 50°C (2 min isothermal) to 240°C at a rate of 7°C $min^{-1}$ (10 min isothermal). The detector temperature was held at 300°C, hydrogen flow was set at 30 ml $min^{-1}$, air flow at 400 ml $min^{-1}$ and make up gas flow (helium) was set at 25 ml $min^{-1}$. Fatty acid methyl esters were quantified with an external standard calibration using standard FAME mixtures (Sigma Aldrich, Steinheim, Germany) containing $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1\Delta9}$, $C_{18:2}$ in the concentration range of 0.4, 0.8, 1, 2, 10, 50, 100, 150, 200 mg $l^{-1}$.

A small set of trimethylsilylated FAME samples (from each block) were selected for the identification of 10-OH-$C_{18:0}$. Analysis was carried out by GC/MS with an Agilent 6890 N gas chromatograph connected to a 5975 B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The instrument was equipped with a DB-5ms Ultra Inert fused silica capillary column (30 m x 250 µm x 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). Helium (99.9995%) was employed as the carrier gas with a constant flow at 1.1 ml $min^{-1}$. The injection port was maintained at 250°C under 84 kPa and an aliquot of 2 µl was injected in split mode (10:1 split ratio, 12.5 ml $min^{-1}$ split flow). The initial oven temperature was held at 80°C for 2 min, then programmed at 10°C $min^{-1}$ to 290°C and held for 5 min. The solvent delay was 5 min and electron ionisation was set at 70 eV in scan mode ranging between *m/z* 50-500.

In all soil samples apart from the reference block samples, 10-OH-$C_{18:0}$ was identified (details in Chapter 2.3). Therefore all trimethylsilylated FAME samples were run on GC/FID to obtain an estimation of the concentration of 10-OH-$C_{18:0}$. The estimation of 10-OH-$C_{18:0}$ was carried out after a method described in Chapter 2.3. The standard response factor (SRF) of 10-OH-$C_{18:0}$ was calculated with the effective carbon number (ECN) of 10-OH-$C_{18:0}$ and $C_{17:0}$ IS I to assess 10-OH-$C_{18:0}$ concentrations. The calculated SRF of 10-OH-$C_{18:0}$ fitted well with the analytical SRF of 12-hydroxystearic acid (12-OH-$C_{18:0}$), as both components show a comparable response on a flame ionisation detector ($SRF_{10\text{-}OH\text{-}C18:0}$=97% of $SRF_{12\text{-}OH\text{-}C18:0}$; Chapter 2.3).

### *4.2.7. Fatty acid salts*

To determine the presence of fatty acid salts in selected soil samples, 5 mg of TLE was accurately weighed into plastic screw top vials and 300 µl each of concentrated nitric acid, concentrated hydrochloric acid and 30% hydrogen peroxide (Carl Roth, Karlsruhe, Germany) was added. Extracts were completely dissolved in an ultrasonic bath at 60°C. Prior to analysis the samples were diluted with distilled water to a volume of 10 ml. Analysis was performed on an inductively coupled plasma optical emission spectrometer (ICP/OES; Spectro Arcos, Kleve, Germany) and quantification was carried out with a calibration solution containing $K^+$, $Na^+$, $Ca^{2+}$ and $Mg^{2+}$ (1, 2, 4, 6, 8, 10 mg $l^{-1}$). This method was previously used to characterise fatty acid salts in adipocere (Zimmermann et al. 2008; Forbes et al. 2005).

## 4.3. Results

Total fatty acid concentrations in the reference soil varied between 14 and 32 µg $g_{soil}^{-1}$ (Fig. 4.2). After a depositional period of 11-18 days of a decomposing human body, concentrations and patterns of fatty acids revealed a large input of exogenous organic matter into the underlying soil close to the proximity of the thorax and the abdomen (Fig. 4.2, Fig 4.3). The total fatty acid concentrations ($FA_t$; sum of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1\Delta9}$ and 10-OH-$C_{18:0}$) of soil samples beneath the thorax ranged between 5 and 330 mg $g_{soil}^{-1}$ having a general decline with increasing soil depth (Fig. 4.2). Beneath the abdomen concentrations of total fatty acids varied between 5 and 52 mg $g_{soil}^{-1}$ showing a decline in concentrations (Fig. 4.2). The difference in total fatty acid concentrations between soil in contact with the decomposing body and the reference soil was particularly pronounced, in some instances with up to ~10,000 fold enrichment (Fig. 4.2). Total concentrations of fatty acids were still enhanced after one year and amounts were comparable in the same order of magnitude of fatty acid concentrations in soil samples taken after 11-18 days beneath the thorax and the abdomen (except $B_T$ 0-0.5 cm; Fig 4.2).

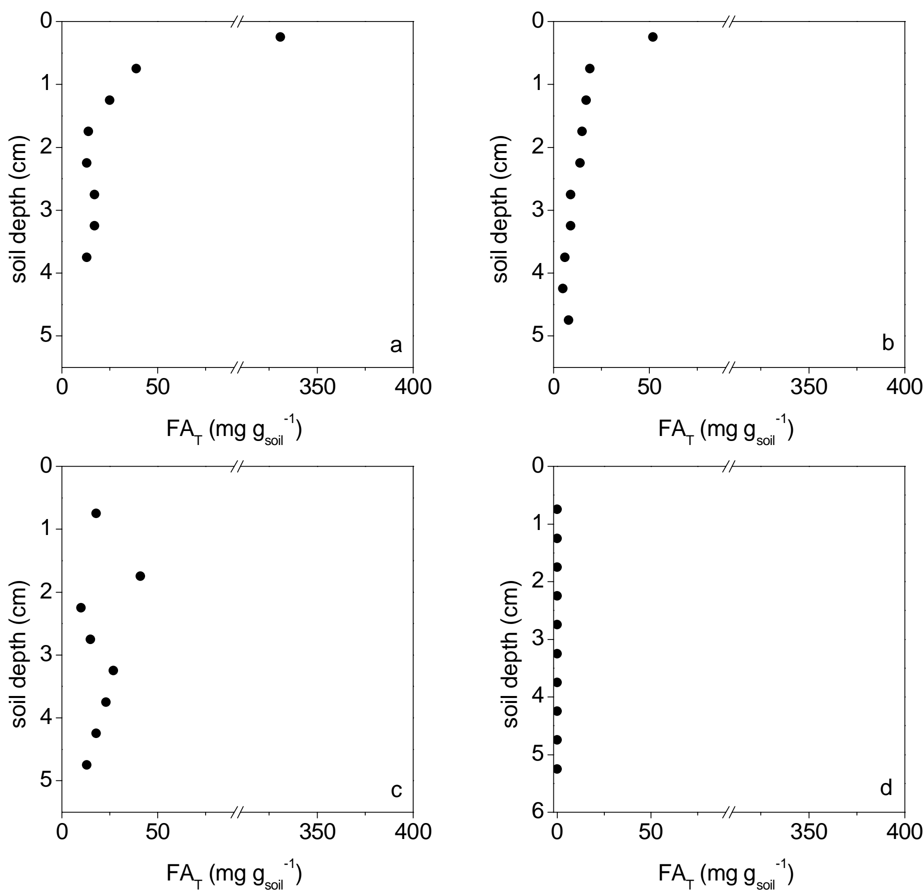


**Figure 4.2:** Summarised fatty acid concentrations (= $FA_t$) including concentration of myristic acid, palmitic acid, stearic acid, oleic acid and 10-hydroxystearic acid of soil block samples taken beneath a decomposing human body: (a) beneath the thorax 11-18 days after deposition of the body and (b) beneath the abdomen 11-18 days after deposition of the body; (c) one year after the body was removed; (d) reference soil, 10 m away from the body.

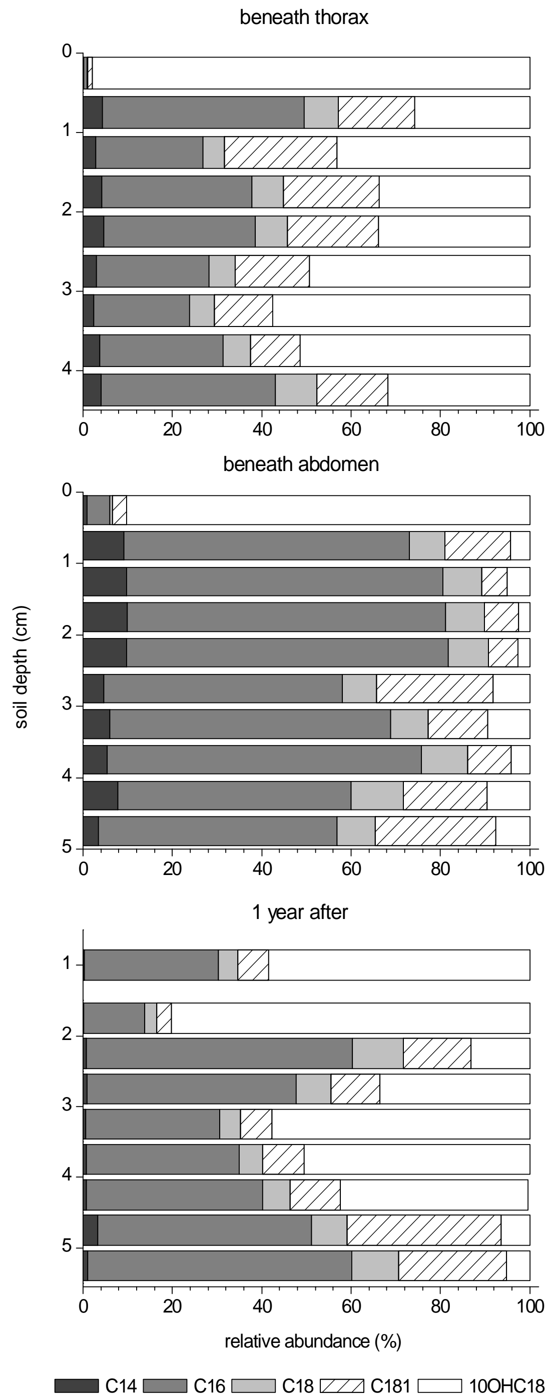


**Figure 4.3:** Relative abundance of fatty acids analysed with soil depth beneath a decomposing body: 11-18 days after deposition of the body beneath the thorax and the abdomen and one year after the body removal beneath the thorax. C14 = myristic acid, C16 = palmitic acid, C18 = stearic acid, C18:1 = oleic acid and 10-OH C18 = 10-hydroxystearic acid.

Although the total fatty acid concentrations did not clearly differ between 11-18 days and one year, patterns of relative abundances revealed mainly saturated fatty acids ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$) over unsaturated fatty acids ($C_{18:1\Delta 9}$) with the unexpected 10-hydroxystearic acid (10-OH-$C_{18:0}$) in all soil blocks under investigation (Fig. 4.2, Fig. 4.3). After

11-18 days, soil beneath the thorax and the abdomen was characterised by a dominance of 10-OH-$C_{18:0}$ in the uppermost 0.5-1 cm of soil depth, followed by $C_{16:0}$, $C_{18:1\Delta9}$, $C_{18:0}$ and $C_{14:0}$ (Fig. 4.3). Beneath the thorax, 10-OH-$C_{18:0}$ remained with $C_{16:0}$ as the most dominant fatty acid to a depth of 5 cm, whereas beneath the abdomen 10-OH-$C_{18:0}$ was a minor constituent of the total fatty acids pattern ≤ 0.5 cm soil depth (Fig. 4.3). Apart from considering the dominant contribution of 10-OH-$C_{18:0}$ to the total fatty acids pattern, the relative abundance of oleic acid ($C_{18:1\Delta9}$) was slightly lower beneath the abdomen, and comparable to oleic acid abundances after one year (Fig. 4.3). Oleic acid ($C_{18:1\Delta9}$) was more abundant in total fatty acids pattern beneath the thorax after 11-18 days (Fig. 4.3). After one year the saturated $C_{16:0}$ and 10-OH-$C_{18:0}$ were the dominant fatty acids to a depth of 5 cm, with minor abundances of $C_{18:1\Delta9}$ and $C_{18:0}$ (Fig. 4.3). Particularly, losses in concentrations and abundances of myristic acid ($C_{14:0}$) was observable over time (Fig. 4.3). The reference soil exhibited minor amounts of linoleic acid (≤ 0.006 mg $g_{soil}^{-1}$) and to a depth of 0-0.5 cm beneath the thorax, minor concentrations of linoleic acid (0.08 mg $g_{soil}^{-1}$) were observed (Appendix 3, Table SI 3.1-3.2).

Summing the means of all analysed cations, there was an increase from 2-7 µg $mg_{TLE}^{-1}$ to 12-18 µg $mg_{TLE}^{-1}$ of fatty acid salts observed over a period of one year (Tab. 4.2). At a depth of up to 1 cm, fatty acid salts were dominated by $Ca^{2+}$ fatty acid salts (up to 16±5 µg $mg_{TLE}^{-1}$) with minor abundances of $Na^{+}$ (≤ 1±0.2 µg $mg_{TLE}^{-1}$), $K^{+}$ (≤ 0.3±0.06 µg $mg_{TLE}^{-1}$) and $Mg^{2+}$ (≤ 0.5±0.2 µg $mg_{TLE}^{-1}$) fatty acid salts in all soil blocks (Tab. 4.2). Higher $Ca^{2+}$ fatty acid salts were found beneath the thorax (3-7 µg $mg_{TLE}^{-1}$) compared to the abdomen (2 µg $mg_{TLE}^{-1}$; Tab. 4.2). Over time $Na^{+}$ and $K^{+}$ fatty acid salts did not show any difference in concentration, whereas $Ca^{2+}$ and $Mg^{2+}$ fatty acid salts increased in concentration (Tab. 4.2).

**Table 4.2:** Potassium ($K^{+}$), sodium ($Na^{+}$), magnesium ($Mg^{2+}$) and calcium ($Ca^{2+}$) salts of fatty acids extracted from total lipid extracts (TLE) from soil samples taken beneath a decomposing body (beneath thorax and abdomen) after it was removed; samples were taken 11-18 days after deposition and one year after the body was removed. Results are presented as mean of two replicates ± standard error.

| sampling time | Position | soil depth cm | $Na^{+}$ | $K^{+}$ | $Mg^{2+}$ | $Ca^{2+}$ | sum |
|---|---|---|---|---|---|---|---|
| | | | µg $mg_{TLE}^{-1}$ | | | | |
| 11-18 days | Thorax | 0-0.5 cm | 1.2±0.2 | 0.3±0.1 | 0.2±0 | 5.4±0.6 | 7.0 |
| | | 0.5-1 cm | 0.5±0.2 | 0.1±0.1 | 0.1±0 | 2.3±0.4 | 3.0 |
| 11-18 days | Abdomen | 0-0.5 cm | 0.1±0 | 0.1±0 | 0.1±0 | 1.6±0.2 | 1.9 |
| | | 0.5-1 cm | 0.2±0.1 | 0.1±0 | 0.1±0 | 1.9±0 | 2.3 |
| 1 year after | Thorax | 0.5-1 cm | 0.8±0.1 | 0.1±0 | 0.4±0 | 10.4±0.5 | 11.7 |
| | | 1-1.5 cm | 1.0±0.1 | 0.2±0.1 | 0.5±0.2 | 16.1±5.4 | 17.8 |

## 4.4. Discussion

In this study, total fatty acid concentrations and fatty acid distributions demonstrated an extremely high input of exogenous organic material beneath a decomposing body. Even after a short term of body deposition (11-18 days) those components were present in the investigated soil to a depth of 5 cm. In previous studies, the presence of fatty acids derived from decomposition fluids had been found in soil samples of a forensic temporary grave (Bull et al. 2009), in cemetery grave soils known to comprise adipocere (Forbes et al. 2003; Forbes et al. 2002) and on a concrete floor where a body was found 30 years earlier (Zimmermann et al. 2008). Bull et al. (2009) found similar composite concentrations of $C_{16:0}$ and $C_{18:0}$ (~1-4.2 mg $g_{soil}^{-1}$; our study $B_A$, $B_T$ and $B_{1ya}$: 5-330 mg $g_{soil}^{-1}$), indicating an input of exogenous fatty acids from a corpse. However, the time since deposition and the elapsed period between removal of the body and the soil sampling were not indicated (Bull et al. 2009).

Fatty acids were not only enriched after the short deposition of the body (11-18 days), but were also found in a comparable order of magnitude 358 days after the body was removed (Fig. 4.2), indicating that fatty acids were stable against decomposition over a period of one year in these conditions. Generally, lipids, including fatty acids, are known to comprise mean residence times in soils in the range of several decades (Wiesenberg et al. 2010; Quénéa et al. 2006; Wiesenberg et al. 2004). The soil block taken after one year in the subsoil (≤ 10 cm) was lighter in colour and less sticky than the fresh soil blocks ($B_T$, $B_A$), which were taken after 11-18 days (Fig. 4.1). This indicates that degradation of the decomposition fluids commenced during the year, presumably from the easily degradable constituents such as proteins and carbohydrates (van Belle et al. 2009; Benninger et al. 2008; Dent et al. 2004) and of fatty acids in the first 1 cm of soil depth (lower $FA_T$; Fig. 4.2). Lower total fatty acid abundances ≤ 1 cm can be also attributed to leaching processes of fatty acids with soil depth over the period of one year (Fig. 4.2). Slight changes were observed in the decline of the relative abundance of $C_{14:0}$ (Fig. 4.3), which was attributed to β-oxidation. Under conditions of sufficient oxygen supply, fatty acids undergo β-oxidation mediated by microorganisms (Michal 2013; van Belle et al. 2009; Madigan et al. 2006; Ratledge 1994; Knopp 1904).

Microorganisms recycle intact fatty acids (e.g. palmitic acid), but also transform and form fatty acids from soils in their cells (Dippold and Kuzyakov 2016). It is known, that during cadaver decomposition soil respiration rates and soil microbial biomass increases during the active decay of a human body (Haslam and Tibbett 2009; Carter and Tibbett 2006; Hopkins et al. 2000). Cobaugh et al. (2015) observed only during the active decay (7-12 days post-mortem) increases of microbial biomass, which dropped after 10-23 days post-mortem. It was therefore assumed, that fatty acids could have been also introduced by microorganisms, as a result of the increased microbial biomass.

Fatty acids seem to be less degraded by β-oxidation after one year, as shown by the minimal loss of total concentrations (≤ 1 cm) and relative abundances (mainly $C_{14:0}$), but patterns of fatty acids revealed a change of relative abundances and distributions of fatty acids over time. The human adipose tissue comprises about 90-99% of triacylglycerols, where one glycerol molecule is attached to three fatty acid molecules (Schoenen and Schoenen 2013; Dent et al. 2004; Takatori 1996). In fresh human adipose tissue oleic acid (~60% of total fatty acids in adipose

tissue) is the dominant fatty acid, followed by palmitic, linoleic (~10%) and palmitoleic acid (Notter et al. 2009; Dent et al. 2004; Makristathis 2002). During the process of decomposition hydrolysis of triacylglycerols yields free fatty acids. They are further hydrogenated to their saturated constituents: oleic, linoleic and palmitoleic acid yields stearic, oleic and palmitic acid respectively (Schoenen and Schoenen 2013; Dent et al. 2004; Evans 1963). The high abundance of palmitic and stearic acid in soil beneath the thorax and the abdomen after 11-18 days showed that extensive hydrogenation of the human fatty acids have occurred inside the body and in the soil. In particular, palmitic acid (except first 0.5 cm of soil depth; Fig. 4.3) displays an overall predominance. Hydrogenation is also indicated by the absence of linoleic acid, which was presumably rapidly hydrogenated successively to oleic and stearic acid (Notter et al. 2009; Dent et al. 2004; Evans 1963). However, oleic acid abundances indicate, that the process of hydrogenation is still in progress. Lower abundances of the unsaturated oleic acid ($C_{18:1\Delta9}$) beneath the abdomen and in soil sampled after one year indicate that hydrogenation of oleic acid was marginally advanced compared to fatty acids found beneath the thorax.

The hydroxylated fatty acid 10-hydroxystearic acid (10-OH-$C_{18:0}$) was mainly observed in high abundances (up to 98%; Fig. 4.3) after 11-18 days in the subsoil (≤ 0.5 cm) beneath the thorax and the abdomen and remained there in relative abundances ≤ 80% after one year (Fig. 4.3). Normally, 10-OH-$C_{18:0}$ is an indicator associated to adipocere formation (Bull et al. 2009; Varmuza et al. 2005; Makristathis 2002; Takatori 1996), which was not identified on the body after discovery. Adipocere, a white greasy substance, can be formed from adipose tissue during incomplete decomposition. Adipocere formation is mainly associated with anaerobic environments (Ubelaker and Zarenko 2011; Evans 1963). It is comprised of free fatty acids ($C_{16:0}$ > $C_{18:0}$ > $C_{14:0}$), $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ fatty acid salts (= soaps), hydroxy-fatty acids, oxo-fatty acids and polyhydroxy-fatty acids (Schoenen and Schoenen 2013; Takatori 2001; Takatori 1996; Takatori et al. 1988; Takatori et al. 1987; Tomita 1984). Microorganisms are involved in the formation of adipocere and it has been shown that some varieties of aerobic and anaerobic bacteria are able to produce 10-OH-$C_{18:0}$ from oleic acid (Schoenen and Schoenen 2013; Takatori 2001; Takatori 1996; Takatori et al. 1987). In well-developed adipocere the 10-OH-$C_{18:0}$ content varies between 2-20% (Makristathis et al. 2002; Takatori and Yamaoka 1977) and even as high as 49% in some instances. Dent et al. (2004) assumed that hydroxy fatty acids can be produced in minor amounts during putrefaction, which was also discussed by O'Brien et al. (2007). In this study, abundances of 10-OH-$C_{18:0}$ can be explained by putrefactive fluids containing 10-OH-$C_{18:0}$ leaching into the soil. It is suggested that additional processes resulted in high abundances of 10-OH-$C_{18:0}$ (up to 82%; Fig. 4.3) in the subsoil.

