Interpreting Anthropogenic Signals From a Clandestine Grave Via Soil Lipid Biomolecular Analysis

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ABSTRACT: The identification of a buried cadaver becomes complicated for remains which badly decomposed as the DNA analysis is not possible. As the potential of cadaveric derived lipids have been recognised to be used as 'biomarkers' to detect a clandestine grave and/or the provenance of a cadaver, subsequently to determine its post-mortem interval (PMI), soils from eleven identified crime scenes were collected. The biomolecular analysis of the soil lipid extracts by GC and GC/MS remarkably shows the shifting in the sources of lipids which can be associated with post-dispositional interval (PDI) and/or PMI. The soils with low PMI contain lipids components that are most likely derived from decomposing fatty flesh, exhibiting a higher concentrations of palmitic (C16:0) and stearic (C18:0) acids, unsaturated analogues palmitoleic (C16:1) and oleic (C18:1) acids, and cholest-5-en-3β-ol (cholesterol). Whilst, the cases with longer PDI demonstrate a large shifts towards higher fatty acid homologues, with the presence of sitosterol, 5α-stigmastanol and β-amyrin indicating the predominance inputs of plant derived organic material. As conclusion, the lipid distributions would provide useful information for forensic investigations.

KEYWORDS: Human decomposition, soil lipids, free fatty acids, postmortem interval, clandestine grave, lipid bimolecular analysis

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INTRODUCTION

The analysis of lipids has been recognised to provide a highly diagnostic means to study the differences of soil organic matter. The fundamental principle behind this organic geochemical analysis is the persistence of many classes of lipid in the soil over geological time, in either their original or diagenatically altered forms (Evershed, 1993). This enables their detection which may potentially to be used in determining the origin of lipid compounds found in the soil of interest. Lipid represents a significant pool of organic carbon in soil. The distribution of the lipid components recovered from the soils would shows a large contribution from the input of terrestrial organic matter and anthropogenically activity (Moucowi *et al*., 1981; van Bergen *et al*., 1997; Bull *et al*., 1999).

The suitability of this organic geochemical analysis is addressed further in investigating the changes on the soil lipid wrought by a decomposing body. Soils have successfully been used in archeological studies to reconstruct aspect of ancient life (Evershed, 1992). This has been achieved through the biomolecular analysis where specific molecules are regarded as biomarkers, in this study is lipid, that are preserved within the soils or artefacts associated with the site under study. This approach has been used in the detection of biomarkers derived from faecal matter within archaeological soils through the use of highly specific biomarkers, i.e. 5β-stanols and bile acids (Bull *et al*., 1999).

It was envisaged that the soils collected from the crime scenes would potentially provide a comprehensive sample set, thereby enabling a robust comparison of alteration and preservation of organic materials, particularly lipids. The introduction of a cadaver into the terrestrial has known to cause the alteration of soil chemical composition (Benninger *et al*., 2008). The body components, e.g. C, N, and P are released during the decomposition into underneath soil, maximum influx is detectable during active decay (Forbes *et al*., 2005; Carter *et al*., 2007). The seeping of these substantial cadaver derived materials into surrounding soils creates a localized patch of nutrients in the environment which has been described as a cadaver decomposition island (CDI) (Vass *et al*., 1992). Furthermore, the presence of a corpse would result in the addition of a range of distinct fatty acid components into the soils. The fatty acyl composition of fresh human tissue, i.e. triacylglycrides (TAGs) and phospholipids, is primarily palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$). Oleic acid is the most abundant (Evershed and Connoly, 1994; Forbes *et al*., 2002).

Eleven cases were identified as being cases of a particular interest for further scientific analysis in order to produce basic information on the persistent of burial activity signal over a much longer period of time. The cases varied in the post depositional interval (PDI), ranging from a few days since death to more than sixty years of interval. PDI is a significant feature that determines the classes of lipids which have been introduced to the burial environment. The exact PMI/PDI of each case was not known, even for the recent cases. The police reports and results of *in situ* examination by a pathologist have been taken into account to estimate an approximate interval (PMI/PDI). However, several samples were not been considered and discussed further due to the very low concentration of lipids recovered from the soils. The most likely explanation to the circumstances is that these crime scenes had experienced heavy flush of the organic material to the underground soil during the precipitations over time.

