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Characterisation of organic and inorganic constituents in cemetery soils and its application as forensic evidence

Sara Filipa de Sousa Queirós

Dissertation for the Master's Degree in Forensic Sciences and Laboratory Techniques

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Submitted to the University Institute of Health Sciences (IUCS)

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Gandra, abril 2022

A Estudante,

Sara Filipa de Sousa Queirós

To my mother, father, sister, and uncle Leonel.

Scientific Production

Scientific article in a peer-reviewed international journal:

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Resumo

O solo é uma matriz complexa, composta por matéria inorgânica e orgânica, utilizada em múltiplos contextos, incluindo na investigação forense. A sua elevada capacidade de retenção permite estudar compostos provenientes de um corpo em decomposição sobre/dentro do solo, associá-los às diferentes fases de decomposição cadavérica, e estimar há quanto tempo o corpo está naquele local. A translação de um corpo humano também pode ser comprovada na eventualidade de se identificarem compostos provenientes da sua decomposição, sem que este esteja no local em análise.

A decomposição cadavérica é afetada por fatores extrínsecos e intrínsecos ao corpo. Ao estudar a decomposição cadavérica, devem considerar-se todas as variáveis envolvidas, para que melhor se entenda a relação entre o processo de decomposição e os compostos orgânicos encontrados no solo. Muitos estudos foram desenvolvidos ao longo dos anos, sendo que, por questões éticas, a maioria foi realizada em animais (principalmente porcos). Contudo, existem diferenças que devem ser consideradas, uma vez que podem influenciar as conclusões dos estudos. Assim, torna-se pertinente a realização de novos estudos com humanos, objetivando-se a ampliação do conhecimento científico nesta área. Posto isto, neste trabalho foram recolhidas 69 amostras de solo de 5 sepulturas humanas em 2 cemitérios portugueses (Monte D'Arcos e Castelo de Mértola). Caracterizou-se inorganicamente o solo das campas (pH, cor e humidade) e determinou-se o seu teor de matéria orgânica. Posteriormente, as amostras recolhidas no interior da sepultura foram analisadas por Cromatografia Gasosa Acoplada a Espectroscopia de Massa para identificar ácidos gordos e esteroides.

Os resultados obtidos mostraram que as amostras recolhidas a norte do país têm maior conteúdo de humidade, e a sul verificaram-se valores mais altos de pH e matéria orgânica; a análise da cor não revelou grandes diferenças entre os cemitérios. Relativamente à caracterização orgânica, não foram definidos nenhuns marcadores orgânicos que se relacionassem com a fase específica da decomposição cadavérica, nem com a parte do corpo proveniente. No entanto, identificou-se a presença de ácidos gordos característicos da decomposição humana (ácido mirístico, palmítico, oleico e esteárico), comprovando a importância do estudo destes lípidos em casos forenses. Adicionalmente, foi possível verificar que solos mais ácidos e menos ricos em humidade e matéria orgânica, apresentam menos ácidos gordos. Por fim, não foram encontrados esteroides biomarcadores da degradação humana, refletindo a necessidade de uma futura otimização

do protocolo aplicado. Futuramente, deverá também alargar-se o estudo a mais cemitérios com diferentes tipologias de solo.

Palavras-chave: ácidos gordos, CG-EM, decomposição cadavérica, esteroides, pedologia, química dos solos

Abstract

Soil is a complex matrix, composed of inorganic and organic matter, used in multiple contexts, including forensic investigation. Its high retention capacity makes it possible to study compounds from a decomposing body on/within the soil, associate them with the different phases of cadaveric decomposition, and estimate how long the body has been there. The translation of a human body can also be proven if compounds from its decomposition are identified, without the body being at the site under analysis.

Cadaveric decomposition is affected by both extrinsic and intrinsic factors to the body. When studying cadaveric decomposition, all the variables involved must be considered to better understand the relationship between the decomposition process and the organic compounds found in the soil. Many studies have been developed over the years, most of which, for ethical reasons, have been carried out on animals (mainly pigs). However, there are differences that should be considered since they may influence the conclusions of the studies. Thus, it becomes pertinent to carry out new studies with humans, aiming expanding scientific knowledge in this area. Therefore, in this study 69 soil samples were collected from 5 human graves in 2 Portuguese cemeteries (Monte D'Arcos and Mértola Castle). The soil of the graves was inorganically characterised (pH, colour and moisture) and its organic matter content was determined. Subsequently, the samples collected inside the grave were analysed by Gas Chromatography Coupled with Mass Spectroscopy to identify fatty acids and steroids.

The results obtained showed that the samples collected in the north of the country have higher moisture content, and in the south, there were higher values of pH and organic matter; the colour analysis did not reveal great differences between the cemeteries. Regarding the organic characterisation, no organic markers were defined that could be related with the specific phase of the cadaveric decomposition, nor with the part of the body provenance. However, the presence of fatty acids characteristic of human decomposition (myristic, palmitic, oleic and stearic acids) was identified, proving the importance of studying these lipids in forensic cases. Additionally, it was possible to verify that soils thar are more acidic and less rich in humidity and organic matter, present less fatty acids. Finally, no biomarker steroids of human degradation were found, reflecting the need for a future optimisation of the applied protocol. In the future, the study should also be extended to more cemeteries with different soil typologies.

Keywords: fatty acids, GC-MS, cadaveric decomposition, steroids, soil science, soil chemistry

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List of Abbreviations, Symbols and Acronyms

%	Percentage
Å	Ampere
µL	Microlitre
1/s	Hertz
10-Keto-C18:0	10-ketostearic Acid
10-OH-C16:0	10-hydroxyhexadecanoic Acid
10-OH-C18:0	10-hydroxystearic Acid
10-OH-C18:0	10-hydroxy stearic Acid
A	Abdomen
ALC	Fraction Containing Sterols, Stanols and Stanones
BAME	Bile Acid Methyl Esters
BG	Bottom Grave
BSTFA	N,O-Bis(trimethylsilyl(trifluoroacetamide))
C14:0	Myristic Acid
C16:0	Palmitic Acid
C16:1	Palmitoleic Acid
C17	Heptadecanoic Acid
C18:0	Stearic Acid
C18:1	Oleic Acid
C18:2	Linoleic Acid
CCM	Cemitério Castelo de Mértola
CDCA	Chenodeoxycholic Acid
CDI	Cadaver Decomposition Islands

cm	Centimetre
CMA	Cemitério Monte D'Arcos
CPR	Cemitério Prado do Repouso
DCA	Deoxycholic Acid
DCM	Dichloromethane
DD	Day
DNA	Deoxyribonucleic Acid
EC	Electric Conductivity
eV	Electron Volt
F	Feet
Fl	Female
FAME	Fatty Acid Methyl Esters
G	Gluteal Region
g	Gram
GC-MS (CG-EM)	Gas Chromatography Coupled with Mass Spectroscopy (Cromatografia Gasosa acoplada a Espectroscopia de Massa)
GLC	Gas-Liquid Chromatography
GPS	Global Positioning System
H	Head
h	Hours
ha	Hectare
HCl	Hydrogen Chloride
IDCA	Isodeoxycholic Acid
ILCA	Isolithocholic Acid
IS	Internal Standard

Km	Kilometre
KOH	Potassium Hydroxide
LCA	Litholic Acid
LG	Left Grave
LT	Left Thigh
m	Metre
M	Molar
Ml	Male
m/z	Mass-to-charge Ratio
m^2	Square Metre
MeOH	Methanol
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
MM	Month
n.a.	Not Available
N_2	Nitrogen
^oC	Celsius
pH	Potential of Hydrogen
PMI	Post-mortem Interval
PSD	Particle Size Distribution
PTFE	Polytetrafluoroethylene
RG	Right Grave

rpm	Rotations per Minute
RSSCC	Revised Standard Soil Colour Charts
RT	Right Thigh
RTm	Retention Time
s	Seconds
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
T	Thorax
TCMS	Trimethylchlorosilane
TG	Top Grave
TLE	Total Lipid Extract
TMS	Trimethylsilyls
TMSI	1-(Trimethylsilyl)imidazole
TSD	Time-Since-Death
UK	United Kingdom
v/v	Volume per volume
W	Wall
YYYY	Year

I. Introduction

In forensic investigations, the inquiry around the circumstances involving death and the estimation of the post-mortem interval (PMI) are highly important. The PMI is particularly relevant given that it can help authorities to identify the body by reducing the timeline of events that led to the individual's death, or even help to identify a suspect in criminal cases such as a homicide. Currently, several methods are used to obtain information to help answer questions that arise with an individual's death. When the remains are skeletonised, and therefore usually involve cadavers a relatively longer PMI, some of the applied techniques are, for example, radioactive dating using carbon-14, Deoxyribonucleic Acid (DNA) degradation, analysis of the organic constituents of bones, the succession of insects (entomology), and the collection of material at the crime scene that may be associated with the cause of death (Buchan and Anderson 2001). Nevertheless, there are some disadvantages regarding the use of these techniques. Most of them have no application in real forensic cases, thus, it is important to work on the development of new techniques that can aid in giving new and more accurate answers to investigators. Soil has been shown to be a very useful tool in forensic investigations helping in answering questions in which other techniques fail (Murray and Tedrow 1992).

1.1.Use of soil within forensic investigations

Soil used as evidence became notable due to the famous books of Sherlock Holmes written by Sir Arthur Conan Doyle (1859-1930), between 1887 and 1927 (Saferstein 1981). In those books, soil is presented as physical evidence used by police forces when conducting criminal investigations, which has influenced procedures in actual cases (Berg 1970). Adding to the practice of forensic soil science in real scenarios, history refers to Edmond Locard (1877-1966), a French forensic medicine student during the early 1900s, who established the Exchange Principle in 1930. Locard stated that whenever two objects contact with each other, an exchange of material always occur and that every contact leaves a trace (Saferstein 1981). This principle is applied in almost all forensic scientific areas, and forensic soil science is no exception. The first time that soil analysis was reported in a real criminal investigation was in 1904 by Georg Popp (1861-1943) (Toomey 1976).

Soil is increasingly being used as evidence in worldwide forensic investigations (Di Maggio et al. 2017). However, some countries are less experienced when it comes to working with soil to its full forensic potential, either due to lack of investment, information, study and lack of soil classification ('Status of the World's Soil Resources - World' 2015). An article published in 2017 summarised the developments of forensic geology worldwide and discussed the use of soil in some European countries such as Italy, the Irish Republic and the United Kingdom (UK) (Di Maggio et al. 2017). The authors explained that due to historical, political, and social differences, the development of this science occurred differently in each country. For that reason, the use of soil in some countries has its focus in mining and agricultural purposes, and others have developed laws and invested in law enforcement to also apply it to criminal situations. In Portugal, for example, the collection and use of soil as evidence in court is a scientific field that needs to be further evaluated and explored. To date, soil has only been legally used in cases concerning construction or agriculture processes, never in cases of abduction, murder, or disappearances. This is despite what happens in other countries such as the UK and Australia, where soil is considered for use in criminal investigations as a common practice (Stam 2004; *BBC News* 2011). In fact, in 2021 an article was published in which the authors presented a missing person case in the UK that was solved using soil science techniques (Donnelly et al. 2021). Perceiving the importance of its use in a forensic context can lead to justified investments in Pedology – the study of soil (Thomas and Goudie 2009) – and forensic science - either by investing in training researchers in countries where this evidence is not fully used and purchasing new equipment.

Soil evidence is applied in many investigations such as murders, abductions, hit and runs, sexual assaults, drug shipment pathways, construction problems, determining the location of a certain crime scene, search area reduction, and proving the presence/absence of a vehicle or suspect in a location (Sangwan et al. 2020). In criminal investigations, practices are usually based on comparisons in which the characteristics of a questioned sample (e.g., mineral content, particle size distribution, pH, colour) are compared to the same characteristics of a reference sample; the former being a soil sample of unknown origin recovered from any natural, artificial or human item (due to the soil ability to be transferred and to persist on clothes and objects), and the latter a well-known documented sample (Saferstein 1981; Sangwan et al. 2020)). A questioned sample could be, for example, a sample recovered from a suspect's shoe. This sample could then be compared to samples on databases and information regarding the origin of the questioned sample

could be achieved. Notwithstanding, the reference sample could also be a sample collected from the place where a certain victim was found dead and compared to the sample collected from a suspect, aiming to test if that individual was, or was not, likely in the place where the victim body was found (Fitzpatrick and Donnelly 2021). In fact, the analysis of soil samples collected at crime scenes mostly test for a link between a suspect, an object, a victim, and/or an alibi to a crime scene (Mistek et al. 2019). However, due to its high capacity of retaining information, soil can allow investigators to estimate how long a body had been decomposing in the area where it was recovered from. Analysing the organic compounds from the breakdown of the cells (e.g., fatty acids and sterols) in a soil where a body was found decomposing can lead to the possibility of predicting how much time has passed since that body started to decompose in that site, helping in answering questions related to the PMI. This is possible by establishing a connection between the phases of decomposition and the biological compounds associated with it (Swann, Forbes, and Lewis 2010; von der Lühe et al. 2017; von der Lühe et al. 2018). Additionally, using the same effective techniques to identify and quantify these organic compounds, soil analysis may aid to ascertain if the body had been moved from another location, helping in cases involving bodies relocation.

Also, when it comes to the forensic context, some questions regarding the events that led to the death of the individual are solved by performing exhumations, since exhuming the body allows researchers to analyse soil surrounding the corpse (Spennemann and Franke 1995). In some countries, such as Portugal, exhumations in cemeteries are common practice to make room for new burials. Performing exhumations in temporary graves is mandatory by Portuguese law and occurs when the organic matter associated with the human body is destroyed, and the body is fully skeletonised. If this is not the case, the body will be again covered with soil and buried for a further period, until it is skeletonised. Understanding the conditions that influence the body decomposition rate is not only important for criminal justice (PMI determination) but also for space management in cemeteries, in countries where exhumation is a common practice. This is relevant given that low decomposition rates can compromise the reuse of graves within the expected time (Fiedler et al. 2015; Ferreira and Cunha 2016).

1.2. Human Body decomposition process

It is estimated that body decomposition commences about four minutes after death and may take up to 15-25 years to be full skeletonised (Fiedler and Graw 2003). However,

the decomposition process duration depends on environmental factors (see chapter 3.1). Although several studies have been carried out over the years, authors differ in their opinions regarding the number of decomposition process stages. The decomposition of a body is a continuous process that leads to the release of biochemical compounds, due to the occurrence of the mortis triad, autolysis, putrefaction, decay, and skeletonisation (Figure 1; Dent, Forbes, and Stuart 2004).

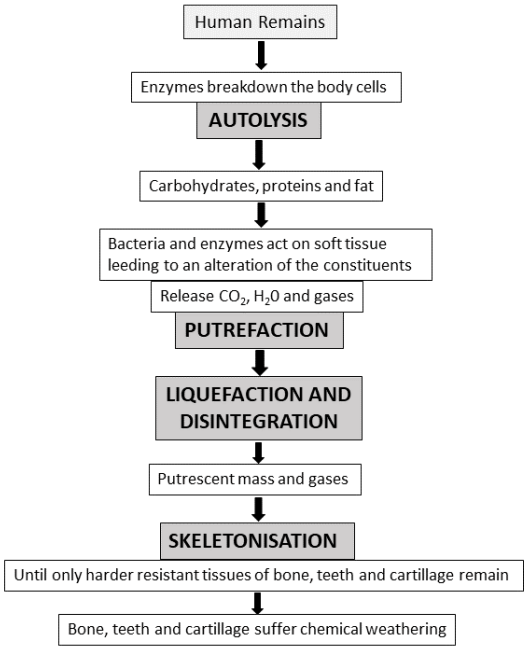

Figure 1: Summary of the human decomposition process (adapted from Dent, Forbes, and Stuart 2004).

Initial observable body changes are normally used to determine the PMI or the time-since-death (TSD). The mortis triad includes *rigor mortis*, *livor mortis* and *algor mortis*. When lactic acid accumulates in tissues after death, muscles stiffen being this process referred as *rigor mortis* (Heron et al. 1995). *Livor mortis* or lividity happens due to gravitational pooling of blood in areas of the body, creating visible red/purple areas (Badden and Hennesse 1989), and *algor mortis* is the body temperature equilibration after death, until it reaches ambient temperature (Perper 1993). In addition to these, other modifications can take place, such as *greenish discoloration* which occurs when the hydrogen sulfide (one of the gasses produced during decomposition) reacts with the hemoglobin in the blood leaving a green colour in the vessels. When a separation of epidermis is observable, due to moist or wet environments that promote production of hydrolytic enzymes, the process is called *skin slippage*. If eyes are exposed after death, the cornea dries and therefore a black discoloration appears – *Tache noir*. Other corpse

observation phenomenon is *marbling*, which has its name due to the mosaic appearance caused by the spreading of anaerobic bacteria. Depending on the environment conditions the body can suffer *mummification* which is defined as being desiccation of body in dry environments due to an inhibition of bacterial activity caused by low levels of humidity. If the body is under wet and anaerobic conditions, *saponification* process is favourable to occur, so hydrolysis of fatty tissues is promoted, leaving the corpse with a wax-like appearance (Goff 2009). *Putrefaction* happens due to high bacterial activity that leads to a break down in the surrounding tissue (Coe 1993). After putrefaction, the body is reduced to a skeletal state – *skeletonisation* –, which is the final stage of decomposition and can last several years hampering the determination of TSD (Fiedler and Graw 2003; Goff 2009).

Specifically, during the saponification phase, and under anaerobic conditions, adipocere ("adipo" = fat and "cere" = wax) could be formed from human tissues. It occurs as a later post-mortem change when the body fat is under moist and anaerobic conditions (Fiedler et al. 2015). Adipocere forms as a result of the degradation of the adipose tissue caused by the hydrolysis and hydration of the adipocytes, the saturated free fatty acids such as myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids being its major constituents (Forbes et al. 2003). This substance constitution differs given the body part where it is formed. Despite various studies that have been carried out through the years, the exact nature of adipocere is still being studied and determined (Magni, Lawn, and Guareschi 2021). Understanding its formation mechanism in grave soils and the conditions that are needed for that formation is of paramount importance for forensic investigators, given that the derived soil compounds can help determining the PMI. Additionally, adipocere formation in some specific conditions can lead to the preservation of the corpse which is particularly helpful in criminal investigations to uncover the victim's identity and/or to determine the cause of death (Mohd Nor and Das 2012; Mohit and Gaurav 2012; Fiedler et al. 2015). However, even eventually preserving some lesions it can also hamper the detection of ecchymosis or wounds due to the activity of microbes (Pinheiro 2006).

One of the major setbacks of the study of adipocere is that in general it is found in very small amounts within graves (Forbes et al. 2003). This is a problem when conducting analysis in real forensic cases and also in mock experimental conditions (Takatori et al. 1987). Therefore, most of the researchers chose to use more sensitive techniques and methods to analyse adipocere, such as gas chromatography–mass

spectrometry (GC-MS). Moreover, GC-MS is also usually used to identify and quantify compounds in soil that emanate from a decomposing body (von der Lühe et al. 2017). These compounds, such as fatty acids or steroids, can either be preserved in the soil matrix or decomposed by microorganisms (Yang et al. 2020).

1.2.1. Factors influencing decomposition

The decomposition process may not always follow a sequential path, meaning that a body can simultaneously be mummified, in putrefaction, and saponified (Pinheiro 2006). Some of the known variables affecting human body decomposition are described below, being discussed as extrinsic (external) or intrinsic (internal) relatively to the body.

1.2.1.1. Extrinsic factors

Environmental factors may affect body decomposition due to their influence on insect activity and microorganisms' growth. The extrinsic factor that seems to influence the most is temperature, with optimal interval for bacterial development ranging between 25ºC and 35ºC (Campobasso, Di Vella, and Introna 2001). That being said, environments with higher temperatures promote bacterial growth and activity and, consequently, contribute to a faster autolysis and putrefaction phenomena (Mann, Bass, and Meadows 1990; Gill-King, H. 1997; Dautartas 2009). On the other side, colder environments usually delay body's decomposition (Goff 2009).

Soil characteristics can also affect body decomposition (Forbes, Dent, and Stuart 2005; Haslam and Tibbett 2009). The range of different particle sizes - soil's texture - influences porosity and permeability, which weighs on the retention of solutions. When dealing with the texture of soil, the inorganic particles can be divided into three classes: clay (< 0.002 mm), silt (0.002 – 0.02 mm) and sand (> 0.02 - 2 mm). Moreover, fine-textured soils, rich in clay particles, will have a higher retention capacity than coarse textured soils, rich in sand particles. When a body is decomposing in a soil mainly composed of fine particles, the compounds will be retained. However, if the soil is composed of large sized particles, the compounds emanating from the body will eluviate through the soil. As a simple guide, clayey soils tend to preserve tissue while sandy soils tend to lead to progressive chemical decomposing reactions (Piepenbrink and Schutkowski 1987). This can be explained by the different rates of gas diffusion. Fine-textured soils will diffuse less gases than a coarse-textured soil impacting the gas exchange and leading to a domination of anaerobic organisms. Since these organisms are

generally less effective decomposers, the decomposition process can be retarded (Tumer et al. 2013). A study developed in 2005 by Forbes, Dent, and Stuart showed how different soil types impact the adipocere formation. X-Ray diffraction was used to analyse the mineral content, and particle size analysis was applied to give a general description of the soil type. The obtained results showed adipocere can form in different soil types, though sandy and silty soils were able to accelerate its formation. However, all soil types were studied in controlled environments; forensic experts must consider moisture content and temperature when performing investigations. Soil pH and moisture content have also proved to influence the decomposition rate by changing the microbial and fungal activity (Alexander 1980; Campobasso, Di Vella, and Introna 2001; Neher et al. 2003; Hansel et al. 2008; Haslam and Tibbett 2009). This happens because cadaver decomposition islands (CDI) are associated with an increase in microbial activity in soil during the corpse cells' breakdown (Carter, Yellowlees, and Tibbett 2006). Usually, fungal communities predominate in acidic soils whilst neutral soils are beneficial to bacteria (Haslam and Tibbett 2009). Low air humidity is associated with a dry environment, promoting desiccation of the tissues while a humid environment slows the degradation of the corpse (Campobasso, Di Vella, and Introna 2001). Additionally, the presence of humic acids (chemicals produced by the decay of plants) in soil contributes to body preservation (Fiedler and Graw 2003).

In addition to differences in decomposition between buried and surface exposed cadavers, burial depth plays an important role in the length of decomposition processes (Dautartas 2009). If the body is placed in an area with difficult access for decomposers, the decomposition rate will decrease. On the other hand, higher temperatures and access to oxygen can hasten decomposition (Dent, Forbes, and Stuart 2004).

Regarding the material that coffins are made of, bodies buried in wooden coffins (*e.g.*, pine, spruce) will decompose faster given the high deterioration rate of the latter, while zinc coffins can slow down the process (Fiedler and Graw 2003). Coffins that contain heavy metals (*e.g.*, lead) can also inhibit microbial activity, therefore contributing to the preservation of the body (Müller et al. 2011).

Clothes, made of natural or synthetic materials, can also influence decomposition. In general, coverings slow down the decomposition processes. Bodies covered with heavier clothes (*e.g.* thick jackets) tend to decay more quickly given that temperature accelerates decomposition (DiMaio and Dana 2006), though garments can sometimes ease adipocere formation by favouring a moist environment. Moreover, diapers usually slow the rate of

decomposition in the pelvic and gluteal regions, given that they retain the gut content, hampering soil direct contact Other types of coverings can influence decomposition, such as plastic bags which can trap moisture and increase adipocere formation (Miller 2002).

1.2.1.2.Intrinsic factors

Individual variation is one of the most relevant factors when it comes to the body decomposition process (Dautartas 2009). Physical conditions of the individual (*e.g.*, body size) must be considered in forensic investigations, though they are not always a decisive factor. Overall, bodies with a higher fat content tend to decompose faster (Campobasso, Di Vella, and Introna 2001). Still, a study conducted in 2017 by Roberts, Spencer, and Dabbs showed that body size had a minor influence on the decomposition rate and pattern. It should be noted that the mentioned study has the limitation of the sample size (n = 12) and future research in this area is required. Sex and age at death are two other factors that should be considered given that decomposition is usually faster in older males (Guebelin et al. 2021). On the other side, putrefaction is known to occur at a slower rate in foetuses and new-borns (Campobasso, Di Vella, and Introna 2001) due to their lack of intestinal flora. After death, bacterial flora of the gastrointestinal tract begins putrefaction, but the former is only acquired during contact with the environment and breastfeeding. Another variable that usually hastens body decomposition is water content. When human tissues are hydrated, putrefaction is faster compared to when individuals who suffered from perimortem diarrhoea and/or vomits (Pinheiro 2006).

Regarding traumatic injuries, there is also a difference between buried corpses and those decomposing on the soil surface given that the latter are more exposed to scavengers leading to easier access to the corpse and consequently fastening the decomposition process (Bachmann and Simmons 2010; Cross and Simmons 2010). Nevertheless, the presence of trauma seems to affect the decomposition pattern more than the decomposition rate (Cross and Simmons 2010; Smith, 2010).

1.3. Lipids released from a decomposing body found in soil

1.3.1. Fatty acids

1.3.1.1. Description of the compounds

Fatty acids are carboxylic acids with hydrocarbon chains of variable sizes between four and thirty-six carbons (Figure 2). Unsaturated fatty acids have double bonds in the hydrocarbon chain while saturated fatty acids have no double bonds. These acid

characteristics (*e.g.*, the melting point and solubility) depend on the number of carbon atoms, the existence of double bonds, rings, hydroxyl groups, and/or branches of methyl groups (Lehninger, Nelson, and Cox 2013).

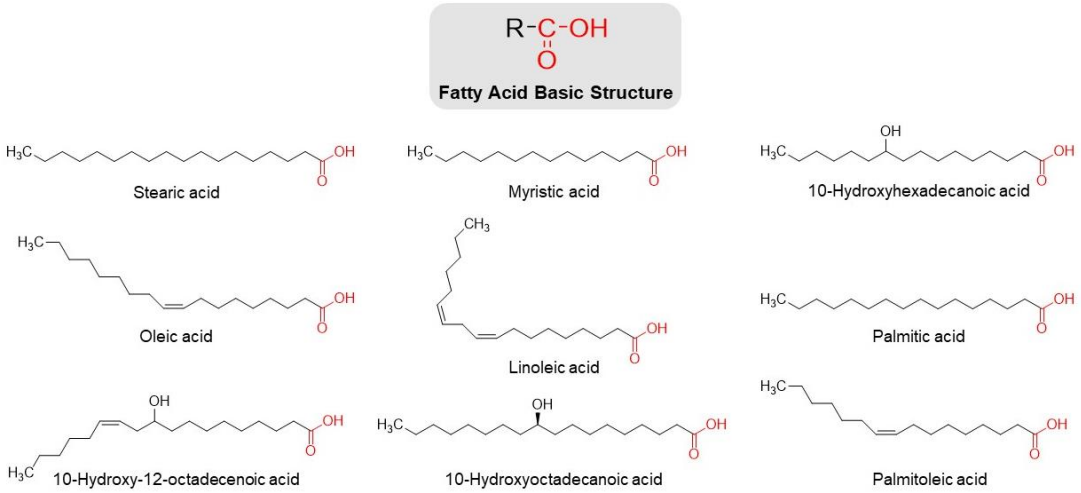

Figure 2: Chemical structure of fatty acids (the hydrocarbon chain (R) and the carboxyl group are highlighted in black and red, respectively).

1.3.1.2. Applicability in forensic investigations

1.3.1.2.1. Identification of fatty acids in adipocere studies

Fatty acids can be found in soils due to human decomposition, being sometimes identified when adipocere is present. Most of the studies in recent literature lay their focus in adipocere constitution.