During the deposition of the body (11-18 days) the environmental and climatic conditions at the study site (precipitation ~20 mm; temperatures between 13-23°C) would not normally favour adipocere formation on the corpse. There are selected reports about adipocere formation under warm or dry conditions; in these studies adipocere was either formed under dry concealment in anaerobic conditions (Nushida et al. 2008) or human remains were partly buried in direct contact with soil during summer time in a temperate forest (Schotsmans et al. 2011).

Therefore, it was presumed that other factors promoted the formation of 10-OH-$C_{18:0}$ at the present study site. Zimmermann et al. (2008) found adipocere on an indoor concrete floor, probably produced by decomposition fluids from a body, which was found and removed 30 years previously assuming that, during the body deposition, the contact areas between the body and the concrete floor had created an anaerobic environment, which favoured adipocere formation. Furthermore, fatty acids from the decomposition fluids formed insoluble salts with $Ca^{2+}$ from the concrete (Zimmermann et al. 2008). In this study, the highest concentrations of 10-OH-$C_{18:0}$ were found directly after the body was removed (11-18 days), presumably having been formed by microorganisms under anaerobic conditions when the body was in contact with the soil creating an anaerobic environment (Takatori et al. 1986; Takatori and Yamaoka 1977). The tissue remnants created an oxygen depleted environment and aeration was prevented during the presence of the body, which was also suggested by Cobaugh et al. (2015). Additionally, the formation of insoluble fatty acid soaps with free $Ca^{2+}$ and $Mg^{2+}$ ions (Tab. 4.2) from the soil solution promoted the preservation of fatty acids over time. Both processes (formation of 10-OH-$C_{18:0}$ and fatty acids salts) are normally attributed to adipocere formation. But the formation of fatty acid salts likely occur naturally in soils, when $Mg^{2+}$ and $Ca^{2+}$ salts are present. Cobaugh et al. (2015) observed the migration of human associated bacteria in soil beneath decomposing human bodies during the active and advanced stages of decomposition. They also detected a change in the microbial community with a proliferation of anaerobes, presuming that oxygen was limited and anaerobic metabolism was more prevalent (Cobaugh et al. 2015). This indicates that an anaerobic environment can be produced directly beneath the carcass during decomposition, where 10-OH-$C_{18:0}$ was produced by microbes from oleic acid. It is therefore concluded that similar conditions promoted anaerobic metabolism, which resulted in the formation of 10-OH-$C_{18:0}$ whilst the body was deposited during the 11-18 day period. After the body was removed, oxygen was available again, which lead to the degradation of 10-OH-$C_{18:0}$ resulting in decreased relative abundances after one year (Fig. 4.3). Cobaugh et al. (2015) showed that intestinal microorganisms disappear after bodies were removed from the soil surface. They concluded, that the removal of the bodies again created an aerobic environment, promoting the predominance of aerobic microorganisms (Cobaugh et al. 2015), where fatty acids might be predominantly degraded by β-oxidation (Dent et al. 2004). However, the increase in the insoluble $Ca^{2+}$ and $Mg^{2+}$ fatty acid salts after one year promotes protection against microbial attack and presumably retarded fatty acid degradation (Zimmermann et al. 2008; Dent et al. 2004; Gill-King 1997).

The presence of 10-OH-$C_{18:0}$ and fatty acid salts in soil samples after the deposition of a female human body for a period of 11-18 days let suggest a fast formation of adipocere. It is dependent on the environmental conditions exactly when adipocere can be observed on decomposing bodies, and reports vary between 22 days (Yan et al. 2001; Simonsen 1977) and several months to years (Nushida et al. 2008; Makristathis 2002), but it is assumed, that in this study the environmental conditions in close contact between the corpse and the underlying soil created an anaerobic environment. It might be possible, that the high nutrient supply (Carter et al. 2007), and the warm temperatures stimulated soil microorganisms to form 10-OH-$C_{18:0}$ rapidly in the topsoil in 11-18 days. Fatty acids were preserved

over one year in the soil samples but, given the fact that oxygen supply is sufficient after the body was removed, the overall abundance of fatty acids will probably decline over the years as fatty acids will be degraded.

## 4.5. Conclusions

This study was conducted in order to monitor the presence of human derived fatty acids over time in a soil environment under natural conditions. Total fatty acid concentrations revealed an exogenous input 11-18 days after the deposition of the body and enhanced concentrations remained in soil samples one year after the body was removed from the crime scene. Relative abundances of fatty acids showed that extensive hydrogenation of unsaturated fatty acids occurred, as indicated by higher abundances of saturated fatty acids after 11-18 days and also after one year. Over time, fatty acid patterns changed slightly, indicated by the decline of myristic acid and 10-hydroxystearic acid likely induced by microbial β-oxidation. The predominant abundance of 10-hydroxystearic acid and fatty acid salts showed that adipocere was formed, presumably in an anaerobic environment resulting from the direct contact of the body to the soil surface during the period of 11-18 days. The removal of the body and subsequent promotion of an aerobic environment resulted in the slow degradation of fatty acids, which is indicated by the decline of myristic acid. However, the increase of the water insoluble $Ca^{2+}$ and $Mg^{2+}$ salts likely prevented the degradation by microbes as access to fatty acids was inhibited. This study therefore concluded that human derived fatty acids were preserved in soil for one year after the removal of the body. Further research should be conducted to determine if the formation of adipocere from decomposition fluids leaching into soil in close contact with a decomposing body is a natural or an exceptional process wholly dependent on the environmental conditions where a body is deposited.

## 4.6. References


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# Chapter 5

# Steroids – promising biomarkers for the identification of temporary graves from World War II

# Abstract

Steroids are widely used for the detection of faecal matter and – in recent years – also for characterising human decomposition in the environment. Until now it was not clear, whether all commonly used faecal (i.e. stanols, stanones and bile acids) and tissue steroids (i.e. cholesterol) could reveal the presence of human decomposition products in temporary graves. In this study, soil was sampled from three putative mass graves where concentration camp prisoners were buried for 10 months at the end of World War II (1944-45). We hypothesised that the putative mass grave exhibits elevated contents of faecal and tissue steroids compared to control samples, thereby reflecting the former input of human decomposition fluids. Steroids ($\Delta^5$-sterols, 5β-stanols, 5α-stanols, stanones and bile acids) from soil samples of the putative mass graves and control soil samples were analysed with gas chromatography/mass spectrometry (GC/MS). Significantly elevated ($p < 0.05$) contents of coprostanol, 5α-cholestanol and 5α-cholestanone were detected in one pit compared to the reference. Lithocholic acid indicated an introduction of faecal material in two soil pits, presumably of human origin. Selected steroids (epicoprostanol, epi-5β-stigmastanol and isolithocholic acid) were unique components for the soil pit fillings as they were not found in the reference. In combination with earlier findings, steroid and bile acid patterns showed an input of faecal and tissue constituents from the former buried bodies at least in one of the three soil pits.

## 5.1. Introduction

A temporary grave can be defined as soil pit, where a human body was deposited for a certain period of time before its subsequent removal. Without a body, a temporary grave is unlikely to be recognisable upon first sight, but the construction of a grave can leave homogenised soil material, changes in vegetation cover or the concealed remains of bodies (e.g. decomposition products; Hunter et al. 2013; Carter and Tibbett 2008; Carter et al. 2007). During decomposition of a buried cadaver it is probable that, during the active and advanced stages of decomposition, tissue constituents are leaching into the surrounding environment (Carter and Tibbett 2008). When a body is deposited on a soil surface or buried in soil, constituents of the decomposition fluids remain as decay markers after removal of the body and may even be preserved for a longer period of time (Forbes et al. 2002; Davies and Pollard 1988). Tissue and faecal steroids are useful decay markers because they are specific constituents as well as relatively stable against decomposition in a soil environment (Bull et al. 2002; Evershed and Connolly 1994).

The common $\Delta^5$-sterol of higher animal tissues is cholesterol, but it is also present as a minor constituent of plants, fungi and other eukaryotes and is thus ubiquitous in soils (Christie and Han 2010; Weete et al. 2010; Mouritsen and Zuckermann 2004). During digestion, cholesterol is microbially reduced to coprostanol (5β-stanol) in the gut of higher animals (Björkhem and Gustafsson 1971). In human faeces coprostanol occurs in large amounts and is therefore utilised as human faecal marker in archaeological and environmental pollution studies (Bull et al. 2002; Leeming et al. 1996; Bethell et al. 1994). The plant derived faecal 5β-stanol 5β-stigmastanol is also found in human faeces (Prost et al. (in prep, a); Leeming et al. 1996). In soils, the usual reduction product of cholesterol is the 5α-stanol 5α-cholestanol, but it can also be found in minor contents in animal tissues, faeces and plants (Noda et al. 1988; Hatcher and McGillivary 1979; Daniellson and Tchen 1968; Murtaugh and Bunch 1967). When coprostanol and 5β-stigmastanol are excreted into the environment, they can be further transformed to epicoprostanol and epi-5β-stigmastanol (Bull et al. 2002; McCalley et al. 1981; Quirk et al. 1980; Wardroper and Maxwell 1978). Epicoprostanol is found in minor concentrations in human faeces (Prost et al. (in prep, a)). Stanones (5α-cholestanone and 5β-cholestanone) are intermediate steroidal ketones of the intestinal and environmental microbial $\Delta^5$-sterol transformation to 5β-stanols and epi-5β-stanols (Grimalt et al. 1990; Gaskell und Eglinton 1975; Björkhem und Gustafsson 1971). In contrast, bile acids are only produced by vertebrates and - compared to other steroids - are highly specific markers for faeces and dead animal remains (Hofmann und Hagey 2008; Haslewood 1967). Primary bile acids (e.g. cholic acid, chenodeoxycholic acid) are produced from cholesterol in the liver of vertebrates. When excreted into the gut they get further converted to secondary bile acids (e.g. lithocholic acid, deoxycholic acid, isolithocholic acid) by microorganisms (Bull et al. 2003; Bull et al. 2002; Stellwag and Hylemon 1979; Hayakawa 1973).

Because animal (including human) faeces comprise different steroid patterns, they have been mainly used to distinguish between faecal sources in environmental and archaeological studies (Prost et al. (in prep, a); Bull et al. 2002; Elhmmali et al. 2000; Elhmmali et al.1997; Leeming et al. 1996). Steroids are constituents of human tissues and

faeces and should therefore be released from a body during decomposition. Recent studies were able to detect either cholesterol and coprostanol up to three months post-mortem (von der Lühe et al. 2013) or cholesterol in graves containing human skeletal remains from 400-700 AD (Davies and Pollard 1988) and 7400-6000 BC (Shillito et al. 2011). Lithocholic acid and deoxycholic acid, characteristic bile acids in human faeces were found in graves by Shillito et al. (2011). Due to their diagnostic potential and their long lasting presence in soils steroids are interesting markers for forensic investigations, e.g. to identify human decomposition fluids in temporary graves. However, in recent studies steroid analyses were performed on soil samples, where bodies were always present during sampling (von der Lühe et al. 2013; Shillito et al. 2011; Davies and Pollard 1988). In temporary graves it is assumed that bodies are removed before they have completely decomposed and, as such, it is not clear (I) whether steroid analyses reveal sufficient information to identify human decomposition fluids in temporary graves and (II) for how long those components remain preserved to enable them to be distinguished from steroids and bile acids present from the natural background.

This study investigated if steroids can be used as markers of human decomposition fluids from a temporary grave. Soil samples were taken from former mass graves in Germany that had been in use for 10 months during World War II (1944-45), exhumed and refilled thereafter. Soil samples were analysed for their steroid ($\Delta^5$-sterols, stanols, stanones and bile acids) patterns and compared to reference soil samples taken in close proximity. We hypothesised that tissue (cholesterol, 5α-cholestanol, 5α-cholestanone and primary bile acids), faecal (coprostanol, 5β-stigmastanol, 5β-cholestanone, epicoprostanol and secondary bile acids) and faecal derived (cholesterol, 5α-cholestanol, 5α-cholestanone and primary bile acids) steroids are enhanced in the mass graves compared to reference soil, thereby identifying the former human decomposition fluids of the temporary graves.

## 5.2. Material and Methods

### *5.2.1. Case study*

During late 1944 to October 1945, 66 concentration camp prisoners were buried for a period of 10 months in a municipal forest close to Stuttgart, Germany. After World War II occupying forces exhumed the bodies and buried them in a cemetery nearby. 64 years later the knowledge about the exact location of those former mass graves was inconsistent (Faltin 2008). Fiedler et al. (2009) were able to locate the position of the mass graves by using a combination of historical evidence and soil mapping, finding three pits (pit 1, pit 2, pit 3) in the area where the mass graves were most likely to have been located (Fig. 5.1; Fiedler et al. 2009). Characteristic soils in this area were Planosols and Gleysols (Schad 2008). Chemical analysis of soil sampled along a transect through pit 2 and 3 (Fig. 5.1) revealed elevated phosphorus and organic carbon contents to a soil depth of 50 and 75 cm compared to soil taken between the two pits (intermediate space; Fiedler et al. 2009). During the construction of the test trench of pit 2, a pivot crown ("Richmond" crown) was found, which was identified as dental prosthesis used in Germany around 1950 (Fiedler et al. 2009). Due to the findings of Fiedler et al. (2009), the possibility that the three discovered soil pits were the actual mass graves is relatively high. By analysing steroids as decomposition markers a highly sensitive method is introduced to identify human decomposition fluids in temporary graves in the present study.

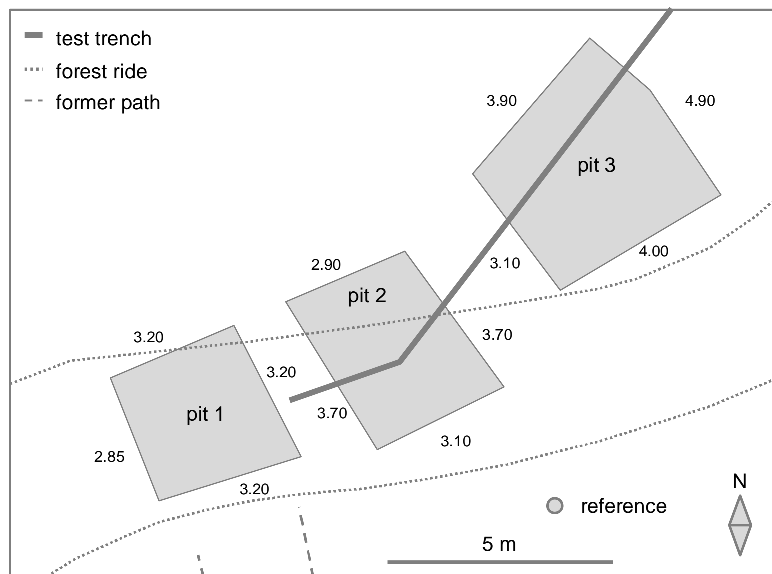


**Figure 5.1:** Map (after Fiedler et al. 2009) of the location of the temporary mass graves (burial of 66 bodies for 10 months) from late 1944 to early 1945 in a municipal forest close to Stuttgart, Germany. The test trench was constructed during the first search of the former mass graves in 2009 (Fiedler et al. 2009).

### *5.2.2. Soil sampling*

In 2012 (67 years after exhumation) soil was sampled for lipid biomarker analysis with a clean auger at two sampling points in each pit (Pürckhauer; 100 cm). Pit samples were taken at a depth of 40-50 cm (n=2) and 50-60 cm (n=2)

below the soil surface (in the following sections referred to as subsoil), where highest total phosphorus and organic carbon contents were previously found (Fiedler et al. 2009). Reference samples were taken at ca. 5 m distance to pit 1 and pit 2, at three sampling points and at two depths of 40-50 cm (n=3) and 50-60 cm (n=3). To determine the natural background of steroids in the topsoil (0-40 cm) additional soil samples from pit 2 and the reference were collected at a depth of 0-10 cm, 10-20 cm and 20-40 cm.

### *5.2.3. Steroid analysis*

Steroid analytics followed the protocol described in Chapter 2, with slight modification according to a method from Birk et al. (2012). Soil samples were freeze-dried for 24 h, sieved ≤ 2 mm, and finely ground in a ball mill (450 rpm, 5 min). The soil samples were weighed (10 g) into fibreglass thimbles and subsequently covered with quartz sand (both combusted at 300°C for 12 h). Extraction was carried out with a Soxhlet apparatus, using a dichloromethane/methanol (2:1, v/v, 150 ml) solvent system. The total lipid extract (TLE) was obtained after an extraction of 36 h. Solvent was removed by using a rotary evaporator (Büchi Rotavapor R-3, Essen, Germany) and the residue TLE was dried under a gentle stream of nitrogen ($N_2$). Steroids analysed in this study are listed in Table 5.1.

A sterol internal standard (IS I) cholesterol-d7 (50 µl; 10 ng $µl^{-1}$), a stanone IS I 5α-pregnan-3-one (250 µl; 2 ng $µl^{-1}$) and a bile acid IS I isodeoxycholic acid (250 µl, 2 ng $µl^{-1}$; Tab. 5.1) was added to the TLE to correct any sample loss during extraction. Samples were dried under nitrogen. Saponification was carried out with 1.5 ml 0.7 M methanolic KOH at room temperature overnight for 12 h. Extracts were heated to 50-60°C on a dry block heater, and neutral lipids (containing sterols, stanols and stanones) were separated by liquid-liquid extraction by adding 4 x 1.5 ml *n*-heptane and 0.5 ml water (18-MΩ grade). To obtain the acid fraction, including bile acids, the water/saponification solution was acidified to pH ≤ 2 with 6 M HCl and bile acids were extracted with 4 x 1.5 ml chloroform. Both fractions were dried under a gentle stream of $N_2$.

The neutral lipid extract was redissolved in 500 µl *n*-heptane and transferred to solid phase extraction (SPE) glass columns containing silica gel (60 Å, 0.063-0.200 mm, Merck, Darmstadt, Germany; 1 cm bed volume, combusted at 380°C for 4 h and activated at 200°C for 6 h, preconditioned with 2 x 1.5 ml *n*-heptane). To elute any hydrocarbons, columns were washed with 2 x 1.5 ml *n*-heptane. The fraction containing steroids and alcohols was collected by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (80:20, v/v). Eluates were dried under nitrogen. To convert steroids to their trimethylsilyl derivates, 50 µl of *N,O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS; 99:1, v/v; Steinheim, Germany)/pyridine (3:1, v/v) was added and extracts were heated at 90°C for 1 h. After derivatisation, the mixture was evaporated and 100 µl of 5α-cholestane (5 mg $l^{-1}$ in toluene, IS II; Tab. 5.1) was added.

The acid fraction was methylated by adding 1 ml dry 1.25 M HCl in methanol (Fluka, Sigma-Aldrich, St-Louis, MO, USA) and heating at 80°C for 2 h. The methyl esters were extracted by adding 1 ml water (18-MΩ grade) and 3 x 1.5 ml *n*-hexane. Extracts were dried under a gentle stream of nitrogen. SPE was carried out to separate bile acid methyl esters from fatty acid methyl esters. Therefore, glass SPE columns containing 1 ml bed volume of silica gel

(Polygoprep 100-130, Macherey-Nagel, Düren, Germany) activated at 120°C for 12 h, were preconditioned with 2 x 1.5 ml *n*-hexane. Extracts were redissolved in 500 µl *n*-hexane and transferred to the SPE columns. SPE columns were preconditioned with 2 x 1.5 ml *n*-hexane. Fatty acid methyl esters were eluted to waste with 4 x 1 ml dichloromethane/*n*-hexane (2:1, v/v). Bile acids were collected by adding 4 x 1 ml of dichloromethane/methanol (2:1, v/v). Eluates were dried under a gentle stream of $N_2$. To convert bile acids methyl esters to their trimethylsilyl derivates, 100 µl of BSTFA/1-(Trimethylsilyl)imidazole (98:2, v/v; Supelco, Bellefonte, PA, USA) and 50 µl of toluene were added, and extracts heated at 90°C for 1 h. After derivatisation, the mixture was evaporated with $N_2$ and 100 µl of 5α-cholestane (5 mg $l^{-1}$ in toluene, IS II; Tab. 5.1) was added.