METHODOLOGY

Sampling and site descriptions

Sampling was carried out over three months and performed with a collaboration of Forensic Laboratory Royal Malaysia Police (RMP). In total 67 soils from different points of each scene were collected from the eleven selected cases. The criteria for choosing these sites were: (i) sites have been recognized as reported crime scenes where the body was found. Therefore, there is complete documentation of the cases, (ii) for cases where no remains were found, the sites have been identified to have been a body disposal area, such as a graveyard. Soils were collected from underneath specific anatomy parts of the deceased, e.g. abdomen, head or leg, which were believed to be areas contributing large quantities of organic matter in the soils. Soils were also collected from other points of each grave, for instance the grave wall and floor, especially for the cases with a longer PDI by where the body was not present during sampling. The location of the body in the grave upon recovery was identified with the assistance of photographs, police report and the police officers who were involved in the investigation of the cases. Soils that were taken approximately one meter distance from the body were regarded as 'control' samples throughout the analysis. Soils were stored in a freezer before been transported to the laboratory for analysis.

Isolation and pre-treatment of lipid compounds

Freeze dried soil was used for the lipid extraction. The lipid extraction was carried out by the modified method of modified Bligh and Dyer. The soils (approx. 2g) transferred into a culture tube

(8 ml), the dry weight obtained and treated with 3 ml DCM/Methanol (2:1 v/v), spiked with 100 µl internal standard tetratriacontane, sonicated for 15 min and centrifuged for 5 min (~3000 rpm). The supernatant was transferred into a clean vial. The process was repeated three times with 2 ml DCM/Methanol (2:1 v/v). The soil then treated with 3 ml of Bligh Dyer solvent, sonicated (15 min) and centrifuged (~3000 rpm, 5 min). The resulting supernatant was transferred into the same vial. The extraction was repeated with 3 x 2 ml Bligh-Dyer solvent. A 2 ml each of buffered water and chloroform were added to the supernatant to break the organic phase, then the mixture was centrifuged for 1 min (~3000rmp). The organic layer then was transferred into a clean vial. This process was repeated with 3 x 2 ml chloroform. The solvent was evaporated from the resulting TLE solution under a gentle flow of nitrogen, and the resultant TLE was stored in a freezer (>-20°C).

Instrument Analysis

Derivatised fractions were analysed using a Hewlett-Packard 58900 series II gas chromatograph (GC) equipped with a fused-silica capillary column (Varian Chrompack CPSil-5CB, 50 m length x 0.32 mm i.d., film thickness 0.12 µm). Samples were dissolved in hexane and injected (1.0µl) on-column. The temperature of GC was programmed from 40^{0}C (2 min isothermal) to 300^{0}C at a rate of 10^{0}C min^{-1}(10 min isothermal). The flame ionization detector (FID) temperature was held at 300^{0}C. Hydrogen gas with head pressure 10 psi was used as carrier gas.

Gas chromatography-mass spectrometry (GC/MS) analyses were performed using a ThermoFinnigan Trace MS equipped with a fused silica capillary column (Phenomenex. ZB1; 60 m length × 0.32 mm i.d., film thickness 0.1 µm). The electron emission current was 300 µA, the ion source temperature was 170ºC, the ionisation energy was set at 70eV with quadrupole analyser scanning the range m/z50-850 with a cycle time of 0.8 s and the GC/MS interface maintained at 300ºC. The column and temperature programs used were identical to those described for GC analyses. Helium was employed as carrier

RESULT AND DISCUSSION

HT-GC and HT-GC/MS results of TLEs treated with BSTFA + 1% TMCS revealed complex and wide range mixtures components for the majority of RMP soils, derived directly from a decomposing body, the soil microbial community and inputs from overlying vegetation. Figure 1 shows a typical example of chromatogram depicting the range of extractable lipid components that were extracted from these soils. Control soils were obtained to ascertain the presence of lipid components at concentrations greater than their 'background' level and will be interpreted as materials deriving from anthropogenically introduced organic matter. The distributions of TLEs may suggest that the TLE concentrations are PDI dependent. Clearly that the lipid components in soil for cases with a shorter PDI (between a few minutes after death and days) are likely to originate from degraded human/animal remains. These soils remarkably contain lipid components at a concentration approximately thousand times greater than their controls. This would confirm a recent introduction of substantial amounts of organic material that shall be interpreted as having derived from a decomposing body. Basically, the total concentrations of extractable lipid components obtained from these soils were found to range in mass of 0.02 g g^{-1} to 2.0 g g^{-1} soil dry weight, with the percentage being 0.08% to 6.2%. Then the lipid components skewed to the dominance of plant-derived lipids for soils with a longer PDI. These soils of a larger PDI exhibited similar lipid distributions with majority of the high molecular weight compounds were identified

as being plant-derived. They contained TLE concentrations ranging between 0.007 g g^{-1} and 0.23 g g^{-1} soil dry weight.