The sequence leading to the adipocere formation and the products resultant from the body decomposition have been studied through years (Magni, Lawn, and Guareschi 2021). The first time this substance was mentioned was in 1786, in a French study of the Holy Innocents' Cemetery that was relocated (due to the fact that the original cemetery was placed in soil with high humidity content) and is now closed (Fourcroy, 1787; Fiedler et al. 2015).

In 1977, a study of adipocere constitution conducted on human remains found at a sea in Japan, allowed the identification of 10-hydroxystearic acid (10-OH-C18:0) and 10-hydroxyhexadecanoic acid (10-OH-C16:0), using Gas-Liquid Chromatography (GLC) and GC-MS techniques (Takatori and Yamaoka 1977).

In 1983, Takatori et al. identified 10-hydroxy-12-stearic acid in human adipocere collected from different regions of human bodies found at a sea in Japan, with an estimated PMI of six to ten months, using GC-MS.

In 1997, Adachi et al. developed a study using GC-MS, in which the relation between the lipid composition and the time of adipocere formation in submerged victims from one month to four years was discussed. Regarding the fatty acid percentages, it was possible to verify samples collected from victims submerged during one to two months contained about 1% of hydroxy fatty acids, and that linoleic acid (C18:2) ranged between 13 to 15%. In adipocere samples from victims submerged during four months to four years, there was an increase in the percentage of hydroxy fatty acids (8%) and a decrease in the percentage of linoleic acid as the 10-ketostearic acid (10-Keto-C18:0) content rose from 0.7% to 3.2%. Additionally, this study demonstrated that keto fatty acids were not detected in victims submerged for less than three months.

In 2002, Forbes et al. published an article about the identification of adipocere in samples collected from cemetery graves, using GC-MS. The samples were taken from dry and wet environments with a wide range of burial times (five to fifty years). By the time the authors opened the grave in dry environments, they found water content inside and a white solid fragment floating in the aqueous coffin environment. Instead of collecting soil in this site the authors decided to collect the white solid fragments and analysed it. In addition, samples were also taken from several graves without knowing the duration of the burial, and from cemetery sites with no proximity to the graves (control samples). It was found that the fatty acid content varied according to the type of environment. Fatty acids were not found in the control samples but were detected in the remaining samples: myristic (C14:0), palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), and 10-hydroxy stearic acid (10-OH C18:0).

In 2003, Forbes et al. published an article describing the development of a new GC-MS method for quantitative analysis of adipocere samples collected from grave soils. The authors performed exhumations in Australian cemeteries and collected soil samples under the lower coffin board. Control samples were collected from the grave walls. The inhumation time spanned between six and twelve years, with one burial duration being unknown. The authors developed a method and applied it to the grave soil samples. Control samples did not present any fatty acids, thus confirming the relation between the fatty acids present in soil under the human remains and body decomposition. In the latter samples, C14:0, C16:0, and C18:0 were identified. Besides these acids, C18:1 and 10-OH

C18:0 were also detected. However, in samples collected from the burial of six years, the concentration of C18:1 was low. This suggests that the hydrogenation process was still occurring, and the conversion was not complete, as it was in the samples collected from burials of twelve years. Additionally, the detection of 10-OH C18:0 only in some samples seemed to be reliant on the conditions of the decomposition environment.

Another study was conducted in 2005 by Forbes, Stuart, and Dent, to determine the effect of the burial method on the adipocere formation, using GC-MS. Unlike the 2002 referred study (Forbes, Stuart, and Dent 2002), the authors did not take samples from human exhumations since it was intended to control the environment as much as possible to reduce the number of variables obtaining more reliable results. Therefore, domestic pigs (*Sus scrofa domesticus*) were used as a human surrogate. Pigs are normally used in forensic studies as an animal model to substitute humans due to ethical reasons that restrict human remains' use and since the digestive systems are more similar, both having monogastric systems. Moreover, pigs provide a homogenous sample that allow isolation of individual variables. However, a study conducted in 2017 by Connor, Baigent, and Hansen showed that fundamental differences exist between the two species and different structural and functional gastrointestinal tract may affect rate, timing, and trajectory of decomposition. Therefore, pigs can provide a homogenous sample that allow isolation of individual variables, but human samples tend to be more variable. In this study, a section of adipose tissue from the abdominal region of pigs subjected to the same diet was cut and then tissue sections were buried in controlled environments for one year. Some samples were also buried in plastic bags to understand the behaviour of adipocere formation in situations where a body is wrapped in plastic. It was verified that C16:0 was present in all samples reaching higher values than other fatty acids, followed by the C18:0. There was also a significant presence of 10-OH-C18:0 in the coffin samples, which is strong evidence of adipocere formation. It was also possible to verify that saturated fatty acids were found in greater quantities than unsaturated fatty acids. However, in samples taken from plastic bags, the amount of unsaturated fatty acids was greater than in other samples. Thus, it seems possible to verify that adipocere does not occur in cases where the body is submitted to these conditions. Moreover, the results obtained in this experiment confirm the previous written observations about adipocere forming more quickly in bodies buried directly in soil, showing that this matrix has a strong interference in the formation of this substance and consequently in the decomposition of a body. These results and conclusions provide new insights regarding adipocere formation.

1.3.1.2.2. Identification of fatty acids in soil studies not adipocere related

Fatty acids can be detected due to the emanation of decomposition fluid into soils. However, soils are complex and require suitable analysis. Throughout the years, forensic scientists have studied soil's composition and characteristics (Swann, Forbes, and Lewis 2010). Fatty acids have been detected in decomposition fluids (Cabirol et al. 1998) and also been successfully extracted from textiles in decomposing bodies (Ueland, Forbes, and Stuart 2018). However, studies regarding the detection of fatty acids collected from soils where a body is decomposing are scarce.In 2008 and 2009, Larizza conducted a study on domestic pig carcasses (*Sus scrofa domesticus*), to analyse physical and chemical soil surface characteristics during decomposition. Thus, the author evaluated inorganic characteristics such as pH, and organic ones such as the fatty acid content of soil samples. GC-MS was used. The trimethylsilyl fatty acid esters identified were C14:0, C16:0, C16:1, C18:0 and C18:1. These compounds were detected in soil due to decomposition fluid that emanate from the pig carcasses. Since C18:1 is generally the most abundant fatty acid in adipose tissue, it was expected that this was the compound found in highest quantities (Pfeiffe, Milne, Stevenson 1998). However, the most abundant fatty acid detected was C16:0 followed by C18:0. This can be explained by the occurrence of an anaerobic putrefactive process within the pig carcasses. This study also evaluated soil depth intervals throughout the decomposition process. For this, several soil samples were collected from 0 cm up to 50 cm from the soil-carcass boundary. As expected, the higher quantities of fatty acids were detected at 0 cm, since it was the region closest to the source of the compounds, the body.

In 2017, von der Lühe et al. studied the changes of fatty acids from soil samples collected underneath a decomposing human body. One of the most relevant conclusions of this study was the demonstration that human derived fatty acids were still preserved in soil one year after the body's removal. In addition, it has been proved that fatty acids derived from the human adipose tissue can be used as indicators of the presence of decomposition fluids.

1.4. Steroids

1.4.1. Description of the compounds

Sterols are structural lipids present in the membranes of most eukaryotic cells and are composed of four joined carbon rings (Figure 3). These compounds, in addition to structural functions, are also the origin of products with relevant and specific biological

activity. Cholesterol is known to be the main sterol in animal tissues, and it is a precursor of a wide range of steroids, such as bile acids, sexual hormones, and adrenocorticoids. This molecule has a structure formed by a polycyclic ring skeleton with 27 carbon atoms. Intestinal cholesterol can either be absorbed by the individual or suffer conversion to other metabolites by the microbiota. One of the main metabolites of that conversion is coprostanol which is unabsorbable and therefore is excreted in the faeces. In vertebrate animals, most of the cholesterol synthesis occurs in the liver. From this organ, bile is exported, a fluid mainly composed of bile acids and their salts. Bile acids are particularly important in the digestive process as they help to convert large fat particles into smaller structures (Lehninger, Nelson, and Cox 2013).

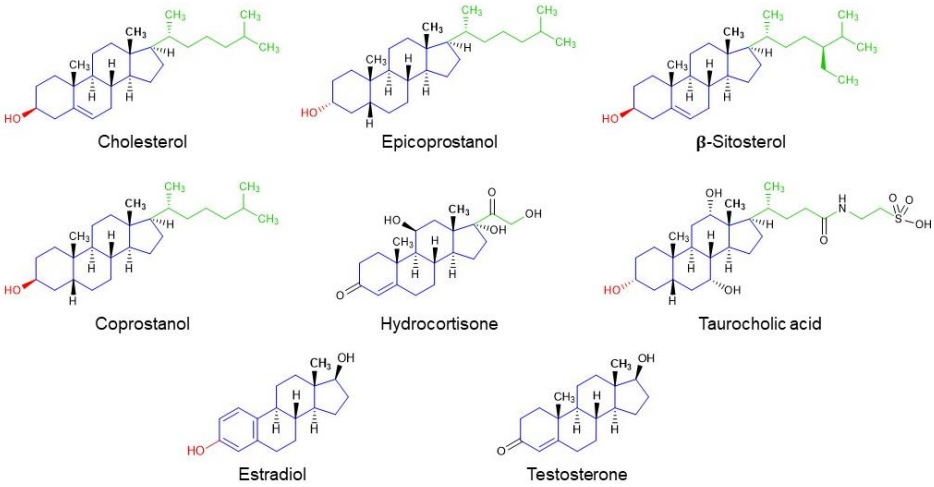

Figure 3: Chemical structure of sterols (the hydroxyl group, steroid nucleus, and hydrocarbon side chain are highlighted in red, blue, and green, respectively).

1.4.2. Applicability in forensic investigations

1.4.2.1. Identification of steroids in adipocere studies

In a study conducted in 1997, and described earlier, the authors analysed adipocere samples using GC-MS (Adachi et al. 1997). Thus, they were able to identify and quantify lipids in samples, documenting for the first time the presence of epicoprostanol in adipocere which is a coprostanol isomer (Figure 4).

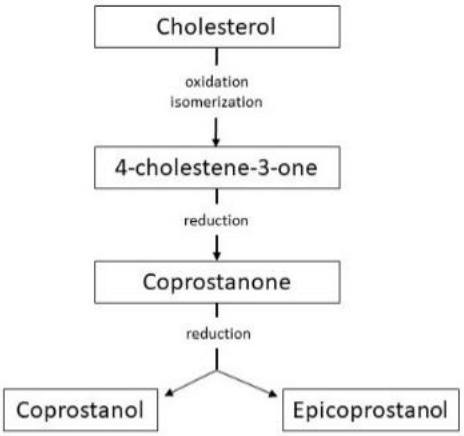

Figure 4: Epicoprostanol formation (Adachi et al. 1997).

The first step of the conversion of cholesterol to coprostanol is its oxidation to 4-cholesten-3-one. Then, cholesten-3-one suffers reduction reactions, being converted to coprostanone. The following coprostanone reduction leads to the formation of coprostanol and its isomer, epicoprostanol (Juste and Gérard 2021). Adachi et al. (1997) discovery is particularly interesting since epicoprostanol is not present in the normal adipose tissue. Once this was the only metabolite of cholesterol found in samples, this could mean that epicoprostanol is more easily produced in adipocere. The ratio of epicoprostanol:cholesterol rose with the longer immersion time, thus showing that epicoprostanol analysis can be useful to determine PMI. However, since epicoprostanol is present in the intestinal content of humans, its presence in seawater samples could be explained by sewage-contaminated sediments.

1.4.2.2. Identification of steroids in soil studies not adipocere related

In 2013, a research was conducted in order to analyse typical animal sterols from grave soil and identify potential biomarkers of decomposition fluids (von der Lühe et al. 2013). The authors collected soil samples below four buried domestic pig carcasses (*Sus scrofa domesticus*) from shallow graves. Two of the carcasses were exhumed after three months, and the other two were exhumed six months after the former. Sample collection was performed under the head, torso, and rear of the pig. Wall samples from the grave were also collected. After soil characterisation, sterols were extracted from samples. The results obtained showed that higher concentrations of cholesterol and coprostanol were detected in the graves of the first two exhumed pigs. This can be explained with putrefaction beginning after three months of burial: the decomposition phase in which the body releases higher amounts of carbohydrates, lipids, and proteins. Given that

cholesterol is present in animal and human cells, its detection in soil can be related to body decomposition and used as a biomarker for the detection of burial sites. Similarly, coprostanol may also be a reliable biomarker; however, the fact that coprostanol can be also present in animal faeces needs to be kept in mind. The presence of β-sitosterol (one of the main plant sterols) was also detected. Its high concentrations can be due to the dietary behaviour of pigs, since this compound was found in samples collected under them, possible coming from gut content. Notwithstanding, this compound could also have come from roots or plant litter.

A more recent study was developed in 2018 by von der Lühe et al. in the context of a real forensic case, where concentrations of tissue and faecal steroids in soil under a human decomposing body were determined. Samples were collected immediately after the cadaver's removal and one year later. Once steroids can derive from various sources besides the human activity, reference samples were also collected, using them to distinguish the compounds source (Bull et al. 2002). Steroids were extracted and then analysed by GC-MS. Despite the translocation and transformation influence on the steroids' quantity, one year after the removal of the corpse they continued high. This study demonstrated that if the analysis of steroids is performed and all the variables are considered, these types of compounds can be a relevant biomarker to forensic soil analysis, since its presence can be indicative of human degradation.

In 2020, von der Lühe et al. presented a study of soil samples recovered from temporary mass graves of World War II. For this, the authors did a geological and historical study of the place where the graves were located and used GC-MS to analyse soil samples. However, given the human impact for sixty-seven years, the natural distribution of the steroids was disturbed. The presence of human decomposition fluids was confirmed with the detection of coprostanol and epicoprostanol. This study reenforced the idea of the utility of using steroids as biomarkers for human decomposition fluids.

Other class of compounds with increasing interest in forensic context are bile acids. These are a group of C24, C27 and C28 steroidal acids produced by the digestive system of animals (Bull et al. 2002). Bile acids, and its respective salts, are synthetised in the liver and derived from cholesterol. These hydrophilic compounds act in the intestine converting big fat particles into small micelles, increasing the interaction surface with the digestive enzymes (Lehninger, Nelson, and Cox 2013). In mammals, these lipids present a C24 structure, thus having twenty-four carbon atoms forming a steroid nucleus and a

five-carbon side chain with a carboxyl group (Gregorio, Cautela, and Galantini 2021). These compounds are usually used in soil studies with pollution detection purposes but given its origin, it may be relevant for forensic investigations to keep studying this type of lipids in forensic contexts, in order to provide helpful information to investigations (Elhmmali, Roberts, and Evershed 2000; Birk et al. 2012). In 2012, Birk et al. quantified faecal sterols, stanols, stanones, and bile acids in terrestrial sediments. The authors were able to purify the compounds and develop a method, using the GC-MS technique. In 2020, von der Lühe et al. detected bile acids - litholic acid (LCA) and deoxycholic acid (DCA) - in high concentrations on samples from mass graves. However, they were also detected in reference samples probably due to animal activity. Bile acids can be a very useful biomarker in soils since these compounds can only be produced by vertebrates and are more stable in soils than stanols and sterols. Notwithstanding, more studies need to be performed since the data regarding its natural distribution is scarce.

With the combined information on these compounds, fatty acids, and/or sterols, investigators can answer several relevant questions regarding the presence of decomposition fluids. Besides that, analysing compounds like sterols, stanols, stanones, and bile acids can also be relevant to study the diet of the individual by analysing the faecal material (Elhmmali, Roberts, and Evershed 2000).

1.5. Other relevant compounds found in soils

In 2019, von der Lühe et al. conducted a study in Southwest Germany reporting for the first time the presence of ambrein in adipocere samples collected from exhumed bodies in a cemetery. Ambrein is a tricyclic triterpenoid usually found in ambergris which is a coprolith mainly produced by sperm whales (*Physeter macrocephalus*) that can also contain minor quantities of steroids (*e.g.*, epicoprostanol, coprostanol, coprostanone), all derived from cholesterol. Ambrein can be a product of intestinal transformations but to date the only known natural source of this compound is the sperm whale. In this article, authors found it in adipocere samples collected from grave soils proving that it can also be formed in terrestrial setting.

The absorptive characteristics of soil allow compounds to be retained for several years. The study of the inorganic and organic characteristics of soil is of upmost relevance to improve our understanding of the human body decomposition processes which occur on

or within soil. Studies that rely on the identification and quantification of the organic compounds that emanate from a decomposing body can provide new inputs for forensic investigators, enriching the scientific community.

II. Aims

Soil is an important forensic tool, being used in criminal investigations worldwide. Its ability to easily transfer from places to people/objects and its capacity to retain, makes it an interesting source of information within forensic science. It is of upmost relevance to keep developing further studies to provide this matrix its full potential in Portugal.

In this work, we sought to consolidate/evolve the state of the art on human body decomposition compounds and their presence in soil over time, also considering extrinsic and intrinsic factors that affect the decomposition process. For that, we aimed to:

- collect soil samples from different cemeteries and different graves where corpses were decomposing (3 years minimum), and also collect control samples from sites with no human decomposition activity in each cemetery;

- characterise some inorganic parameters (pH, colour, and moisture content) and the organic matter content of the topsoil where human bodies went into decomposition, in order to perceive how these particularities could have influenced the organic compound results;

- mainly characterise organic constituents, namely fatty acids and steroids (sterols, stanols, stanones and bile acids), aspiring to find new biomarkers that will allow to prove that a decomposing human body was in contact with a specific soil. The possibility of relating them with their human body provenance (different body parts) and of finding time dependent constituents was also aimed.

The knowledge obtained might also contribute for a more efficient management of cemeteries in Portugal. Additionally, the obtained results might help to ascertain the correct time for performing exhumations in order to execute only one, being the body already skeletonised, thus reducing the family's suffering.

III. Materials and Methods

3.1. Framing of cemetery sites

3.1.1 Geographic and Geological context

Portuguese cemeteries were selected with no geographic restrictions, in order to study different geological backgrounds. Several city councils were contacted and 3 of them (Braga, Porto and Mértola) authorised soil sampling during exhumations.

The city of Braga, in Braga district, is composed by 37 parishes, being in the north region. It is north limited by Amares, east limited by Póvoa do Lanhoso, south limited by Vila Nova de Famalicão, and west limited by Barcelos. The average annual temperature and average annual precipitation are 15ºC and 1448.6 mm, respectively (Source: Instituto Português do Mar e da Atmosfera (Portuguese Institute of the Sea and Atmosphere) - based on data collected from 1981 to 2010).

The city of Porto, in Porto district, is composed by 7 parishes, being also in the north region. It is north limited by Matosinhos and Maia, east limited by Gondomar, south limited by Vila Nova de Gaia, and west limited by the Atlantic Ocean. The average annual temperature and average annual precipitation are 15.2ºC and 1186 mm, respectively (Source: Instituto Português do Mar e da Atmosfera (Portuguese Institute of the Sea and Atmosphere)- based on data collected from 1971 to 2000).

The city of Mértola, in Beja district, is also composed by 7 parishes, although being in the south region. It is north limited by Beja and Serpa, east limited by Spain, south limited by Alcoutim, and west limited by Almodôvar and Castro Verde. The average annual temperature and average annual precipitation are 16.5ºC and 571.8 mm, respectively (Source: Instituto Português do Mar e da Atmosfera (Portuguese Institute of the Sea and Atmosphere) - based on data collected from 1971 to 2000).

3.1.1.1 Geographic context

The Portuguese cemeteries that were sampled were Monte D'Arcos (CMA), in Braga; Prado do Repouso (CPR), in Porto; and Castelo de Mértola (CCM), in Mértola (Figure 5).

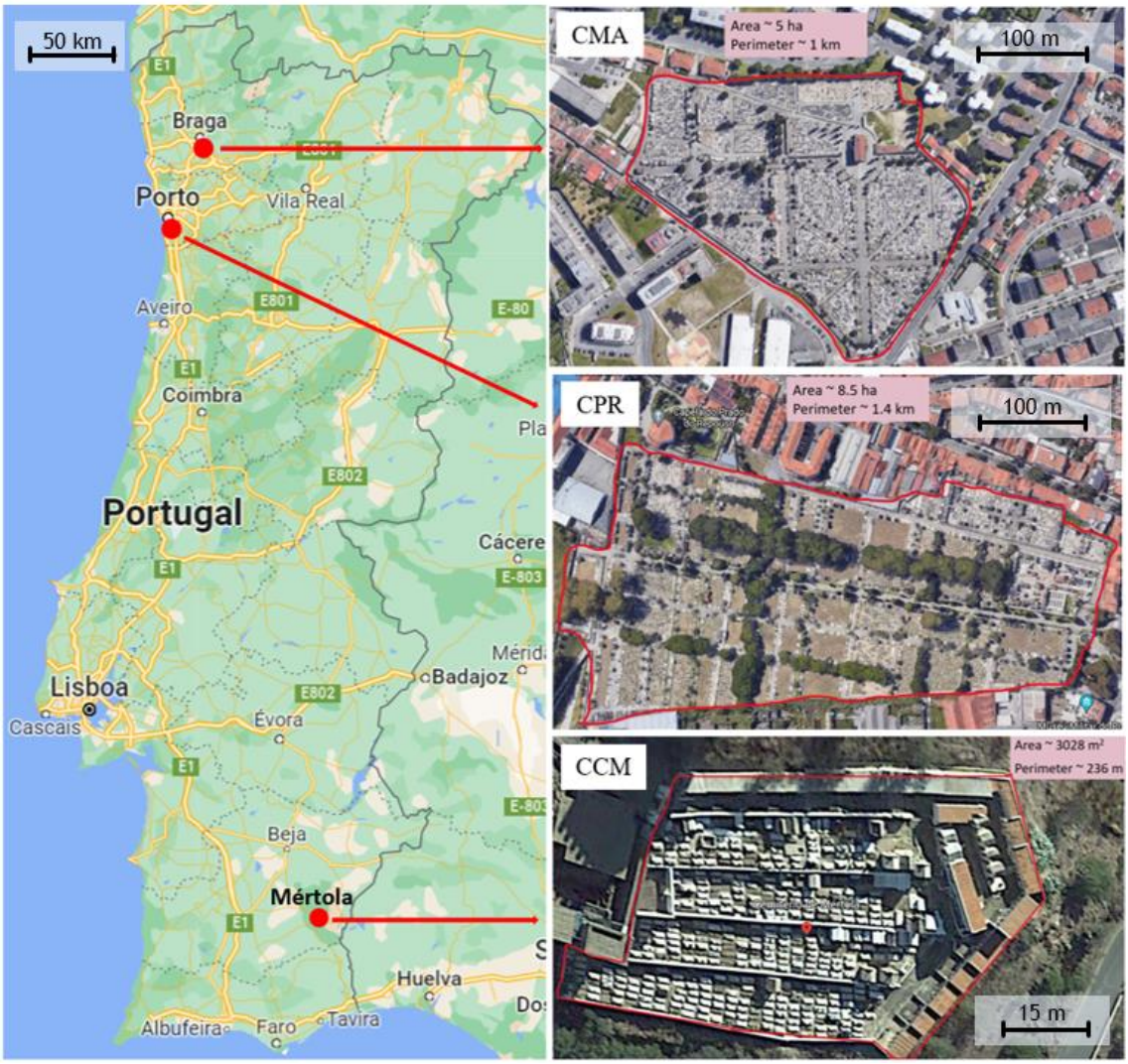

Figure 5: Geographic location of Monte D'Arcos (CMA), Prado do Repouso (CPR) and Castelo de Mértola (CCM) cemeteries, and their satellite images also referring their areas and perimeters (ha = hectares; Km = kilometres; m^2 = square metre) [Images retrieved from Google Maps in June 2021].

CMA is in Largo de Monte d'Arcos, 4710-300, Braga (Datum WGS84: 41º 33.471540'N, -8º 24.843360'E). It is a municipal cemetery with approximately 5 ha and over 150 years of existence (inaugurated on July 1, 1870). This cemetery belongs to São Victor parish, located in an urbanistic area. It is limited north by Travessa São José, east by Rua São José, south by Rua Dom António Bento Martins Júnior, and west by Rua do Areal de Baixo (the Braga sheet of the Portugal´s Military Letter is not available).

CPR is in Largo do Padre Baltazar Guedes, 4300-059, Porto (Datum WGS84: 41º 8.491500'N, -8º 35.742000'E). It has approximately 9 ha and it was the first public cemetery in the city (inaugurated on December 1, 1839). This cemetery belongs to Bonfim parish, located in an urban area. It is limited north by Rua Joaquim António de Aguiar, east by Centro Empresarial, south by Colégio dos Salesianos, and west by the

Rua do Duque do Saldanha. The location of the Prado do Repouso Cemetery is included in the extract of the 122 - Porto sheet of the Portugal´s Military Letter at 1:25.000 scale, of the Army's Geographic Institute (Figure 6).

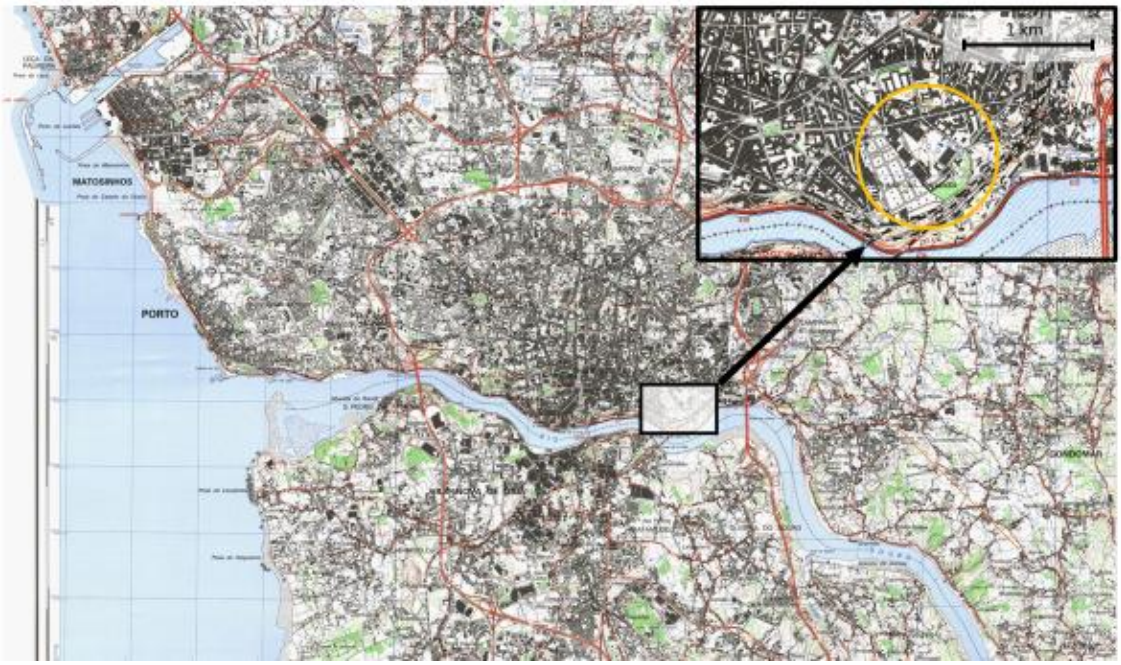

Figure 6: Prado do Repouso Cemetery, Porto (yellow circle), indicated in the extract of the 122 - Porto sheet of Portugal's military letter of the Army's Geographic Institute, at 1:25.000 scale.

CCM is in Rua Alves Redol, 7750-336, Mértola (Datum WGS84: 37º 38.367420'N, -7º 39.795780'E). Is is the smallest sampled cemetery with only approximately 3000 m^2 and it was built in the 20th century. This cemetery belongs to Mértola parish, located in a rural area, being placed in a slope of an archaelogical castle – Mértola Castle (*Castelo de Mértola*). It is limited north by IC27, east by Estrada da Ribeira, south by Rua Elias Garcia, and west by Rua Alves Redol (the Beja sheet of the Portugal´s Military Letter is not available).