**Table 5.1:** List of steroids analysed in the pit and reference soil samples of the putative mass graves. Steroids were measured in SIM-mode with listed quantifier and qualifier ions.

| trivial name | substance | compound group | RT[a] min | quantifier *m/z* | qualifier *m/z* | |
|---|---|---|---|---|---|---|
| 5α-pregnan-3-one[b] | | IS I stanones | 19.0 | 302 | 217 | |
| 5α-cholestane[c] | | IS II | 20.3[f], 22.9[g] | 217 | 357 | 372 |
| coprostanol[c] | 5β-cholestan-3β-ol | 5β-stanol | 26.6 | 370 | 355 | 215 |
| epicoprostanol[c] | 5β-cholestan-3α-ol | 5β-stanol | 27.2 | 355 | 370 | 215 |
| 5β-cholestanone[b] | 5β-cholestan-3-one | stanone | 28.2 | 386 | 231 | 107 |
| cholesterol-d7[d] | | IS I sterols, stanols | 28.6 | 336 | 375 | 465 |
| cholesterol[c] | cholest-5-en-3β-ol | $\Delta^5$-sterol | 28.7 | 329 | 368 | 458 |
| 5α-cholestanol[c] | 5α-cholestan-3β-ol | 5α-stanol | 29.0 | 445 | 355 | 460 |
| 5α-cholestanone[c] | 5α-cholestan-3-one | stanone | 29.3 | 386 | 231 | |
| 5β-stigmastanol[e] | 5β-stigmastan-3β-ol | 5β-stanol | 31.5 | 398 | 383 | 215 |
| epi-5β-stigmastanol[e] | 5β-stigmastan-3α-ol | 5β-stanol | 32.2 | 398 | 383 | 215 |
| IDCA[b] | isodeoxycholic acid | IS I bile acids | 24.8 | 255 | 355 | 370 |
| ILCA[b] | isolithocholic acid | bile acid | 27.3 | 215 | 257 | 357 |
| LCA[c] | lithocholic acid | bile acid | 28.2 | 215 | 257 | 372 |
| DCA[c] | deoxycholic acid | bile acid | 29.1 | 255 | 345 | 370 |
| CDCA[c] | chenodeoxycholic acid | bile acid | 30.0 | 255 | 355 | 370 |

[a]retention time │Purchased from [b]Steraloids (Newport, RI, USA) │[c]Sigma Aldrich (Steinheim, Germany) │[d]Avanti (Alabaster, AL, USA) │[e]Chiron (Trondheim, Norway) │[f]retetion time of the $\Delta^5$-sterol, stanol and stanone method │ [g]retention time of the bile acid method

### 5.2.4. GC/MS analysis and quantification

Analysis was performed on an Agilent 6890 gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). A DB-5ms Ultra Inert (Agilent, Santa Clara, CA, USA) fused silica capillary column (30 m length x 250 µm internal diameter x 0.25 µm film thickness) was used with helium (99.9995%) as a carrier gas at 1.1 ml $min^{-1}$ constant flow. For steroids, the injection port was maintained at 250°C and 1 µl was injected in splitless mode. The initial oven temperature was held at 80°C for 1.5 min, then programmed at 12°C $min^{-1}$ to 265°C, at 0.8°C $min^{-1}$ to 280°C and at 10°C $min^{-1}$ to 300°C, and held for 12 min. For the analysis of bile acids, a comparable method was used except that the injector port was set at 290°C and the column flow was 1.0 ml $min^{-1}$. The

temperature program for bile acids was as follows: 80°C initial time, hold for 1.5 min, 20°C $min^{-1}$ to 250°C, 1.2°C $min^{-1}$ to 280°C and 10°C $min^{-1}$ to 300°C. For both lipid fractions, the solvent delay was 20 min and electron ionisation was set at 70 eV. Samples were run in selected ion monitoring mode (SIM); quantification ions in Tab. 5.1. Quantification was carried out by comparing the integrated peak area of the quantification ion with the injection internal standard quantification ion (IS II 5α-cholestane *m/z* 217). Concentrations in ng $g_{freezed\ dried\ soil}^{-1}$ (in all following sections µg $g_{soil}^{-1}$) were calculated with an external calibration ($R^2 \geq 0.98$) of each individual analyte in respect of the injection IS II. Recovery surrogates of the sterol and stanol IS I was 127±7%, the stanone IS I was 109±19% and the bile acid IS I was 100±19%.

Partial SIM-chromatograms of the characteristic ions (Tab. 5.1) of each silylated steroid are shown in Figure 5.2 and Figure 5.3. In Figure 5.2, epicoprostanol eluted at similar retention times than the secondary alcohol nonacosan-10-ol having characteristic mass fragments at *m/z* 229 and 369 (Dragota und Riederer 2007). Therefore, the quantifying ion of epicoprostanol was modified from *m/z* 370 to *m/z* 355, which did not interfere with the mass spectrum of nonacosan-10-ol.

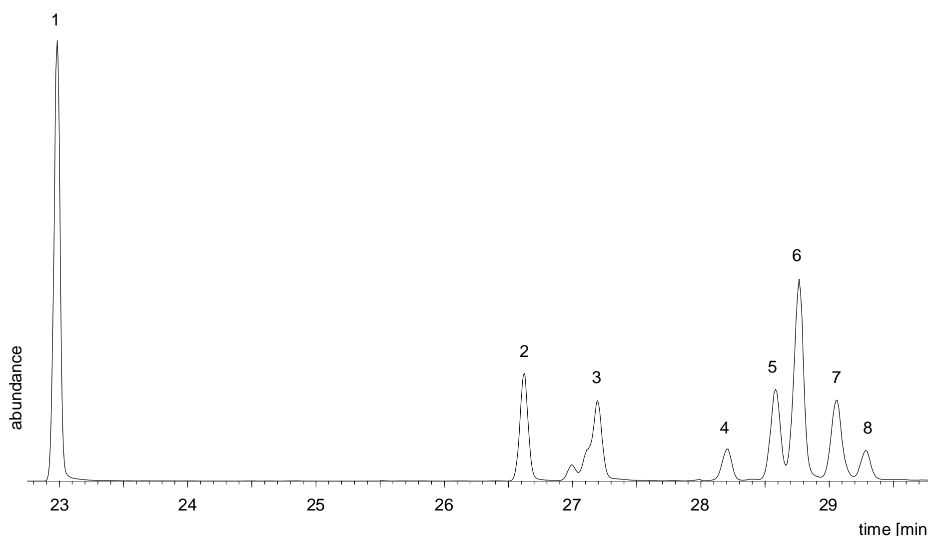


**Figure 5.2:** Partial SIM-chromatogram of analysed characteristic ions of silylated Δ5-sterols, stanols and stanones of one sample from pit 2. Peaks (1) 5α-cholestane IS II, (2) coprostanol, (3) epicoprostanol, (4) 5β-cholestanone, (5) cholesterol-d7 IS I, (6) cholesterol, (7) 5α-cholestanol, (8) 5α-cholestanone.

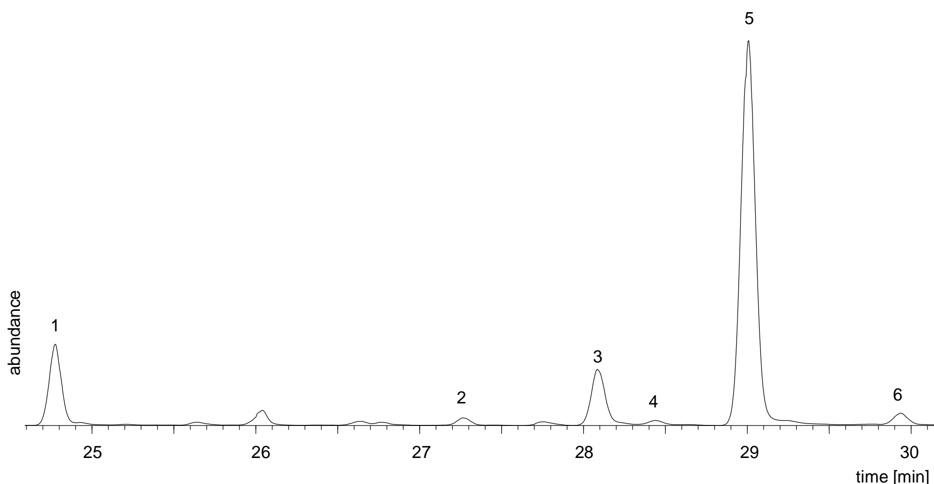


**Figure 5.3:** Partial SIM-chromatogram of analysed characteristic ions of silylated bile acid methyl esters of one sample from pit 2. Peaks (1) isodeoxycholic acid IS I, (2) isolithocholic acid, (3) lithocholic acid, (4) unidentified, (5) deoxycholic acid, (6) chenodeoxycholic acid.

### *5.2.5. Statistical analysis*

Statistical analyses were performed on summarised data obtained from the analysis of soil samples taken at a depth of 40-60 cm (pits: 2 x 40-50 cm and 2 x 50-60 cm to n=4, reference 3 x 40-50 cm and 3 x 50-60 cm to n=6) with SPSS (Version 22, IBM Corporation, New York, USA). A one-way analysis of variance (ANOVA) was carried out, after the data was tested for normal distribution with the Shapiro-Wilk test and homogeneity of variances (Levene test). The Dunnett's post-hoc test was applied, to test for significant differences of lipid concentrations from pit 1, pit 2 and pit 3 compared to the reference soil. A significance level of 0.05 was selected for all tests.

## 5.3. Results

### *5.3.1. Topsoil (0-40 cm) of pit 2 and reference*

The dominant Δ5-sterols and stanols found in the reference and pit 2 were cholesterol and 5α-cholestanol, showing a decline with increasing soil depth (0-40 cm; Fig. 5.4a-c). Further stanols and stanones found in the reference and pit 2 were 5α-cholestanone and coprostanol (0-40 cm; Fig. 5.4a-c). Additionally, 5β-cholestanone (0-10 cm), 5β-stigmastanol and epi-5β-stigmastanol (0-40 cm) were only detected in pit 2 (Fig. 5.4a-c).

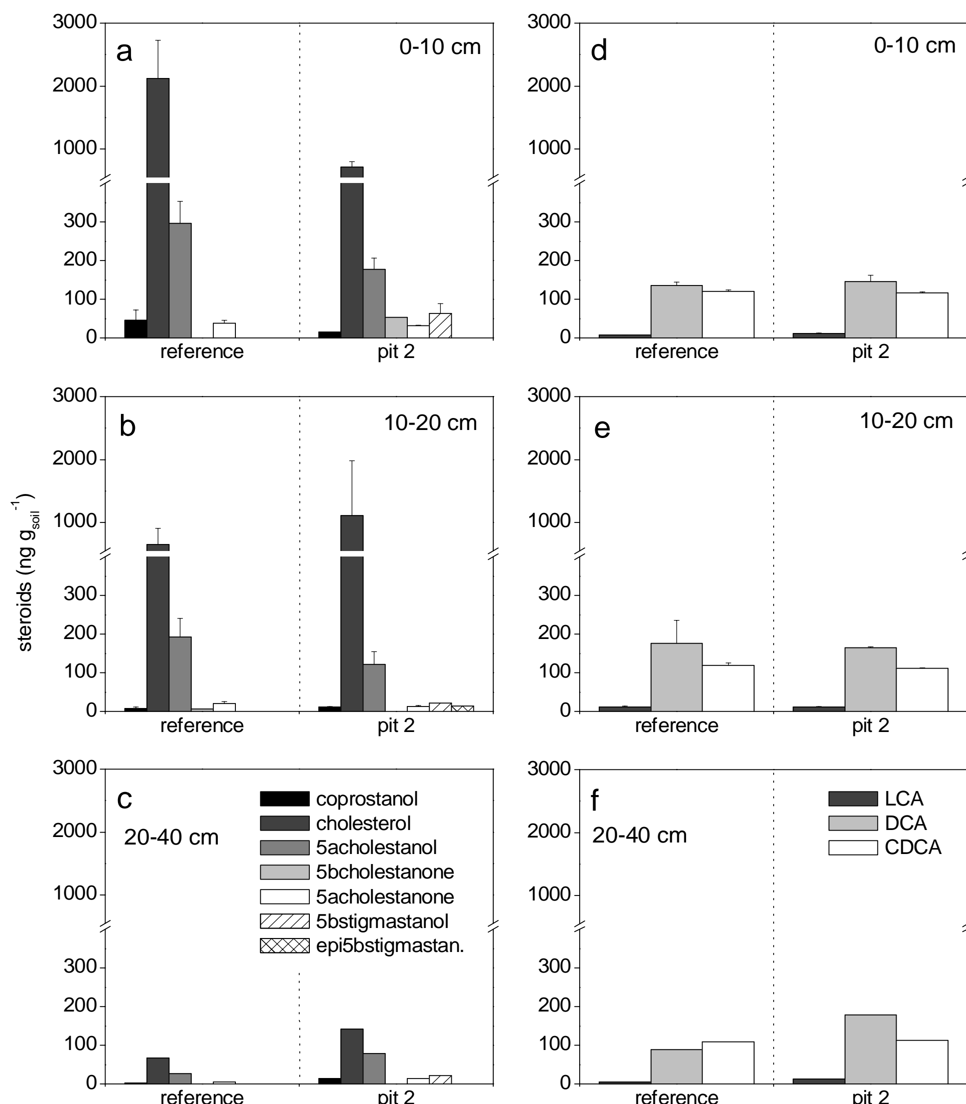


**Figure 5.4:** Δ5-sterols, stanols, stanones and bile acids in ng $g_{soil}^{-1}$ from reference topsoil and pit 2 topsoil: (a) Δ5-sterols, stanols, stanones, 0-10 cm, reference n=3, pit 2 n=2; (b) Δ5-sterols, stanols, stanones, 10-20 cm, reference n=3, pit 2 n=2; (c) Δ5-sterols, stanols, stanones, 20-40 cm, n=1; (d) bile acids, 0-10 cm, reference n=3, pit 2 n=2; (e) bile acids, 10-20 cm, reference n=3, pit 2 n=2; (f) bile acids, 20-40 cm, n=1; error bars = standard deviation (n=3), standard error (n=2); epi-5β-stigmastan. = epi-5β-stigmastanol; LCA = lithocholic acid; DCA = deoxycholic acid; CDCA = chenodeoxycholic acid.

Bile acid distributions did not considerably differ between reference and pit 2 in a depth of 0-20 cm (Fig. 5.4d-f). Bile acids further decreased in the reference topsoil between 20-40 cm while bile acid patterns in pit 2 in a depth of 20-40 cm were comparable to concentrations detected ≤ 20 cm (Fig. 5.4d-f).

### *5.3.2. Subsoil (40-60 cm) of pit 1-3 and reference*

Cholesterol and deoxycholic acid (DCA) had the largest contents of all analysed steroids in all pit fillings and the reference (Fig. 5.5a, b). However, regarding summed concentrations of faecal steroids and their epimers significant for faecal matter (i.e. coprostanol, 5β-stigmastanol, epicoprostanol, epi-5β-stigmastanol, 5β-cholestanone and bile acids) only pit 2 revealed significantly elevated ($p < \mathbf{0.05}$) concentrations compared to the reference (Fig. 5.6).

Significantly greater concentrations ($p < \mathbf{0.05}$) of the faecal steroid coprostanol and the cholesterol transformation products 5α-cholestanol and 5α-cholestanone were found only in one pit (pit 2) compared to the reference (Fig. 5.5a). Additionally, 5β-cholestanone was elevated in pit 2 compared to the reference, where it was found in traces in one (2 ng $g_{soil}^{-1}$; Fig. 5.5a) of the six samples. Epicoprostanol was exclusively detected in pit 2 (15±4 ng $g_{soil}^{-1}$; Fig. 5.5a). Traces of the faecal stanols 5β-stigmastanol and its environmentally produced epimer epi-5β-stigmastanol were found in pit 1 and pit 3, whereas larger concentrations of 5β-stigmastanol were detected in pit 2 (Fig. 5.5a).

The bile acids lithocholic acid (LCA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) were found in the reference soil and in the three pit fillings (Fig 5.5b). Highest DCA concentrations were detected in all pit fillings compared to the reference (Fig. 5.5b). The LCA concentrations were significantly larger ($p < \mathbf{0.05}$) in pit 2 and pit 3 than in the reference (Fig. 5.5b). ILCA was exclusively found in pit 2 and pit 3, and was not detected in the reference samples (Fig. 5.5b).

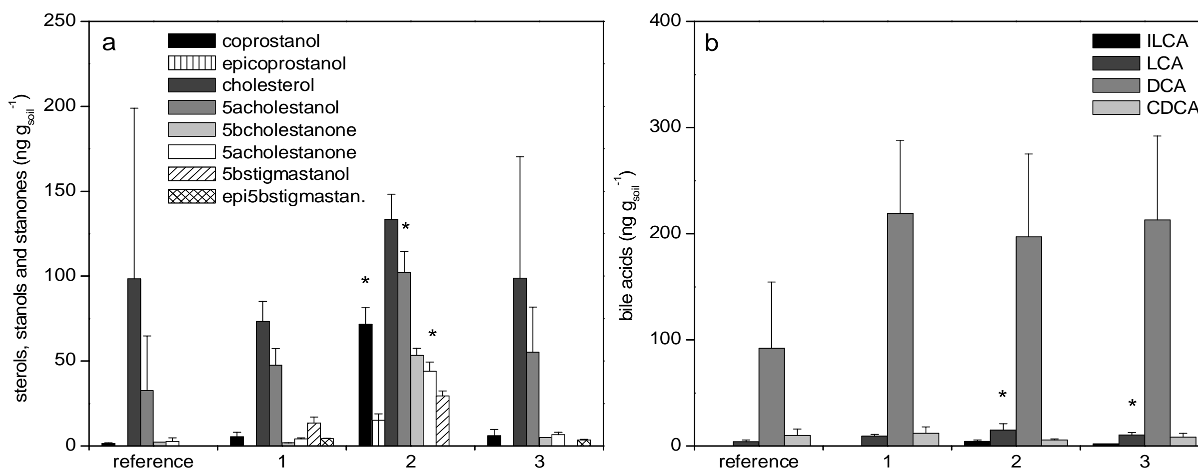


**Figure 5.5:** (a) Δ⁵-sterols, stanols and stanones and (b) bile acids (ng $g_{soil}^{-1}$ ± standard deviation, n=4) extracted from putative temporary mass graves (pit 1, pit 2 and pit 3) and reference soil at a depth of 40-60 cm; * = significant difference to the reference ($p < \mathbf{0.05}$). epi-5β-stigmastan. = epi-5β-stigmastanol; ILCA = isolithocholic acid; LCA = lithocholic acid; DCA = deoxycholic acid; CDCA = chenodeoxycholic acid.

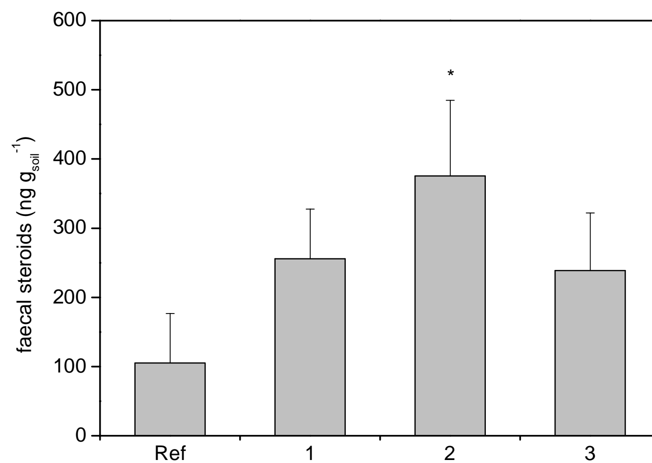


**Figure 5.6:** Summed concentrations (ng $g_{soil}^{-1}$ ± standard deviation, n=4) of faecal steroids and their epimers (coprostanol, epicoprostanol, 5β-cholestanone, 5β-stigmastanol, epi-5β-stigmastanol, isolithocholic acid, lithocholic acid, deoxycholic acid, chenodeoxycholic acid) extracted from soil sampled in a depth of 40-60 cm of predicted temporary mass graves (pit 1, pit 2, pit 3) and from reference soil. * = significant difference to the reference ($p < 0.05$).

## 5.4. Discussion

The aim of this study was to characterise the potential use of tissue (cholesterol, 5α-cholestanol, 5α-cholestanone and primary bile acids), faecal (coprostanol, 5β-stigmastanol, 5β-cholestanone, epicoprostanol and secondary bile acids) and faecal derived (cholesterol, 5α-cholestanol, 5α-cholestanone and primary bile acids; see also Table 1.1 in Section 1.1.2) steroids as markers of human decomposition fluids in soils. Their suitability was tested on soil pit fillings from putative mass graves from World War II.

The mass graves represent areas which have undergone a high level of disturbance during digging and filling of the graves and the reopening after a period of 10 months. The natural distribution of steroids along a soil profile represented by the reference soil was found to be disturbed by the human impact associated with the graves. As a result, an all over homogenisation of steroids from the natural background (reference) and the decomposition fluids occurred. This includes the probability that high abundances of cholesterol, 5α-cholestanol and 5α-cholestanone were introduced into lower soil depth. The ubiquitous presence of cholesterol in the pit fillings and the reference (Fig. 5.5a) could also be explained by additional sources and entry paths, like root exudates, plant material, fungi and other eukaryotes (Christie and Han 2010; Weete et al. 2010; Mouritsen and Zuckermann 2004; Thompson and Hale 1983). Cholesterol is not a stable component in soils and is further microbially reduced to 5α-cholestanol (Bull et al. 2002). 5α-cholestanone was also found in anoxic depositional sediments (Boudou et al. 1986; Robinson et al. 1986; Robinson et al. 1984), derived from microbial reduction of cholesterol, indicating that these reduction processes can occur outside the human gut and tissues. Because cholesterol, 5α-cholestanol and 5α-cholestanone are naturally present in soil environments, they were not suitable markers to identify decomposition fluids of human bodies in this study.