Majority of the TLE extracts of the RMP soils contain appreciable concentrations of intact acyl lipids of diacylglycerides (DAGs) and triacylglycerides (TAGs), with trace amounts of monoacylglycerides (MAGs). The distributions of TAGs are very narrow, where only the C_{46} to C_{54} homologous were detected. Similar distributions are observable in most of the extracts, with the C_{52} homologue being the most abundant component. Soils of shorter PDI are characterized by the presence of high concentrations of TAGs. Their measured concentrations are approximately 100 to 4000 times higher than their controls and of those cases with longer intervals. The high concentrations indicate the recent introduction of a substantial amount of TAGs into the soil, presumably from a decomposing cadaver. The low concentrations of TAGs detected in the soils of longer PDI would be an indicative of the higher level of hydrolytic degradation of TAGs into simpler components, such as fatty acids, with time. It is known that the hydrophobic properties of lipids contribute to their preservation in soil (Evershed *et al*, 1992). However, under certain burial conditions, protection within the soil matrix is not sufficient to prevent the degradation of intact acyl lipids, for example by oxidation or enzymatic hydrolysis (Evershed *et al*., 1992). The concentrations of MAGs and DAGs recovered in the majority of the soils, especially for those with longer intervals, are too low to be detected, for soils of both shorter and longer PDI. A rapid degradation of these compounds into simpler organic and inorganic components with burial is being the most possible explanation.

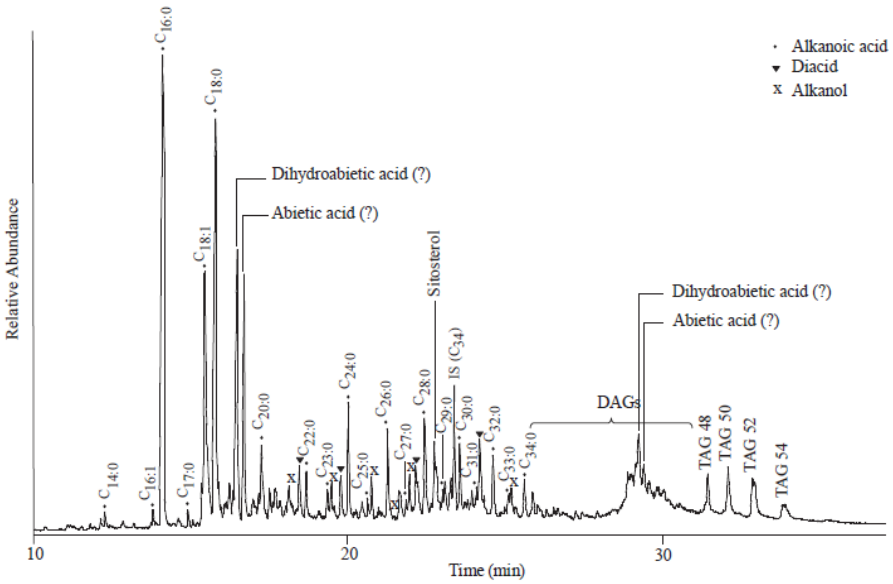

Figure 1. Example of TLE chromatogram obtained from a crime scene. This chromatogram shows a typical distribution of solvent extractable lipids, occurring in most of the forensic soils, particularly of those with a longer PDI. The labelled peaks correspond to; IS = internal standard (C-34 alkane); WE = wax ester; TAG = triacylglyceride; DAG = diacylglyceride