3.1.1.2 Geological context

Braga is mainly represented by granitic lands, with the predominance of a biotitic monzogranite with rare muscovite with porphyroid tendency, and with a medium to fine grain, commonly known as the Braga Granite.

The location of the CMA is included in the extract of the 05D – Braga sheet of the Portugal´s Geologic Letter at 1:50.000 scale, of the Geologic Services (Figure 7).

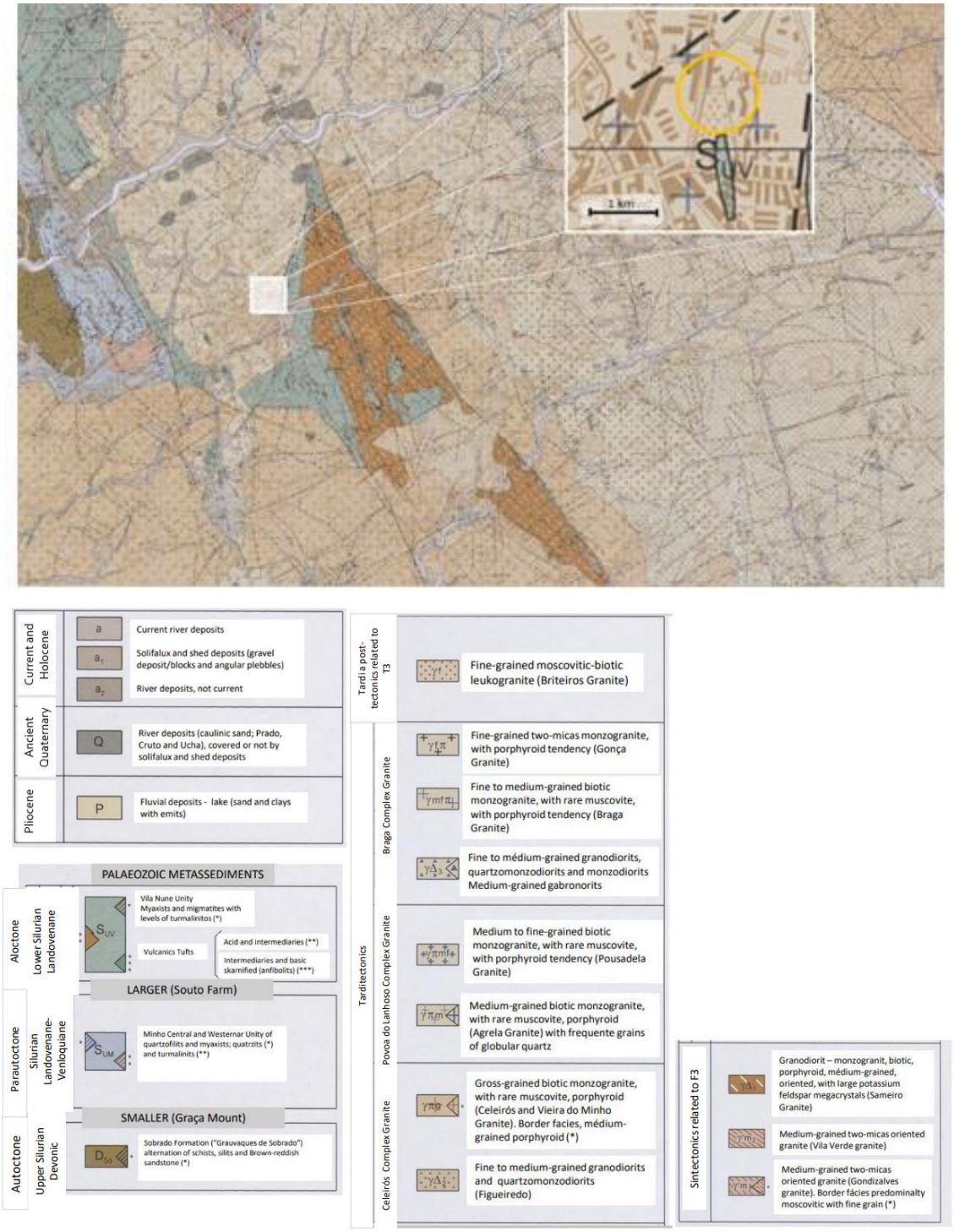

Figure 7: Monte D'Arcos Cemetery, Braga (yellow circle), indicated in the extract of the 05D – Braga sheet of Portugal's Geologic letter of the Geologic Services, at 1:50.000 scale.

Porto is mostly represented by granitic formations, similarly to Braga, with the predominance of a two-mica leucocrat medium to coarse-grain alkaline granite, also known as the Porto granite.

The location of the CPR is included in the extract of the 09C – Porto sheet of the Portugal´s Geologic Letter at 1:50.000 scale, of the Geologic Services (Figure 8).

Mértola region is mainly represented by greywackes, which alternate with clay and siltstones, as well as levels of conglomerates. The Castelo de Mértola Cemetery area has classical dominant turbidites (pelites, greywackes and conglomerates), finely stratified turbidites (pelites and fine greywackes), phyllites, silites and quartzites.

The location of the CCM is included in the extract of the 46D – Mértola sheet of the Portugal´s Geologic Letter at 1:50.000 scale, of the Geologic Services (Figure 9).

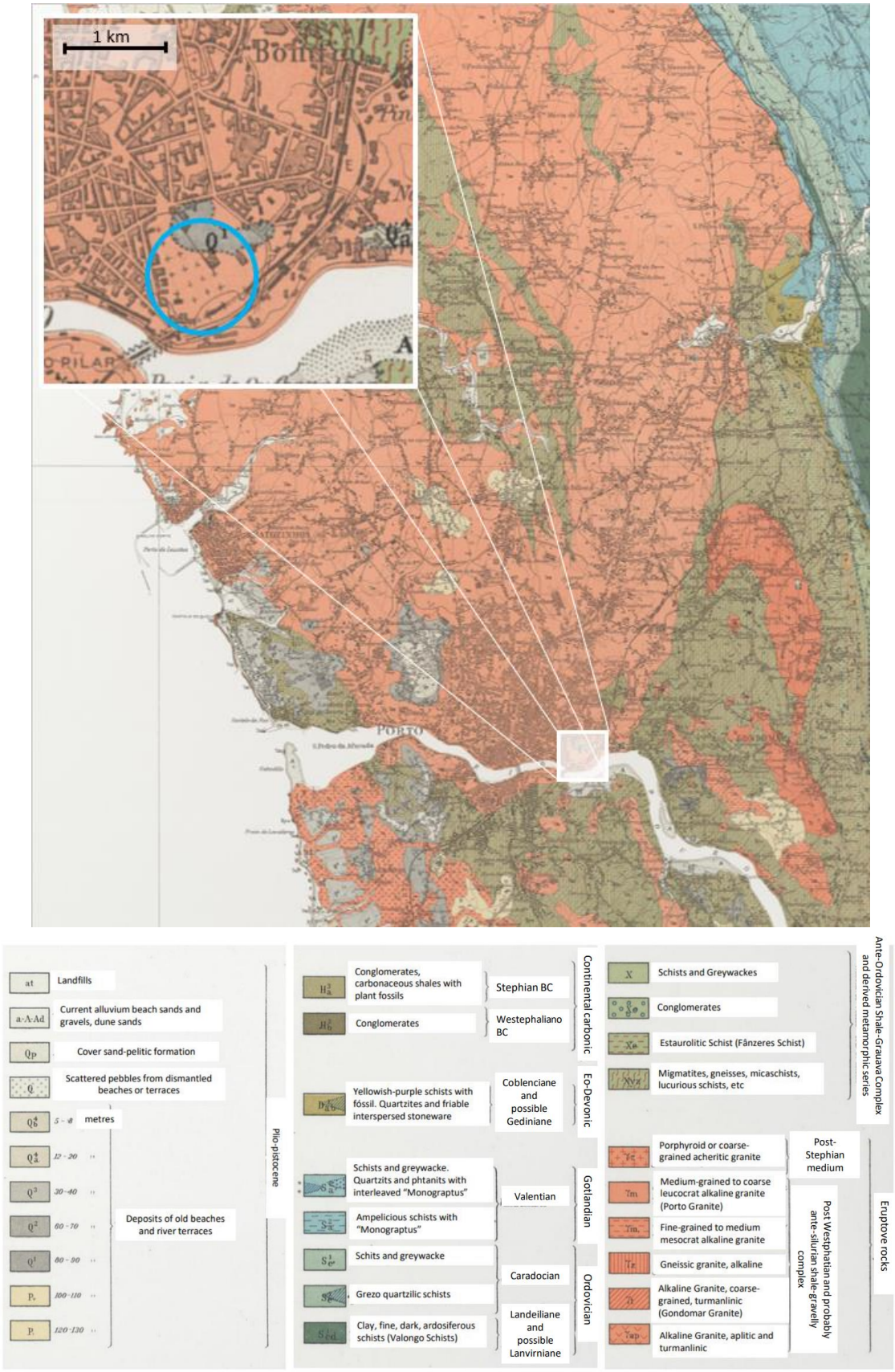

Figure 8: Prado do Repouso Cemetery, Porto (blue circle), indicated in the extract of the 09C – Porto sheet of Portugal's Geologic letter of the Geologic Services, at 1:50.000 scale.

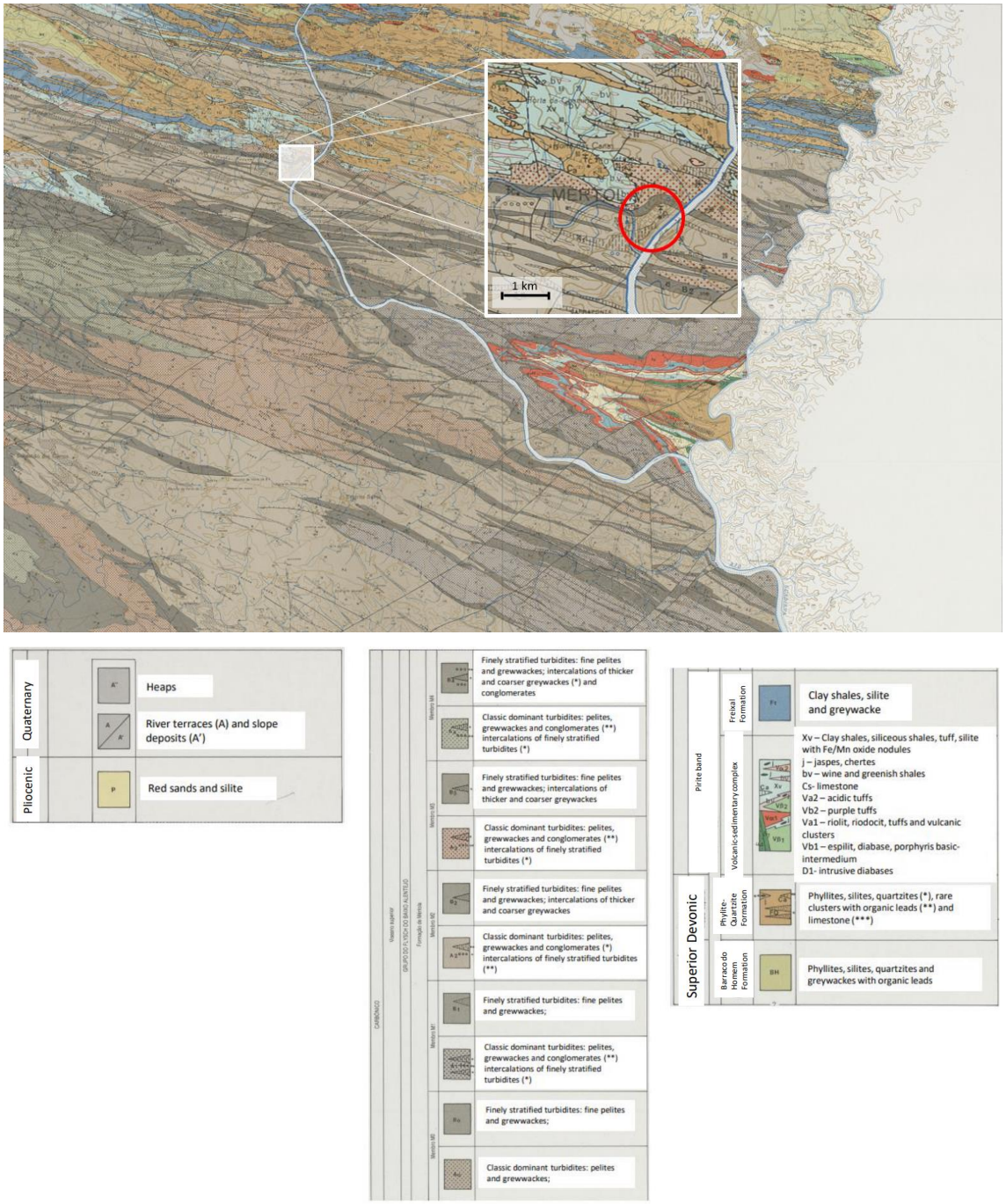

Figure 9: Castelo de Mértola Cemetery, Mértola (red circle), indicated in the extract of the 46D – Mértola sheet of Portugal's Geologic letter of the Geologic Services, at 1:50.000 scale.

Despite the geological setting of the 3 sampled cemeteries, it is important to borne in mind while studying soil from these sites, that it might have different origins since, in CCM, the soil that was used to build cemeteries was soil collected from construction sites where residues from civil construction works (e.g., rock fragments and bricks) were placed (Figure 10).

Figure 10: Grave soil in Castelo de Mértola Cemetery with visible large angular rocks and bricks.

3.2 Sampling, labelling, and transport procedures

The Northern cities (Braga and Porto), having cemeteries with larger areas and more buried bodies, also have more often exhumations. The southern city (Mértola), on the other hand, with a cemetery with a smaller area and less buried bodies, also had less often exhumations. Despite that, Portuguese cemeteries have different exhumation policies depending on the environmental and geological conditions of each geographic location. Usually, in the northern region, exhumations are performed in 3 to 7 years after burials. However, in warmer areas like the southern ones, exhumations are performed with a bigger time interval (e.g., Mértola - normally after 12 years). Sometimes it can take over 20 years to truly exhume a body, since it must be fully skeletonised at that time. This usually happens in the southern regions given that are warmer and the soil characteristics does not allow a fast decomposition process. If it is not the case, the grave is closed and reopened every 2 years minimum until the full decomposition process has occurred. In CMA, an enzymatic biological product is added on the top of the deceased to fasten tissues degradation. Nevertheless, it was brought to our attention by some cemetery's employees that most of the times this product does not work as it is supposed.

Soil samples were collected from burial graves in three different Portuguese cemeteries, during exhumations that occurred between March 2021 and June 2021 (Table

1) and considering different corpse regions and grave walls (Figure 11). Additional soil samples were also collected from sites with no human decomposition activity nearby (in CMA and CCM), being the sampling places selected according to each cemetery space.

Table 1: Portuguese cemeteries, their city and region, sampling dates and respective exhumation number.

Cemetery	City and Region	Sampling dates (DD/MM/YYYY)	Exhumation numbers
Monte D'Arcos	Braga – North of Portugal	03/03/2021	1
		09/03/2021	2
		27/04/2021	6
		28/04/2021	7
		11/05/2021	8
		20/05/2021	9
		26/05/2021	10
		02/06/2021	12
Prado do Repouso	Porto – North of Portugal	20/04/2021	5
		01/06/2021	11
Castelo de Mértola	Mértola – South of Portugal	17/03/2021	3
		22/03/2021	4

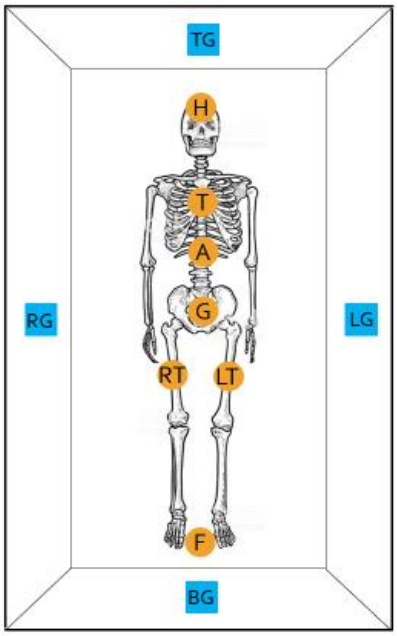

Figure 11: Representation of the soil sampling strategy. Orange circles represent soil samples collected under the corpse (H-Head, T-Thorax, A-Abdomen, G-Gluteal region, RT-Right Thigh, LT-Left Thigh, F-Feet); Blue squares represent soil samples collected from the grave walls (TG- Top Grave, RG- Right Grave, LG-Left Grave, BG-Bottom Grave).

At first instance, in all exhumations, a grave topsoil sample was collected into a plastic bag, using a plastic spatula, being posteriorly labelled with the exhumation number.

This soil collection did not follow any rigorous procedure thus, it was only aimed to fulfil at least 1/3 of the bag capacity to insure a minimum quantity of soil for the analysis.

Then, each grave and corpse were measured, and other information about each deceased was registered (sex, age at death, years of burial, type of clothes/accessories and other relevant information), being given by cemetery employees. In some cases, the information about the deceased was written on old papers, being therefore difficult for the cemetery employees to provide some related data (birth date/age at death and years of burial). Identification data were pseudo-anonymised, since individuals were all codified with the number of their exhumation, as exhumations were being performed.

At each grave, after the removal of the human body/skeletons by the cemetery workers, a 100 centimetres (cm) auger (04010330V, Eijkelkamp Soil & Water) was used on the right hand corner (the head side of the corpse (H)), to observe the soil's profile that was also photographed whenever possible and collect samples of 10 cm interval (Figure 12). The soil was sampled and placed in plastic bags and was stored at -20ºC for further subsequent analyses if required.

Figure 12: 100 centimetre sampling Auger (04010330V, Eijkelkamp Soil & Water) sampling soil´s profile at the right corner of the grave (head side of the corpse).

The sampling related to the first exhumation (CMA, 03/03/2021) was a trial to test equipment and improve the sampling protocol. In this first exhumation, grave inner soil samples (under the head, thorax, abdomen, gluteal region, right and left thigh, feet, and on the 4 walls) were collected using a 15 cm length stainless steel auger, with 5 cm diameter, specially constructed by *Arte e frio* (Portuguese company) for this purpose and study. Having realised that it would be more advantageous to collect 20 cm of soil, and that using the same auger in all different graves and cemeteries would increase the change of sample contamination (besides that cleaning the auger between every sampling was time-consuming), 20 stainless steel augers with 25 cm length and 5 cm diameter were then produced by the same company (Figure 13a-c). In this way, to study the behaviour of the human body decomposition compounds through soils' depth, grave inner soil samples were all collected using the 25 cm stainless steel auger (trial exception), being then split into four subsamples: 0-5, 5-10, 10-15 and 15-20 cm depth (Figure 13d). The augers were pushed into soil with the aid of a hammer (Figure 13e) with a length of 37 cm and, during sampling, nitrile gloves and feet protection were used. All reusable material was properly cleaned (distilled water and ethyl alcohol) between each use.

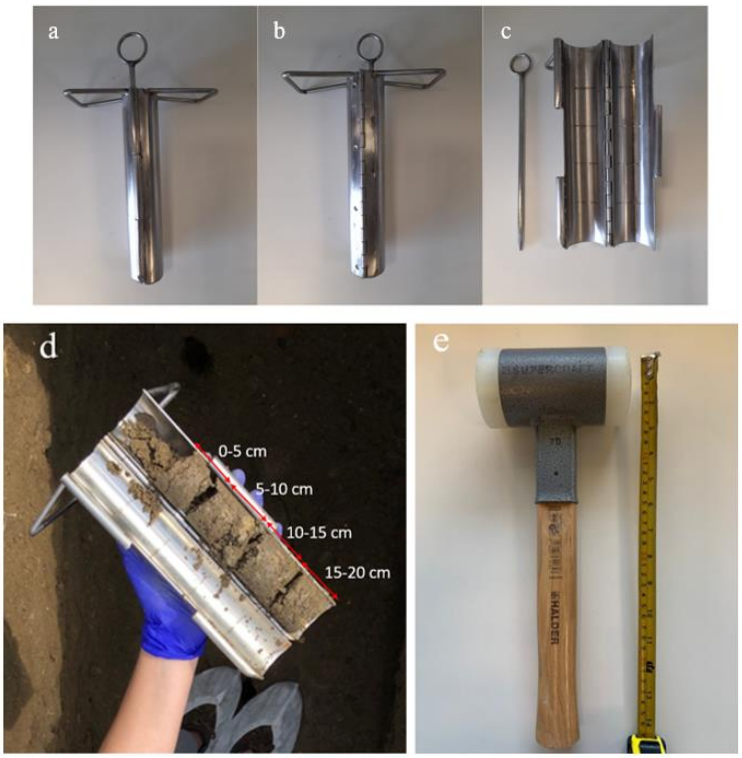

Figure 13: 25-centimetre length stainless steel auger: (a) closed front view, (b) closed back view, (c) open view showing 5 cm length internal marks, (d) one soil sample being subsampled into four parts, and (e) the auger hammer.

All grave inner soil samples were collected into plastic bags, being then labelled considering the exhumation number, the part of the body/wall from which they were collected and the depth of the subsample. Thus, each subsample was labelled with a number from 1 to 12, plus the letter of the part of the grave where it was collected, plus the greatest depth (e.g., 2H10: subsample collected under the head of the individual number two, from the 5-10 cm depth). Samples collected from the walls followed the same logic, however a "W" was written before the exhumation number so no misspellings would happen and the samples from corpses and walls were easily distinguished (e.g., W7TG5: subsample collected from the top grave wall of individual 7, from the 0-5 cm depth).

Control soil sampling was also performed at sites with no human decomposition activity in the CMA and CCM. Given the different exhumation process in the CPR, it was decided not to analyse its soil samples, which led to no control samples being collected. In CPR, clothes of the deceased are buried in their graves after the removal of the skeleton. If these samples were studied, other variables had to be taken into consideration and solid conclusions could be harder to be achieved. However, for future reference, this source of contamination must be taken into consideration. Additionally, in CPR, graves are only open enough to remove corpses. In the other two cemeteries (CMA and CCM) more soil is removed, having wider graves which makes the soil sampling much easier.

For the collection of the control samples in the CMA and CCM, the 25 cm length stainless steel auger was stuck into the soil and 20 cm of soil were also collected, being then also split into four subsamples and stored in plastic bags. The samples were collected from at least 3 different locations at each cemetery, and the distance from each site to the nearest grave was measured. To label the control samples, the different sites were referred by an L (meaning Local) and by a number given the sampling order. Then, a C was added (meaning Control), followed by the exhumation number that took place the same day. Plus, the distance to the nearest grave and, finally, the soil greatest depth. For example, the control sample collected at the second local and in the same day as the exhumation 1, being 5.5 m away from the nearest decomposition site, and belonging to the 5-10 cm soil depth, was labelled as "L2/C1/5.5/10" (Local/Exhumation/Distance/Depth). In CMA the control samples were collected from four different sites all 15-16 m away from the nearest grave and near to the tanatory of the cemetery. In CCM, control samples were collected in three different sites: L1, L2 and L3 - 2 m, 5.5 m and 8 m apart from the nearest grave,

respectively. These three sites are located in a part of the cemetery that has a wall with a thickness of 50 cm separating them from the graves. It was planned to collect control samples from a fourth site, outside the cemetery, however, the soil was filled with large stones making impossible to collect soil samples from there.

The transport of all soil samples to the laboratory occurred inside a thermic transport case, also filled with ice cuvettes to keep the samples cold (Figure 14).

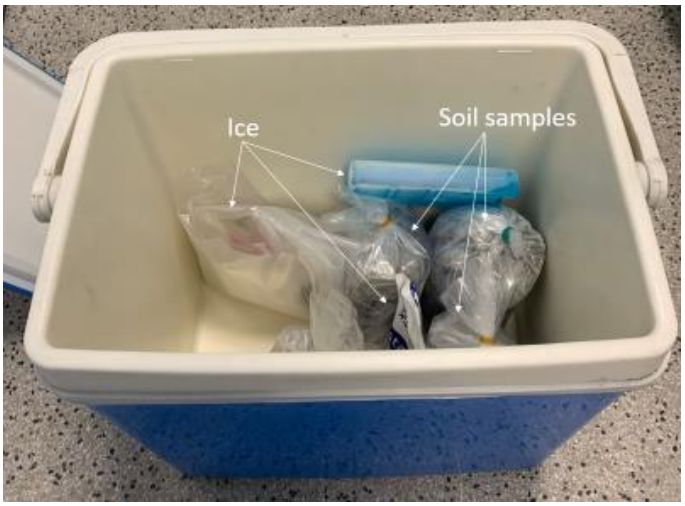

Figure 14: Thermic transport case with ice cuvettes and plastic bags with soil samples.

The plastic bags with samples within them which had been collected under each corpse were packed within 2 additional empty plastic bags; the plastic bags with samples collected from the walls and from the right corner of each grave were packed within one other empty plastic bag, and the plastic bags with the control samples of each cemetery were also packed within 1 other empty plastic bag (Figure 15). All soil samples were stored in the dark, at -20ºC, until being analysed.

Figure 15: Plastic bags with grave inner soil samples packed within other different plastic bags (CORPSE - two bags with the samples collected under the corpse; WALL + 100 centimetre Auger - one bag with the samples collected from the grave walls and from the right corner of the grave; CONTROL - one bag with the samples collected from locations with no human decomposition activity).

This project was submitted to the Ethics Committee of the University Institute of Health Sciences and received a favourable recommendation (Attachment 3). It is also important to highlight that when requested by cemeteries and/or desirable by the deceased families, some relatives were present during exhumations (exhumations number 4, 5, 11 and 12). In these cases, an explanation of the soil sampling procedure was previously given to each family member before their consent for soil collection. Additionally, every soil sampling was accompanied by the cemetery employees.

3.3. Laboratory analyses

The covid-19 pandemic caused some setbacks during the development of this research work.

First, due to the restrictions applied in Portugal during the lockdown, sampling was not allowed to be performed in the early planned dates. Additionally, this phenomenon prevented, on two occasions, the realisation of an Erasmus to have a quicker practical rapport with the line of investigation. Moreover, due to several delays in shipments, the starting of the laboratorial activities had to be significant postponed. Therefore, the planning of the laboratory analyses was forced to be adapted to the available time.

Initially, soil samples from 12 exhumations from 3 different cemeteries were collected, hoping to analyse soil samples from the best 10 (trial exception) which were the 10 exhumations where we were able to collect the biggest number of samples. Due to the limitations already described, the number of exhumations considered for laboratory analyses was reduced to half: 3 from CMA and 2 from CCM. Consequently, the soil samples intended to be analysed were from exhumations number 3, 4, 7, 10 and 12 (Table 1). Thus, topsoil samples from the graves of the referred 5 exhumations were submitted to inorganic characterisation (pH, colour, and moisture content; texture was firstly intended, however, due to the shipment delay of the materials it was not carried out) and analysis of their total organic matter content. Then, the soil samples collected within each of the 5 graves were intended to be analysed to characterise their organic constituents, namely fatty acids and steroids (sterols, stanols, stanones and bile acids). However, also due to the limitations referred, the number of samples to be analysed was reduced. Thus, soil samples under the individual's left tight and feet were not intended to be analysed, being this decision supported in literature that consider these sites as having lower quantities of lipidic compounds since these body parts have less adipose tissue (von der Lühe et al. 2017). Additionally, only the first 10 cm of soil were intended to be analysed from the samples under corpses and the first 5 cm from the wall samples. The samples from the soil profile and several of the control samples were retained for potential analysis in the future.