Cholesterol was found in association with decomposing human bodies in previous studies. Davies and Pollard (1988) found cholesterol levels enhanced three-fold beneath a skeleton in an Anglo-Saxon grave compared to reference soil samples (c. 400-700 AD). Shillito and co-workers (Shillito et al. 2011) detected cholesterol (2.6 $\mu g\ g^{-1}$) in burials containing skeletons at Neolithic Çatalhöyük (c. 7400-6000 BC) suggesting an origin of animal derived organic material. No reference samples were determined to indicate increased cholesterol levels in the burial samples (Shillito et al. 2011). Both studies demonstrated that cholesterol was present in close proximity of skeletons over a long period of time, whereas no differences were found in the present study between the reference soil and cholesterol levels in the temporary mass graves. One reason for the long-term presence of cholesterol in both of the previously detailed studies is that the entire body was able to decompose, whereas in the present study bodies were removed after 10 months, only allowing a partial decomposition.

The abundance of 5α-cholestanol (102±12 ng $g_{soil}^{-1}$) and 5α-cholestanone (44±5 ng $g_{soil}^{-1}$) in pit 2, suggests that higher cholesterol levels were previously present (Fig. 5.5a). Similar processes were found in a study where cholesterol from decomposition fluids was rapidly converted to 5α-cholestanol (0-5 cm soil depth) of soil sampled one year after a body was removed from the soil surface (Chapter 3). Evershed and Connolly (1994) found cholesterol,

5α-cholestanol and 5α-cholestanone in the skin of a bog body. They assumed that enzymes from the surrounding peat were involved in the post-mortem decay of cholesterol to 5α-cholestanol and 5α-cholestanone (Evershed and Connolly 1994). In our study, the presence of cholesterol, 5α-cholestanol and 5α-cholestanone in the reference soil shows, that the transformation of cholesterol to 5α-cholestanol is a natural microbial reduction process. However, contributions of 5α-cholestanol and 5α-cholestanone to the soil might also derive from the decomposition of human tissue and from human faeces, where they are known to be minor constituents (Prost et al. (in prep, a); Daniellson and Tchen 1968).

Soil samples from pit 2 contained substantial amounts of faecal stanols and stanones (coprostanol, epicoprostanol, 5β-cholestanone and 5β-stigmastanol) with coprostanol having significant larger concentrations compared to the reference. In contrast, stanol and stanone contents (coprostanol, 5β-cholestanone and 5β-stigmastanol) of pit 1 and pit 3 were partly enhanced in a depth of 40-60 cm. These results indicate an input of faecal material, presumably derived from the intestinal contents of the decomposing bodies. Coprostanol and 5β-stigmastanol are the major stanols in human faeces, epicoprostanol is a minor constituents (Prost et al. (in prep, a); Gérard 2014; Bull et al. 2002; Bartlett 1987; Korpela 1982). Similar findings beneath decomposing pig carcasses and a human body (von der Lühe et al. 2013; Chapter 3) confirmed the presence of faecal 5β-stanols (coprostanol, epicoprostanol and 5β-stigmastanol) in decomposition fluids. Because of the near absence of faecal stanols and stanones in the reference samples, the presence of faecal stanols and stanones in the soil pits was more indicative of decomposition fluids than cholesterol, 5α-cholestanol and 5α-cholestanone.

Epicoprostanol could only be found in pit 2, indicating its important role as a single biomarker for the introduction of external organic matter in this study. Amongst faecal matter from the corpses, which was presumably one source of epicoprostanol, epicoprostanol found to be produced from coprostanol under anaerobic conditions (Bull et al. 2002; McCalley et al. 1981). Epicoprostanol was found in aged sewage sludge (Wardroper and Maxwell 1978) and in soils (Simpson et al. 1998). Furthermore, epi-5β-stigmastanol was found in pit 1 and pit 3 in a depth of 40-60 cm. Epi-5β-stigmastanol was detected during compost formation (Prost et al. (in prep, b)) and in aged sewage sludge (McCalley et al. 1981), presumably formed from 5β-stigmastanol. In this study, the predominately waterlogged soils (Planosols and Gleysols) likely favoured the microbial epimerisation of coprostanol to epicoprostanol and 5β-stigmastanol to epi-5β-stigmastanol (Bull et al. 2002; Bartlett 1987; McCalley et al. 1981; Quirk et al. 1980; Wardroper and Maxwell 1978).

The ketone 5β-cholestanone is an intermediate product of the conversion of cholesterol to coprostanol and epicoprostanol (Eyssen et al. 1973; Björkhem and Gustafsson 1971; Rosenfeld and Hellmann 1971) and was found in the subsoil (40-60 cm) of pit 2. Low coprostanol and 5β-cholestanone concentrations in the reference top- and subsoil suggested that the conversion to 5β-stanols at the study site was not a natural process, but derived from an input of faecal material in pit 2. Similar findings were observed by Evershed and Connolly (1994) who found, amongst others, cholesterol, coprostanol, 5α-cholestanol, 5α-cholestanone and 5β-cholestanone to be well preserved in bog bodies,

but no conversion of cholesterol to its 5β-stanol in the surrounding peat. They suggested that the reaction to 5β-stanols is minor, because the associated microorganisms are not present in the peat (Evershed and Connolly 1994). In the environment, 5α-cholestanol is preferentially produced from cholesterol, whereas microorganisms in the intestines are able to convert $\Delta^5$-sterols to their corresponding 5β-stanols due to their stereospecific enzymes (Eyssen et al. 1973; Björkhem and Gustafsson 1971; Rosenfeld and Hellmann 1971). Therefore, the presence of 5β-cholestanone in the topsoil and the subsoil of pit 2 likely indicated an input of faecal matter from the corpses.

In contrast to $\Delta^5$-sterols, stanols and stanones, bile acids are very specific biomarkers for the input of faecal material as they are only produced by vertebrates. If combined with stanol analytics, it may even be possible to determine the source of the faecal material (Tyagi et al. 2007; Simpson et al. 1999; Leeming et al. 1997; Leeming et al. 1996; Gülaçar et al. 1990; Knights et al. 1983). Furthermore, bile acids are supposed to be more stable against decomposition compared to 5β-stanols (Elhmmali et al. 1997) and are hence valuable biomarkers to detect ancient faecal material in soils (Evershed 2008; Bull et al. 2002; Bull et al. 1999a; Bull et al. 1999b). As far as the authors know, there is only one study that determined bile acids in a grave containing human skeletal remains. Shillito et al. (2011) found LCA in one and DCA in two of five burial samples at Neolithic Çatalhöyük (c. 7400-6000 BC). However, no reference sample was analysed in that study; it is therefore uncertain if DCA and LCA from the burials were from the decomposition of the bodies or naturally present (Shillito et al. 2011).

In this study, the proposed addition of human bile acids from faeces and tissues to the natural existing bile acids deriving from wildlife has to be considered. The absence of hyodeoxycholic acid (HDCA) in all analysed soil samples excludes a contribution of pig faeces (Matschiner 1971). CDCA is a primary bile acid and produced from cholesterol in the liver (Bull et al. 2002). CDCA is found in high abundances in bird faeces, but it is also a ubiquitous bile acid and widely distributed among mammalian species (Prost et al. (in prep, a); Matschiner 1971). It was detected in all soil pits and the reference and did not show any particular difference. Hence, CDCA is either introduced by the homogenisation of steroids during mass grave construction or it was additionally introduced by the decomposition of the bodies. The abundance of LCA and DCA in the reference soil is attributed to faecal matter input from wildlife. As there is no published data on natural bile acid distributions in forest soils, it is difficult to evaluate bile acid concentrations found at the study site. Hence, it cannot be ruled out that the former areas were used as agricultural fields, where bile acids might have been introduced by organic fertilisers.

In terms of concentrations, the order of bile acids in human faeces is DCA > LCA > ILCA > CDCA (Prost et al. (in prep, a)). High abundances of DCA (pit 1, pit 2, pit 3; Fig. 5.5b) and significantly enhanced LCA (pit 2, pit 3; Fig. 5.5b) suggest an input of bile acids, presumably derived from the decomposing bodies. DCA and LCA are used as typical indicators of human and cattle faeces (Elhmmali et al. 1997; Subbiah et al. 1976; Matschiner 1971), but cattle faeces has considerably lower contents of LCA (Tyagi et al. 2009; Tyagi et al. 2007). Therefore, a contribution of cattle bile acids can be ignored, because cattle faeces have dominant abundances of 5β-stigmastanol over coprostanol (Prost et al. (in prep, a); Bull et al. 2002), which was not detected in the reference soil of the study site (Fig. 5.4d-f, Fig. 5.5b).

Furthermore, the secondary bile acid ILCA was solely found in pit 2 and pit 3, which further strengthens the notion of an input of human derived bile acids to those pits. Consequently, bile acids revealed that human decomposition fluids might have been introduced at least into pit 2 and pit 3, due to the higher abundances of LCA and the presence of DCA, CDCA and ILCA.

Combining the results of faecal derived stanols and their epimers, stanones and bile acids (Fig. 5.6) only one (pit 2) of the three pits contained elevated steroid contents and indicative steroids for the input of human decomposition fluids. In pit 3 and pit 1, little evidence was found in steroid analyses to ascertain an external matter introduction through decomposing bodies. Presumably pit 2 contained most or all of the corpses buried during the period of 10 months and the other two pits were rarely used, if at all. Calculations revealed that a volume of 8.7 $m^3$ in pit 2 with a depth of 80 cm is sufficient to bury 66 male corpses with a volume of 9.5 $m^3$ (1.8 m length x 0.4 m width x 0.2 m height). The steroid analytics support the findings of Fiedler et al. (2009), indicating the former use of pit 2 as a mass grave, as shown by elevated total organic carbon, phosphorous and the discovery of a dental evidence.

## 5.5. Conclusions

The aim of this study was to evaluate if steroids are indicative for human decomposition fluids in temporary graves. Steroidal biomarkers were used to identify temporary mass graves, where concentration camp prisoners were buried for approximately 10 months (~late 1944-October 1945). It was shown that several tissue, faecal derived and faecal steroids were found in the mass graves and differed to some instances from the undisturbed reference soil. The steroid patterns suggest that pit 2 was used as a temporary mass grave. Bile acid patterns further revealed that two pits (2 and 3) were likely to have been used as temporary mass graves. As cholesterol, 5α-cholestanol and 5α-cholestanone are ubiquitously present in soils they were not suitable as decomposition markers in this study. The presence of steroids (epicoprostanol, epi-5β-stigmastanol) and bile acids (ILCA) being absent in reference soil were indicative for an input of decomposition fluids. However, due to high abundances of steroids in the reference soil, faecal steroids and their epimers were most indicative of decomposition fluids in pit 2. Combined with the evidence of Fiedler et al. (2009), the bile acid and steroid patterns established here, strengthen the evidence that at least one (pit 2) of the soil pits were most likely used as mass grave. Fiedler et al. (2009) used phosphorous and organic carbon as decomposition markers comparing concentrations in pit fillings with reference material. The occurrence of phosphorus and organic carbon is relatively unspecific for cadaver decomposition in soils because they have various origins (e.g. parent material, plant litter, soil flora, soil fauna). In contrast steroids used in this study were specific for the decomposition fluids of the human bodies and therefore represent a highly sensitive method for future investigations. A multi-molecular approach with additional biomarkers such as fatty acids and their $\delta^{13}C$ patterns might support the identification of temporary graves and the human origin of decomposition products.

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# Chapter 6

## Adipocere decomposition: a comparative case study on Arenosol and Calcisol soils

# Abstract

Adipocere forms on human and animal tissues as a result of bacterial hydrolysis of fat in environments where anaerobic conditions prevail. Research has focused on processes that lead to formation of adipocere, but little is yet known about the factors that affect its decomposition. Our study is aimed at investigating adipocere degradation in two vastly differing soil types (Calcisol and Arenosol) using minicontainers covered with gauze of three different mesh sizes (20, 500 and 2000 µm), which are accessible by soil micro-, meso- and macrofauna species. Adipocere pellets were incubated in these two soils at a depth of 10 cm for 234 and 419 days.

Regardless of soil type, mesh size and/or incubation period, the amount of adipocere still present in the minicontainers after removal from the soil varied considerably. Less adipocere was, however, present after 419 than after 234 days. In general, more adipocere was degraded in the Arenosol, suggesting that the soil's sandy texture and high air capacity had a positive effect on adipocere degradation. The Calcisol, which has a clayey texture and hence low air capacity, is influenced by groundwater, which suggests that the air balance was clearly impaired and led to the less effective decomposition of adipocere. The study also showed that the loamy Calcisol, where macrofauna species were found, had an impact on adipocere metabolisation.

Future studies will need to address the effect of physical and chemical parameters of soils in greater detail in order to assist forensic investigators in assessing decomposition processes in soils where a corpse is discovered.

## 6.1. Introduction

A detailed understanding of how buried cadavers decompose in various soil types is important from both a basic and applied perspective (Mondor et al. 2012). On the one hand, cadaver decomposition studies provide information on how terrestrial ecosystems function (Carter and Tibbett 2008). On the other hand, such information is also instrumental for determining decomposition rates (Carter et al. 2010, Carter et al. 2008). The latter is a key prerequisite to resolving forensic challenges surrounding the unexplained deaths of humans (Schotsmans et al. 2011; Carter et al. 2009).

Decomposing burial remains are a food source for soil organisms and are thus recycled into the ecosystem. Earlier studies have shown that microbial colonisation and break down of buried bodies occurs in a predictable sequential order (Mondor et al. 2012). It has been shown that post-mortem intervals (PMI) can be reliably estimated to within two to three days using high-throughput nucleotide sequencing techniques and bioinformatics analyses (Metcalf et al. 2013). Forensic entomology is widely used for the estimation of PMI, but is not always as precise as desired (Catts and Goff 1992).

Over the past few years, several studies have addressed the decomposition of carrion in relatively undisturbed environmental settings (Lauber et al. 2014; Olakanye et al. 2014; Carter et al. 2010; Wilson et al. 2007). Little focus has yet been put on adiopocere and other parameters that delay the decomposition of organic material. Adipocere (adipo = fat, cere = wax) is a white wax-like substance that forms on human and animal tissues as a result of the enzymatic hydrolysis of fat (Dent et al. 2004; Evans 1963; Strassmann and Fantl 1926). This phenomenon occurs especially in moist environments where anaerobic conditions prevail (Betts et al. 2014). The formation of adipocere has been studied in detail under laboratory (Moses 2012) and field conditions (Forbes et al. 2003), but little is yet known about the factors that affect its decomposition. In particular, the impact of the various biotic and abiotic parameters of special soil environments and the interactions of soil organisms on adipocere decomposition are still poorly understood.

Lack of this knowledge hampers remediation approaches to cemeteries that suffer from the problem of adipocere building up on corpses, thus preventing their decomposition and the reuse of graves after regular burial time (Fiedler and Graw 2005). From a more applied point of view, the PMI of corpses covered with adipocere is difficult to determine (Ubelaker and Zarenko 2011).

The current understanding is that adipocere decomposes best under aerobic conditions (Schoenen and Schoenen 2013; Fründ and Schoenen 2009; Froentjes 1965). Fiedler et al. (2015) have shown that poikiloaerobic conditions favour anoxic and facultatively anaerobic microbial and chemical degradation processes. Other researchers have shown that oxygen exposure time (Hartnett et al. 1998) and alternating redox conditions (Aller 1994) greatly affect preservation of organic matter.

Moreover, as shown for the formation of adipocere (Forbes et al. 2005), it is also assumed that adipocere degradation heavily depends on the environment (Fründ and Schoenen 2009; Pfeiffer et al. 1998). The so-called litterbag method is widely used for studying the decomposition of organic material in the field (Bocock and Gilbert 1957). Fründ and Schoenen (2009) used a minicontainer system, which is a modification of the litterbag technique, to study adipocere decomposition (Eisenbeis et al. 1999). The authors observed that adipocere buried in field or 'living' soil (i.e. field soil that includes animals) degraded faster than for example in sterilised quartz sand and air depleted soils. Thus, one may assume that not only microorganisms but also meso- and macrofauna have an effect on adipocere degradation. Fiedler et al. (2015) carried out investigations in a municipal cemetery in the Black Forest, Germany, and were able to link the presence of a number of earthworms (*Dendrobaena rubida*), collembola and small arthropods in humic and rooted black soil to the effective degradation of adipocere.

However, no study has yet attempted to assess in detail the contribution of micro-, meso- and macrofauna on the decomposition of adipocere in soils. In order to glean information on this issue, a case study was performed in two different soil types, Arenosol and Calcisol (Schad 2008). The test sites are located in close vicinity to each other, but the soils differ considerably in their chemical and physical properties.

Our study addresses the following hypotheses:

1. Adipocere decomposition takes place along the organic matter necrophage food chain, starting with microfauna/flora and increasing in complexity to higher trophic levels.
2. Adipocere decomposition is faster when meso- and macrofauna occur in soil alongside microfauna, rather than when only microbes are present.
3. Adipocere decomposition takes place at a higher rate in slightly alkaline loamy soil (Calcisol) with high levels of microbial biomass than in acidic sandy soil (Arenosol) with low levels of microbial biomass.

## 6.2. Material and Methods

### 6.2.1. *Case study*

The forest "Heißfeld" close to the German village of Berkach, approximately 20 km south of Frankfurt International Airport was chosen as the experimental site because the soil types in the area had already been studied in detail by Dambeck (2005). In this area, several different soil types can be found in close proximity, including sandy Arenosols and loamy Calcisols (Schad 2008), which developed from Holocene wind-borne sand and clayey high-flood sediments, respectively. At the chosen study site, the two soil types are found only around 120 m from each other, so it is possible to compare soils with significantly different physical and biochemical properties that would otherwise be exposed to quite different abiotic parameters such as precipitation and sunlight levels (Dambeck 2005).

A reanalysis of the properties of the two soil types in summer 2012 confirmed Dambeck's soil characterisations (Tab. 6.1; methods and extended soil description in Appendix 4, Table SI 4.1). The major differences between Calcisol and Arenosol that are considered most relevant for the current case study are the following: total organic carbon (TOC) and total nitrogen ($N_t$) levels were 9 and 12 times higher in the Calcisol than the Arenosol. The loamy Calcisol was characterised by medium air capacity and high field capacity. Approximately one third of the soil water was strongly bound in finepores (< 0.2 µm) and thus unavailable to plants. Furthermore, the Calcisol was influenced by groundwater (around 60 to 80 cm below the surface) and characterised by a 30 cm thick horizon of calcareous topsoil, immediately above mineral subsoil horizons with redoximorphic properties and secondary calcium carbonate precipitations. In contrast, the acidic Arenosol had a sandy texture with an Ah horizon of just 3 cm. The soil had a comparatively high air capacity of between 38 to 42 vol.%, which accounts for a large proportion of total pore volume (46-54 vol.%) and resulted in a low field capacity of only 3-10 vol.%.

**Table 6.1:** Physical and biochemical characteristics of the Calcisol and Arenosol soils at the study site in the "Heißfeld" forest at a depth of 10 cm. Further details are provided as supplementary information (Appendix 4, Table SI 4.1).

| | Calcisol* | Arenosol* |
|---|---|---|
| Height above sea level | 87 m | 90 m |
| Soil horizon* | Akh1 | Bw |
| Particle size distribution (sand│silt │clay) (%) | 35│31│34 | 95│3│3 |
| pH (in $CaCl_2$) | 7.3 | 3.7 |
| $CaCO_3$ (g $kg^{-1}$) | 58 | n.d. |
| Total organic carbon (TOC) (g $kg^{-1}$) | 65 | 7 |
| Total nitrogen ($N_t$) (g $kg^{-1}$) | 4.8 | 0.4 |
| C/N ratio | 14 | 18 |
| Microbial biomass carbon ($C_{mic}$) (µg C $g^{-1}$ soil), mean ± standard error | 579±77 | 33±4 |
| Microbial biomass nitrogen ($N_{mic}$) (µg N $g^{-1}$ soil), mean ± standard error | 83±11 | 3±2 |
| Bulk density (g $cm^{-3}$) | 0.9 | 1.2 |
| Total pore volume (vol.%) | 67.2 | 54.3 |
| Field capacity (vol.%), equivalent pore size 0.2-50 µm | 23.8 | 3 |
| Air capacity (vol.%), coarse pore volume; equivalent pore size ≥ 50 µm | 20.9 | 42.4 |

*Soil horizon and soil type classified according to Schad (2008) │ n.d. not detected

### *6.2.2. Minicontainer experiments*

Adipocere degradation in soil was investigated at a depth of 10 cm, i.e. Akh1 and Bw horizons (Tab. 6.1), respectively. They represent the biologically most active soil zones. To enable better comparison of results with those obtained by Fründ and Schoenen in 2009, the adipocere samples were buried using the same method as Fründ and Schoenen (2009).

The adipocere samples came from the corpse of a female, approximately 50 years of age, found in water. The PMI was estimated at around 9 years (Munich University forensic collection). All adipocere samples were pelleted and dried at 30°C for 16 h. In order to estimate the average ash content of the adipocere, seven randomly chosen pellets were ashed at 550°C for 24 h and weighed.