The presence of sterols is observed in some of TLE extracts with the relative abundances and concentrations varying considerably. Noticeably, the majority of sterol compounds recovered is derived from plant matter. However, because of the complex nature of soil extracts, any input from animals would likely to be obscured. Similar sterol compounds can be identified in most of the extracts, particularly of those with longer intervals. The most ubiquitous steroidal component detected is 24-ethyl-cholest-5-en-3β-ol (sitosterol). Cholest-5-en-3β-ol (cholesterol), 24-ethyl-5α-cholestan-3β-ol (5α-stigmastanol) and β-amyrin, a pentacyclictriterpenoid, are also present in

lower concentrations in some soils. Both sitosterol and 5α-stigmastanol are common components of terrestrial vascular plants (Goad, 1991; Bull *et al*., 2000). However, there has been a substantial loss of sterol components and only sitosterol was identified with a modest abundance. Eventhough these sterol components are relatively more resistant to degradation than other lipids, the loss of these compounds is possibly occurring *via* either direct assimilation, complete mineralization, condensation of steroid moieties, degradation or/and transformation to modified sterol. The reduction process is a common pathway for the fate of sterols in soils and sediments (Bull *et al*., 2000).

The presence of a corpse would result in the addition of a range of distinct fatty acid components into the soil. The occurrence of a homologous series of normal, saturated chain fatty acids was observed in most of the soil extracts. The detected-components of saturated fatty acids ranged from dodecanoic acid (C_{12}) to hexatriacontanoic acid (C_{36}), with a number of shorter unsaturated compounds as minor components. Majority of soils of a shorter PDI, exhibit a maxima of either palmitic acid ($C_{16:0}$) or stearic acid ($C_{18:0}$), together with the presence of their unsaturated analogues; palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$). These monounsaturated fatty acids are major components of animal (including human) adipose tissue (Hilditch, 1940). Furthermore, an appreciable amount of the monounsaturated fatty acids in the soil extracts tends to confirm that there has been hydrolytic biodegradation of TAGs (Hita *et al*., 1996). The extracts of these soils contain unusually high concentrations of palmitic (17.3 mg g^{-1} to 2.75 mg g^{-1} soil dry weight), palmitoleic (1.56 mg g^{-1} to 1.87 mg g^{-1} soil dry weight), stearic (2.75 mg g^{-1} to 17.3 mg g^{-1} soil dry weight) and oleic (0.61 mg g^{-1} to 1.87 mg g^{-1} soil dry weight) acids. Their concentrations were 15 to 1000 times higher to the control, and were approximately about 18 to 200 times higher than the extracts of longer PDI. The even chain fatty acid homologues (C_{14}–C_{18}), unsaturated monohydroxy and keto analogues of saturated C_{16} and C_{18} components have also been perceived in the extracts, varying in concentrations.

Whilst, the soils with a longer PDI exhibit bimodal distribution with maximum at either palmitic acid ($C_{16:0}$) or stearic acid ($C_{18:0}$), and a latter maximum typically at the C_{28} or C_{32} fatty acid. However, unsaturated fatty acids were undetectable in these extracts. The potential explanation for the absent of unsaturated fatty acids extracts of longer PDI are due to unsaturated fatty acid moieties being more prone to the oxidative degradation than the saturated components (Voet and Voet, 1995). It had been reported that the unsaturated oleic acid ($C_{18:1}$) is rapidly decomposed in all soils, evolving a large amount of CO_2, indicating a rapid mineralization of the substrate (Moucawi *et al*., 1981). The occurrence of even chain fatty acids homologues, unsaturated monohydroxy and keto analogues of the saturated C_{16} and C_{18} components have been observed in the extracts of the soils suspected of having comprised a temporary grave of a missing person, with a corresponding higher concentration of the C_{16} and C_{18} components of about 500 times compared to the control soils (Bull *et al*., 2009). Therefore, these phenomena are significant, which indicates a large input of fatty acids such as might have_arisen from the decay of a buried body. In addition, the higher relative concentration of lower molecular weight fatty acids in extracts of shorter PDI reveals that a substantial source of fatty acids has recently been introduced to the soil.

CONCLUSION

The observed wide range of TLE concentrations highlights the variable level of decomposition across different PDIs and soil environmental factors. The soils of shorter PDI demonstrated the predominant of human/animal derived lipids with concentration remarkably higher than their control soils. The higher molecular weight fatty acids were almost undetectable in the extracts of

soils of shorter PDI, but they were detectable in those derived from soils of longer PDI where the plant derived compounds were predominant and easier to detect. The presence 5α-stanols within the soil extracts provides conclusive evidence of the input from plant material.

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