Unfortunately, even having already reduced the number of soil samples to be analysed for organic constituents, at a 3rd instance, the available time showed to be insufficient for all of these secondly intended analyses, since some of them had to be repeated, while optimising the Gas Chromatography Coupled to Mass Spectroscopy (GC-MS) method. This work presents the organic constituent results of the analysis of fatty acids, sterols, stanols, stanones and bile acids from the exhumations number 7, 10 and 12, and only the analysis of sterols, stanols and stanones from the exhumation number 3, exhumation number 4 not being analysed. Wall samples were not laboratory analysed at this stage.

3.3.1. Grave topsoil samples

3.3.1.1 *Inorganic characterisation*

The inorganic characterisation was performed through the analysis of pH, colour and moisture content.

3.3.1.1.1 *pH*

pH represents potential hydrogen and is used to specify the acidity or basicity of a solution or water-soluble substance, being a numerical scale from 0 to 14. This parameter can also be defined as the logarithm of hydronium ion activity (Blackstock 1989).

To quantitatively evaluate the pH, topsoil samples were oven-dried in a WTB Binder 78532 (Tuttlingen/Germany) at 40ºC for 24h, and then sieved (<2mm; Cisa). Afterwards, the Hanna Instruments pH meter HI99121 protocol was applied. Thus, 10 grams (g) of each sample were weighted into a beaker and 25 (millilitre) mL of Soil Sample Preparation Solution HI7051 was added. Each sample was then stirred for 30 seconds (s) and, after 5 minutes resting, stirred again. Using the pH meter HI99121, the pH values were obtained.

3.3.1.1.2 *Colour*

The colour of a soil is an important morphological property in soil's characterisation and can be easily determined even in the field. This parameter gives information regarding the relative amount of organic matter and/ or moisture in a given soil, as well as a general notion of its mineralogical composition (Owens and Ruthledge 2005).

Soil colour was qualitatively evaluated, based on the Revised Standard Soil Colour Charts (RSSCC), from Münsell. In this way, dry and wet topsoil sample colours were determined by the evaluation of three attributes: hue (which represents the reddish or yellowish tones of the soil samples), value (which evaluates how dark or light soil samples are) and chroma (which means how dull or bright soil samples are). For this, each topsoil sample was oven-dried in a WTB Binder 78532 (Tuttlingen/Germany)) at 40ºC for 24h, and then sieved (<2mm; Cisa). The colour of the samples was then compared with the RSSCC under a natural light environment. After evaluating the colour in dry samples, the same analysis was performed after having wet them (no visible water). In both processes, a colour code was obtained for each soil sample.

3.3.1.1.3 *Moisture Content*

The moisture content of a soil sample represents the percentage of water within it by its mass (Heddam 2021).

To quantitatively evaluate the moisture content, 1 g of each topsoil sample was weighed in an analytical balance (ALC-210.4, Acculab), being posteriorly placed into an

oven, a WTB Binder 78532 (Tuttlingen/Germany)) at 105ºC, for 24h. Afterwards, each sample was reweighed, the mass loss meaning the moisture content that was evaporated.

The remaining samples were then stored in a desiccator, for future determination of their organic matter content.

3.3.1.2. *Organic Matter Content*

The total organic matter content of a soil represents its organic fraction which is composed of living and dead organisms, and humus. Its amount is related to 4 factors: quantity of organic material in soil, its decomposition rate, the mineralisation rate, and the soil's texture (Johnston, Poulton and Coleman 2009).

To quantitatively evaluate the total organic matter content, the loss-on-ignition method was used (Hoogsteen et al. 2018). In this way, after performing the moisture content analysis, the remain of each topsoil sample mass was placed into a furnace (Nabertherm LT 9/11) at 450ºC for 8h. Then, after being removed from the furnace and having cooled down, soil samples were weighed in an analytical balance (ALC-210.4, Acculab), and the total organic matter content was calculated by analysing the mass loss.

3.3.2. Grave inner soil samples

To characterise the organic compounds (fatty acids, sterols, stanols, stanones and bile acids) of each grave inner soil sample, the protocol presented in von der Lühe 2016 was used.

First, all samples were pre-treated for being posteriorly analysed. In this way, samples were freeze-dried for 24h in an Edwards Modulyo 4K Freeze Dryer, being then milled using a MM400 mill from Retsch, at a frequency of 28 (hertz) 1/s for 10 min. The used mill allowed the simultaneous milling of two samples, being the mortars and spheres made of agate.

Afterwards, 1 g of each soil sample was weighed in an analytical balance (ALC-210.4, Acculab) and put into centrifuge tubes, being 3 mL of Dichloromethane (DCM):Methanol(MeOH) added to each tube. Then, samples were placed into an ultrasonic bath (USC 300 T, VWR) at room temperature for 10 min, and centrifuged (Z 206 A, Hermle) at 2000 rpm for 5 min. The supernatant was removed to 4 mL vials and dried under a gentle stream of N_2 using a SpeedVac (CentriVap Concentrator, Labconco). The extraction was repeated two more times: one with 3 mL of DCM and one with 3 mL of n-hexane. This ultrasonic extraction allowed to obtain the total lipid extract (TLE).

These extracts could either keep being analysed or be kept at -20ºC for several weeks, being only further analysed.

The first internal standard (IS) addition was the next step, for enabling compounds quantification. IS stock solution mixture was prepared (Table 2) and 100 µL were added to each sample. This mixture was prepared by adding 100 µL of each stock solution of the internal standards (all with the concentration of 1 (mg/1mL) to a 10 mL volumetric flask and by adding n-hexane. The IS mixture was then stored at -20ºC in a recipient with silica gel with orange indicator.

Table 2: First internal standard mixture for soil sample organic constituent analysis.

Lipid	**Internal Standard**
Fatty acid	Heptadecanoic acid (c17)
Sterol and Stanol	Pregnanol
Stanone	Pregnanone
Bile Acid	Isodeoxycholic acid (IDCA)

Besides this IS mixture, a second internal standard was added to the samples right before they were injected into the GC-MS equipment (GC-2010 Plus coupled to a MS QP2020, Shimadzu, Japan): 100 µL of 5α-cholestane (from a stock solution with a concentration of 5 ng/µL). This second IS allows the detection of any syringe inaccuracy.

Concerning the separation of neutral lipids from the lipidic fraction, saponification and liquid-liquid extraction were carried out. For this, 1.5 mL of 0.7 M potassium hydroxide (KOH) in MeOH was added to the TLE, and samples were saponified for 16h at room temperature. Then, to redissolve the extracts, each sample was placed on a dry block heater (Fisherbrand Isotemp Digital Dry Block Heater) at 50-60ºC, and 0.5 mL of ultrapure water was added. After a vigorous shaking, 1.5 mL of n-heptane was also added. Succeeding this, two different layers appeared in each sample: the lower one (aqueous phase) containing acidic lipid fraction (with fatty and bile acids) and the upper layer (organic phase) containing neutral lipidic fraction (with alkanes and alcohols). The upper layer was removed and transferred into a new vial. The extraction was then repeated using more 1.5 mL of n-heptane, the upper layer being again transferred into the neutral fraction vial, which was dried under a gentle stream of N_2. Both vials, with the neutral and acidic fraction were stored at -20ºC until further analysis.

Using the vial with the remaining aqueous phase, extraction of the acidic lipid fraction was conducted. Each soil sample was acidified by adding drops of a 6 M hydroxide chloride (HCl) until the achievement of a pH ≤ 2. Then, 1.5 mL of chloroform was added, and two layers appeared: the lower layer (organic phase) containing fatty and bile acids and the upper layer (aqueous phase) that should not contain any lipids. The lower layer was removed, transferred into a new vial, and extracts were dried under a stream of N_2.

After the obtention of the neutral fraction, a separation of alkanes and alcohols is needed. For this, a solid phase extraction (SPE) was carried out using 3 mL glass SPE columns. Columns were prepared using polytetrafluoroethylene (PTFE) frits and 1 cm of silica gel (60 Å, 0.063–0.200 mm particle size). Then, they were preconditioned using 1.5 mL of n-heptane, twice. To redissolve samples, 500 µL of n-heptane were added to them, being placed in a dry block heater at 50-60ºC. Then, samples were transferred into the SPE columns, and the redissolving step was repeated twice using 1.5 mL of n-heptane, to ensure the transfer of all lipids into the columns. These washing steps carries to waste the fraction containing hydrocarbons such as alkanes. After the removal of alkanes and alcohols, 1.5 mL of n-heptane:ethyl acetate (80:20, v/v) was added twice to the vials (containing the lipidic extracts) on the dry block heater. Before transferring its content to the columns, new vials were placed below the SPE columns to collect the fraction containing sterols, stanols and stanones (designated as ALC fraction). After SPE, samples were dried under a gentle stream of N_2 and stored at -20ºC.

Since the aim was to identify and quantify the lipidic compounds using GC-MS, there was a need to perform derivatisation of the ALC fraction from the previous step to obtain trimethylsilyl (TMS) derivatives. For this, a derivatisation agent was added - N,O-Bis(trimethylsilyl(trifluoroacetamide) (BSTFA) + Trimethylchlorosilane (TCMS); 99:1)/pyridine (3:1 v/v) – to a GC-MS vial with the sample extract already dissolved in n-heptane and dried with N_2. Samples were placed on a dry block heater at 90ºC for 1 h. Then, the derivatisation agent was evaporated with N_2; 100 µL of the second internal standard, 5α-cholestane, were added, and the samples were analysed with GC-MS. With this derivatisation process, peak separation is ensured, and a well-defined chromatography is achieved.

To perform SPE and GC-MS analysis of the acidic fraction, a methylation of the carboxylic group of fatty and bile acids was required. Thus, 1.0 mL of 1.25 M HCl in MeOH was added to the dried extracts that were then put into a dry block heater at 80ºC

for 2 h. The methyl esters were later extracted by performing a liquid-liquid extraction with 1 mL of ultrapure water and then 1.5 mL of n-hexane, thrice. The upper layers (containing the methyl esters) were transferred into a new vial and the extracts were dried using N_2 and stored at -20ºC.

A new SPE was performed aiming to separate the Fatty Acid Methyl Esters (FAME) fraction from the Bile Acid Methyl Esters (BAME) fraction. Columns were prepared using PTFE frits and 1 cm of Polygoprep 100-130 silica. Then, they were preconditioned using 1.5 mL of n-hexane, twice. Afterwards, 500 µL of n-hexane were added to the samples and the extracts were warmed in a dry block heater at 50-60ºC. Then, 1 mL of DCM/n-hexane (2:1, v/v) was added to the samples on the dry block heater and 1 mL was transferred from the vials to the columns. At this point, new vials labelled with FAME were placed below the columns and the washing step was repeated thrice with 1 mL of DCM/n-hexane. Thus, FAME fraction was obtained. Additionally, new vials were put below the columns to collect the BAME fraction. For this, washing was performed with DCM/MeOH. FAME and BAME extracts were ultimately dried with N_2 stream.

Similarly, to the ALC fraction, also FAME and BAME fractions needed to be derivatised. The derivatisation of the FAME fraction is important if samples contain any hydroxy fatty acids, which are compounds frequently found in soils. For this, the derivatisation followed the same protocol as the one used for the ALC fraction. To derivatise the BAME fraction the protocol differs in the derivatisation agent: BSTFA/1-(Trimethylsilyl)imidazole (TMSI; 98:2, v/v), adding also toluene. Samples were placed on a dry block heater at 90ºC for 1 h, and the agent was evaporated using N_2. Then, 5α-cholestane was added and GC-MS analysis was performed.

The GC-MS conditions were different for the three fractions: ALC, FAME and BAME (Table 3).

Table 3: Gas Chromatography Coupled with Mass Spectroscopy conditions to analyse the ALC fraction (fraction containing sterols, stanols and stanones), FAME (fatty acid methyl esters) and BAME (bile acid methyl esters) in soil samples (m = metre; µm = micrometres; mL = mililitre; ºC = celsius degrees; µL = microlitre; min = minutes; eV = electron volt).

Features	*ALC fraction*	*FAME fraction*	*BAME fraction*
Column	DB-5ms Ultra Inert (or other supplier), 30 m length x 250 µm internal diameter x 0.25 µm film thickness		
Carrier gas	Helium		
Column flow	1.1 mL, min^{-1}; constant flow		1.0 mL, min^{-1}; constant flow
Injection port temperature	250ºC		290ºC
Injection volume	1 µL		
Injection mode	splitless		
Temperature program	80°C for 1.5 min 12°C / min to 265°C 0.8°C / min to 280°C 10°C / min to 300°C and held for 12 min		80°C for 1.5 min 20°C / min to 250°C 1.2°C / min to 280°C 10°C / min to 300°C and held for 12 min
Solvent delay	20 min	10 min	20 min
Electron ionisation	70 eV		

All samples were analysed in full scan and Selected Ion Monitoring (SIM) mode. The ions used in SIM mode for the ALC and BAME fractions are presented in Table 4.

Table 4: Ions used in the Selected Ion Monitoring mode of the Gas Cromatography Coupled to Mass Spectroscopy (from von der Lühe, 2016) (RTm = retention time; min = minutes; m/z = mass-to-charge ratio; IS I = first internal standard; IS II = second internal standard, "-" = no data).

Common name	Compound group	RTm (min)	Qualifier (m/z)		Quantifier (m/z)
5α-preganan-3-one	IS I stanones	19.0	215	-	300
5α-cholestane	IS II	10.3, 22.9	357	372	217
Coprostanol	5β-stanol	26.6	355	215	370
Epicoprostanol	5β-stanol	27.2	370	215	355
5β-cholestanone	stanone	28.2	231	107	386
Cholesterol-d7	IS I sterols, stanols	28.6	375	465	336
Cholesterol	$\triangle^5$-sterol	28.7	368	458	329
5α-cholestanol	5α-stanol	29.0	355	460	445
5α-cholestanone	stanone	29.3	231	-	386
5β-stigmastanol	5β-stanol	31.5	383	215	398
Epi-5β-stigmastanol	5β-stanol	32.2	383	215	398
Isodeoxycholic acid	IS I bile acids	24.8	355	370	255
Isolithocholic acid	Bile acid	27.3	257	357	215
Lithocholic acid	Bile acid	28.2	257	372	215
Deoxycholic acid	Bile acid	29.1	345	370	255
Chenodeoxycholic acid	Bile acid	30.0	355	370	255

IV. Results and Discussion

4.1 Sampling, labelling, and transport procedures

A total of 12 exhumations were carried out: 8 in Braga, 2 in Porto and 2 in Mértola, with corpses presenting different decomposition rates and patterns (Table 5).

Table 5: Exhumation and Deceased information (CMA = Monte D'Arcos Cemetery; CCM = Castelo de Mértola Cemetery; CPR = Prado do Repouso Cemetery; Fl = Female; Ml = Male; cm = centimetres; ºC = Celsius degrees; n.a. = not available).

Exhumation information					**Deceased information**					
Cemetery	Number	Date (day/month /year)	Weather temperature (ºC)	Grave size (length x width x depth) (cm)	Sex	Height (cm)	Age at death (years)	Years of burial	Type of clothes/ acessories	**Other relevant information**
CMA	**1**	03/03/2021	13	200 x 70 x 80	Fl	160	n.a.	n.a.	Dress, catholic rosary on hands	Fully skeletonised
	2	09/03/2021	15	200 x 70 x 80	Ml	180	84	6	Coat, shirt, pants, sneakers, diaper	Presence of water in the grave; Corpse in saponification phase
	6	27/04/2021	15	200 x 75 x 110	Fl	165	n.a.	13	Suit, vest, shirt, shoes, tie, catholic rosary, belt, diaper	Dental prothesis buried in a plastic container; hospital's bracelet; plastic flowers; fully skeletonised
	7	28/04/2021	16	210 x 75 x 80	Ml	175	89	7	Suit, shoes, tie, catholic rosary	Heavily raining before exhumation; coffin not degraded; corpse partially putrefied (back and gluteal region); remain parts skeletonised

(Table 5. Continued)

Exhumation information					Deceased information					
Cemetery	Number	Date (day/month /year)	Weather temperature (ºC)	Grave size (length x width x depth) (cm)	Sex	Height (cm)	Age at death (years)	Years of burial	Type of clothes/ acessories	**Other relevant information**
	8	11/05/2021	15	210 x 65 x 97	Fl	150	n.a.	7	Coat, blouse, skirt, pantyhose	Presence of a pacemaker equipment; fully skeletonised
	9	20/05/2021	21	200 x 75 x 100	Ml	175	76	5	Suit, shirt, shoes, tie, diaper	Presence of flowers in the grave; haemodialysis bag; died in an elderly care facility; skeletonised (presence of some tissue in the lower back)
	10	26/05/2021	22	200 x 70 x 90	Ml	170	94	5	Suit, shirt, shoes, catholic rosary, diaper	Skeletonised; the back region of the thorax was mummified and putrefied
	12	02/06/2021	16	200 x 75 x 90	Ml	180	83	7	Suit, shirt, shoes, tie, diaper	Corpse was exhumed to be transferred to other cemetery; soil above the coffin had flowers, sponges, and plastic wrapping material; the cause of death was a lung cancer; individual not submitted to chemotherapy; fully skeletonised

(Table 5. Continued)

Exhumation information					Deceased information					
Cemetery	Number	Date (day/month /year)	Weather temperature (ºC)	Grave size (length x width x depth) (cm)	Sex	Height (cm)	Age at death (years)	Years of burial	Type of clothes/ acessories	**Other relevant information**
CPR	**5**	22/04/2021	14	200 x 70 x 120	Fl	130	13	7	n.a.	Raining during the exhumation; the cause of death was a brain tumor; fully skeletonised
	11	01/06/2021	16	200 x 70 x 120	Fl	165	78	8	Coat, blouse, skirt, pantyhose, catholic rosary, diaper	Fully skeletonised
CCM	**3**	17/03/2021	24	220 x 80 x 110	Fl	160	77	26	Suit, shirt, shoes, belt, diaper	Soil above the coffin had bones of other human remains; flowers wrapped in plastic; fully skeletonised
	4	22/03/2021	20	185 x 50 x 98	Ml	165	93	20	Coat, shirt, pants, socks, shoes, belt	No underwear; dental prosthesis; presence of moisture; flowers wrapped in plastic; fully skeletonised (bones easily deteriorated)

Regarding the Monte D'Arcos Cemetery (CMA) exhumations, individuals in the exhumations 6, 8 and 12 were fully skeletonised (with years of burial being 13, 7, and 7, respectively). The individual from the trial exhumation (exhumation number 1) was fully skeletonised and the time since burial was not provided. The individual in the exhumation 2, showed presence of adipocere in some parts of the corpse and was still in the saponification phase of the decomposition process. This could be explained by the high amount of water inside the grave and around the corpse. In normal circumstances, the grave would be closed since the body was not skeletonised, but this exhumation had the purpose of translating the body to a family tomb. For that reason, the body was exhumed,

an enzymatical product was placed all over the body surface hoping to fasten the decomposition process, and then it was placed into the new grave. The individual of exhumation 7, with 7 years of burial, was putrefied in the back and gluteal region, being the rest of the corpse skeletonised. The individual from the exhumation number 9 was decomposing in that site for 5 years and was almost fully skeletonised excepting for the lower back region that showed the presence of some tissue. Individual from exhumation 10, 5 years buried, was skeletonised but showed mummification and putrefaction in the back part of the thorax region.

In the Prado do Repouso Cemetery (CPR) exhumations number 5 and 11, both corpses were fully skeletonised, having had a similar number of years of burial (7 and 8, respectively).

Regarding the Castelo de Mértola Cemetery (CCM) exhumations number 3 and 4, with 26 and 20 years of burial, respectively, both corpses were found to be fully skeletonised. However, the bones of the last individual easily disintegrated when touched, indicating an advanced stage of decomposition.

In relation to the topsoil sample collection, in each exhumation different soil mass was collected (Table 6).

Table 6: Mass of the topsoil samples collected in all exhumations (CMA – Monte D'Arcos Cemetery; CPR – Prado do Repouso Cemetery; CCM – Castelo de Mértola Cemetery; g = grams).

Cemetery	Exhumation number	Mass of topsoil sample (g)
CMA	1	107.78
	2	172.31
	6	135.92
	7	104.43
	8	144.81
	9	111.61
	10	127.30
	12	118.72
CPR	5	112.30
	11	116.20
CCM	3	120.20
	4	110.77

Concerning soil's profile of each exhumation, photographs were taken in during exhumations (Figure 16).

Figure 16: Soil's profiles, collected using the 100 centimetre auger, from exhumations in Monte D'Arcos Cemetery (a-c) and from exhumations in Castelo de Mértola Cemetery (d and e).

Relatively to the grave inner soil sampling in each exhumation, as it was expected, the 25-centimetre length stainless steel auger allowed the collection of four soil

subsamples and their efficient separation. However, in some exhumations, due to the presence of many rocks within the soils, resistance was found while sampling, thus, the deepest samples were not able to be collected. Also, in the graves that had multiple buried coffins (exhumations number 6 and 8), sometimes the coffins were not separated by more than 25 cm of soil. For that reason, the auger would hit the coffin, and the deepest subsamples were also not able to be collected.

Table 7 shows the soil samples obtained under each corpse from exhumations number 7, 10, 12 and 3 indicating all the samples that were analysed during this work. The other obtained but not analysed grave inner soil samples are shown in the Attachment 4.

Table 7: Soil samples collected under the corpse during exhumations number 3, 7, 10 and 12 and their laboratory analysis state (cm = centimetres; CCM = Castelo de Mértola Cemetery; CMA = Monte D'Arcos Cemetery; H = head; T = thorax; A = Abdomen; G = gluteal region; RT = right thigh; W = wall; TG = top grave; RG = right grave; BG = bottom grave; LG = left grave).

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Sample collection	Laboratorial analysis
7	CMA	Head	0-5	7H5	Yes	Yes
			5-10	7H10	Yes	Yes
			10-15	7H15	Yes	No
			15-20	7H20	Yes	No
		Thorax	0-5	7T5	Yes	Yes
			5-10	7T10	Yes	Yes
			10-15	7T15	Yes	No
			15-20	7T20	No	No
		Abdomen	0-5	7A5	Yes	Yes
			5-10	7A10	Yes	Yes
			10-15	7A15	Yes	No
			15-20	7A20	Yes	No
		Gluteal Region	0-5	7G5	Yes	Yes
			5-10	7G10	Yes	Yes
			10-15	7G15	Yes	No
			15-20	7G20	Yes	No

(Table 7. Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Sample collection	Laboratorial analysis
		Right Thigh	0-5	7RT5	Yes	Yes
			5-10	7RT10	Yes	Yes
			10-15	7RT15	Yes	No
			15-20	7RT20	Yes	No
10	CMA	Head	0-5	10H5	Yes	Yes
			5-10	10H10	Yes	Yes
			10-15	10H15	Yes	No
			15-20	10H20	Yes	No
		Thorax	0-5	10T5	Yes	Yes
			5-10	10T10	Yes	Yes
			10-15	10T15	Yes	No
			15-20	10T20	Yes	No
		Abdomen	0-5	10A5	Yes	Yes
			5-10	10A10	Yes	Yes
			10-15	10A15	Yes	No
			15-20	10A20	Yes	No
		Gluteal Region	0-5	10G5	Yes	Yes
			5-10	10G10	Yes	Yes
			10-15	10G15	Yes	No
			15-20	10G20	Yes	No
		Right Thigh	0-5	10RT5	Yes	Yes
			5-10	10RT10	Yes	Yes
			10-15	10RT15	Yes	No
			15-20	10RT20	Yes	No
12	CMA	Head	0-5	12H5	Yes	Yes
			5-10	12H10	Yes	Yes
			10-15	12H15	Yes	No
			15-20	12H20	Yes	No
		Thorax	0-5	12T5	Yes	Yes
			5-10	12T10	Yes	Yes
			10-15	12T15	Yes	No
			15-20	12T20	Yes	No
		Abdomen	0-5	12A5	Yes	Yes
			5-10	12A10	Yes	Yes
			10-15	12A15	Yes	No
			15-20	12A20	Yes	No

(Table 7. Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Sample collection	Laboratorial analysis
		Gluteal Region	0-5	12G5	Yes	Yes
			5-10	12G10	Yes	Yes
			10-15	12G15	Yes	No
			15-20	12G20	Yes	No
		Right Thigh	0-5	12RT5	Yes	Yes
			5-10	12RT10	Yes	Yes
			10-15	12RT15	Yes	No
			15-20	12RT20	Yes	No
3	CCM	Head	0-5	3H5	Yes	Yes
			5-10	3H10	Yes	Yes
			10-15	3H15	No	No
			15-20	3H20	No	No
		Thorax	0-5	3T5	Yes	Yes
			5-10	3T10	Yes	Yes
			10-15	3T15	Yes	No
			15-20	3T20	Yes	No
		Abdomen	0-5	3A5	Yes	Yes
			5-10	3A10	Yes	Yes
			10-15	3A15	No	No
			15-20	3A20	No	No
		Gluteal Region	0-5	3G5	Yes	Yes
			5-10	3G10	Yes	Yes
			10-15	3G15	Yes	No
			15-20	3G20	No	No
		Right Thigh	0-5	3RT5	Yes	Yes
			5-10	3RT10	Yes	Yes
			10-15	3RT15	No	No
			15-20	3RT20	No	No

A total of 40 grave inner soil samples were laboratory analysed.

The collected, although not laboratory analysed control soil samples are presented in the Appendix 5. Also, control samples could had been collected from other places in the CMA, since there were more places with soil where no human corpses were

decomposing. However, in the CCM this is not possible. Moreover, it would be interesting to also collect samples using the 100 cm auger and analyse the soil profile from the control sites.

4.2 Laboratory analyses

Unfortunately, due to time restrictions caused by the Covid-19 pandemic, the results presented in this thesis are far from being the ones expected at the beginning of this ambitious project. However, it is still desired to leave a solid base and line of work for future researchers.

Regarding the inorganic characterisation and organic matter content analysis, results will be presented from exhumations number 7, 10, 12, 3 and 4. However, the organic constituent characterisation results will be only related to exhumations 7, 10, 12 and 3.

4.2.1 Grave topsoil samples

4.2.1.1 Inorganic characterisation and Organic matter content

The inorganic characterisation of the topsoil samples was performed analysing the soil's pH, colour and moisture content. The total organic matter content was also analysed (Table 8).

Table 8: Inorganic features (pH, Colour and Moisture content) and total organic matter content of the topsoil samples collected in graves from the Castelo de Mértola (CCM) and the Monte D'Arcos (CMA) cemeteries (% = percentage).

Exhumation number	Cemetery	pH	Colour		Moisture Content (%)	Total organic matter content (%)
			Dry state	Wet state		
7	**CMA**	5.47	10YR 6/3	10YR 4/4	21.02	2.75
10		4.09	10YR 8/4	10YR 5/6	12.27	1.25
12		5.70	10YR 5/3	10YR 3/3	18.09	3.52
3	**CCM**	7.79	10YR 5/3	10YR 3/3	11.21	4.09
4		7.22	10YR 6/4	10YR 4/4	11.84	3.56

Cemeteries are located in different country regions, therefore, their geological composition differs. Performing an inorganic characterisation of the topsoil samples of each grave allowed to perceive some of those differences.

Topsoil samples from the CMA are considered acidic in the pH scale, being the most acidic soil the one collected in the exhumation 10. In acidic soils, the fungal community predominates, whilst neutral soils shown a predomination of the bacterial community (Haslam and Tibbett 2009). pH values of the topsoil samples from exhumations 3 and 4 (CCM), in the Southern region, are like each other and closer to neutral pH values, but higher than the pH values from the samples collected in Braga (CMA), in the Northern Region. This difference in the pH of the soils can be explained due to differences in mineral content and pH values of the residual waters that are in contact with the graves.