Polyethylene minicontainers with a volume of about 1.5 $cm^3$ were filled with 140 mg adipocere and closed at either end with plastic gauze discs (technical design by Eisenbeis et al. 1999). Litterbag mesh size is easily adapted to the issue under investigation (Kampichler and Bruckner 2009). In accordance with Lenz and Eisenbeis (1998), gauze discs with three different mesh sizes, 20 µm, 500 µm and 2000 µm, were used in the present project in order to restrict access to different size classes of soil fauna and enable the effect of different soil biota on adipocere decomposition to be compared.

Minicontainers covered with plastic gauze discs with pores with a diameter of 20 µm are accessible to microorganisms (e.g. bacteria, fungi, protozoa), while minicontainers covered with gauze with 500-µm pores will also allow mesofauna specimens (e.g. Acari, Collembola) to enter. Those covered with gauze with 2000-µm pores enable also macrofauna species (e.g. Enchytraeidae, Diplopoda, Chilopoda, Arthropoda, Annelida, Mollusca, Diptera larvae) to enter.

Six minicontainers were randomly placed in a polyvinylchloride bar. Six bars were placed horizontally into each soil type at a depth of 10 cm. Three bars per soil type (18 adipocere samples) were recovered after 234 days (from 30$^{st}$ November 2012 to 22$^{nd}$ July 2013), and the remaining three bars (18 adipocere samples per soil type) were left for 419 days (from 30$^{st}$ November 2012 to 23$^{rd}$ January 2014).

Temperature sensors equipped with data loggers (PT1000 1/3 DIN EcoTech, Bonn, Germany) were placed alongside the minicontainer bars, and the soil temperature was measured at one-hour intervals over the entire incubation period. Accumulated degree days (ADDs) were calculated from average daily temperatures according to Megyesi et al. (2005). Daily precipitation values were obtained from the nearby weather station at Frankfurt International Airport (Deutscher Wetterdienst 2014).

After removal from the soil, the samples were stored at -20°C until further processing. The adipocere material was air-dried, weighed and ash determined as described above. The half-lives of adipocere pellets were calculated according to a simple first-order kinetic (Gisi 1997).

## 6.3. Results and Discussion

The current study is aimed at deepening the understanding of adipocere degradation in natural soils in two different soil types, namely sandy Arenosol and loamy Calcisol.

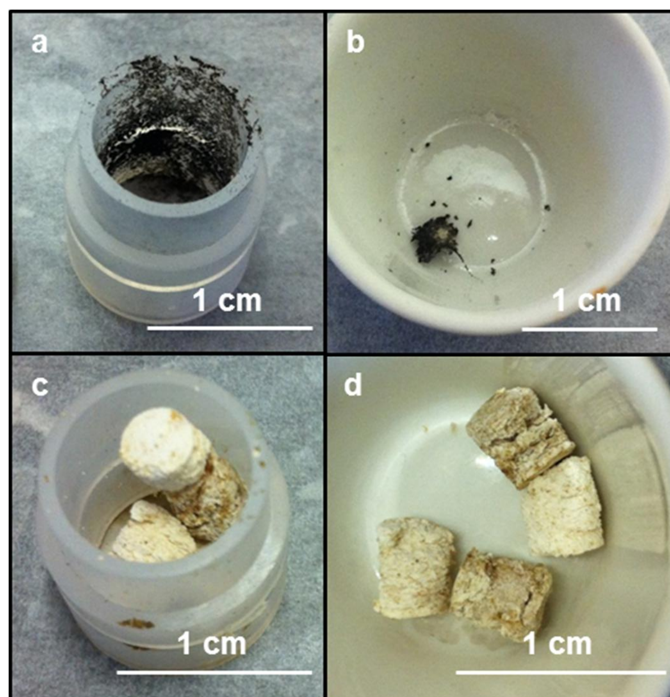
Given the close geographic proximity of the two soils, there were, as expected, no substantial differences in the recorded temperatures. Accordingly, the accumulated degree days (ADD) only differed to a minor degree between the Calcisol and Arenosol soils (Tab. 6.2). Temperature could thus be ruled out as a key parameter for explaining differences in adipocere degradation in Calcisol and Arenosol soils. Furthermore, the recorded precipitation values indicated that there were no noticeable dry periods during the incubation periods of the adipocere samples (Appendix 4, Table SI 4.1). In addition to being composed of different soil materials (wind-borne sand and clayey high-flood sediments), the Calcisol was located in a slight depression and closer to the water table than the Arenosol (Dambeck 2005). While the Arenosol did not show any redoximorphic characteristics up to a depth of 1 m, the Calcisol was characterised by the presence of iron mottles and concretions at a depth of as little as 30 cm, which suggests that the soil's air balance was clearly impaired. Moreover, the presence of secondary lime precipitates in the upper Calcisol horizons is a sign of a rather thick capillary fringe.

**Table 6.2:** Average weight loss of adipocere pellets (n=6) in minicontainers (total number: 72) covered with plastic gauze of different pore size in Calcisol and Arenosol soils at a depth of 10 cm soil depth after a burial time of 234 and 419 days (i.e. 36 minicontainers were removed after 234 days, 36 after 419 days), respectively. Accumulated precipitation, mean average adipocere half-lives and accumulated degree days (ADD) are also given.

| Soil type | | **Calcisol** | | | **Arenosol** | |
|---|---|---|---|---|---|---|
| Gauze pore size (µm) | 20 | 500 | 2000 | 20 | 500 | 2000 |
| | | | **Analysis after 234 days (30/11/2012-22/07/2013)** | | | |
| **Adipocere loss (%)** | | | | | | |
| Mean ± SD | 33.6±17.8 | 31.8±18.5 | 31.6±18.9 | 68.4±18.1 | 66.3±39.4 | 76.8±21.0 |
| 25%-quantile | 22.0 | 19.8 | 18.7 | 61.2 | 41.7 | 43.9 |
| 50%-quantile (median) | 37.5 | 31.3 | 21.4 | 65.9 | 84.8 | 85.8 |
| 75%-quantile | 47.3 | 35.7 | 42.4 | 77.5 | 94.2 | 64.0 |
| Accumulated precipitation (mm) | | | 417.4 | | | |
| ADD | | 1873 | | | 1898 | |
| | | | **Analysis after 419 days (30/11/2012-23/01/2014)** | | | |
| **Adipocere loss (%)** | | | | | | |
| Mean ± SD | 27.7±12 | 34.8±6.6 | 56.8±17.2 | 80.7±25.7 | 83.4±15.1 | 86.4±8.0 |
| 25%-quantile | 23.5 | 33.1 | 42.9 | 70.4 | 73.7 | 86.5 |
| 50%-quantile (median) | 24.8 | 37.1 | 52.5 | 95.4 | 90.3 | 88.2 |
| 75%-quantile | 35.4 | 39.2 | 67.6 | 96.9 | 93.7 | 89.3 |
| Accumulated precipitation (mm) | | | 740.5 | | | |
| ADD | | 3906 | | | 3975 | |
| Mean half-life | 3.1±2 | 2±0.6 | 1.1±0.5 | 0.6±0.6 | 0.5±0.3 | 0.4±0.1 |

At the end of the incubation periods, the amount of adipocere still present in the minicontainers varied considerably among the replicates, regardless of soil type and/or incubation period (Tab. 6.2, Fig. 1). In some minicontainers, all adipocere had turned into blackish material while other minicontainers still contained apparently fully preserved adipocere pellets. Accordingly, there was a high standard deviation for all estimates, which hampered straightforward interpretation of the results (Appendix 4, Table SI 4.2). In general, more adipocere was degraded in the Arenosol than in the Calcisol.

**Figure 6.1:** Examples of adipocere pellets after incubation in different soil types; (a) and (b): Decomposed adipocere from minicontainers covered with 20 µm gauze after a 419-day incubation period in the Arenosol; (c) and (d): Nearly intact adipocere pellets from minicontainers covered with 20 µm gauze after 419 days in the Calcisol.




This study is not the first to observe huge variations in adipocere decomposition rates. Schotsmans et al. (2011) even observed adipocere formation and desiccation on a single body. In a comprehensive exhumation series, Mant (1987) observed the presence of adipocere and completely skeletonised bodies in close spatial proximity. The reasons for this variability are barely understood. However, it seems unlikely that the huge differences in the degree of degradation between replicate samples can be attributed to soil type. In the current case study, similar variation levels were observed in both soils investigated. Even minicontainers located in immediate vicinity (1 cm) of one another differed considerably with regard to the amount of adipocere degraded. It must therefore be assumed that factors other than the soils' physical and biochemical properties account for the observed variation in the degree of adipocere decomposition.

Adipocere decomposition is a continuous process that can take up to several years - depending on the environment (Fründ and Schoenen 2009). The decomposition process may also proceed in different phases, all of which depend on the particular physical/biochemical characteristics of the soil environment and the presence of particular organisms. In the current case study, less adipocere remained after 419 than after 234 days. This was not really surprising. Adipocere decomposition was much more efficient in the sandy Arenosol than in the loamy Calcisol. After 234 days, twice as much adipocere was degraded in the 20-µm minicontainers buried in the Arenosol than in those buried in the Calcisol. While roughly 30% of the adipocere material in the Calcisol minicontainers had disappeared, as much as 70% had disappeared in the Arenosol after 234 days and 85% after 419 days. Adipocere degradation did not occur at a constant rate over the entire incubation period. While the decomposition of adiopocere in the Calcisol came virtually to a standstill after the first 234 days (20, 500 µm), further decomposition occurred in the Arenosol from day 235 to 419, and 1.2 times more adipocere was degraded in the Arenosol than in the Calcisol in this period. Nevertheless, most of the decomposition also took place in the Arenosol during the first 234 days. Although these observations are difficult to explain, they might be the result of the fluctuating water table and the thick capillary fringe that left the Calcisol water-logged at times, and hence in an environment that prevents decomposition.

Amongst other characteristics, the sandy Arenosol and the loamy Calcisol differed with respect to TOC and $N_t$ concentrations as well as pH (Tab. 6.1). The hypothesis that adipocere decomposition occurs at a higher rate in slightly alkaline loam soil with high levels of microbial biomass than an acidic sandy soil with low levels of microbial biomass was to our surprise not observed in the current case study. In contrast, more than twice as much adipocere disappeared in Arenosol than in Calcisol, which had much higher TOC and $N_t$ concentrations and biomass. This result thus refutes hypothesis 3.

Adipocere decomposes along the detritus/necrophage food chain. Microbes are expected to be the first and main organism group involved in the degradation of carcasses and the primary regulators of terrestrial carbon (Crowther et al. 2012; Mondor et al. 2012; Rapp et al. 2006), regardless of the soil environment. In the current case study, we addressed the effect of accessibility of adipocere pellets to differently sized organisms by closing the incubation minicontainers with gauzes of different pore size. Significant differences in the amount of remaining adipocere (Tab. 6.2) were not detected in either of the two soil types.

However, the values for adipocere loss in the minicontainers covered with gauze with 2000-µm pores tended to be the highest. These results did not meet the expectations of hypotheses 1 and 2, although they did not formally reject them either. We tentatively calculated the effect macrofauna might have had on decomposition. These calculations suggest that macrofauna had a 22% (Tab. 6.2) involvement in adipocere degradation in the Calcisol and only a 3% involvement in the Arenosol after 419 days. From this it can be further deduced that the biodiversity in the Calcisol was higher than in the Arenosol. Soil moisture and pH have been identified as important abiotic factors for decomposer communities (Rousk et al. 2009). In addition, Enchytraeidae are generally found in very high densities in any types of soil apart from rather dry soils such as Arenosol. Abrahmason (1972) observed in soils with pH values of

between 3.9 and 6.4 that the number of Enchytraeidae increased as the pH increased. Earthworms prefer soils that are neither too wet nor too dry. In addition, the pH of the soil is of great significance for earthworm distribution. Most earthworm species prefer a neutral to slightly alkaline pH (Römbke et al. 1997). Scheu (1990) found that acidification of the soil led to a reduction in the saprophagous macrofauna.

The lack of significant differences in terms of adipocere degradation in relation to pore size raises the question as to whether the experimental setup following Eisenbeis et al. (1999) was indeed suitable for studying adipocere decomposition in the selected soils. Technically, it cannot be excluded that under the given circumstances medium-sized sand particles clogged a large number of pores (minicontainers with 500-µm pores) and prevented soil organisms that would theoretically be able to pass through meshes of the respective pore sizes from accessing the minicontainers and feeding on the adipocere.

Are there also ecological rather than technical reasons that may explain the observations? Bååth and Anderson (2003) found that the composition of the microbial community is to a large extent determined by soil pH. Bååth and Anderson (2003), Blagodatskaya and Anderson (1998) and Gong et al. (2001) observed more fungi at slightly acidic pH than slightly alkaline pH. Based on the findings of these authors, it can be assumed that more fungi were present in the Arenosol than in the Calcisol, where in turn a somewhat higher bacterial content was expected. Given that fungi have hyphae that allow them to bridge air-filled pores, they may colonise and degrade surface litters more easily, whereas soil bacteria may be unable to come into contact with the adipocere pellets (Carter et al. 2010; Bailey et al. 2002). If this is correct, hypothesis 3 needs to be refined. This hypothesis relates adipocere degradation to microbial biomass, following for example a study carried out by Schotsmans et al. (2012) which found that high carbonate concentrations delay degradation. Similarly, we showed in a previous case study that microbial adipocere degradation was associated with a reduction of the C/N ratio from 200 to 19 (Fiedler et al. 2015). These studies suggested that increased availability of N attracted a higher diversity of macrofauna. Nevertheless, the current study suggests that the ratio of bacterial and fungal biomass needs to be taken into account too. Since this ratio is difficult to determine, the pH value - as a proxy for adipocere degradation - deserves more attention. For the time being, this remains speculative and addressing this question in detail requires a different experimental approach.

The results obtained are just as likely to suggest that the onset of efficient adipocere decomposition was delayed in the Calcisol. Although the experiment lasted for more than 400 days, the time span may still be too short for a conclusive comparison. Fründ and Schoenen (2009) calculated a half-life of between 11 and 82 years (mean = 37 years) under anaerobic conditions and between 0.7 and 10 years (mean = 2.8 years) in aerated environments. Thus, it may also be plausible that adipocere decomposition simply has an extended "ignition" period in the Calcisol. We nevertheless consider such an explanation unlikely, as the data obtained do not point to the delayed onset of adipocere degradation in the Calcisol. It was much more surprising that the degradation process came to a standstill relatively early.

Finally, soil pore size might be another reason for the delay in the onset of adipocere degradation in the Calcisol. Elliott et al. (1980) and Carter et al. (2010) found that the habitable space in each trophic level is influenced by pore size and the diameter of water-filled pores, which also has an effect on adipocere decomposition. Lipids such as stearic and palmitic acid are attractive energy sources for microbial decomposers. However, microorganisms require approximately three times more oxygen for metabolising these fatty acids than a carbohydrate molecule of roughly comparable size (Kleber 2010). The availability of oxygen depends on soil texture and structure (Neira et al. 2015; Moldrup et al. 1998) and is the reason why adipocere buried in the sandy Arenosol degraded much faster than in the loamy textured Calcisol. The sandy texture gives the soil a high air capacity, thereby ensuring that sufficient oxygen is available, and enables the effective degradation of adipocere. In contrast, the thick Ah horizon of the Calcisol may only allow for limited microbial decomposition of organic substances. The observed C richness supports this interpretation, since microbes are usually very efficient in metabolising C sources. In the Calcisol, the groundwater table varied between 60 and 80 cm below surface, and given the clayey texture of the soil one might expect that the pores of the Ah horizon to be frequently filled with water, and the availability of oxygen to be extremely limited during such periods. Moreover, the thick Ah horizon (30 cm compared to 3 cm) seems to be the major reason for the less effective decomposition of adipocere in the Calcisol compared to the Arenosol.

The present study shows that the decomposition of adipocere depends on numerous factors, and future studies will therefore have to address the effects of physical and chemical parameters of the soil in greater detail.

## 6.4. Conclusions

The incubation of adipocere in Arenosol and Calcisol soils with pronouncedly different physical and biochemical properties yielded the unexpected result that decomposition of the material proceeded faster in the Arenosol, which represented the soil type that was expected to be less favourable for the process. Although some technical limitations may have prevented the comprehensive assessment of the impact of various faunas on the decomposition process, there was a clear trend towards accelerated metabolisation when the samples were accessible to somewhat smaller soil macrofauna species. Aeration of the soil and presence of fungi seemed to have the greatest impact on decomposition rate. Future studies will need to address the effects of physical and chemical parameters of the soil in greater detail. The maximum mesh size of about 2000 µm limits the access of larger soil macro- and megafauna. In order to obtain a comprehensive picture of the contribution of different decomposers, future studies will have to specifically investigate the effect of earthworms.

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# Chapter 7

# Synthesis

## 7.1. Summary of the research objectives

In the present work the fate of cadaveric lipids from the decomposition of human remains in/on soils were investigated. This study also considered the impact of biotic and abiotic soil parameters on the decomposition of adipocere. As adipocere formation received substantial attention in the past (Ubelaker and Zarenko 2011; Fiedler and Graw 2003), little is known about the fate of adipocere under oxidative soil conditions with the access of micro-, meso- and macrofauna. Hereby, two different types of materials were used in this work: soil samples which were in contact with decomposing bodies (surface samples, mass grave samples) and adipocere from one individual which was formed under anaerobic conditions for 9 years in a lake. Soil samples which were temporarily in contact with decomposing bodies (11-18 days and 10 months) were investigated over several time periods: 11-18 days, 1 year (Chapter 3 and 4) and 67 years (Chapter 5). The decomposition of adipocere in soils was determined over two time periods: 234 days and 419 days after the insertion of adipocere (Chapter 6). Laboratory methods adapted from previous work (von der Lühe et al. 2013; Birk et al. 2011; Dove and Mayes 2006; Isobe et al. 2002) were tested prior application to soil samples which were subjected to decomposition fluids. Recoveries of steroids ($\Delta^5$-sterols and stanols) were assessed in matrix samples extracted from reference soil. Steroids were added by standard addition in the order of magnitude of expected steroid concentrations in soil samples containing decomposition fluids. Furthermore, ECN factors were applied to estimate a SRF for the quantification of 10-hydroxystearic acid in soil samples containing decomposition fluids. Minicontainers, an improved method of the commonly used litter bags, were used to assess decomposition of adipocere in a field experiment. Adipocere samples were provided from the Institute of Forensic Medicine in Munich (Chapter 6) and soil samples of the surface CDI (Chapter 3 and 4) were taken in cooperation with the police department of Rhineland-Palatinate, Germany. It should be noted, that research on human decomposition in Germany is complicated by the access to respective crime scenes and soil material. The increased interest in understanding forensic taphonomic processes in outdoor areas resulted in establishing body farms, e.g. the Forensic Anthropology Centre in Tennessee, USA. Using pigs as human surrogates is often an alternative method in forensic taphonomic studies (Notter et al. 2009; Schoenly et al. 2006; Payne 1965), but direct application to human crime scenes (Bull et al. 2009) might fail in certain issues (e.g. body size and weight; Notter et al. 2009). It was therefore decided to carry out fundamental research on human bodies from case studies as those are by far closest to forensic questioning.

This study focused on the use of steroids ($\Delta^5$-sterols, stanols, stanones and bile acids) and fatty acids for the identification of decomposition fluids for several reasons: (I) steroids are specific for their source and (II) delivery of lipids from other sources (e.g. plants, fungi, animals) differs considerably in patterns and concentrations to lipids from human decomposition fluids (III) as demonstrated in archaeological research steroids and fatty acids are preserved in soils for longer periods (IV) lipids are rather immobile components in soils (Lloyd et al. 2012) (V) fatty acids can be used as identifiers of adipocere formation in soils (see also Chapter 4).

In addition adipocere degradation was assessed which demonstrated a further investigation of the impact of soil on the decomposition of human remains. The influence of soil organisms (micro-, meso-, and macrofauna) and soil types on adipocere decomposition was the main focus in this investigation.

The specific objectives of the present work were (I) to test laboratory methods which can then be applied to soil samples containing human decomposition fluids. (II) The investigation of steroids and fatty acids in soil samples comprising decomposition fluids. (III) The study of the temporal patterns, the spatial distribution, the chemical transformation and degradation processes of cadaveric lipids in soils. (IV) Adipocere degradation in undisturbed environmental settings was assessed to propose abiotic and biotic factors which influence its disappearance over time. Considering those objectives (see also Chapter 1.2.) following results are recapitulated:

**(I) To develop suitable laboratory methods which can then be applied to detect cadaveric lipids in soil samples containing human decomposition fluids by gas chromatography/flame ionisation detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS).**

As mentioned above, laboratory methods were tested on reference lipid extracts and applied to soil samples which received consistently high amounts of decomposition fluids. For $\Delta^5$-sterol and stanol analysis a combined method (von der Lühe et al. 2013; Birk et al. 2011; Dove and Mayes 2006; Isobe et al. 2002) was tested on reference matrix extracts by standard addition (TLE from reference soil; 0-0.5 cm depth; Chapter 2) in order to quantify sample loss during the laboratory procedure. With this method, steroid recoveries between 78-97% (precision $R^2$ 0.94-0.99) and relative standard deviations (RSD) of 2-11% (of 8 steroids, four replicate assays) were achieved. By using selected ions with GC/MS low detection limits (LOD 0.2-1.0 ng $g_{soil}^{-1}$) allowed analysis of traces (e.g. epicoprostanol LOD=0.8 ng $g_{soil}^{-1}$). Application of the combined method on soil samples enriched with human decomposition fluids showed low standard errors between laboratory replicates (below 10% deviation of standard error from mean). The standard addition experiments worked well for all steroids in the range of the standard amounts added. However, in a few cases selected TLEs of cholesterol exceeded amounts of the added cholesterol standard (Chapter 2). It has been shown that the method established for the extraction and purification of CDI samples is simple, sensitive and can deal with a large set of samples.

Fatty acids are normally extracted from adipocere to characterise its formation processes (Fiedler et al. 2009b; Notter et al. 2009; Forbes et al. 2005; Forbes et al. 2002). Amongst saturated and unsaturated fatty acids, also found in fresh human tissues, 10-hydroxystearic acid was indicated as characteristic fatty acid found in adipocere tissues. In the surface decomposition study, 10-hydroxystearic acid was found and validated by its mass spectrum with GC/MS (Chapter 2). Quantification of 10-hydroxystearic acid was not possible, as an analytical standard was not available. Therefore, ECN factors were applied to estimate a $SRF_{TMS\text{-}10\text{-}OH\text{-}C18:0}$ of 10-hydroxystearic acid with internal standardisation. To validate the quality of the calculated $SRF_{TMS\text{-}10\text{-}OH\text{-}C18:0}$ an analytical standard of 12-hydroxystearic acid was analysed by GC/FID. As flame ionisation detectors have a linear response to carbon chains and functional groups, the SRF of both components must be identical. The accordance of estimated $SRF_{TMS\text{-}10\text{-}OH\text{-}C18:0}$ and the

analytical $SRF_{TMS-12-OH-C18:0}$ was 97%, showing a well estimate of $SRF_{TMS-10-OH-C18:0}$. The SRF estimation by using ECN factors does not replace an analytical standard, but it is a suitable alternative when no analytical standards are available.

**(II) To discover the presence of $\Delta^5$-sterols, stanols, stanones and bile acids in soil samples comprising decomposition fluids and test (I) their suitability as diagnostic markers of decomposition fluids and (II) to identify the human origin.**

Two different soil sample sets comprising decomposition fluids were analysed. The first soil sample set was from a case study, were a human body was found decomposing aboveground 11-18 days after deposition. After discovery, the body was removed from the crime scene. Soil samples were taken, 11-18 days after the body was deposited and one year (358 days) after the body was removed. In soil samples taken after 11-18 days, steroids (cholesterol, 5α-cholestanol, coprostanol, epicoprostanol, 5β-stigmastanol, epi-5β-stigmastanol and 5α-stigmastanol) and fatty acids (myristic acid, palmitic acid, oleic acid, stearic acid and 10-hydroxystearic acid) had larger contents than the reference. After one year amounts of steroids and fatty acids were comparably decreased, but still larger than lipids analysed in the reference samples. Hence, lipids from both tissue degradation products and faecal material were released 11-18 days after deposition of a body and were indicative for the former presence of a decomposing body over one year.

The second soil sample set came from putative mass graves (pit 1-3) from World War II. Concentration camp prisoners, who died of starvation, were buried for a period of 10 months (~late 1944-October 1945) in a municipal forest close to Stuttgart, Germany. Steroid analytics ($\Delta^5$-sterols, stanols, stanones and bile acids) was applied to demonstrate the presence of cadaveric lipids from the former buried concentration camp prisoners. One (pit 2) of the three putative mass graves investigated had larger contents of 5α-cholestanol, epicoprostanol, coprostanol, 5β-cholestanone, 5α-cholestanone, 5β-stigmastanol, epi-5β-stigmastanol, deoxycholic acid and lithocholic acid in a depth of 40-50 cm than the reference. Selected steroids (epicoprostanol and epi-5β-stigmastanol) and bile acids (isolithocholic acid) were unique components for pit 2 as they were not found in the reference. Because cholesterol and 5α-cholestanol did not differ between reference and soil pits, they were not suitable as identifiers of human decomposition fluids in the mass graves. Faecal steroids and their environmental conversion products (coprostanol, epicoprostanol, 5β-stigmastanol, epi-5β-stigmastanol, 5β-cholestanone, LCA, DCA, ILCA) were specific for pit 2 indicating the input of decomposition fluids. In combination with other evidence (large TOC and phosphorus contents, pivot crown) an input of cadaveric lipids from the concentration camp prisoners in pit 2 was most likely (Fiedler et al. 2009a). It was demonstrated, that the combined analysis of $\Delta^5$-sterols, stanols, stanones and bile acids can aid to identify temporary graves.

**(III) To investigate the fate of cadaveric lipids in soil: the (I) temporal presence (II) the spatial distribution and (III) transformation and degradation processes of steroids and fatty acids in soils to propose factors which influence their preservation.**

Steroids and fatty acids in soil samples from the surface case study (11-18 days, one year later; Chapter 3 and 4) showed temporal changes in patterns and concentrations. The dominance of faecal steroids over tissue steroids found in soil beneath the abdomen (11-18 days) can be explained by the position of the intestinal tract. An early leaching of decomposition fluids through burned shoulder parts of the body, might explain the high abundances of tissue and faecal steroids beneath the thorax (11-18 days). After 11-18 days, the vertical distribution of steroids with highest concentrations found close to the soil surface is likely caused by the free leaching of decomposition fluids, because the soil was oversaturated. After one year (358 days), decreased steroid concentrations were attributed to microbial degradation and bioturbation. Microbial transformation of cholesterol and β-sitosterol to the respective 5α-cholestanol and 5α-stigmastanol was observed after one year. Fatty acid patterns revealed that after a short deposition of the body (11-18 days) an extensive hydrogenation of fatty acids already took place (Chapter 4). The presence of 10-hydroxystearic acid in close proximity to the decomposing body (0-0.5 cm soil depth) indicated adipocere formation under anaerobic conditions during the presence of the body (11-18 days). The increase of fatty acid salts over one year showed preservation mechanisms of fatty acids from decomposition fluids. Decreased abundances of myristic acid after one year likely indicated fatty acid degradation by β-oxidation. The analysis of cadaveric lipids over time showed that several processes have an impact on their spatial distribution and concentrations. Knowledge obtained from Chapter 5, let suggest that steroids found in the putative mass grave (pit 2) were extensively degraded and microbially transformed to 5α-stanols and epi-5β-stanols. 5β-cholestanone was exclusively introduced by the decomposing bodies in pit 2, as microbial transformation of coprostanol to 5β-cholestanone was not evident in the reference.

**(IV) To explore the impacts of biotic and abiotic parameters on the decomposition of adipocere in a soil environment.**

Adipocere degradation was determined in two differing soil types (Calcisol and Arenosol) with the access of micro-, meso- and macrofauna. Adipocere residuals varied considerably, regardless of soil type, mesh size and/or incubation period. A decrease in adipocere abundances was found from 234 to 419 days. In the Arenosol, more adipocere was degraded which was attributed to the sandy texture and the high air capacity that likely promoted adipocere degradation. The clayey texture and influence of groundwater in the Calcisol probably hampered a rapid adipocere degradation. The study also showed that the loamy Calcisol, where macrofauna species were found, had an impact on adipocere metabolisation.

## 7.2. Synthesis

The analysis of steroids and fatty acids with GC/MS and GC/FID demonstrated sensitive **laboratory methods** in the characterisation of decomposition fluids in soils. Components were detectable in trace concentrations ($\Delta^5$-sterols and stanols LOD 0.1-1 ng $g_{soil}^{-1}$, Chapter 2) and with GC/MS it was possible to identify 10-hydroxystearic acid in lipid extracts (Chapter 2). The selection of suitable lipid biomarkers to identify human decomposition products in soils depends on their residence time, the natural abundance of lipids in reference soil and on the properties of the depositional site. Therefore, the choice of lipid biomarkers and laboratory methods must be adjusted to the specific forensic case. For instance, fresh (11-18 days, Chapter 3) and aged cadaver decomposition sites (67 years, Chapter 5) require different extraction methods: an ultrasonic extraction is sufficient for soil samples which were recently subjected to human decomposition fluids (Chapter 3), whereas Soxhlet extraction is required when traces of decomposition fluids are expected in soil samples (Chapter 5).

**Cholesterol** was one of the most evident markers of human decomposition fluids in soils (Chapter 3). In the surface decomposition case study (Chapter 3) it was the dominant steroid found after 11-18 days and after one year (385 days). After one year, highest abundances of cholesterol were found in a soil depth of 8-13 cm, because cholesterol was likely microbially reduced to **5α-cholestanol** close to the soil surface (0-5 cm, Chapter 3). The spatial distribution of cholesterol and 5α-cholestanol after one year must be considered in forensic case studies during soil sampling. Several soil samples must be collected in the CDI in different soil depths to ensure a complete assemblage of cadaveric steroids. It was presumed that 5α-cholestanol was also introduced as tissue and faecal component during cadaver decomposition (Chapter 3 and 5). Over longer residence times of cadaveric lipids in soils, the importance of 5α-cholestanol as cadaveric marker increases because it represents former cholesterol levels (Chapter 5). In combination with cholesterol and **5α-cholestanone**, higher levels were thus present in the temporary mass graves compared to the reference (Chapter 5). However, cholesterol, 5α-cholestanol and 5α-cholestanone undergo not only transformation but also degradation over time, and they were not suitable to indicate cadaveric lipids in the mass graves (Chapter 5). Furthermore, cholesterol and its environmental reduction products 5α-cholestanol and 5α-cholestanone do have additional natural sources (Christie and Han 2010; Weete et al. 2010; Mouritsen and Zuckermann 2004), which complicates the identification of the origin of these compounds. In both case studies human bodies were temporarily deposited and removed before soft tissue degradation was completed. In archaeological investigations cholesterol was indicative for decomposition fluids where human bodies completely decomposed (Shillito et al. 2011; Davies and Pollard 1988). It therefore arises the question, if cholesterol can be an indicator of human decomposition fluids in cases where soft tissues completely decomposed.

Amongst tissues steroids (cholesterol, 5α-cholestanol and 5α-cholestanone), **faecal** stanols and stanones and their environmental epimers were also useful for the identification of human decomposition fluids in soils. The analysis of soil from the surface decomposition (Chapter 3) and the mass grave case studies (Chapter 5) showed the presence of coprostanol, epicoprostanol, 5β-stigmastanol, epi-5β-stigmastanol and 5β-cholestanone. **Coprostanol** is an important

faecal marker to identify human faeces. It was successfully applied in archaeological and environmental pollution research (Birk et al. 2011; Isobe et al. 2002; Elhmmali et al. 2000; Bull et al. 1999; Leeming et al. 1998; Leeming et al. 1996; Bethell et al. 1994; Lin et al. 1978). Coprostanol was found in soil samples beneath decomposing pig cadavers, demonstrating the abundance of faecal material in decomposition fluids (von der Lühe et al. 2013). In both case studies (Chapter 3 and 5) it was an important constituent of human decomposition fluids, it was found after short periods (11-18 days) and longer periods of time (one and 67 years) in soils which were temporarily subjected to decomposing bodies (11-18 days and 10 months). The detection of coprostanol was mainly attributed to faecal material. Traces of coprostanol could have been formed under anaerobic conditions from intestinal microorganisms inside the body (Evershed and Connolly 1994; Gülaçar et al. 1990) and in the soil during the presence of the body (Cobaugh et al. 2015). Furthermore, the presence of **5β-cholestanone** in pit 2 (mass grave; Chapter 5) did not only indicate the presence of faecal material (Gérard 2014; Eyssen et al. 1973; Björkhem and Gustafsson 1971; Rosenfeld and Hellmann 1971), but also showed that no environmental transformation of cholesterol to coprostanol occurred in the reference (Evershed and Connolly 1994). The abundance of **epicoprostanol** in both case studies was attributed to faecal material (Chapter 3 and 5), but also from the in-situ transformation of coprostanol (Birk et al. 2011; Bull et al. 2002; Bethell et al. 1994; Wardroper and Maxwell 1978). Epicoprostanol (15±4 ng $g_{soil}^{-1}$) was unique in pit 2 of the mass grave case study (Chapter 5). It was suggested that epicoprostanol derived from human faecal material and from the microbial epimerisation of coprostanol over the 67 years. In summary, coprostanol, epicoprostanol and 5β-cholestanone were highly specific for the input of faecal material from the decomposing human bodies.

**β-sitosterol** was included in this study, because it is ubiquitous in soils and expected to remain at similar concentration levels between reference and soil material at a decomposition site. It was overprinted by the input of organic carbon (µg $g_{TOC}^{-1}$) from the decomposing body in the surface decomposition case study (Appendix 2, Chapter 3). When β-sitosterol was normalised to soil, a higher input was found after 11-18 days beneath the thorax. It is still unknown, if β-sitosterol derived from intestinal contents or from decomposed plant material below the body (Chapter 3). β-sitosterol is digested as part of the diet (Prost et al. (in prep)) and was found in soil below decomposing pig carcasses (von der Lühe et al. 2013). It was either derived from intestinal contents or introduced in soil during the construction of graves where topsoil enriched with organic material is homogenised with subsoil material (von der Lühe et al. 2013). The faecal derived **5β-stigmastanol** and its environmental epimer **epi-5β-stigmastanol** were both present in soil beneath the decomposing body of the surface decomposition case study (Chapter 3). Epi-5β-stigmastanol is the environmental epimer of 5β-stigmastanol, it thus indicates an former input of the faecal 5β-stigmastanol (Bull et al. 2002; McCalley et al. 1981). Their presence is attributed to faecal material (5β-stigmastanol), to the production during decomposition (5β-stigmastanol) and in-situ reduction and epimerisation (5β-stigmastanol and epi-5β-stigmastanol). Furthermore, spatial patterns of β-sitosterol and 5α-stigmastanol proved the environmental reduction of $\Delta^5$-sterols to 5α-stanols as it was proposed for cholesterol and 5α-cholestanol in the surface decomposition case study after one year (Chapter 3). 5β-stigmastanol and epi-5β-stigmastanol were an additional evidence for faecal material and the environmental epimerisation of 5β-stigmastanol in the mass graves, as

reference samples did not or only contain traces of plant stanols (Chapter 5). In the mass graves, 5β-stigmastanol and epi-5β-stigmastanol contributed to the identification of human decomposition fluids in pit 2 (Chapter 5). After coprostanol, 5β-stigmastanol is the second most abundant steroid found in human faeces (e.g. 26% of total sterol, stanol and stanone content; coprostanol 57%; Prost et al. (in prep)). The predominant 5β-stanol of herbivores is 5β-stigmastanol, it can be thus used as marker of herbivorous faeces (Bull et al. 2002). 5β-stigmastanol can be included in steroid analysis of decomposition products in soils, but it must be considered that coprostanol should be dominating 5β-stigmastanol to exclude the input of herbivorous faeces (Prost et al. (in prep)).

**Bile acids** haven't been used yet as identifiers of human decomposition fluids in soil samples. They were therefore included in steroid analysis to identify human decomposition products in the mass graves (Chapter 5). Lithocholic acid and deoxycholic acid are important markers of human faeces, other bile acids found in human faeces are chenodeoxycholic acid and isolithocholic acid (Prost et al. (in prep); Bull et al. 2002). Lithocholic acid was significantly increased ($p < 0.05$) in pit 2 and pit 3 compared to the adjacent reference soil (Chapter 5). Deoxycholic acid was higher in all three soil pits, but due to high reference levels there were no significant differences detected. An input of lithocholic acid, deoxycholic acid, chenodeoxycholic acid and isolithocholic acid in pit 2 and pit 3 as constituents of human decomposition fluids is most likely. Isolithocholic acid was unique in pit 2 and pit 3, which further strengthen an input of human derived bile acids (Chapter 5). Evidence of human derived bile acids is hampered by the high background of bile acids found in the reference. Concentration levels and sources of bile acids in forests soils are still unknown. Hence, the high background can be attributed to various sources (e.g. wildlife, former agricultural use with organic fertilisation). Several archaeological studies demonstrated that bile acids are relatively stable against microbial degradation compared to stanols (Lauer et al. 2014; Elhmmali et al. 1997). They are only produced by vertebrates and in combination with stanols the identification of the origin of faeces is more precise (Tyagi et al. 2007; Simpson et al. 1999; Leeming et al. 1997; Gülaçar et al. 1990). It has been shown, that the high diagnostic potential of bile acids is worthwhile to include them in forensic investigations to identify decomposition fluids from human origin.

In the surface decomposition case study, differences in steroid patterns beneath body positions were detected after 11-18 days of body deposition. The dominance of faecal steroids over tissue steroids beneath the abdomen was attributed to the location of the intestinal tract (Chapter 3). The shoulder of the victim was burned, which probably promoted insect colonisation and hence leaching of decomposition fluids through the burned body part (Chapter 3). This process might explain highest concentrations of steroids in soil beneath the thorax. As there were no field replicates, because all soil blocks (11-18 days, 358 days after) were taken at different positions in the CDI, it is still unknown which impact body positions do have on steroid patterns in soils. In forensic investigations it can be useful to take more than one soil sample in a CDI to reconstruct the body alignment. Although lipids are hydrophobic and associated to organic particulate matter in soils, a property that usually inhibits a transfer along a soil profile, they were found to be vertically distributed in the surface decomposition study. The characteristic soil at the crime scene exhibited a high proportion of sand (~80%) with a high air capacity (~36%; Chapter 3), which presumably promoted free drainage of fluids with soil depth. After one year, steroids were also found in a depth ≥ 10 cm. Steroids were

probably degraded and spatially distributed by soil organisms (= bioturbation) which was previously proposed by Evershed et al. (1997) and Bethell et al. (1994). The depth distribution of steroids in the mass graves was disturbed during the construction, the burial of the bodies and reopening of the graves. Steroids from decomposition fluids of the bodies were introduced into the soil during the burial period of 10 months. In the mass graves steroids indicative for decomposition fluids were found in a soil depth of 40-60 cm, where highest TOC and phosphorus concentrations were found previously (Fiedler et al. 2009a). Hence, soil containing decomposition fluids was homogenised and presumably preserved against microbial degradation in the subsoil. It is still unknown, where highest concentrations of steroids in graves can be found and future studies should include the analysis of soil from the base of a grave.

Archaeological studies demonstrated that steroids were preserved in soils over long periods of time. However, the nutrient rich fluids from a cadaver do also stimulate microbial activity (Cobaugh et al. 2015; Carter et al. 2008; Wilson et al. 2007; Hopkins et al. 2000) and steroids are thus recycled in the soil system. Decreased concentrations were observed from 11-18 days to one year in the surface decomposition case study. Interestingly steroid patterns revealed substantial changes in absolute abundances over time. Most of the selected steroids decreased over one year. 5α-cholestanol and 5β-stigmastanol were the only steroids increasing over time due to $\Delta^5$-sterol reduction in-situ. In the mass graves analysis of steroids was performed 67 years after removal of the bodies, which were temporarily buried (10 months). The mass grave case study demonstrated that human derived steroids are preserved over longer periods up to 67 years. Archaeological investigations proved the long-term preservation of steroids in soils from faecal origin e.g. 1500 BC (Shillito et al. 2011) and 400-700 AD from human tissue origin (Davies and Pollard 1988; Newman and Parkin 1986). Abundances of increased steroids is dependent on the amounts introduced into soil, and skeletons with increased cholesterol levels revealed that cadaveric lipids can be preserved over several hundreds of years (Davies and Pollard 1988). Bile acids are promising biomarkers as they are known to be relatively stable in soils compared to 5β-stanols (Lauer et al. 2014; Elhmmali et al. 1997).

Compared to stanols and bile acids, **fatty acids** are widely spread in soils and their abundances are attributed to various sources (Bull et al. 2000). Analysis of fatty acids was performed on soil samples from the surface decomposition case study (Chapter 4). High concentrations of fatty acids ($FA_T$ 5-330 mg $g_{soil}^{-1}$; Chapter 4) were detected beneath both body positions (11-18 days, thorax and abdomen) compared to reference soil. Oleic acid is the most abundant fatty acid in human adipose tissues, followed by palmitic, linoleic, and palmitoleic acid (Notter et al. 2009; Dent et al. 2004; Makristathis 2002). After 11-18 days high abundances of saturated fatty acids (palmitic and stearic acid) revealed, that a rapid hydrogenation of adipose tissues occurred during that period. Furthermore, 10-hydroxystearic acid was unique for soil sampled beneath the body (11-18 days and one year). After one year, losses of total fatty acids (≤ 1 cm) and myristic acid (≤ 5 cm) were attributed to degradation by β-oxidation and to leaching processes (Michal 2013; Dent et al. 2004; Knopp 1904). Because fatty acids derive from various natural sources, their diagnostic potential is thus restricted; it is rather useful to analyse fatty acids in combination with steroids because it has been shown that 10-hydroxystearic acid indicates further processes (i.e. adipocere formation)

involved in cadaver decomposition. Additionally, it was shown that the diagnostic potential of human derived fatty acids can be improved by analysing $\delta^{13}C$ signals of fatty acids (Bull et al. 2009).