The evaluation of soil colour showed that all topsoil samples share the same value of yellow/red tones in the different humidity states (dry and wet – 10YR). However, the topsoil samples differ slightly from each other in terms of darkness (the first value after 10YR) mainly when comparing states of humidity. Higher darkness values mean less darker samples, being normally associated with less humidity and less organic matter content. Given this, all dry samples presented lesser darkness. Concerning brightness (the second value after 10YR), lower values mean weaker colours, however, dry and wet samples presented relatively similar brightness. Despite the slightly differences showed in Table 8, colour is considered quite similar in both cemeteries, in each state of humidity.

Regarding the moisture content it is possible to conclude that the humidity in the topsoil samples from exhumations 3 and 4 is similar but lower than in the topsoil samples from the CMA. This goes into accordance with the geographical context of the cemeteries given that Mértola is warmer than Braga. The sample collected from exhumation 7 presented the highest value of moisture content, however, it was raining on the day the samples were collected which could help understand this value. Also, exhumation 10 presented the lower value which could be explained by the fact that on the sampling day the weather temperature at the time of the collection (22ºC) was higher than on the other two collections from CMA (7 and 12, both collected with the average weather temperature of 16ºC).

The total organic matter evaluation showed that the topsoil samples from the CCM have the highest organic matter content levels, followed by exhumations 12, 7 and 10, respectively. The association between the organic matter content and the value of darkness of a soil sample is proved since samples with more organic matter content are, by order, 3 > 4 > 12 > 7 > 10. The samples with higher darkness values are 3/12 > 4/7 > 10. Comparing these two properties is possible to see that the only value that is not in

accordance to what was stated before (darker soil colour is associated with more organic matter) is the one from exhumation 4.

4.2.2. Grave inner soil samples

The compounds extracted from the samples collected within the burial graves were analysed using Gas Chromatography Coupled with Mass Spectroscopy (GC-MS), to be identified and quantified. Unfortunately, to the date of the submission of this thesis, only qualitative work was able to be performed due to the restricted time due to COVID. However, quantification is still yet to be carried out. Besides this setback, and as written before, also the number of exhumations presented in this organic characterisation chapter is lower than what was intended. Herein, will be presented a comparison between the organic results obtained from the identification of fatty acids and steroids in exhumations 7, 10 and 12. Given that these exhumations were collected in CMA, a comparison and discussion between different types of soils is not going to be carried out as it was intended. However, the results of the sterols, stanones and stanols fraction (ALC) from exhumation 3 will be presented.

During the laboratory procedure to analyse fatty acids and steroids, sample analysis had to be repeated and some steps of the protocol had to be optimised. This did not allow the identification of any individual compound in the first set of analyses. It was realised that the used split ratio (1:5) needed to be lower since the samples appeared to be too diluted. Given this change required, samples were reanalysed using the split ratio 1:1. Additionally, in the CMA, the initial sample mass (0.5 g) used to perform the total lipid extract (TLE) was increased (1 g) since not enough lipids were being extracted. In the samples collected from the CCM, the used mass was always 2 g since it was expected to be more difficult to extract compounds from this soil which presented more elements that could made difficult the compound retention, such as rock fragments and bricks.

4.2.1. Identification of Fatty Acids

The first analysis of the Fatty Acid Methyl Ester (FAME) fraction of exhumation 7 had to be repeated. A modification in the GC-MS method was required since the solvent delay was to high (20 min). Given this, the solvent delay was modified to 10 min, to allow the detection of fatty acids.

A sample with the fatty acid internal standard - heptadecanoic acid (C17), was also analysed to enable the comparison of this peak, and its retention time, to the remain

peaks in the grave inner soil samples and identify the internal standard to allow quantification (Figure 17).

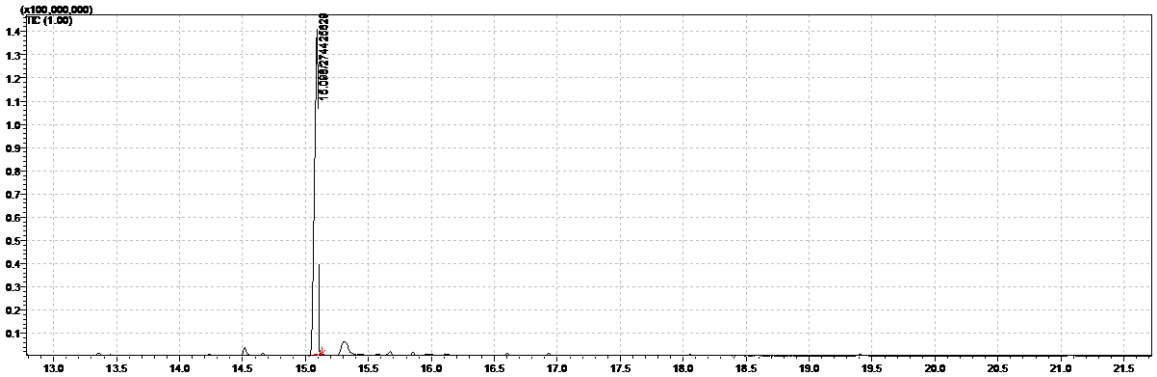

Figure 17: Heptadecanoic acid (C17 - fatty acid internal standard) gas chromatogram coupled with mass spectroscopy.

The heptadecanoic acid was identified with a retention time of 15.098 minutes.

After running the grave inner soil samples, a post-run analysis was performed. For this, similar compounds were searched in the GC-MS software. This similarity search is performed by comparing spectra in the organic compound libraries of the GC-MS. However, this identification method is not usually used since most of the times the known compounds do not present a high similarity percentage with the unknown compounds, leading to mistakes in the identification. Also, GC-MS libraries are limited, meaning that some compounds are not contemplated within them and, for that reason, "no hit" compounds may occur. For the identification of fatty acids in this work, a similarity percentage above of 80% was required to guarantee an accurate identification. Then, mass spectra from lipid library were extracted and compared to the mass spectra obtained from the analysis performed in the soil samples to ensure, even more, an accurate identification.

Regarding the FAME fraction of the grave inner soil samples collected from the exhumation 7, it was possible to verify differences in the compounds present at 0-5 cm and 5-10 cm of soil. Also, it was verified an inter and intra-exhumation variation, meaning that the compounds that were found vary not only from exhumation to exhumation but also depending on the soil provenance under the same corpse (Table 9).

All soil samples collected under the corpse of the exhumation number 7 contained fatty acid. Generally, the most abundant compound is palmitic acid trimethylsilyl ester, which appeared in all samples except in the sample collected under the abdomen at 5-10 cm soil depth, corresponding to 40.1% of the total amount of fatty acids in the grave inner

soil samples from the exhumation 7. The second most abundant compound is stearic acid trimethylsilyl ester corresponding to 14.2% of the total amount of fatty acids (14.2%).

Table 9*:* Fatty acids found within the different grave inner soil samples from exhumation 7 (Monte D'Arcos Cemetery), their retention time, percentage of their peak's area, and their percentage in the total fatty acid content of the grave (min = minutes ; % = percentage; H5 – head at 0-5 cm; H10 – head at 5-10 cm; T5 – thorax at 0-5 cm; T10 – thorax at 5-10 cm; A5 – abdomen at 0-5 cm; A10 – abdomen at 5-10 cm; G5 – gluteal region at 0-5 cm; G10 – gluteal region at 5-10 cm; RT5 – right thigh at 0-5 cm; RT10 – right thigh at 5-10 cm).

Fatty acid	Retention time (min)	Identification in the chromatograms	Sample labelling	Peak's area percentage (%)	Compound's percentage in the total fatty acid content (%)
Myristic acid methyl ester	13.4	A	H5	1.47	9.90
			G10	5.67	
Palmitic acid trimethylsilyl ester	15.3	B	H5	3.91	40.1
			H10	1.99	
			T5	2.6	
			T10	0.95	
			A5	6.48	
			G5	1.62	
			G10	5.67	
			RT5	2.08	
			RT10	3.54	
Oleic acid methyl ester	15.7	C	H5	2.6	10.0
			G10	4.57	
Stearic acid trimethylsilyl ester	16.8	D	H5	2.42	14.2
			G10	3.49	
			A5	3.04	
			RT5	1.24	
Oleic acid tetradecyl ester	25.4	E	H5	2.63	12.6
			H10	1.00	
			T5	3.72	
			A10	1.72	

The chromatograms obtained in the GC-MS analysis the FAME fraction of soil samples H5 and H10 (Figure 18), T5 and T10 (Figure 19), A5 and A10 (Figure 20), G5 and G10 (Figure 21) and RT5 and RT10 (Figure 22) are presented below.

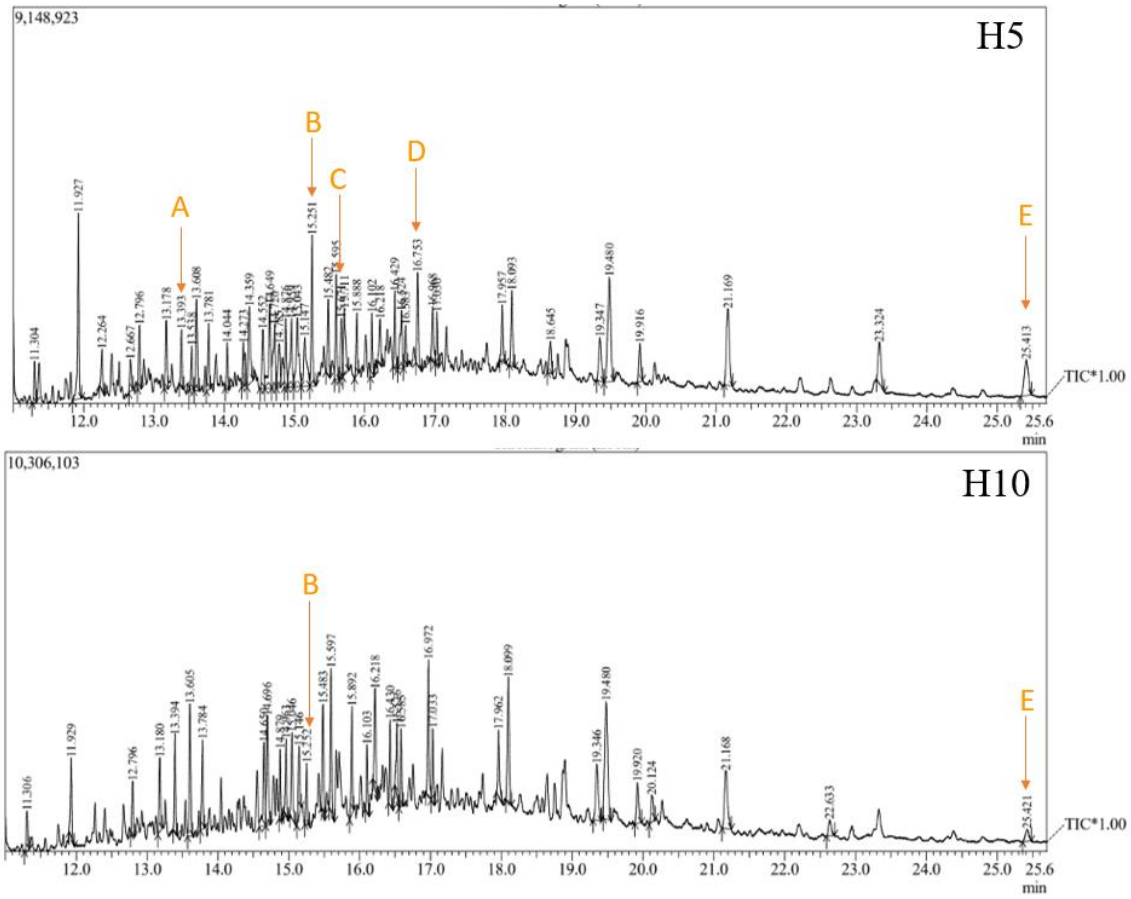

Figure 18: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the head of the individual from the exhumation 7 (Monte D'Arcos Cemetery), at 0-5 cm (H5) and 5-10 cm (H10) soil depth.

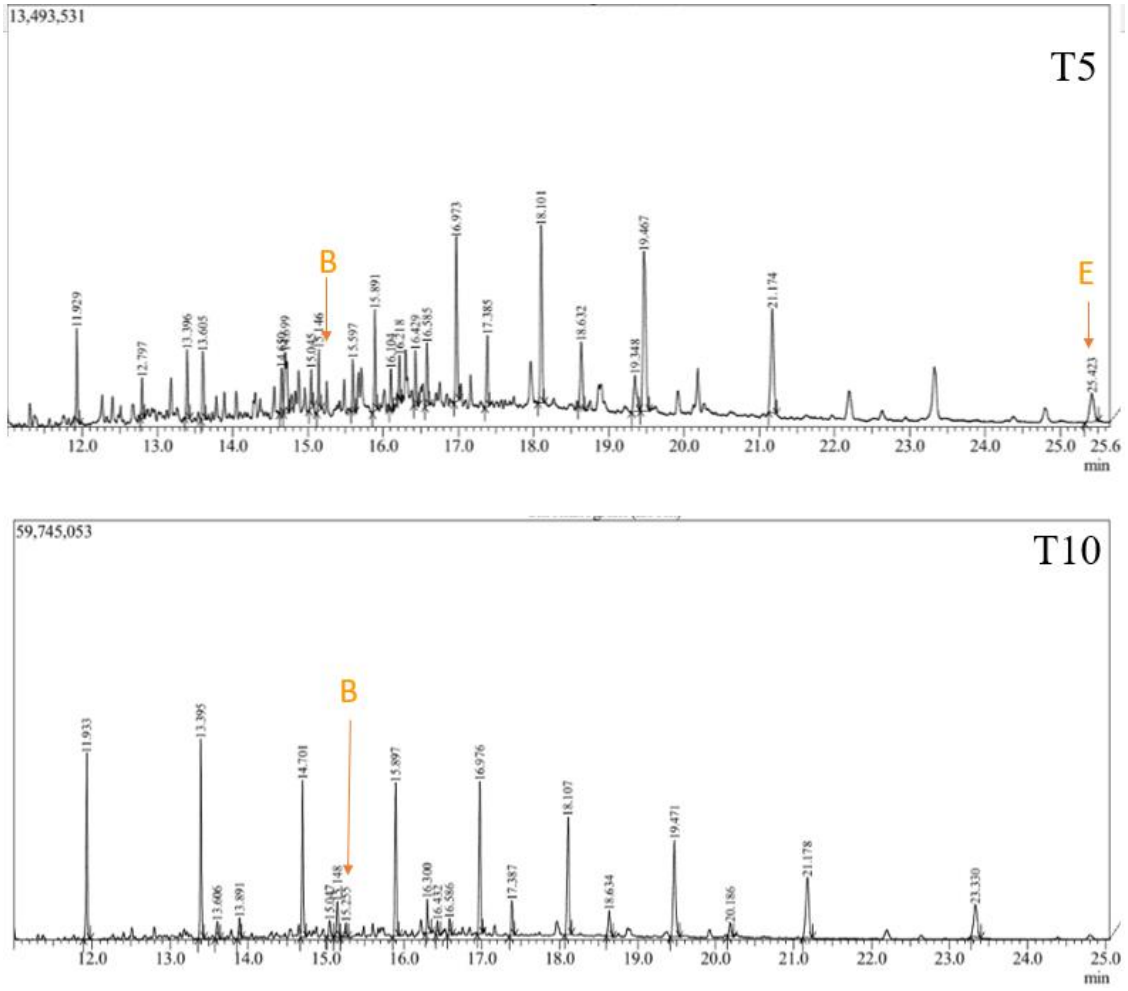

Figure 19: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the thorax of the individual from the exhumation 7 (Monte D'Arcos Cemetery), at 0-5 cm (T5) and 5-10 cm (T10) soil depth.

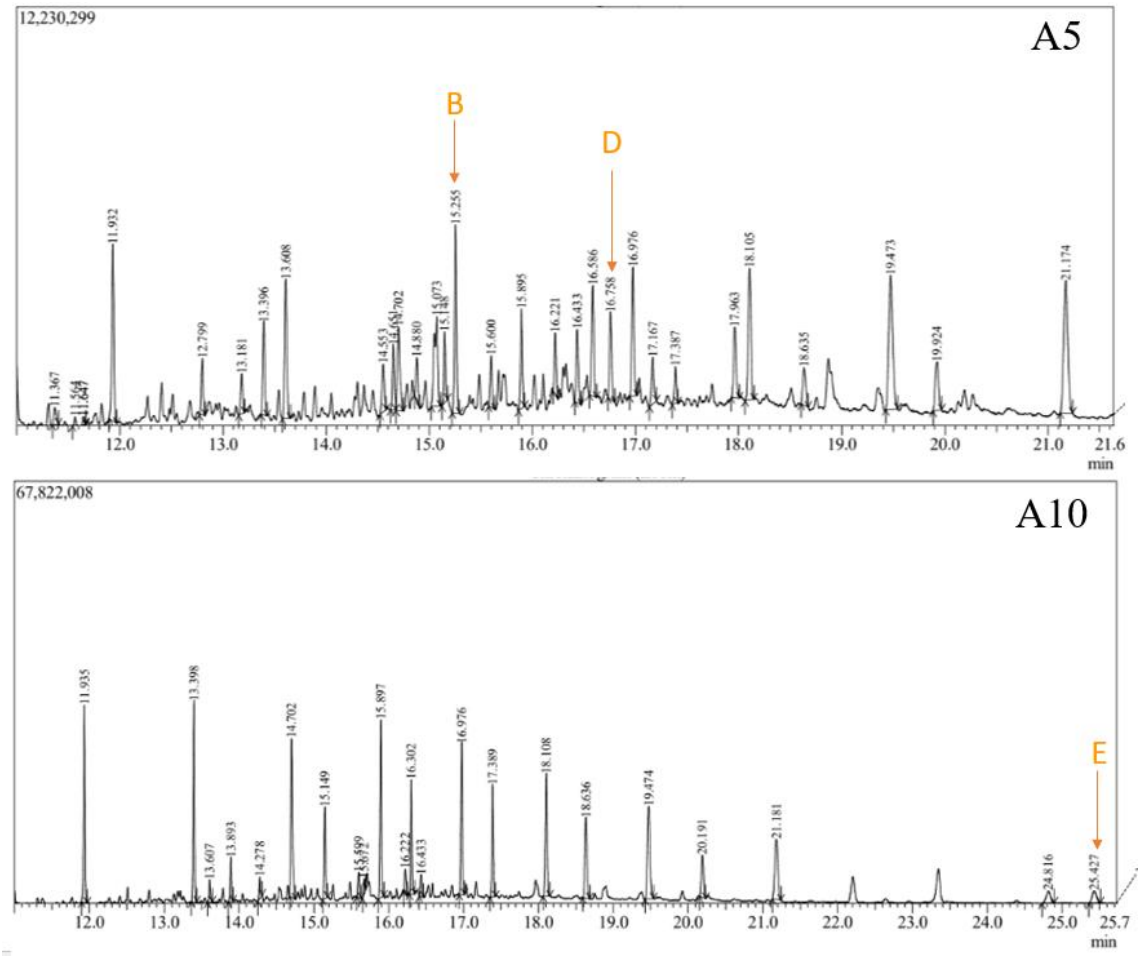

Figure 20: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the abdomen of the individual from the exhumation 7 (Monte D'Arcos Cemetery), at 0-5 cm (A5) and 5-10 cm (A10) soil depth.

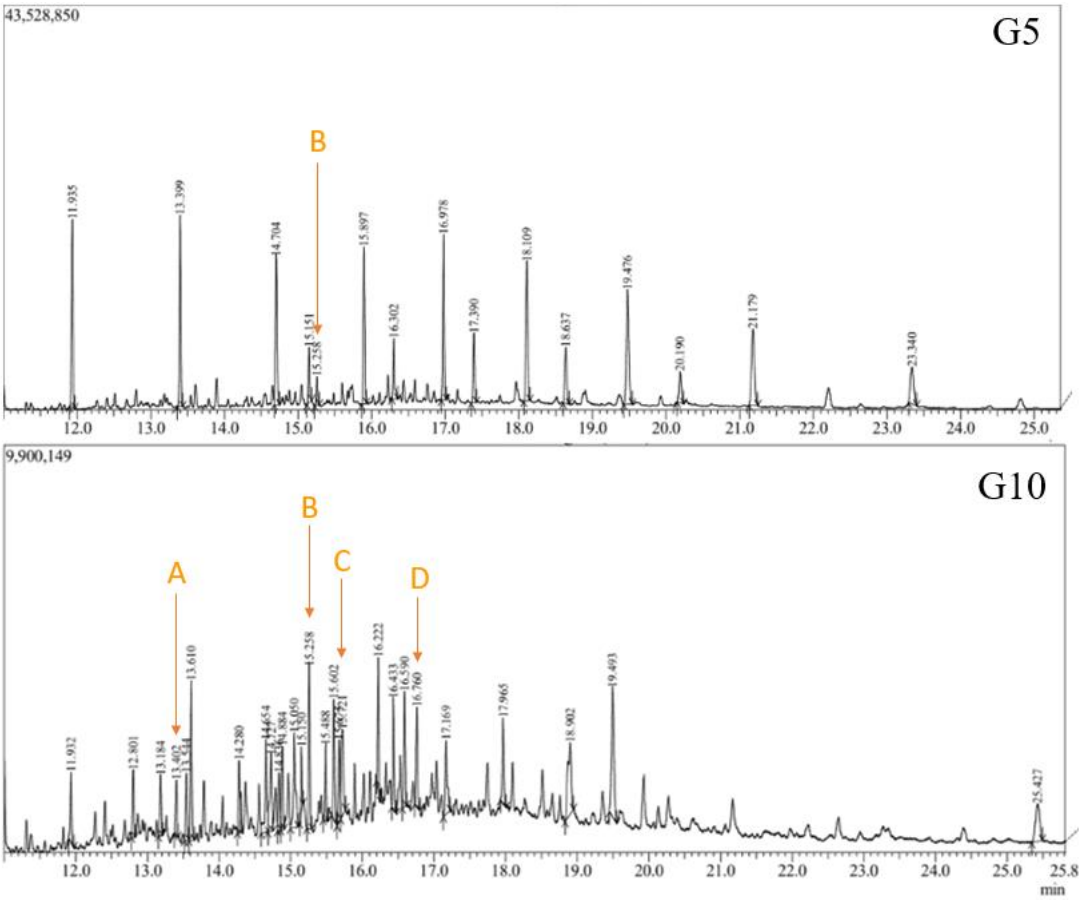

Figure 21: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the gluteal region of the individual from the exhumation 7 (Monte D'Arcos Cemetery), at 0-5 cm (G5) and 5-10 cm (G10) soil depth.

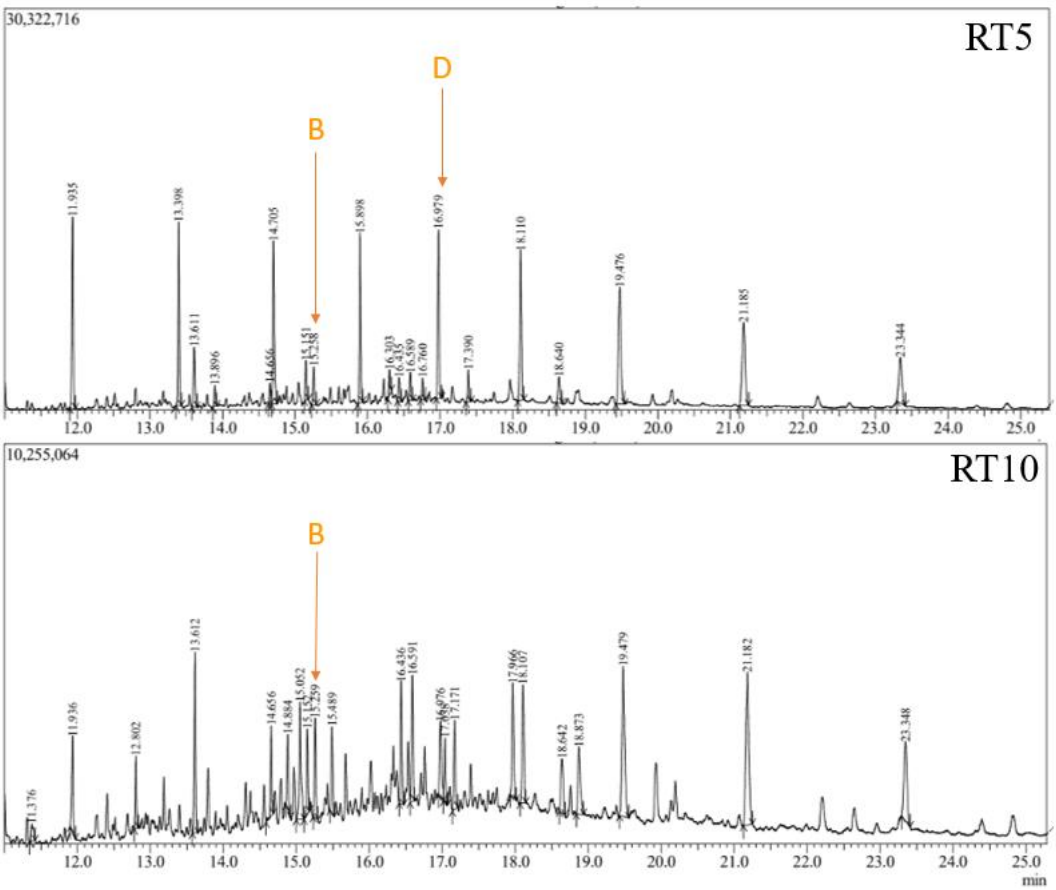

Figure 22: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the right thigh of the individual from the exhumation 7 (Monte D'Arcos Cemetery), at 0-5 cm (RT5) and 5-10 cm (RT10) soil depth.

Table 10 presents the obtained FAME fraction of the grave inner soil samples collected from the exhumation 10.

Table 10*:* Fatty acids found in the samples collected from internal grave soil of exhumation 10, its retention time (min = minutes), the percentage of the peak's area (%) and the total of each fatty acid content in the sample (H5 – head at 0-5 cm; H10 – head at 5-10 cm; T5 – thorax at 0-5 cm; T10 – thorax at 5-10 cm; A5 – abdomen at 0-5 cm; A10 – abdomen at 5-10 cm; G5 – gluteal region at 0-5 cm; G10 – gluteal region at 5-10 cm; RT5 – right thigh at 0-5 cm; RT10 – right thigh at 5-10 cm).

Fatty acid	Retention time (min)	Identification in the chromatograms	Sample labelling	Peak's area percentage (%)	Compound's percentage in the total fatty acid content (%)
Palmitic acid ethyl ester	14.8	F	T5	0.95	11.8
			A5	0.69	
			RT10	1.91	
Palmitic acid trimethylsilyl ester	15.2	B	G5	1.03	3.43
Oleic acid methyl ester	15.7	C	H5	1.04	36.8
			H10	2.58	
			T5	1.08	
			T10	0.47	
			A5	0.33	
			A10	0.31	
			G5	0.83	
			G10	2.46	
			RT5	1.54	
			RT10	1.94	

Similarly to the results of the exhumation 7, all soil samples exhibit fatty acids. Moreover, the most abundant fatty acid in the samples is Oleic acid methyl ester (36.8% of the total amount of fatty acids in the grave inner soil samples), being present in all samples, followed by palmitic acid ethyl and trimethylsilyl esters (3.43 and 36.8 % of the total amount of fatty acids, respectively). Docosanoic acid (also referred as Behenic acid) ethyl ester, was found in all soil samples excepting the samples from thorax and abdomen at 0-5 cm soil depth and the sample from the right thigh at 5-10 cm soil depth. However, this fatty acid has a role in the metabolism of plants, having no direct connection to the human decomposition process. This compound could be originated from the flowers present within the grave or in the vegetation in the cemetery. Reference samples would had been importance to clarify.

The chromatograms obtained in the GC-MS from the analysis of the FAME fraction of the soil samples collected in exhumation 10 from H5 and H10 (Figure 23), T5 and T10 (Figure 24), A5 and A10 (Figure 25), G5 and G10 (Figure 26) and RT5 and RT10 (Figure 27) are presented below.