Recent studies demonstrated the presence of anaerobic areas between a decomposing body and the underlying ground (Cobaugh et al. 2015; Zimmermann et al. 2008). The evidence of anaerobic zones was suggested by the formation of **adipocere** (Zimmermann et al. 2008) and the detection of intestinal anaerobic microorganisms invading the soil below a carcass (Cobaugh et al. 2015). In the present work the findings of 10-hydroxystearic acid and fatty acid salts (mainly $Ca^{2+}$-salts) beneath a decomposing body (Chapter 4) let suggest analogous processes. Beneath the body, an anaerobic environment was created in the time frame of 11-18 days, where 10-hydroxystearic acid and fatty acid salts were formed. 10-hydroxystearic acid is a typical marker of adipocere and can be formed by several body and soil microorganisms (Takatori 2001; Takatori et al. 1988; Takatori et al. 1986). The presence of fatty acid salts further presumed the formation of adipocere-like material from decomposition fluids in the soil below the carcass. The increase of $Ca^{2+}$- and $Mg^{2+}$-salts showed preservation processes of the cadaveric fatty acids in the soil sampled after one year. During adipocere formation fatty acids usually take up $K^{+}$ and $Na^{+}$ from the internal body environment to form fatty acid salts (Fiedler and Graw 2003; Gill-King 1997). In soils, $K^{+}$- and $Na^{+}$-salts are replaced by $Ca^{2+}$- and $Mg^{2+}$, which are water insoluble and prevent microbial access (Dent et al. 2004). This process is not entirely attributed to adipocere formation. It is rather a process, which commences when $K^{+}$ and $Na^{+}$ are replaced by $Ca^{2+}$ and $Mg^{2+}$ in fatty acid salts. The formation of $Mg^{2+}$- and $Ca^{2+}$-salts is dependent on the availability of these ions in the soil environment. Acidic soils lack in basic cations and no formation of fatty acid salts might take place. In summary, results in this study let suggest that adipocere formation from decomposition fluids might be a natural process, because anaerobic zones beneath the body are created by oxygen depletion. This observation is relatively novel as it shows preservation of decomposition fluids by adipocere formation in soil. It is normally not expected under climatic conditions (summer, dry, temperatures 13-23°C) at the study site. However, when soft tissue degradation is completed or the body remains are removed, it was presumed that oxidative degradation of fatty acids commence and thus the overall loss of cadaveric fatty acid abundances over time.

Adipocere decomposition was assessed under different biotic and abiotic soil factors. Because of similar ADDs and precipitation levels, the climatic conditions were ruled out as factors on adipocere decomposition (Chapter 6). Regardless of the treatments (soil type, incubation period, gauze size) amounts of adipocere assessed varied considerably, which complicated straightforward interpretation of the results (Chapter 6). Variability of adipocere degradation was repeatedly observed (Schotsmans et al. 2011; Mant 1987), but reasons are still poorly understood. However, it was shown that adipocere decomposition proceeded faster in the Arenosol (after 419 days 85% loss) than in the Calcisol (after 419 days 30% loss). Redoximorphic characteristics and secondary lime precipitates indicated the close water table which might have influenced the aeration of the Calcisol and thus microbial degradation. An involvement of macrofauna (2000 µm minicontainer) in adipocere decomposition was observed in the Calcisol after 419 days (22%; Chapter 6). The low involvement of macrofauna on adipocere degradation in the Arenosol was attributed to the low pH value (Römbke et al. 1997; Scheu 1990; Abrahmason 1972) and the low soil moisture (Rousk

et al. 2009). It was assumed that the main drivers of adipocere decomposition in the Arenosol was the involvement of fungi (Bååth and Anderson 2003; Gong et al. 2001; Blagodatskaya and Anderson 1998) and the consistent aeration of the topsoil (Neira et al. 2015; Moldrup et al. 1998). Fungi might have better abilities to bridge air-filled spaces between adipocere, soil and the minicontainers, while bacteria were prevented in access, because their distribution dependents on water. In summary, selected time periods were relatively short (Fründ and Schoenen 2009) to ascertain the involvement of adipocere degradation in the Calcisol and future studies should address longer observation periods.

## 7.3. Final conclusion

In the present study, it has been shown that selected markers were suitable as indicators for decomposition fluids in soils. The first indices that an input of cadaveric material into soil occurred are increased concentrations of lipids compared to an adjacent reference soil (Chapter 3-Chapter 5). In the mass grave case study it was demonstrated, that faecal steroids rather than tissue steroids were indicative for human decomposition fluids. A time sequence of 11-18 days and 358 days in the surface decomposition study and 67 years in the mass grave study showed that cadaveric lipids decrease in total abundances, but remain indicative for soil comprising human decomposition fluids (Chapter 6).

In addition the absence of lipids in reference samples such as 10-hydroxystearic acid (Chapter 4), epicoprostanol, 5β-cholestanone, 5β-stigmastanol, epi-5β-stigmastanol and isolithocholic acid (Chapter 6) clearly indicated an introduction of external organic matter. In both case studies it was known that human bodies were present. Because lipids could also derive from the decomposition of other animals (e.g. pigs), methods must be developed to increase a precise assessment of the human origin of decomposition fluids.

Abundances of fatty acid salts and 10-hydroxystearic acid in the surface decomposition case study, demonstrated additional processes in-situ (Chapter 4). 10-hydroxystearic acid is an indicator for adipocere formation, and anaerobic zones between the body and the soil surface during the presence of the body (11-18 days) might have caused its formation (Cobaugh et al. 2015; Zimmermann et al. 2008). The presence of fatty acids salts can be attributed to adipocere formation, but also demonstrated mechanisms of fatty acid preservation in soils comprising decomposition fluids and $Mg^{2+}$ and $Ca^{2+}$ ions.

In summary, the mass grave study showed, that a multiple lipid approach and an increased range of convincing biomarkers enables a close proximate of identifying human decomposition fluids in soils. The selection of suitable lipids must always be adjusted to the specific forensic case.

Adipocere degradation was less effective in slightly alkaline, loamy soils (Calcisol) with a high capillary fringe, which impaired aeration and thus adipocere degradation (Chapter 6). The impact of macrofauna on adipocere decomposition was assessed in the Calcisol. In summary, the main factors in adipocere degradation were the involvement of fungi and the aeration of soils, which had a positive effect on adipocere degradation in the Arenosol. Well aerated soils are important for the reuse of graves at cemeteries, to ensure a complete decomposition of human remains within the regular resting time (15-25 years; Fiedler and Graw 2003).

### 7.3.1. Perspectives – the human fingerprint

The present work aimed in understanding the fate of human decomposition products, namely cadaveric lipids in soils. During the study further future objectives had arisen, which could be subjects for future research:

- To extend the present laboratory methods ($\Delta^5$-sterols and stanols) with stanones, bile acids and fatty acids and validate methods by using standard addition. With this method a wider range of cadaveric lipids can be analysed from a single soil sample.
- Field replicates are important to strengthen knowledge about the fate of cadaveric lipids in soils. It promotes a detailed assessment of processes involved in the distribution of steroids and fatty acids in soils beneath decomposing bodies. Such investigations can be performed on body farms (e.g. Forensic Anthropology Centre in Tennessee, USA), where soil types and climatic conditions are relatively similar.
- Bile acid analysis of the mass graves had shown that high abundances of bile acids are present in the natural background. Natural sources of bile acids in forest soils are unknown. It is thus helpful to determine steroid distributions and abundances in natural undisturbed forests soils and to predict their origins.
- A detailed assessment of adipocere formation in anaerobic areas between the body and the underlying soil might help in understanding if adipocere is usually formed from decomposition fluids between a body and the underlying soil, or under specific circumstances.
- To study adipocere decomposition in association with soil macrofauna under particular consideration of the impact of earthworms.
- "The human fingerprint": a multi-molecular approach including $\Delta^5$-sterols, stanols, stanones, bile acids and fatty acid analysis of soil samples is a further step to identify human decomposition fluids in temporary graves.

In the present work, the analysis of soils comprising decomposition fluids of human cadavers facilitated the identification of decomposition products of a human origin. The input of degradation products of other animals has to be considered. Furthermore it is important to distinguish between the input of faecal sources (e.g. organic fertilisers, latrines) and tissue sources combined with faecal sources (e.g. decomposition products). In environmental and archaeological research, ratios between different steroids were introduced to distinguish between different animal sources, humans included (Prost et al. (in prep); Birk et al. 2011; Bull et al. 2002). Prost et al. (in prep) proposed an improved analytical approach to differentiate faeces from old livestock breed for archaeological purposes. It is possible to distinguish faecal material of omnivores from herbivores with a ratio (coprostanol/(corpostanol+5β-stigmastanol)) introduced by Leeming et al. (1997). Faecal sources of humans and pigs can then be distinguished with hyodeoxycholic acid (HDCA) which is absent in human faeces but the dominant bile acid in pig faeces (Prost et al. (in prep)). To ascertain the input of human decomposition fluids, a ratio can then be developed which identifies

tissue steroids which should dominate faecal steroids (e.g. cholesterol/coprostanol). In soils, where decomposition fluids were preserved over longer periods, such as in the mass graves, the environmental 5α-stanols (e.g. 5α-cholestanol) and the epimers of faecal 5β-stanols (e.g. epicoprostanol, epi-5β-stigmastanol) can be included in such ratios. In comparison with ratios calculated for reference soil, these ratios might aid in identifying the human origin of decomposition products in soils.

## 7.4. References

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# Appendices

# Appendix 1 (Chapter 2)

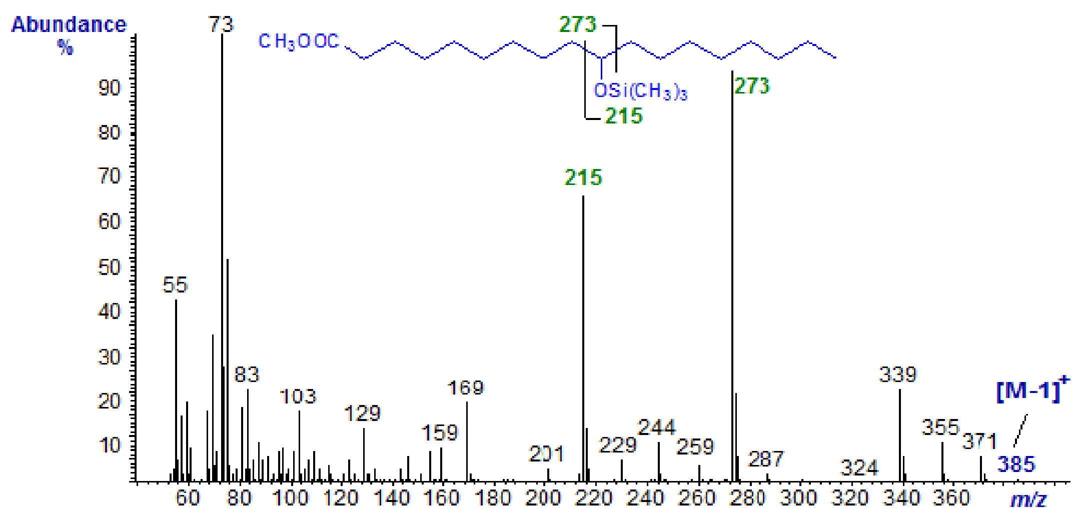


**Figure SI 1.1**: Mass spectrum of TMS 10-hydroxystearate (Christie 2014).

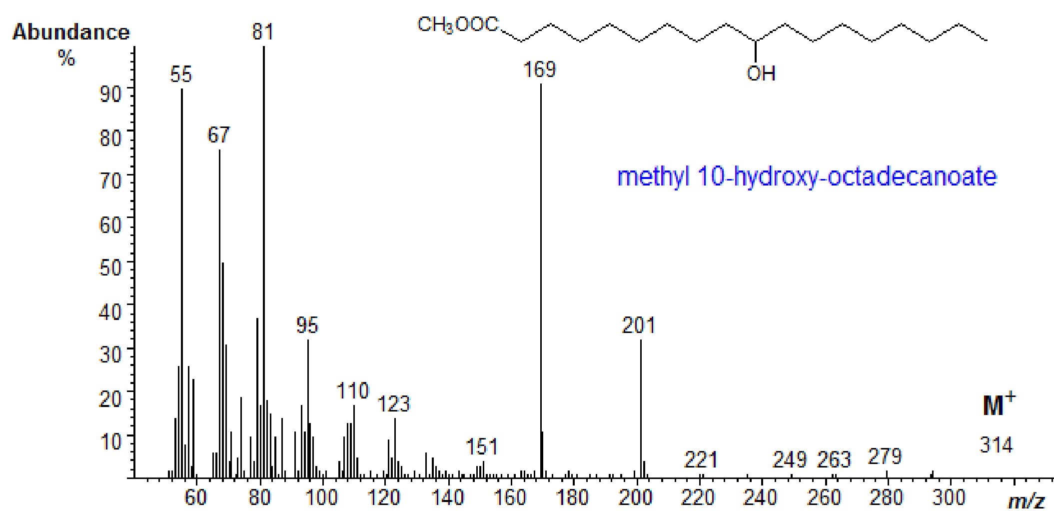


**Figure SI 1.2**: Mass spectrum of methyl 10-hydroxy-octadecaonate (= methyl 10-hydroxystearic acid (Christie 2016)).

# Appendix 2 (Chapter 3)

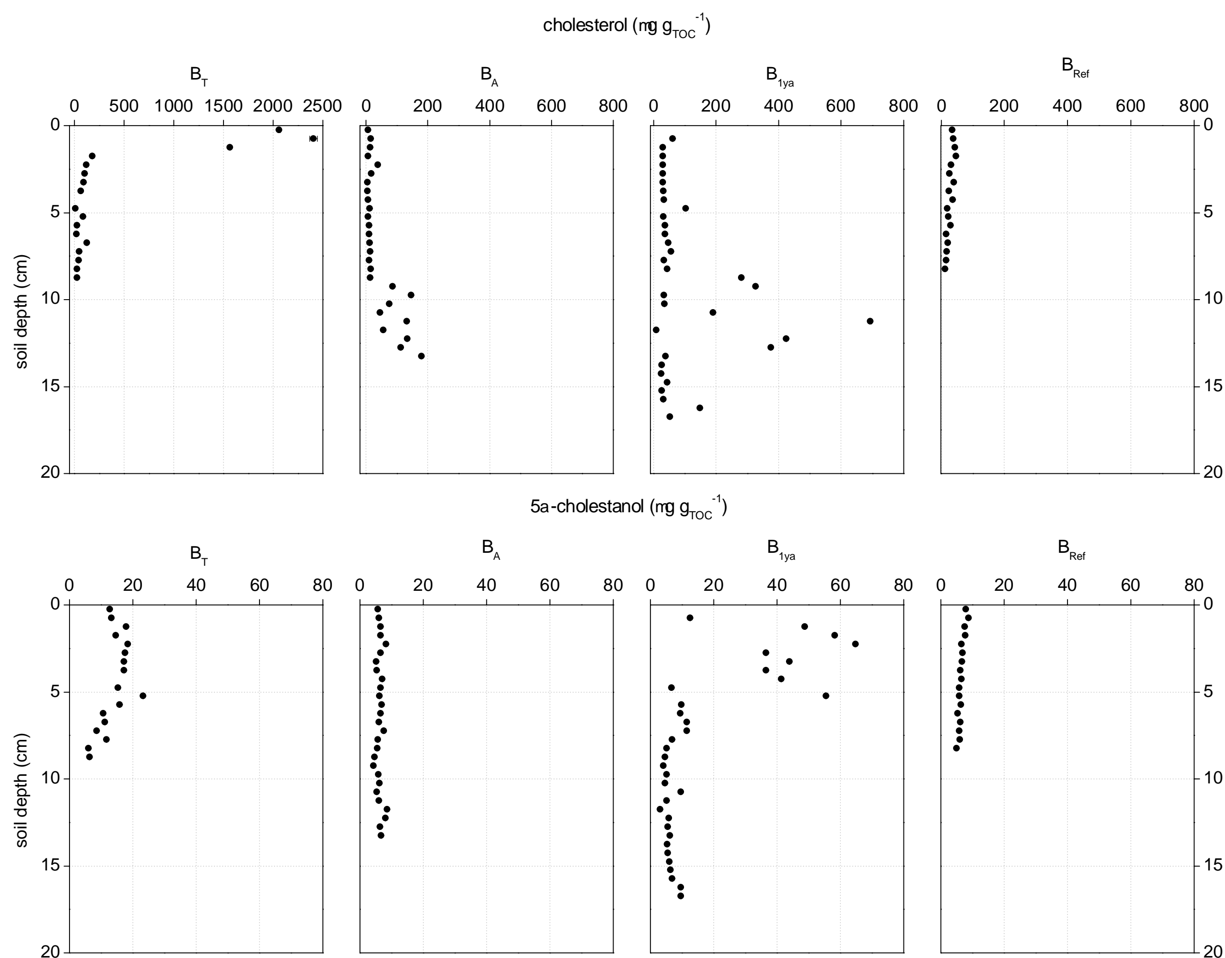


**Figure SI 2.1:** Depth distributions of cholesterol and 5α-cholestanol (μg $g_{TOC}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

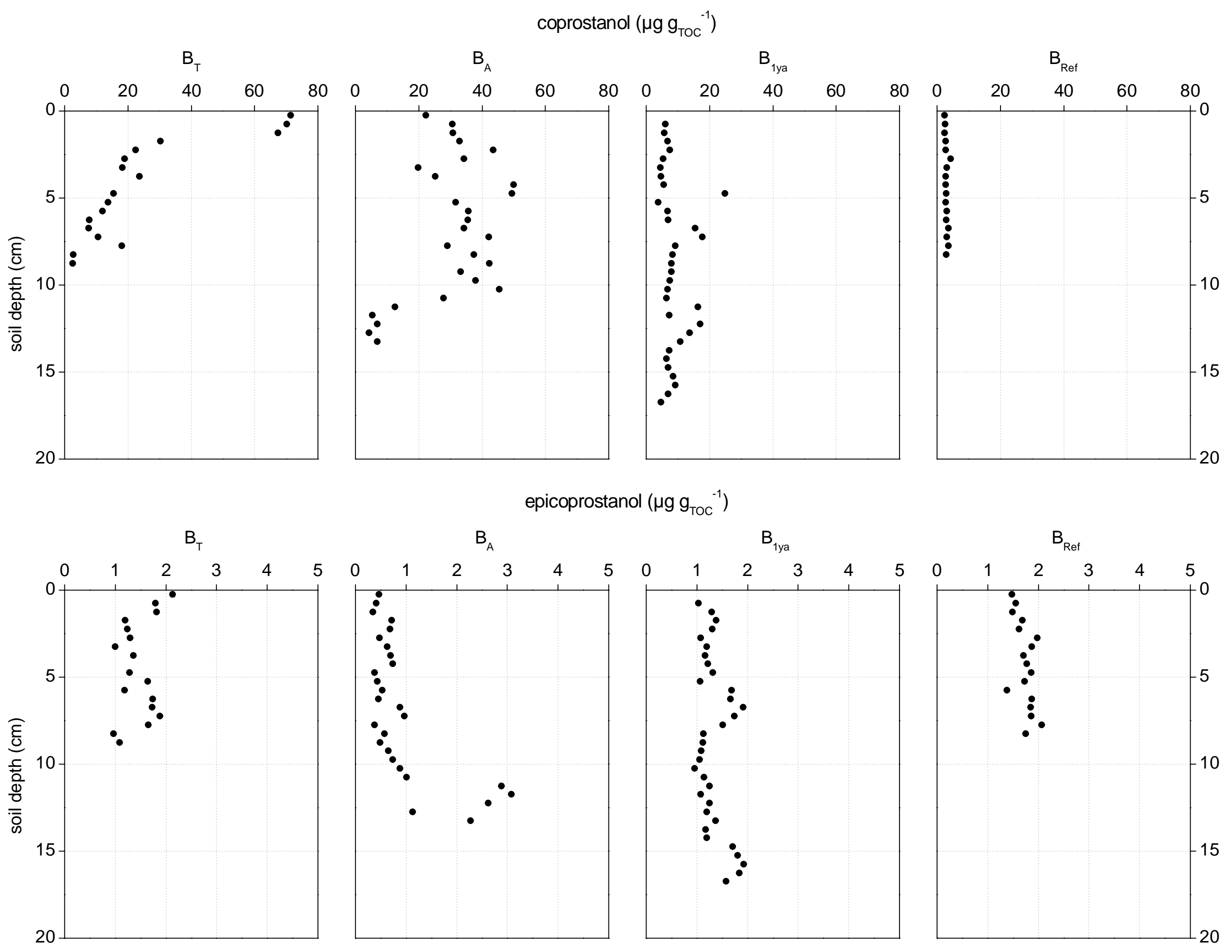


**Figure SI 2.2:** Depth distributions of coprostanol and epicoprostanol (µg $g_{TOC}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