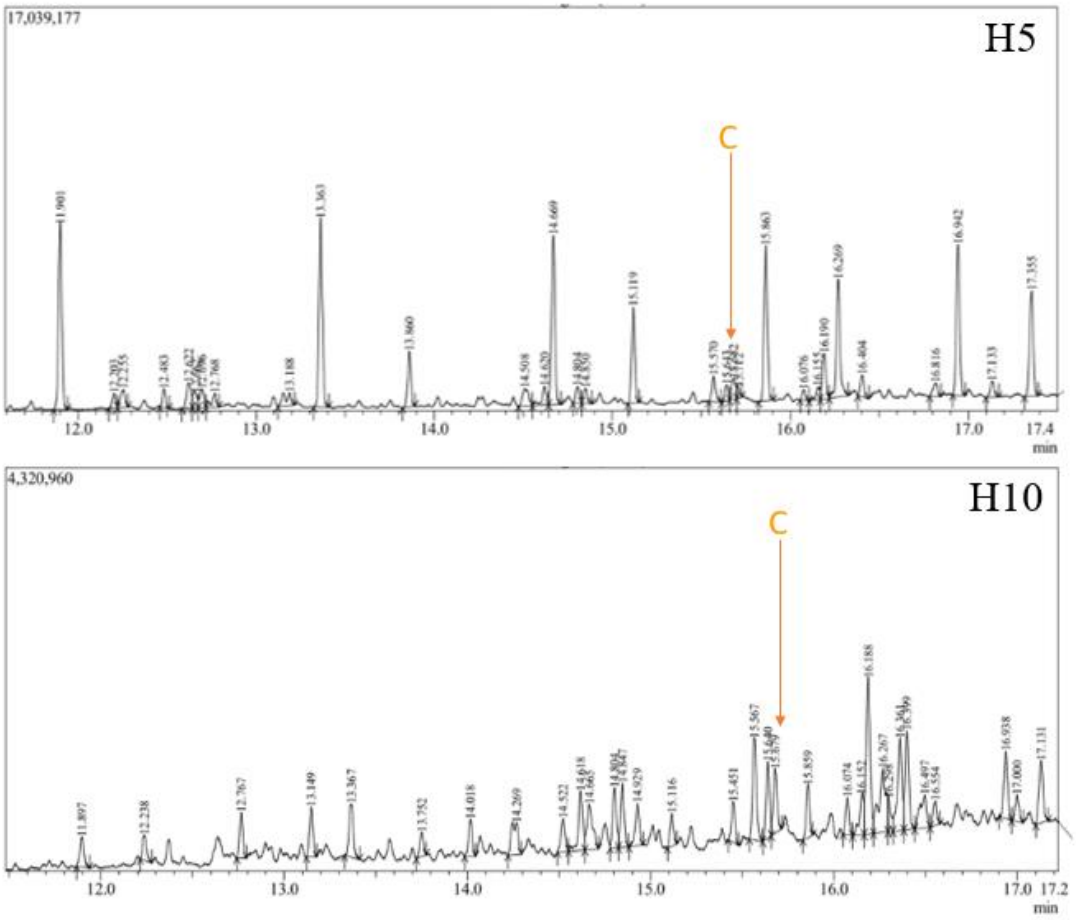

Figure 23: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the head of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (H5) and 5-10 cm (H10) soil depth.

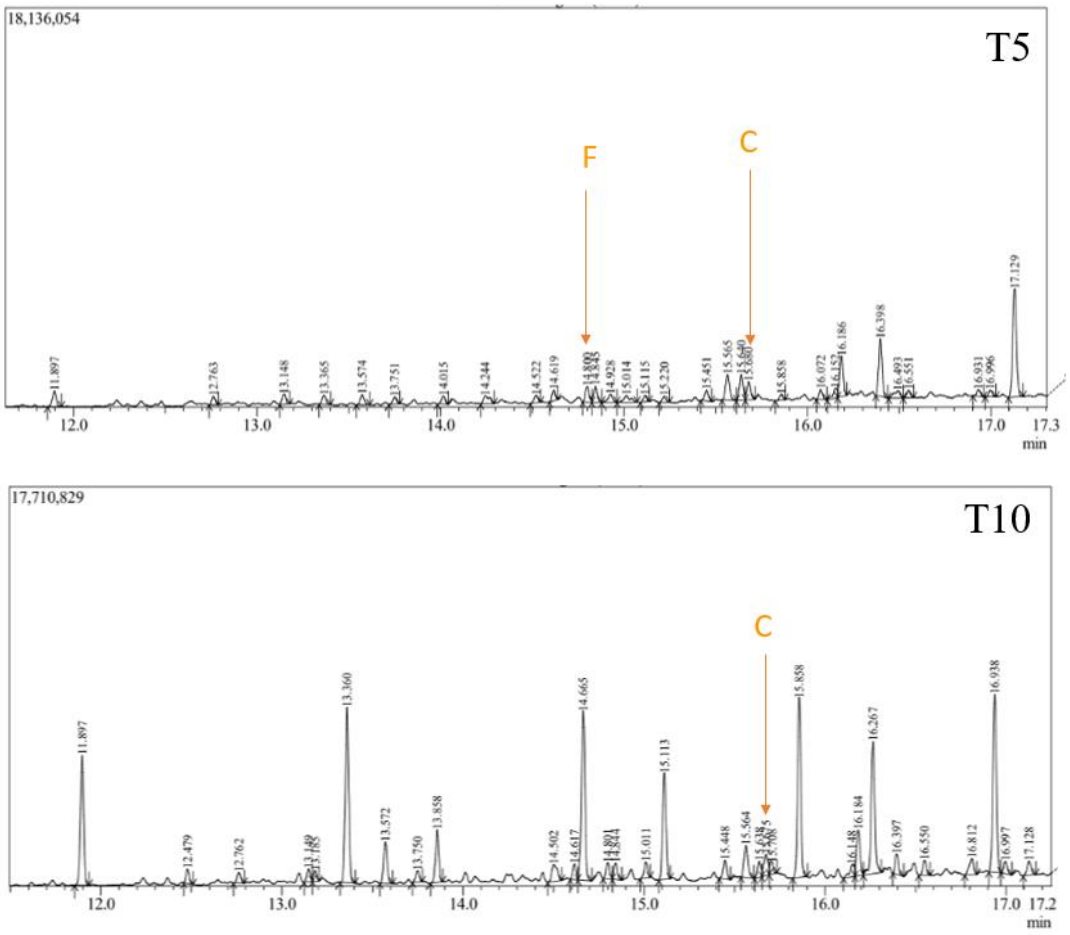

Figure 24: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the thorax of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (T5) and 5-10 cm (T10) soil depth.

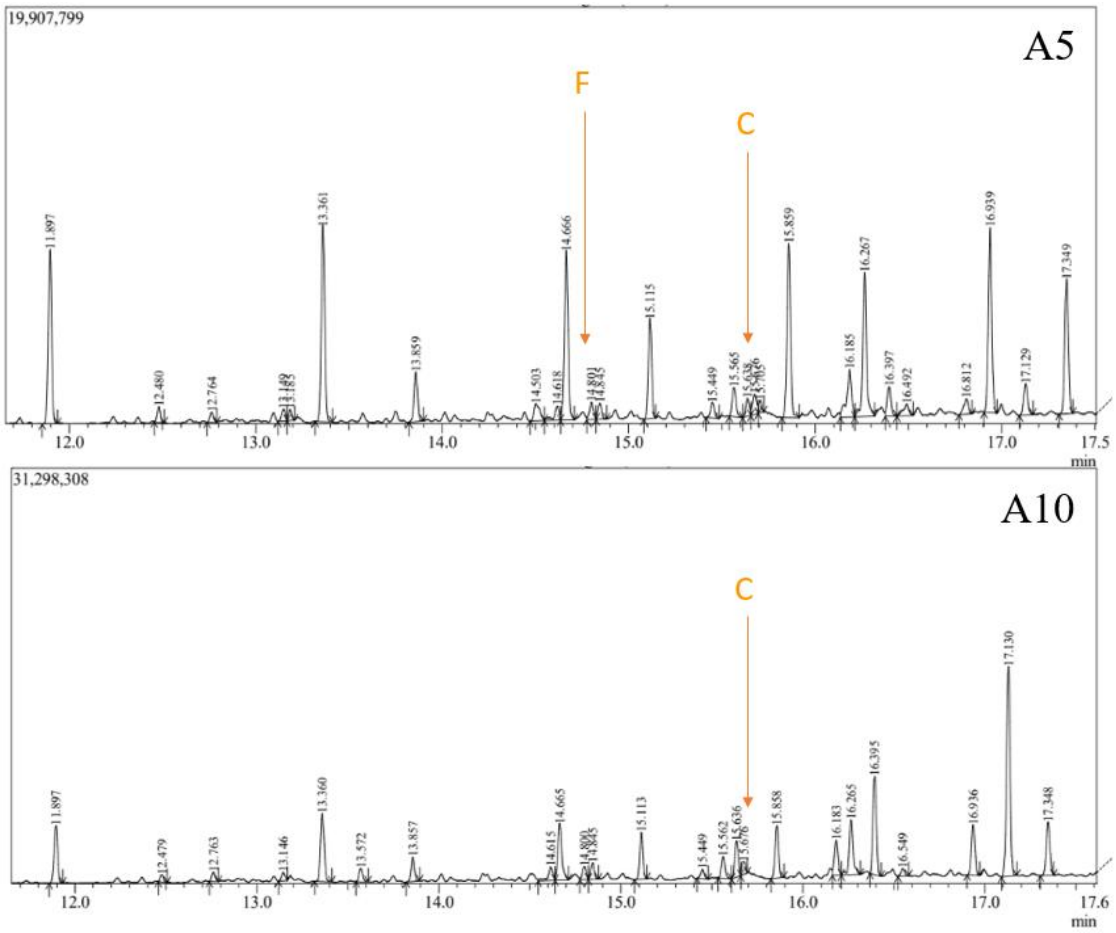

Figure 25: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the abdomen of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (A5) and 5-10 cm (A10) soil depth.

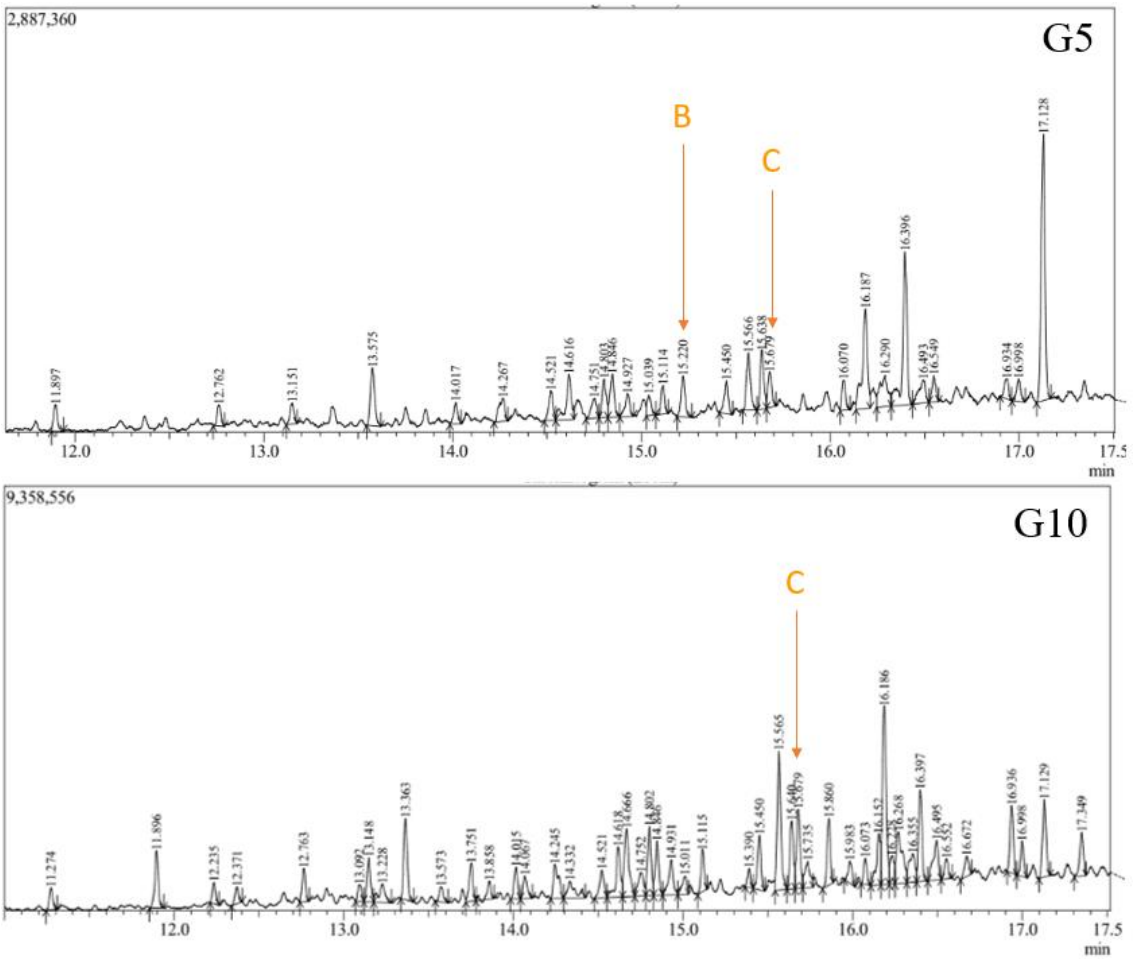

Figure 26: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the gluteal region of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (G5) and 5-10 cm (G10) soil depth.

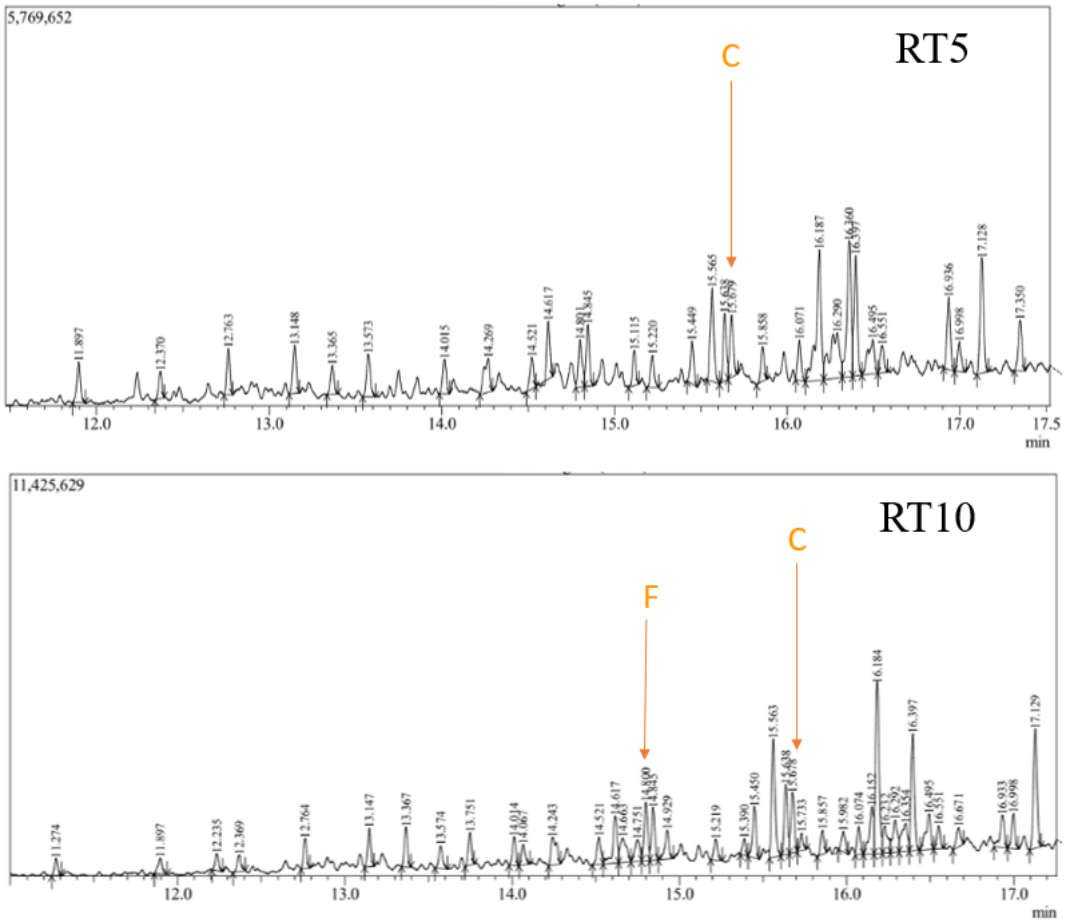

Figure 27: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the right thigh of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (RT5) and 5-10 cm (RT10) soil depth.

Regarding the analysis of the FAME fraction of the grave inner soil samples collected from the exhumation 12, results are presented in Table 11.

Table 11: Fatty acids found in the samples collected from internal grave soil of exhumation 12, its retention time (min = minutes), the percentage of the peak's area (%) and the total of each fatty acid content in the sample (H5 – head at 0-5 cm; H10 – head at 5-10 cm; T5 – thorax at 0-5 cm; T10 – thorax at 5-10 cm).

Fatty acid	Retention time (min)	Identification in the chromatograms	Sample labelling	Peak's area percentage (%)	Compound's percentage in the total fatty acid content (%)
Myristic acid trimethylsilyl ester	13.6	G	H5	3.37	6.78
			H10	3.73	
			T5	3.74	
			T10	5.25	
Palmitic acid trimethylsilyl ester	15.3	B	H5	15.6	27.6
			H10	19.7	
			T5	4.67	
			T10	25.5	
Oleic acid trimethylsilyl ester	16.6	H	H5	15.3	27.6
			H10	26.5	
			T5	4.38	
			T10	19.2	
Stearic acid trimethylsilyl ester	16.8	D	H5	16.3	35.8
			H10	21.1	
			T5	20.9	
			T10	26.7	

Contrary to what has been related so far, in the grave inner soil samples from the exhumation 12, fatty acids were identified only in soil samples collected under the head and thorax. The most abundant fatty acid is stearic acid (35.8% of the total amount of fatty acids in the grave inner soil samples), present in both samples collected under the head and thorax, at 0-5 cm and 5-10 cm soil depth, being followed by Palmitic acid and Oleic acid (27.6 % of the total amount of fatty acids, both). These three compounds plus myristic acid were identified in all the four mentioned soil samples (H5, H10, T5, T10).

The chromatograms obtained in the GC-MS from the analysis of the FAME fraction of the soil samples collected in the exhumation 12 from H5 and H10 (Figure 28), and T5 and T10 (Figure 29) are presented below. The remain chromatograms, from A5 and A10, G5 and G10 and RT5 and RT10 are presented in Appendix 6 (6.1, 6.2 and 6.3, respectively), since no relevant compounds were detected in these samples.

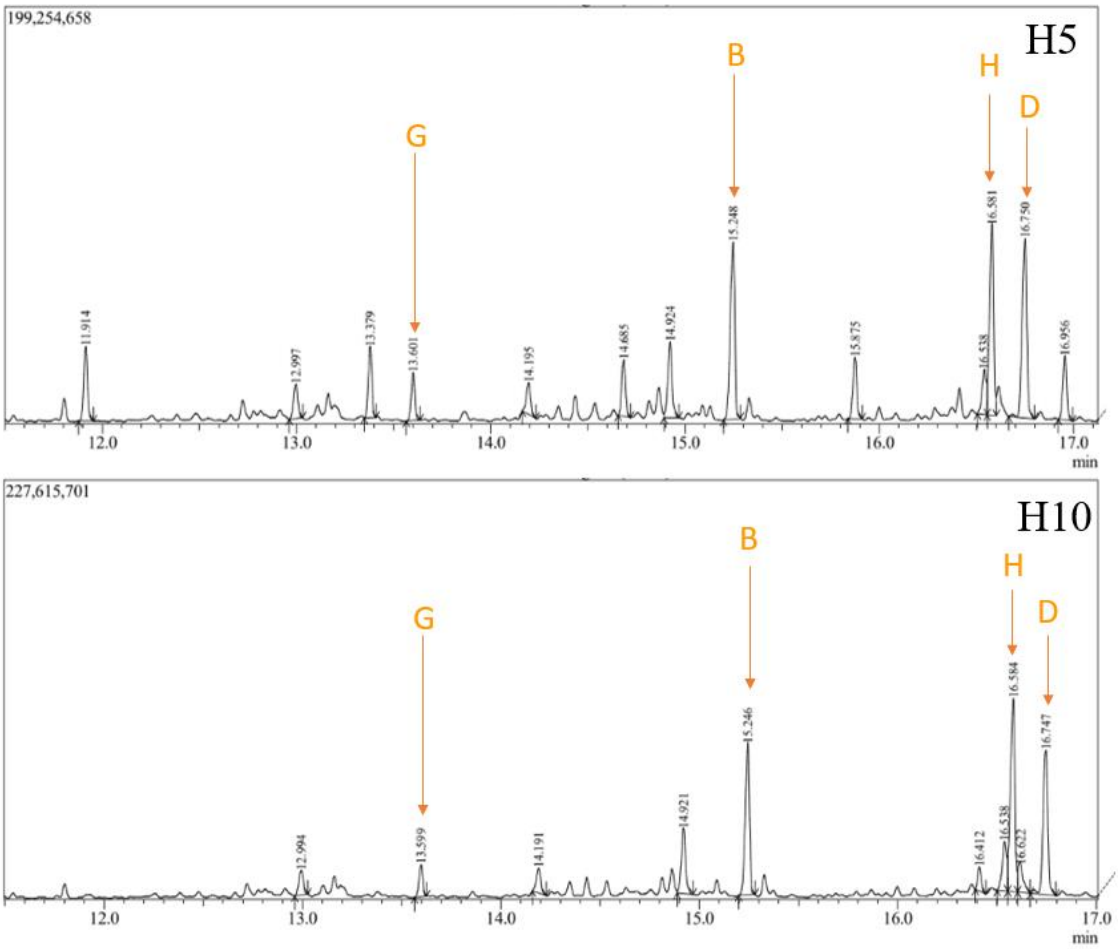

Figure 28: Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the head of the individual from the exhumation 12 (Monte D'Arcos Cemetery), at 0-5 cm (H5) and 5-10 cm (H10) soil depth.

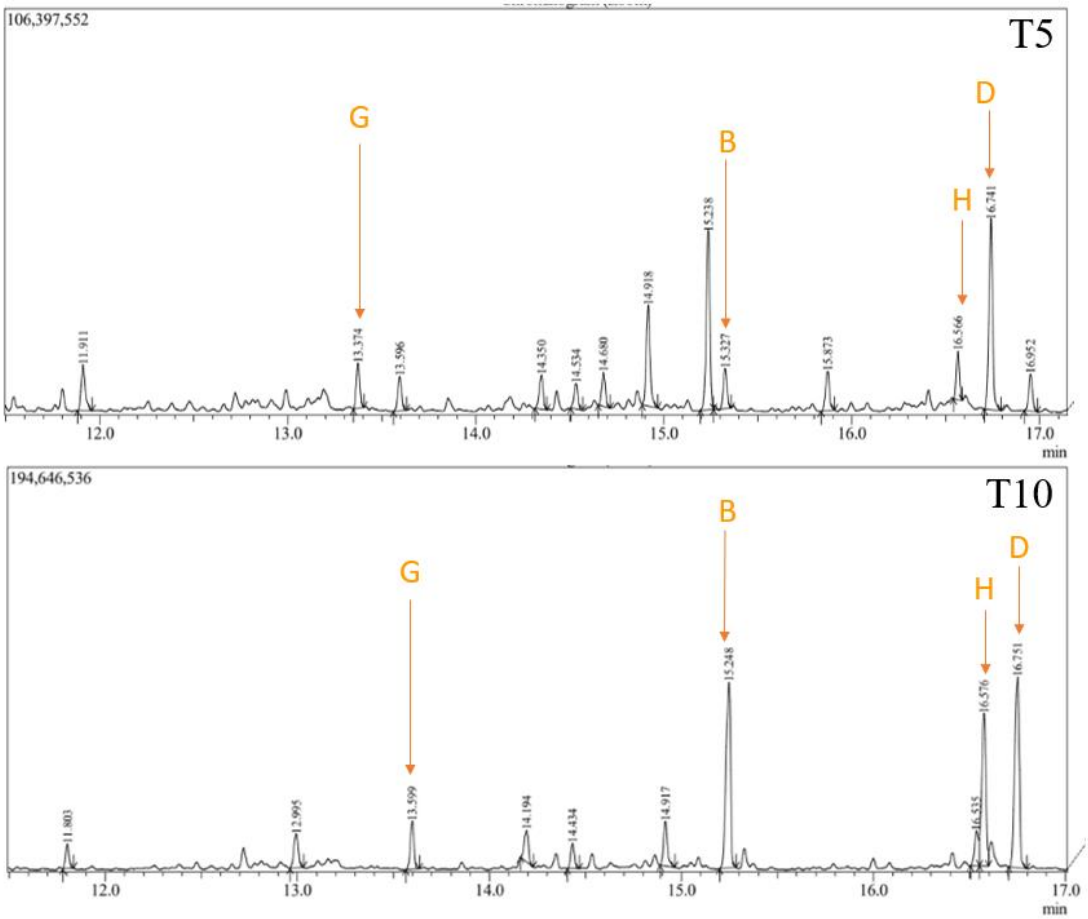

Figure 29*:* Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the thorax of the individual from the exhumation 12 (Monte D'Arcos Cemetery), at 0-5 cm (T5) and 5-10 cm (T10) soil depth.

In all the analysed soil samples from the Monte D'Arcos Cemetery it was expected to detect the internal standard heptadecanoic acid, since it was added to the samples in the second step of the laboratorial procedure. However, this did not happen, probably meaning that the sample is being lost during the process, maybe during the solid phase extraction. Without the detection of the heptadecanoic acid, is not possible to quantify the compounds.

In addition to the fatty acids, other compounds were present in the soil inner grave samples collected from these 3 exhumations. Most of the compounds, roughly identified (*e.g.*, silanes), could have had origin in the associated material related to burial. Others compounds (*e.g*, Phthalic Acid), could have had an origin the contamination within the grave, particularly derived from plastics which had been used to wrap grave flowers.

The most abundant fatty acid in the exhumation 7 was the palmitic acid which goes into accordance with the literature that states this compound as being one of the major constituents of the decomposition fluids (Larizza 2010). However, in the exhumation 10 the most abundant fatty acid was the oleic acid. This finding also makes sense since this compound is found in high quantities in the adipose tissue (Pfeiffe, Milne and Stevenson 1998). In the exhumation 12 the most abundant compounds were the palmitic, oleic and also the stearic acid which is also stated by Larizza in 2010. The latter had the biggest percentage which means it was present in more samples. However, it cannot be ascertained quantity results given the quantification of the fatty acids was not performed. For that reason, the fatty acids found could be present in more samples, although not being the one in bigger quantity in the grave inner soil samples. In the exhumation 7, samples under the head and the gluteal region had more identified fatty acids. Regarding the differences between soil depths (0-5 cm and 5-10 cm), it is observable that, generally, deeper samples have more concentration of compounds. Also, excepting the samples collected under the gluteal region of exhumation 7, the soil samples collected between 0-5 cm presented more fatty acids. In exhumation 10, this was also observable but lesser fatty acids were identified in these grave samples. About exhumation 12, it is not possible to conclude regarding the depth of the samples since the fatty acids were identified in samples collected under the head and thorax at both depths.

The different fatty acids that were found (the methyl esters: myristic and oleic; the trimethylsilyl esters: palmitic, stearic, myristic and oleic; the ethyl ester: palmitic; the tetradecyl ester: oleic) can also be associated with the different patterns of the decomposition, since individuals from exhumations 7 and 12 had the same time of burial (7 years) but did present different patterns of decomposition (individual from exhumation 7 had the back and gluteal region in putrefaction and individual 12 was fully skeletonised). Exhumation 10 occurred 5 years after the death of the individual (lower time since death) and the back region of the thorax mummified and putrefied. This could be explained by the inorganic soil differences. However, in this both exhumations, inorganic results were not very different. The inorganic results obtained, showed that pH was higher in the topsoil sample collected from exhumation 12 which also had higher organic matter content. Individual 10, showing also differences in the analysed inorganic soil parameters (lower pH and lower moisture content), and a lower percentage of total organic matter content. Individual 7 had the higher moisture content, however the accuracy of this value

is compromised given that it was heavily raining at the day of the collection. Looking at the putrefied regions in the corpses (back and gluteal region from individual 7 and thorax from the individual 10, it is possible to see that the fatty acids found closest to the body (which is 0-5 cm) were, in the case of the gluteal region of the individual 7, palmitic acid trimethylsilyl ester, being the only fatty acid found in gluteal region of individual 7, at 0-5 cm. The palmitic acid is the most abundant fatty acid present in decomposition fluids (Larizza 2010) and knowing it was present in all the remain samples from exhumation 7 and that G5 only presented this compound, is possible to conclude that the remain fatty acids found in the grave may be associated to advanced decays stage of decomposition, given that the rest of the corpse was skeletonised. Moreover, the coffin in this exhumation was not degraded leaving the thought the decomposition process could be happening at a slower rate that what was expected. Also, it could be due to the coffin material which information is not available. Given the thorax region, on exhumation 10, in the T5 sample (collected under the thorax at 0-5 cm), palmitic acid ethyl ester and oleic acid methyl ester were found. However, these compounds were also identified in other samples collected from different parts of the corpse making difficult to conclude about its identification and the state the body was. However, it is important to note that individual from exhumation 10 was wearing a diaper (nappy) which could retains the gut contents and delay the decomposition process, being this a possible explanation for the different pattern of decomposition from this individual. One interesting aspects is that, in exhumation 12 the individual was also wearing a diaper, and the grave was filled with plastic wrap material, and the time of burial of 7 years and the body was found to be fully skeletonised. There was not observed any signs of putrefied or mummified region in the corpse leaning to the idea that the decomposition process occurred faster than the other two exhumations (7 and 10). This individual was known to have died from lung cancer but, according to the relatives, he was not subjected to any chemotherapy treatment. This known cause of death could explain why the body decomposed faster than the others. The trauma on the thorax region (lung) might have attracted insects which helped speed up the decomposition process. Cemetery employees said that according to their experience, people who died of cancer usually decompose faster. However, not literature was found to back this up. Moreover, it would be interesting to better understand the association between cancer (and chemotherapy) and the decomposition process. For this, soil from the graves where individuals that died from cancer are decomposing would have to be inorganic and

organically characterised. This may be hard to achieve given that in most cases the cemeteries employees do not have such information in relation to the cause of death.

Furthermore, in these 3 exhumations common compounds, palmitic acid and oleic acid, were identified. Given their identification in soil samples from different individuals which had different decomposition patterns, it seems possible to say that these two compounds are the most promising to be used as indicators of decomposition. However, further investigation is required. Seems to be inaccurate to relate the results obtained to the individual variation factors such as body size or sex, given that the number of corpses analysed was low. For that reason and knowing the impact that these intrinsic factors have in the decomposition process, it is important and advised to increase the number of bodies examined to be able to better conclude about the effect of these factors.

4.2.2. Identification of Sterols, Stanols and Stanones

When soil samples were analysed using GC-MS, a sterols, stanols and stanones standard mix (SSS mix) sample was also analysed. This was done to allow the comparison between chromatograms and mass spectra to identify the sterols, stanols and stanones of the soil samples.

To perform the identification of the chromatogram peaks, characteristic ions were used (Table 12).

Table 12: Compounds expected to be present in the sterols, stanols and stanones standard mix (SSS mix) and their qualifier and quantifier ions (m/z = mass-to-charge ratio).

COMPOUND	QUALIFIER ION (m/z)	QUANTIFIER ION (m/z)
Pregnanol	271, 286	361
Pregananone	215	300
5α-cholestane	357, 372	217
Coprostanol	355, 215	370
Epicholestanol	355, 370	215
Epicoprostanol	355, 370	215
Coprostanone	316, 386	231
d7-cholesterol	375, 465	336
Cholesterol	368, 458	329
5α-cholestanol	355, 445	215
Cholestanone	386	231
5β-stigmastanol	398, 383	215
Epi-5β-stigmastanol	398, 383	215
Sigmasterol	394, 484	255
β-sitosterol	396, 486	129
5α-stigmastanol	383, 473	215

First, the mass spectrum of each peak was analysed by trying to detect the reference ions. The compounds present in the previous table are aligned with the increasing of their retention time. For example, in cases where the mass spectrum shows the presence of ions 215, 355 and 370 m/z, it could either be epicholestanol or epicoprostanol. Notwithstanding, the one with the lowest retention time would be epicholestanol (appears first in the table) and the other with the highest retention time would be epicoprostanol (appears secondly).

Since four ALC fractions (sterols, stanols and stanones) from grave inner soil samples were analysed (3 from Braga – exhumation 7, 10 and 12 -, and 1 from Mértola – exhumation 3), four chromatograms of SSS mix analysed with the set of samples of each exhumation are presented.

In the SSS mix analysed with the grave inner soil samples of the exhumation 7 (Figure 30), it was not possible to identify the peak at 23.837 min since the ions did not match to the reference ones. At 22.930 min, pregnanone was identified; coprostanol at 30.577 min; epicholestanol at 30.767 min; cholesterol at 33.297 min; 5β-stigmastanol at

36.537 min; epi-5β-stigmastanol at 36.697 min; stigmasterol at 37.903 min; β-sitosterol at 39.507 min, and 5α-stigmastanol at 39.767 min.

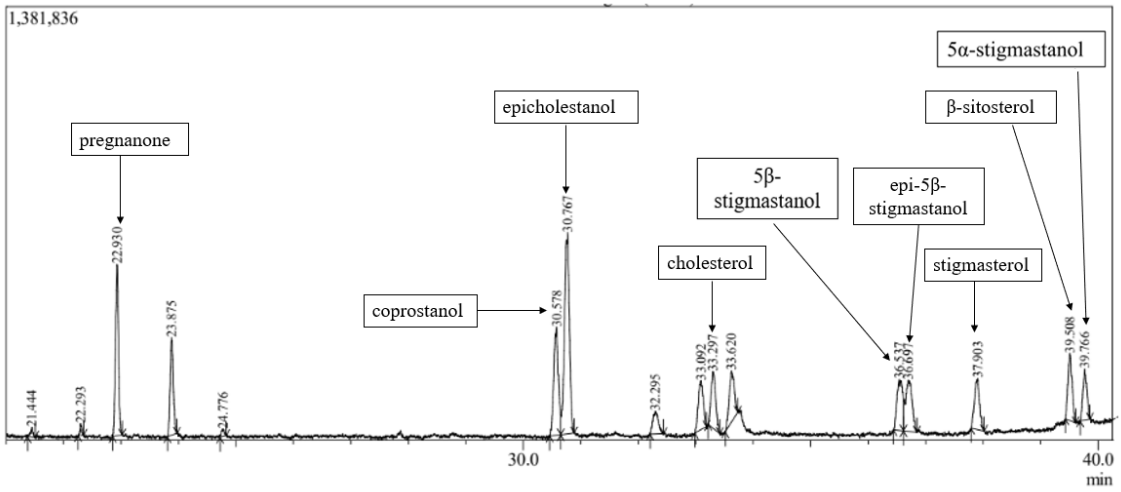

Figure 30: Gas chromatogram coupled with mass spectroscopy obtained in the analysis of sterols, stanols and stanones standard mixture when conducting the analysis of the grave inner soil samples of exhumation 7 (Monte D'Arcos Cemetery).

In relation to the SSS mix analysed with the grave inner soil samples of the exhumation 10 (Figure 31), the compounds identified were: preganone at 22.853 min; coprostanol at 30.433 min; epicoprostanol at 30.613 min; d7-cholesterol at 32.943; cholesterol at 33.147 min; 5α-cholestanol at 33.493 min; cholestanone at 33.603 min; 5β-stigmastanol at 36.383 min; β-sitosterol at 39.397 min, and 5α-stigmastanol at 39.650 min. Other peaks did not show the presence of any reference ion, thus not being identified.

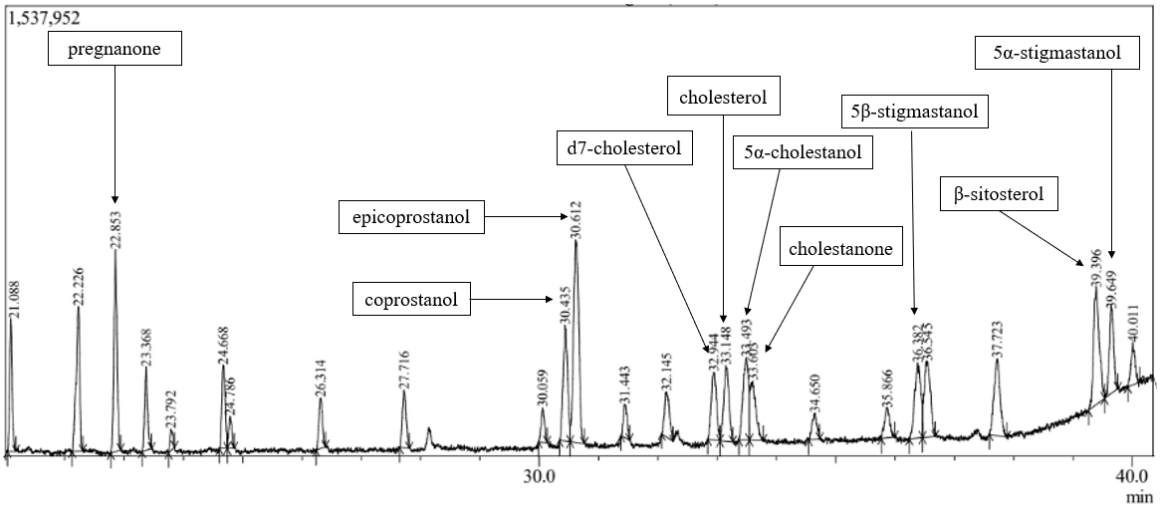

Figure 31: Gas chromatogram coupled with mass spectroscopy obtained in the analysis of sterols, stanols and stanones standard mixture when conducting the analysis of the grave inner soil samples of exhumation 10 (Monte D'Arcos Cemetery).

In the SSS mix analysed at the same time of the grave inner soil samples of individual 12 (Figure 32), less peaks were identified: epicholestanol at 31.120 min; coprostanone at 32.307 min; cholestanone at 33.743 min, and 5β-stigmastanol at 37.350 min. Other peaks did not show the presence of any reference ion, thus not being identified.

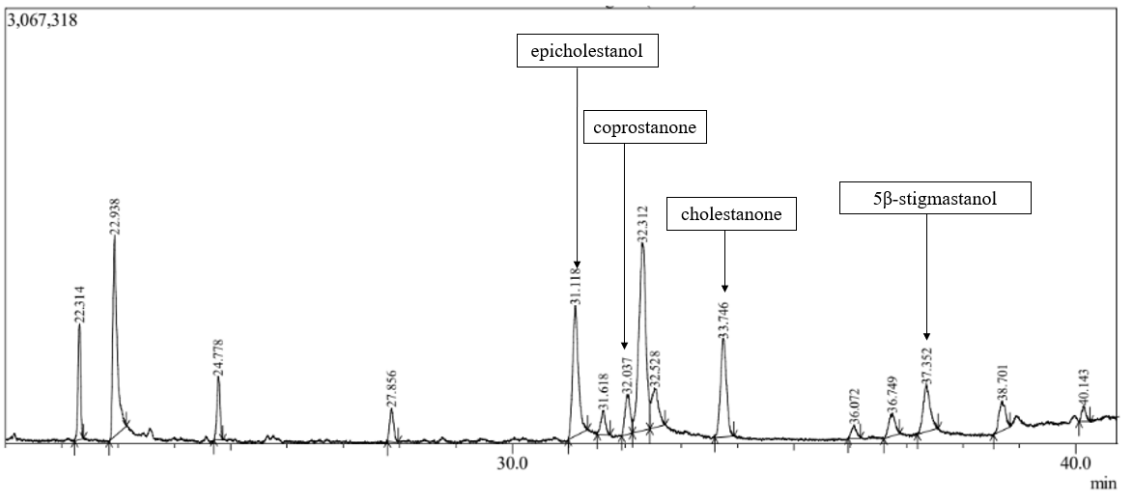

Figure 32: Gas chromatogram coupled with mass spectroscopy obtained in the analysis of sterols, stanols and stanones standard mixture when conducting the analysis of the grave inner soil samples of exhumation 12 (Monte D'Arcos Cemetery).

In the SSS analysed at the same time of the grave inner soil sample collected from CCM, exhumation 3, (Figure 33) the peaks with a retention time (RTm) of 21.092, 22.177, 22.230 and 23.370 min did not show any of the reference ions. The pregnanone was identified at 22.850 min; coprostanol at 30.443 min; epicholestanol at 30.640 min; d7 cholesterol at 32.940 min; cholesterol at 33.167 min; 5α-cholestanol at 33.477 min; 5β-stigmastanol at 36.393 min; epi-5β-stigmastanol at 36.550 min; stigmasterol at 37.710 min; β-sitosterol at 39.393 min, and 5α-stigmastanol at 39.653 min.

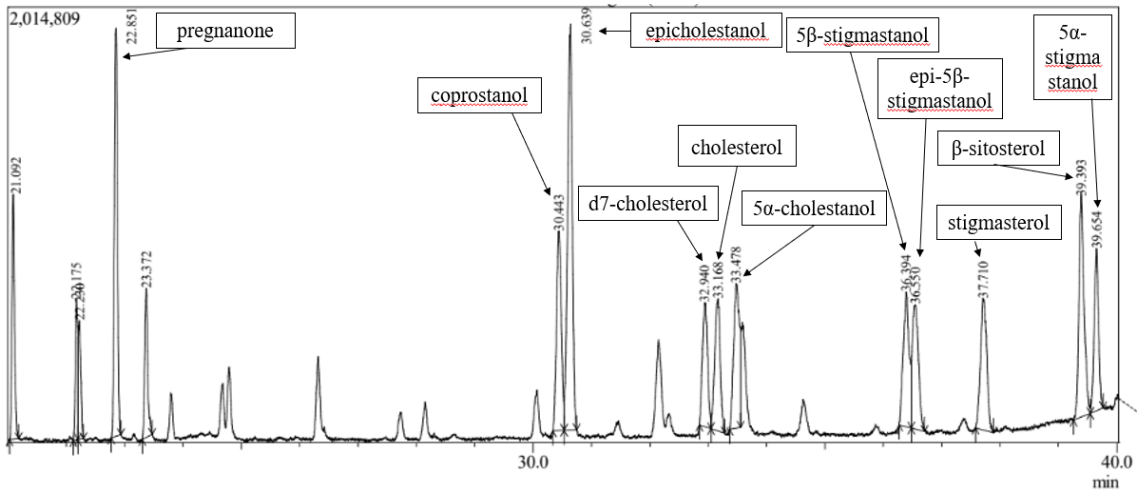

Figure 33: Gas chromatogram coupled with mass spectroscopy obtained in the analysis of sterols, stanols and stanones standard mixture when conducting the analysis of the grave inner soil samples of exhumation 3 (Castelo de Mértola Cemetery).

Similar spectra in all the SSS mix samples analysed were expected, since the solution of the standard mixture used was the same in all analysis. However, this did not happen. The spectra differ in the present compounds and also in their peak areas. This can be probably explained by loss of sample during the derivatisation process, namely when the samples were dried using the SpeedVac equipment.

Given all the sterols, stanols and stanones results, the average value of the retention time of each compound identified in the SSS mix was calculated (Table 13).

Table 13: Average of the retention times of the compounds identified in the sterol, stanol and stanones standard mix (SSS mix) in four repeats (SSS 2 = 7ALC, SSS 3 = 10 ALC, SSS 4 = 12ALC, SSS1= 3ALC) and their mean (min = minutes; "-" = not found).

	Retention times (min)			
COMPOUND	SSS 2	SSS 3	SSS 4	SSS 1
Pregnanol	-	-	-	-
Pregnanone	22.93	22.85	-	22.85
5α-cholestane	-	-	-	-
Coprostanol	30.58	30.43	-	30.44
Epicholestanol	30.77	-	31.12	30.64
Epicoprostanol	-	30.61	-	-
Coprostanone	-	-	32.31	-
d7-cholesterol	-	32.94	-	32.94
Cholesterol	33.30	33.15	-	33.17
5α-cholestanol	-	33.49	-	33.48
Cholestanone	-	33.60	33.74	-
5β-stigmastanol	36.54	36.38	-	36.39
Epi-5β-stigmastanol	36.70	-	-	36.55
Sigmasterol	37.90	-	-	37.71
β-sitosterol	39.51	39.40	-	39.39
5α-stigmastanol	39.77	39.65	37.35	39.65

Using the information relatively to the reference peaks (Table 13), and the chromatograms obtained in the SSS mix analysis (Figure 30, 31, 32, 33), the results of the grave inner soil samples collected from exhumations 7, 10, 12 and 3 were compared using the Data Comparison feature of the GC-MS equipment.

The ALC fraction was not found to contain any sterol, stanol or stanone in the samples collected from exhumation 7. The samples showed peaks corresponding to fatty acids (oleic and palmitic acids) which may be explained by a poor separation of the neutral and acidic fraction. Given these results, the ALC fraction of individual 7 analysis was repeated. However, the new results were in accordance with the previous results showing, no sterol, stanol or stanone in the samples. The comparison between the chromatogram of the SSS mix and the chromatograms of the analysis of samples collected under the head, thorax, abdomen, gluteal region and right thigh of the individual 7, at 0-5 and 5-10 cm, are presented in Attachment 7 (7.1, 7.2, 7.3, 7.4 and 7.5, respectively).

Similar to the ALC fraction of exhumation 7, also in the ALC fraction of the individual 10 no sterols, stanols or stanones were identified. The comparison between the SSS mix chromatogram and the remain chromatograms (head, thorax, abdomen, gluteal region and right thigh, at 0-5 and 5-10 cm) are shown in Attachment 8 (8.1, 8.2, 8.3, 8.4 and 8.5, respectively).

Furthermore, in the internal grave soil samples collected from the exhumation 12 5α-cholestane (second internal standard – IS II) was found in all samples. However, this compound was not present in the SSS mix. The figures comparing chromatograms from the SSS mix and the soil samples collected from the head, thorax, abdomen, gluteal region and right thigh at 0-5 and 5-10 cm, from exhumation 12 are presented in Attachment 9 (9.1, 9.2, 9.3, 9.4 and 9.5, respectively).

Regarding the samples collected under the head of the individual from the exhumation 3, at 0-5 cm (H5) and 5-10 cm (H10) soil depth, the comparison between the chromatogram obtained in the SSS mix (in black) and the chromatogram of H5 and H10 analyses (both in pink) is presented in Figure 34.

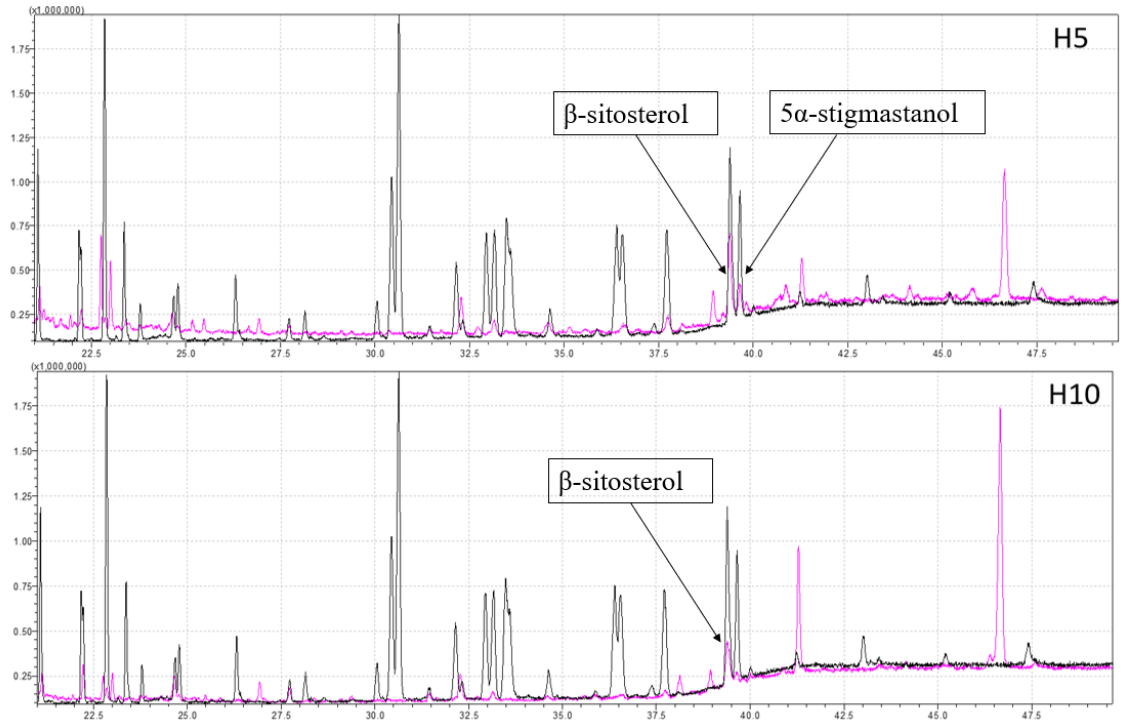

Figure 34: Comparison between the sterols, stanols and stanones standard mixture gas chromatogram coupled with mass spectroscopy (in black) and the chromatograms obtained in the analysis of the grave inner soil samples collected under the head of the individual from the exhumation 3 (Castelo de Mértola Cemetery), at 0-5 cm (H5) and 5-10 cm (H10) soil depth (both in pink).

At first inspection, the chromatogram obtained in the analysis of the SSS mix does not overlap with most of the peaks present in the analysis of the soil samples H5 and H10. However, in the sample H5, it was possible to identify the presence of β-sitosterol (RTm = 39.39 min) and 5α-stigmastanol (RTm = 39.65 min). Figure 35 presents the mass spectra of these peaks.

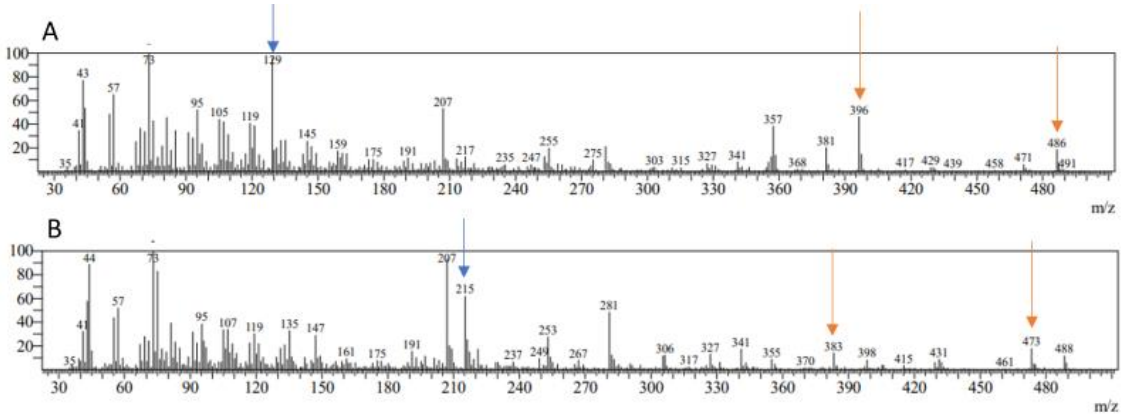

Figure 35: Mass spectra of peaks at 39.39 minutes (A) and 39.65 minutes (B) of retention time, from the grave inner soil sample collected under the head of the individual from the exhumation 3 (Castelo de Mértola Cemetery), between 0 and 5 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

In sample H10, only β-sitosterol was identified at 39.393 min using the reference ions (Figure 36). Other peaks did not show the presence of any reference ion, thus not being identified.

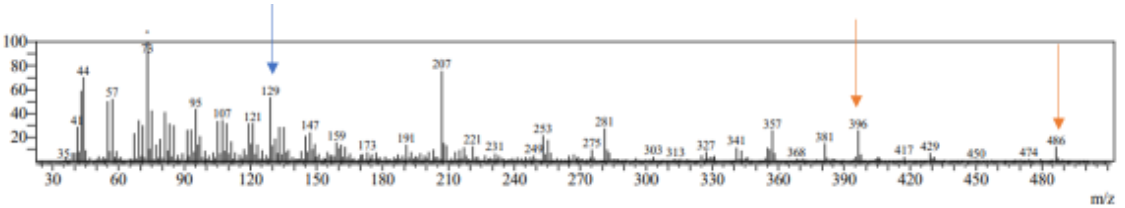

Figure 36: Mass spectra of peaks at 39.39 minutes of retention time, from the grave inner soil sample collected under the head of the individual from the exhumation 3 (Castelo de Mértola Cemetery), between 5 and 10 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

Regarding the samples collected under the thorax of the individual from the exhumation 3, at 0-5 cm (T5) and 5-10 cm (T10) soil depth, the comparison between the chromatogram obtained in the SSS mix (in black) and the chromatogram of T5 and T10 analyses (both in pink) is presented in Figure 37.

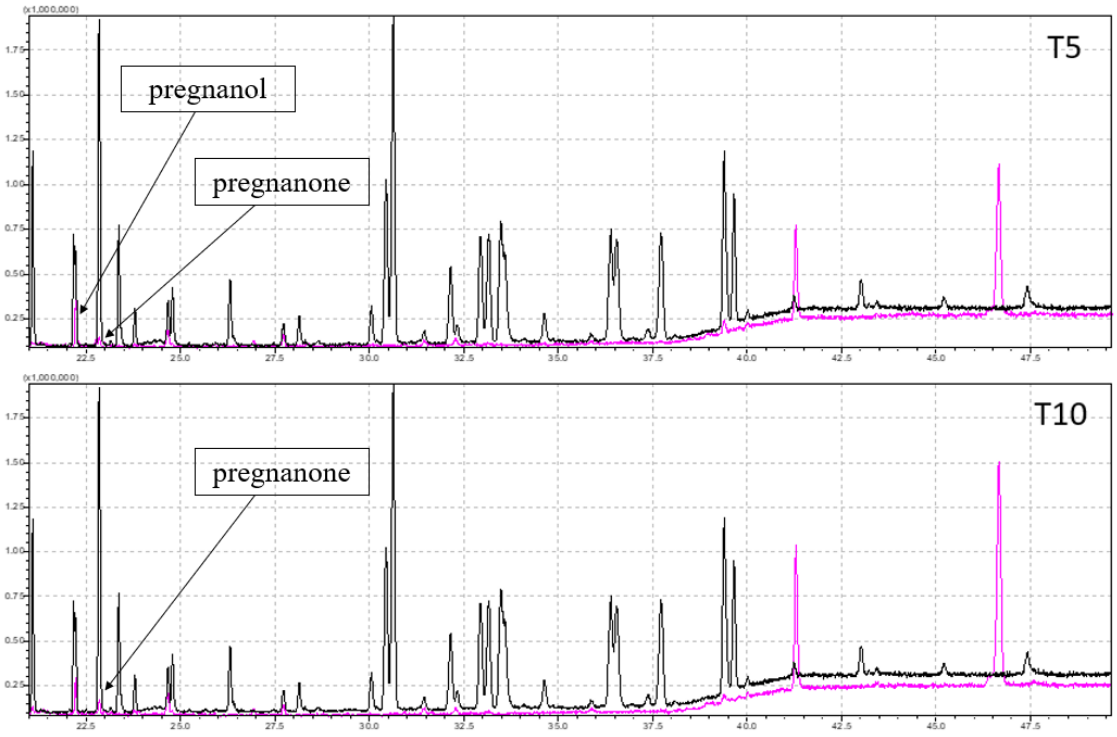

Figure 37*:* Comparison between the sterols, stanols and stanones standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the thorax at 0-5 cm (T5) and 5-10 cm (T10) (both in pink) of the internal grave soil samples collected in exhumation 3.