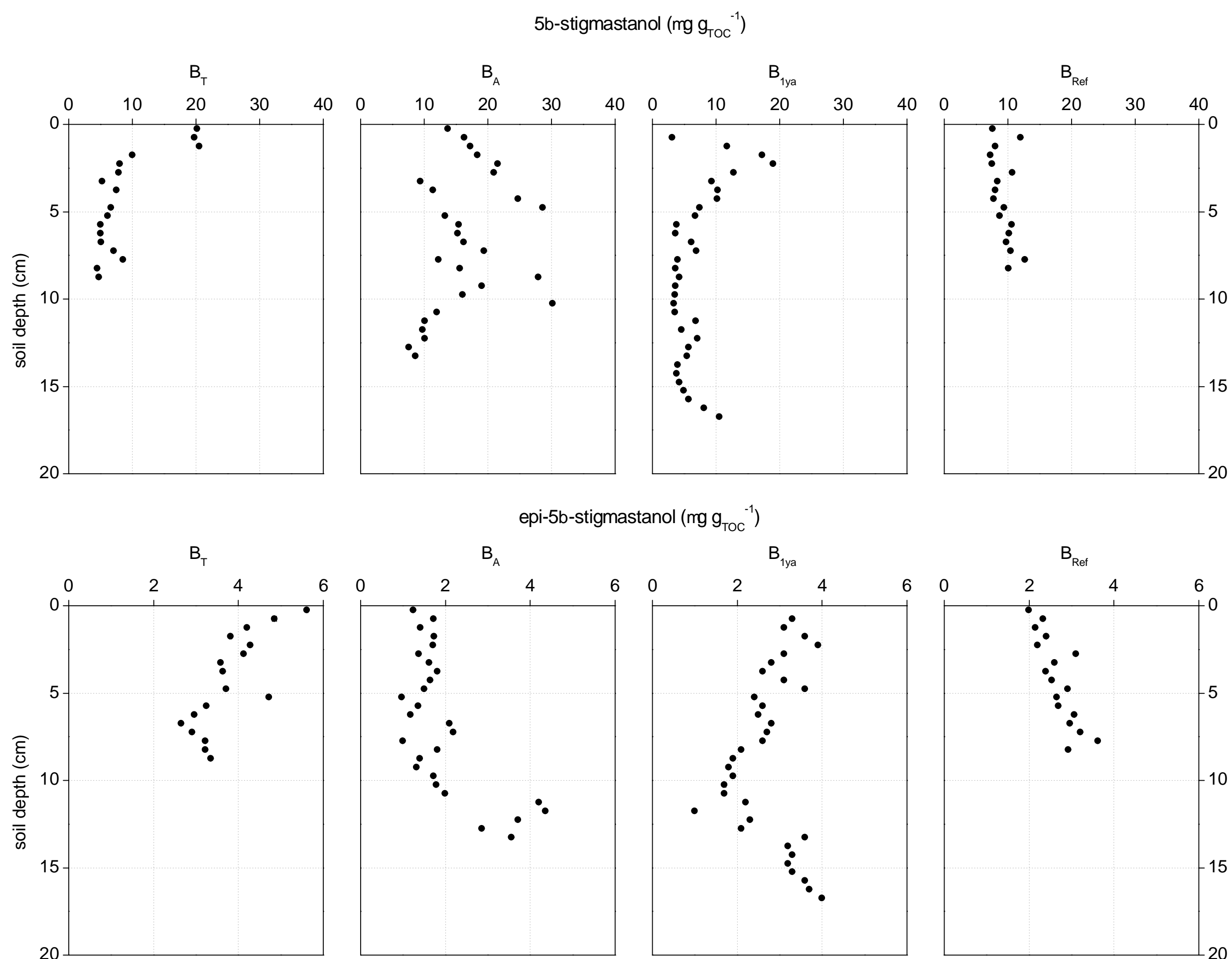


**Figure SI 2.3:** Depth distributions of 5β-stigmastanol and epi-5β-stigmastanol (μg $g_{TOC}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

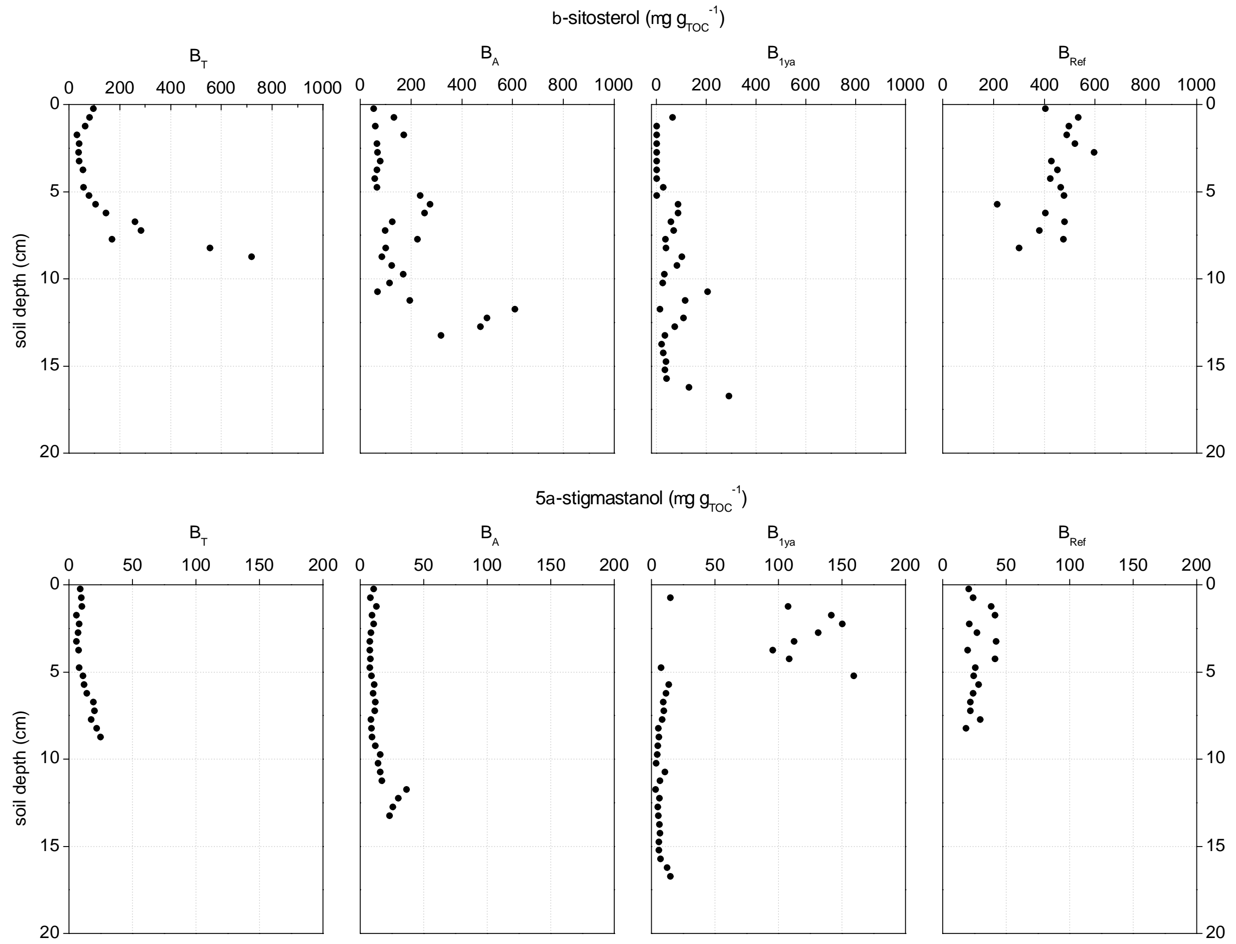


**Figure SI 2.4:** Depth distributions of β-sitosterol and 5α-stigmastanol (µg $g_{TOC}^{-1}$) of soil samples from block $B_T$ (beneath thorax), $B_A$ (beneath abdomen), $B_{1ya}$ (beneath thorax, 358 days after body removal) and $B_{Ref}$ (distance to the body 10 m) from the cadaver decomposition island (CDI). Error bars represent standard error of 2 laboratory replicates.

# Appendix 3 (Chapter 4)

**Table SI 3.1:** Fatty acids in mg $g_{soil}^{-1}$ (n=2) analysed with soil depth beneath a decomposing body: 11-18 days after deposition of the body beneath the thorax ($B_T$) and the abdomen ($B_A$). $C_{14:0}$ = myristic acid, $C_{16:0}$ = palmitic acid, $C_{18:0}$ = stearic acid, $C_{18:1\Delta9}$ = oleic acid, $C_{18:2}$ = linoleic acid and 10-OH-$C_{18:0}$ = 10-hydroxystearic acid.

| position | soil depth | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1\Delta9}$ | $C_{18:2}$ | 10-OH-$C_{18:0}$ |
|---|---|---|---|---|---|---|---|
| | cm | mg g soil$^{-1}$ mean ± standard error | | | | | |
| $B_T$ | 0-0.5 | 0.373 | 2.759 | 0.549 | 3.425 | n.d. | 323.668 |
| thorax | 0.5-1 | 1.689 | 17.553 | 2.963 | 6.664 | 0.078 | 10.022 |
| | 1-1.5 | 0.697 | 5.914 | 1.172 | 6.232 | n.d. | 10.622 |
| | 1.5-2 | 0.607 | 4.852 | 1.006 | 3.098 | n.d. | 4.854 |
| | 2-2.5 | 0.611 | 4.393 | 0.918 | 2.648 | n.d. | 4.394 |
| | 2.5-3 | 0.513 | 4.340 | 1.003 | 2.874 | n.d. | 8.502 |
| | 3-3.5 | 0.413 | 3.650 | 0.940 | 2.216 | n.d. | 9.780 |
| | 3.5-4 | 0.481 | 3.500 | 0.771 | 1.417 | n.d. | 6.522 |
| | 4-4.5 | 0.347 | 3.274 | 0.788 | 1.342 | n.d. | 2.669 |
| $B_A$ | 0-0.5 | 0.439 | 2.617 | 0.361 | 1.587 | n.d. | 46.549 |
| abdomen | 0.5-1 | 1.750 | 12.288 | 1.521 | 2.820 | n.d. | 0.827 |
| | 1-1.5 | 1.622 | 11.845 | 1.436 | 0.973 | n.d. | 0.834 |
| | 1.5-2 | 1.478 | 10.569 | 1.312 | 1.126 | n.d. | 0.372 |
| | 2-2.5 | 1.393 | 10.332 | 1.292 | 0.934 | n.d. | 0.389 |
| | 2.5-3 | 0.417 | 4.758 | 0.685 | 2.316 | n.d. | 0.736 |
| | 3-3.5 | 0.539 | 5.683 | 0.757 | 1.207 | n.d. | 0.845 |
| | 3.5-4 | 0.344 | 4.460 | 0.648 | 0.623 | n.d. | 0.261 |
| | 4-4.5 | 0.382 | 2.584 | 0.575 | 0.930 | n.d. | 0.468 |
| | 4.5-5 | 0.273 | 4.245 | 0.688 | 2.140 | n.d. | 0.606 |

**Table SI 3.2:** Fatty acids in mg $g_{soil}^{-1}$ (n=2) analysed with soil depth beneath a decomposing body: 358 days (one year) after discovery of the body ($B_{1ya}$) and the reference ($B_{Ref}$) taken 10 m away from the body. $C_{14:0}$ = myristic acid, $C_{16:0}$ = palmitic acid, $C_{18:0}$ = stearic acid, $C_{18:1\Delta9}$ = oleic acid, $C_{18:2}$ = linoleic acid and 10-OH-$C_{18:0}$ = 10-hydroxystearic acid.

| **position** | **soil depth** | **$C_{14:0}$** | **$C_{16:0}$** | **$C_{18:0}$** | **$C_{18:1\Delta9}$** | **$C_{18:2}$** | **10-OH-$C_{18:0}$** |
|---|---|---|---|---|---|---|---|
| | cm | mg g soil$^{-1}$ mean ± standard error | | | | | |
| $B_{1ya}$ | 0.5-1 | 0.047 | 5.447 | 0.808 | 1.243 | n.d. | 10.624 |
| 358 days | 1.5-2 | 0.038 | 5.571 | 1.077 | 1.384 | n.d. | 32.591 |
| | 2-2.5 | 0.078 | 5.672 | 1.087 | 1.438 | n.d. | 1.257 |
| | 2.5-3 | 0.147 | 7.039 | 1.182 | 1.648 | n.d. | 5.068 |
| | 3-3.5 | 0.169 | 8.007 | 1.216 | 1.901 | n.d. | 15.389 |
| | 3.5-4 | 0.177 | 7.862 | 1.191 | 2.153 | n.d. | 11.588 |
| | 4-4.5 | 0.136 | 6.982 | 1.088 | 1.991 | 0.060 | 7.428 |
| | 4.5-5 | 0.412 | 6.047 | 1.008 | 4.360 | n.d. | 0.799 |
| | 5-5.5 | 0.122 | 7.384 | 1.297 | 3.026 | n.d. | 0.639 |
| $B_{Ref}$ | 0.5-1 | n.d. | 0.012 | 0.006 | 0.005 | n.d. | n.d. |
| reference | 1-1.5 | n.d. | 0.010 | 0.004 | 0.005 | 0.003 | n.d. |
| | 1.5-2 | n.d. | 0.021 | 0.005 | 0.006 | n.d. | n.d. |
| | 2-2.5 | n.d. | 0.008 | 0.004 | 0.004 | n.d. | n.d. |
| | 2.5-3 | n.d. | 0.008 | 0.004 | 0.004 | 0.004 | n.d. |
| | 3-3.5 | n.d. | 0.007 | 0.006 | 0.005 | n.d. | n.d. |
| | 3.5-4 | n.d. | 0.007 | 0.005 | 0.005 | n.d. | n.d. |
| | 4-4.5 | n.d. | 0.008 | 0.006 | n.d. | n.d. | n.d. |
| | 4.5-5 | n.d. | 0.006 | 0.005 | 0.005 | 0.004 | n.d. |
| | 5-5.5 | n.d. | 0.008 | 0.005 | 0.005 | 0.005 | n.d. |

## Appendix 4 (Chapter 6)

The physical and biochemical soil parameters were determined using standard laboratory protocols. In November 2012, the two soil types were analysed based on reference soil samples taken from each soil horizon. All soil analyses data given in Tab. SI 4.1 were determined using the methods of Blume et al. (2011). Standard soil analyses were performed on the fine earth fractions (≤ 2mm): particle-size distribution, pH ($CaCl_2$) and soil carbonate ($CaCO_3$). Total organic carbon (TOC) and nitrogen ($N_t$) were determined by dry combustion (Euro EA, Eurovector, Milan, Italy). Microbial biomass ($C_{mic}$, $N_{mic}$) carbon and nitrogen were extracted from fresh soil samples taken at each horizon using the chloroform fumigation extraction method (CFE; Joergensen 1996; Vance et al. 1987). Carbon and nitrogen containing compounds of fumigated and unfumigated extracts were analysed with a TOC-TNb multi-N/C® 2100S analyser (Analytik Jena AG, Germany). Exchangeable $Ca^{2+}$ was determined at pH 7 according to Lavkulich (1981).

The physical parameters were determined using undisturbed soil cores (100 $cm^3$, n=5). Bulk density (BD) was calculated from dry weight divided by core volume. Water can be bound to pores by hydration as well as capillary and osmotic forces. The intensity of these forces is expressed as suction tension or pF value (= - log (cm water column); in soils as pF 0-7). In addition to soil texture, pore size is an important physical feature. In general, soil pores are divided into four categories: finepores (< 0.2 µm), mesopores (0.2-10 µm), fine macropores (10-50 µm) and coarse macropores (> 50 µm). In order to drain the pores from water, water-saturated soil cores were placed in a pressure container on saturated ceramic plates (Eijkelkamp, Giesbeek, the Netherlands) and exposed to overpressure equivalent to the values of different pore-size classes (pF > 4.2 = finepores, pF 2.5-4.2 = medium-sized pores, pF 1.8-2.5 = fine macropores, pF < 1.8 = coarse macropores). The soil cores were weighed and saturated between pressure intervals. After drainage, the soil cores were dried at 105°C for 12 h and weighed (= dry weight). The total pore volume ($PV_t$, vol.%) was calculated taking into account dry weight of the cores and substance density of mineral soils (absolute term 2.65 g $cm^{-3}$). The weight of the drained water between pF intervals is equivalent to the pore volume (vol.%).

Thus, the air capacity (AC, vol.%) correlates with the volume of coarse macropores which cannot keep the water that drains away under the influence of gravity. In contrast, smaller soil pores can hold water by capillary action. AC was calculated by subtracting the pF 1.8 volume from the $PV_t$. Other important soil parameters are: field capacity (FC = pF 1.8 – pF 4.2, vol.%) and wilting point (WP at pF 4.2). Plants cannot use water below WP as it is strongly bound to soil surfaces (Blume et al. 2011).

SI 4.1: Field parameters, physical, chemical and microbial characteristics of the Calcisol and Arenosol in which the minicontainers were buried.

| Soil type | depth | Particle size distribution | | | pH | $CaCO_3$ | TOC | $N_t$ | $C_{mic}$ | $N_{mic}$ | $Ca^{2+}$ | BD | AC | FC | $PV_t$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | sand | silt | clay | | | | | | | | | | | |
| horizon | (cm) | (%) | | | | (g $kg^{-1}$) | | | (µg $g^{-1}$) | | (mg $kg^{-1}$) | (g $cm^{-1}$) | (vol.%) | | |
| **Calcisol** | | | | | | | | | | | | | | | |
| Akh1 | -12 | 35 | 31 | 34 | 7.3 | 58 | 65 | 4.8 | 579±77 | 83±11 | 8 | 0.9 | 20.9 | 23.8 | 67.2 |
| Akh2 | -30 | 36 | 31 | 33 | 7.8 | 66 | 31 | 2.3 | 278±20 | 36±1 | 8 | 1.1 | 18.6 | 23.1 | 57.7 |
| Bkg | -68 | 26 | 44 | 30 | 7.8 | 540 | 7.6 | 0.4 | n/a | n/a | 7 | 1.3 | 10.4 | 21.4 | 50.9 |
| 2Ckr | -83 | 80 | 13 | 7 | 7.9 | 81 | 2.3 | 0.1 | n/a | n/a | 5 | 1.6 | 11.4 | 22.8 | 39.6 |
| **Arenosol** | | | | | | | | | | | | | | | |
| Ah | -3 | 92 | 3 | 4 | 3.4 | n.d. | 43 | 2 | n/a | n/a | 0.40 | 1.2 | 42.4 | 3 | 54.3 |
| Bw | -12 | 95 | 3 | 3 | 3.7 | n.d. | 7 | 0.4 | 33±4 | 3±2 | 0.05 | | | | |
| 2Ahb | -32 | 92 | 3 | 5 | 3.5 | n.d. | 7 | 0.4 | 30±13 | 3±1 | 0.05 | 1.3 | 37.8 | 9.5 | 51.1 |
| 2Bwb | -74 | 96 | 2 | 2 | 4.0 | n.d. | 1 | 0.1 | n/a | n/a | 0.02 | 1.4 | 37.6 | 5.9 | 46.1 |

TOC, total organic carbon | $N_t$, total nitrogen| $C_{mic}$, microbial carbon (mean ± standard error, n= 3) | $N_{mic}$, microbial nitrogen (mean ± standard error, n= 3) | $Ca^{2+}$, exchangeable calcium ions | BD, bulk density | AC, air capacity | FC, field capacity | $PV_t$, total pore volume | n.d., not detected | n/a, not analysed

**Table SI 4.2:** Weight lost by the adipocere pellets over a period of 234 and 419 days, respectively. The adipocere samples were enclosed in containers covered with plastic gauze of different pore size.

| | Calcisol | | | Arenosol | | |
|---|---|---|---|---|---|---|
| Gaze pore size (µm) | 20 | 500 | 2000 | 20 | 500 | 2000 |
| **Adipocere loss (%)** | **Analysis after 234 days (30.11.2012-22.07.2013)** | | | | | |
| Sample 1 | 51.9 | 17.3 | 48.8 | 95.0 | 98.6 | 82.1 |
| Sample 2 | 7.4 | 63.6 | 19.6 | 42.5 | 92.5 | 92.5 |
| Sample 3 | 35.6 | 35.8 | 61.8 | 65.9 | 77.0 | 89.4 |
| Sample 4 | 17.5 | 35.5 | 18.3 | 65.9 | 94.8 | 94.9 |
| Sample 5 | 50.0 | 27.2 | 17.7 | 81.3 | 4.9 | 57.9 |
| Sample 6 | 39.3 | 11.1 | 23.3 | 59.7 | 30.0 | 43.9 |
| Mean ± SD | 33.6±17.8 | 31.8±18.5 | 31.6±18.9 | 68.4±18 | 66.3±39.4 | 76.8±21 |
| | **Analysis after 419 days (30.11.2012-23.01.2014)** | | | | | |
| Sample 1 | 23.4 | 38.3 | 83.1 | 36.3 | 68.4 | 88.0 |
| Sample 2 | 10.5 | 39.5 | 40.7 | 97.8 | 89.7 | 89.6 |
| Sample 3 | 23.9 | 35.8 | 40.7 | 94.3 | 94.6 | 94.9 |
| Sample 4 | 25.7 | 40.1 | 55.5 | 97.0 | 90.6 | 86.0 |
| Sample 5 | 44.1 | 32.1 | 49.5 | 62.5 | 60.4 | 71.3 |
| Sample 6 | 38.7 | 22.7 | 71.6 | 96.5 | 96.4 | 88.4 |
| Mean ± SD | 27.7±12 | 34.8±6.6 | 56.8±17.2 | 80.7±25.7 | 83.4±15.1 | 86.4±8 |

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# Lebenslauf