In the soil sample collected under the thorax at 0-5 cm soil depth it was possible to identify pregnanol at 22.240 min and pregnanone at 22.860 min, by analysing the peak's mass spectra (Figure 38).

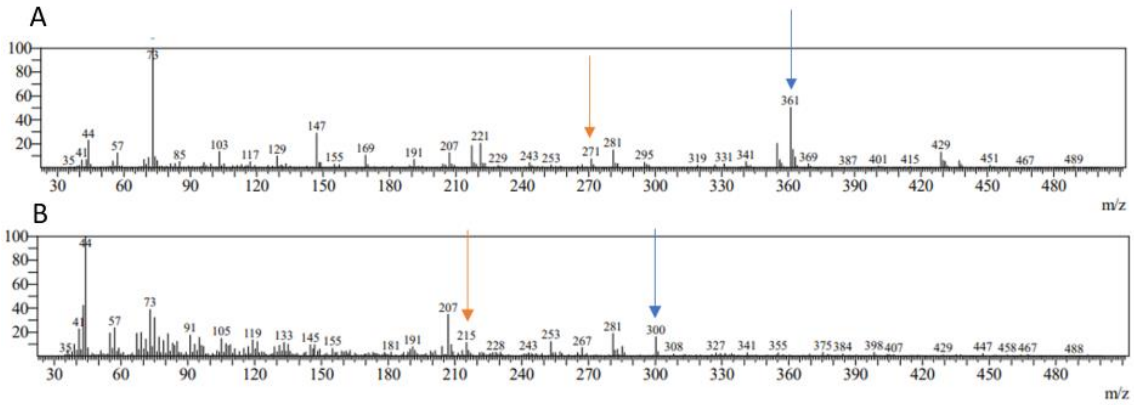

Figure 38*:* Mass spectra of peaks at 22.24 minutes (A) and 22.86 minutes (B), from the grave inner soil sample collected under the thorax of the individual from the exhumation 3 (Castelo de Mértola Cemetery), between 0 and 5 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

In the soil sample T10 collected under the thorax at 5-10 cm soil depth, only pregnanone was identified with a retention time of 22.853 min (Figure 39).

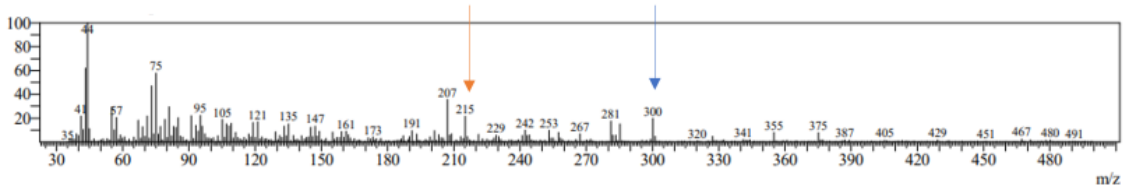

Figure 39: Mass spectra of peaks at 22.85 minutes, from the grave inner soil sample collected under the thorax of the individual from the exhumation 3 (Castelo de Mértola Cemetery), between 5 and 10 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

Regarding the samples collected under the abdomen of the individual from the exhumation 3, at 0-5 cm (A5) and 5-10 cm (A10) soil depth, the comparison between the chromatogram obtained in the SSS mix (in black) and the chromatogram of A5 and A10 analyses (both in pink) is presented in Figure 40.

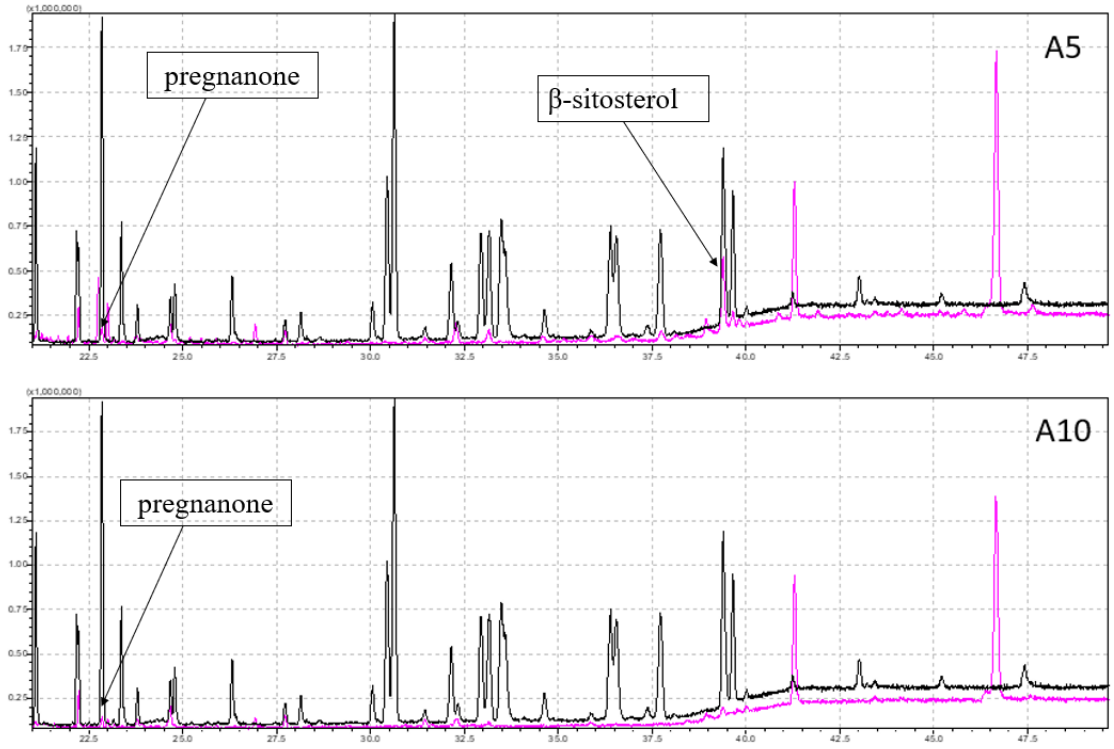

Figure 40*:* Comparison between the sterols, stanols and stanones standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the abdomen at 0-5 cm (A5) and 5-10 cm (A10) (both in pink) of the internal grave soil samples collected in exhumation 3.

In the soil sample collected under the abdomen at 0-5 cm soil depth it was possible to identify pregnanone at 22.853 min and β-sitosterol at 39.387 min (Figure 41).

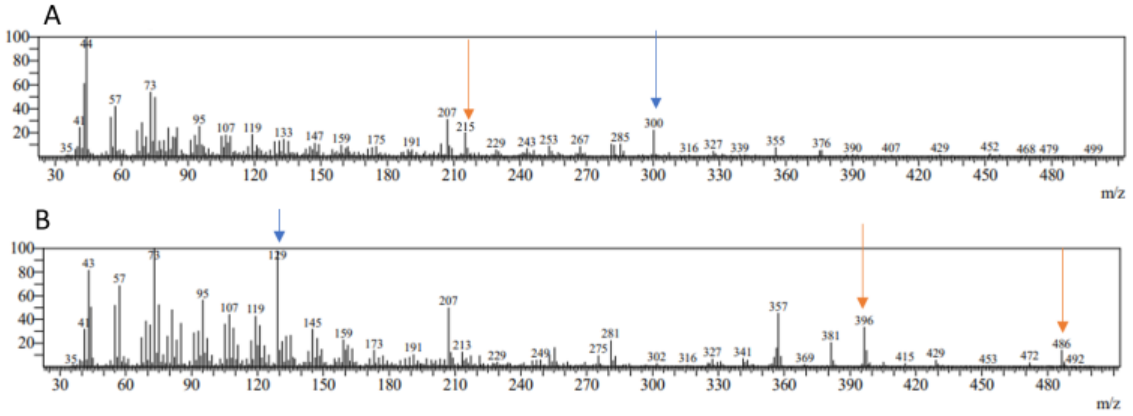

Figure 41: Mass spectra of peak at 22.85 minutes (A) and 39.39 minutes (B) of retention time, from the grave inner soil sample collected under the abdomen of individual 3 (Castelo de Mértola Cemetery), between 0 and 5 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

Regarding the soil sample collected under the abdomen at 5-10 cm soil depth, it was possible to identify, at 22.857 min, the stanone pregnanone (Figure 42).

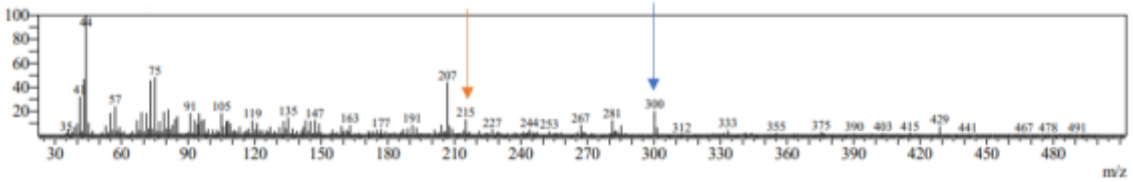

Figure 42: Mass spectra of peak at 22.86 minutes (A) of retention time, from the grave inner soil sample collected under the abdomen of individual 3 (Castelo de Mértola Cemetery), between 5 and 10 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

Regarding the samples collected under the gluteal region of the individual from the exhumation 3, at 0-5 cm (G5) and 5-10 cm (G10) soil depth, the comparison between the chromatogram obtained in the SSS mix (in black) and the chromatogram of G5 and G10 analyses (both in pink) is presented in Figure 43.

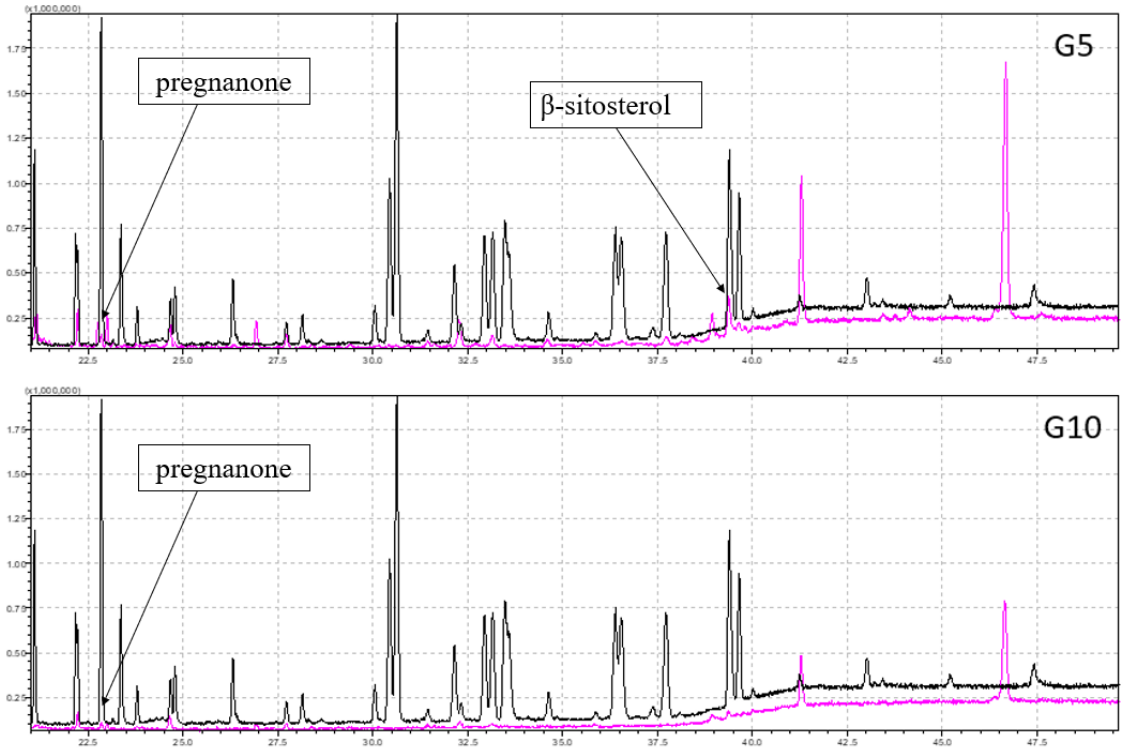

Figure 43: Comparison between the sterols, stanols and stanones standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the gluteal region at 0-5 cm (G5) and 5-10 cm (G10) (both in pink) of the internal grave soil samples collected in exhumation 3.

In the G5 sample, pregnanone and β-sitosterol, with the retention times of 22.850 and 39.370 min, respectively, were identified. Their mass spectrum and the reference ions are presented in Figure 44.

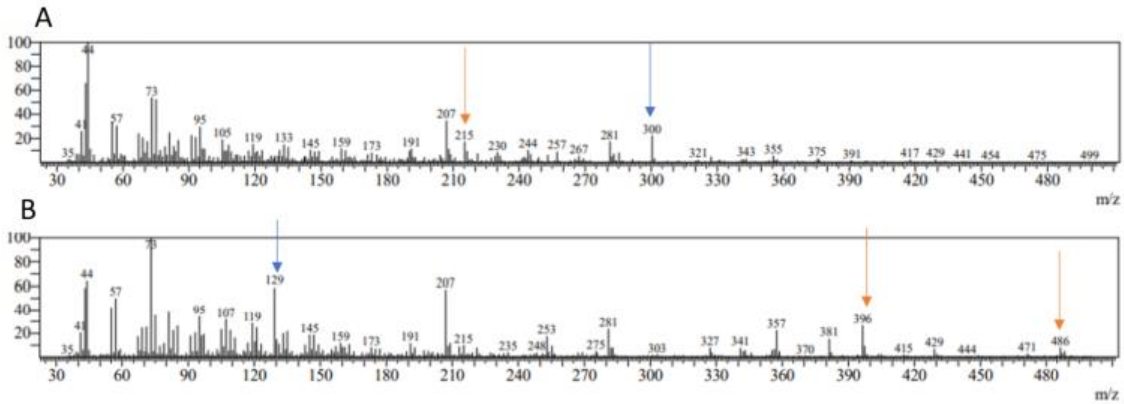

Figure 44: Mass spectra of peak at 22.85 minutes (A) and 39.37 minutes (B) of retention time, from the grave inner soil sample collected under the gluteal region of individual 3 (Castelo de Mértola Cemetery), between 0 and 5 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

In the sample G10, only pregnanone was identified with a retention time of 22.850 min (Figure 45).

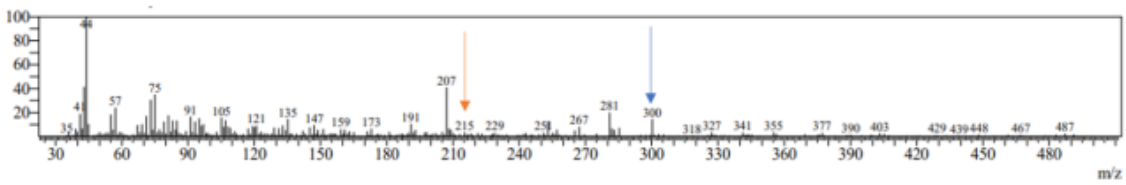

Figure 45: Mass spectra of peak at 22.85 minutes of retention time, from the grave inner soil sample collected under the gluteal region of individual 3 (Castelo de Mértola Cemetery), between 5 and 10 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

Regarding the samples collected under the right thigh of the individual from the exhumation 3, at 0-5 cm (RT5) and 5-10 cm (RT10) soil depth, the comparison between the chromatogram obtained in the SSS mix (in black) and the chromatogram of RT5 and RT10 analyses (both in pink) is presented in Figure 46.

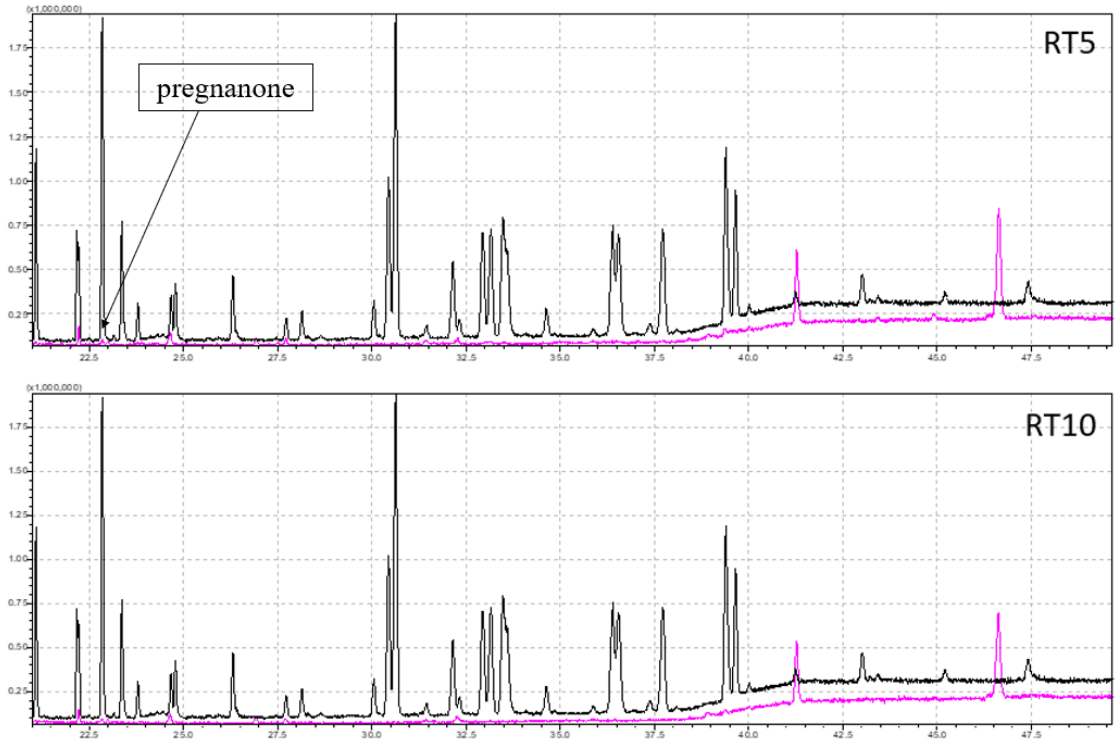

Figure 46: Comparison between the sterols, stanols and stanones standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the right thigh at 0-5 cm (RT5) and 5-10 cm (RT10) (both in pink) of the internal grave soil samples collected in exhumation 3.

In RT5 sample, only pregnanone was identified with a retention time of 22.867 min (Figure 47). The sample collected from 5-10 cm (RT10) did not show the presence of any sterol, stanol or stanone.

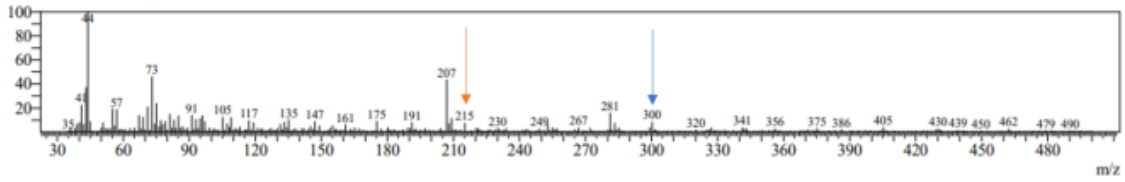

Figure 47: Mass spectra of peak at 22.87 minutes of retention time, from the grave inner soil sample collected under the right thigh of individual 3 (Castelo de Mértola Cemetery), between 0 and 5 cm soil depth (blue arrow =quantifier reference ion; orange arrow = qualifier reference ion).

To sum up, in the samples collected from the grave of individual 3, only four relevant compounds were found: 5α-stigmastanol, pregnanol, pregnanone and β-sitosterol. From these, only pregnanol has human source giving that 5α-stigmastanol and β-sitosterol are compounds originated from plant activity and pregnanol and pregnanone were the internal standards added to the samples.

5α-cholestane, the second internal standard, did not appear in any other samples apart from the ones analysed from the exhumation 12 which was not expected. This could be explained by the fact that the soil samples from exhumation 12 were analysed with a different stock solution of 5α-cholestane and this solution could be, for human error in the preparation, more concentrated than the previous. Another possible explanation is that the compound could had suffer degradation in the first stock solution. Furthermore, also the first internal standard (pregnanone) should have been found in all samples since it was added to perform the quantification of the sterols and stanols found. Since no relevant compound was found, this quantification was not determined. The stock solution used was the same for all samples which goes against to the results obtained since pregnanone was only found in the internal soil samples collected from the exhumation 3.

Given the results obtained from the analysis of the ALC fraction of three exhumations in CMA (numbers 7, 10 and 12) and from one exhumation in CCM (number 3), only samples collected from CCM enable the identification of sterols, stanones and stanols. However, the compounds found are not associated with human decomposition but β-sitosterol and 5α-stigmastanol can have a plant origin. Furthermore, given that these compounds were not identified in the samples from CMA it could mean that the plant

activity in Mértola soil is different from the soil in the cemetery in Braga. Given the inorganic and organic matter content results obtained, in the soil from exhumation 3 the results showed that the samples collected in this exhumation had a lower moisture content and a higher organic matter content which can be linked to the plant activity in the grave. The moisture within the grave could be low due to plant activity, given the retention of water by them, which could also influence the decomposition of the corpses, delaying the decomposition process. The time since burial in this exhumation was bigger than the ones from the CMA exhumations meaning that if the soil samples were analysed earlier in the decomposition process more sterols, stanols and stanones could had been identified. By the time this exhumation occurred the corpse was in an advanced decay process and the bones were deteriorating. The steroids could had also suffered degradation with time. Regarding the samples from CMA, no relevant compound was found. However, it would be advisable to repeat the analysis and tuned the method used. Moreover, the time of burial of the individuals from these exhumations was lower than the one from Mértola. Ideally, in these exhumations we should be able to identify sterols, stanols and stanones, at least the ones associated to plant activity given that in CMA there are more trees and plants than in CCM.

4.2.3. Identification of Bile Acids

Similar to the ALC fraction analysis, to analyse Bile acids a Bile Acid Standard Mix (BA mix) was also placed in the GC-MS. Before analysing the BA mix, a methylation process had to be carried out to ensure that bile acids in the mixture would be chemical transformed into methyl esters, then being properly detected.

The analysis of the Bile Acid Methyl Ester fraction (BAME) was only performed in grave inner soil samples from the exhumations 7, 10 and 12 (Monte D'Arcos Cemetery).

The reference ions that were used to identify bile acids are presented in Table 14. The mass spectra of the peaks were analysed to properly identify the bile acids present in the samples. Similar to the identification of sterols, stanols and stanones the identification relied on the mass spectra and no conclusion was obtained from the library search of the GC-MS. Furthermore, in the library most of the compounds were "unknown" compounds since the library did not recognise them.

Table 14: Compounds expected to be present in the bile acid standard mix (BA mix) and their qualifier and quantifier ions (m/z = mass-to-charge ratio).

COMPOUND	QUALIFIER (m/z)	QUANTIFIER (m/z)
Isodeoxycholic acid (IDCA)	355, 370	255
Isolithocholic acid (ILCA)	257, 357	215
Lithocholic acid (LCA)	257, 372	215
Deoxycholic acid (DCA)	345, 370	255
Chenodeoxycholic acid (CDCA)	355, 370	255

The BA mix mass spectra' interpretation (Figure 48, Figure 49 and Figure 50) did not allow the identification of any bile acid.

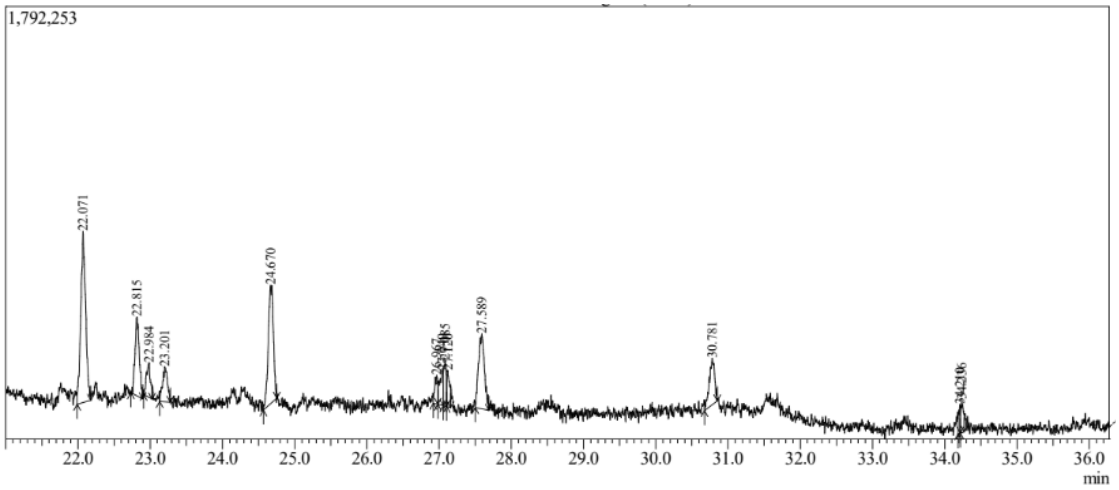

Figure 48: Chromatogram obtained in the Gas Chromatography Coupled to Mass Spectroscopy (GC-MS) analysis bile acids standard mixture (BA mix) when conducting the analysis of internal grave soil samples of exhumation 7.

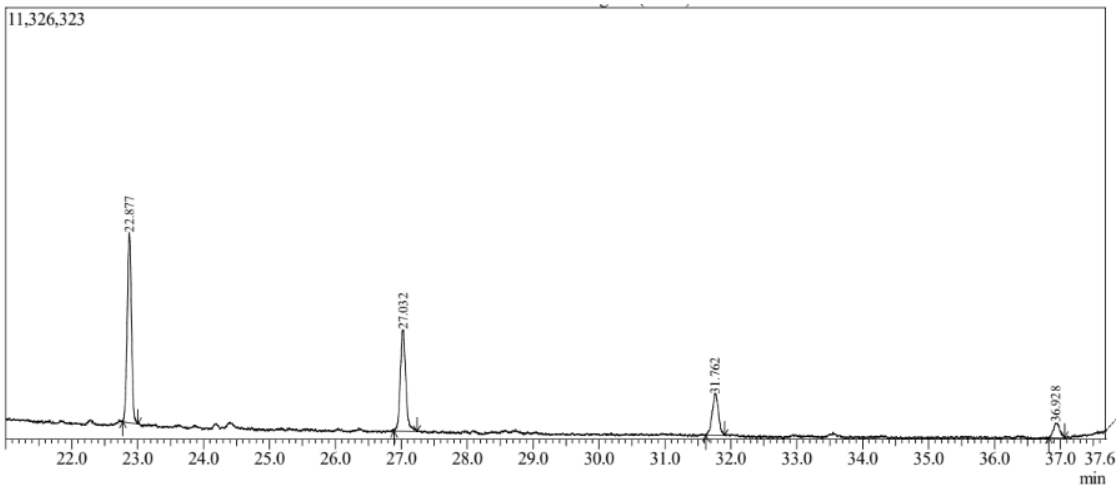

Figure 49: Chromatogram obtained in the Gas Chromatography Coupled to Mass Spectroscopy (GC-MS) analysis bile acids standard mixture (BA mix) when conducting the analysis of internal grave soil samples of exhumation 10.

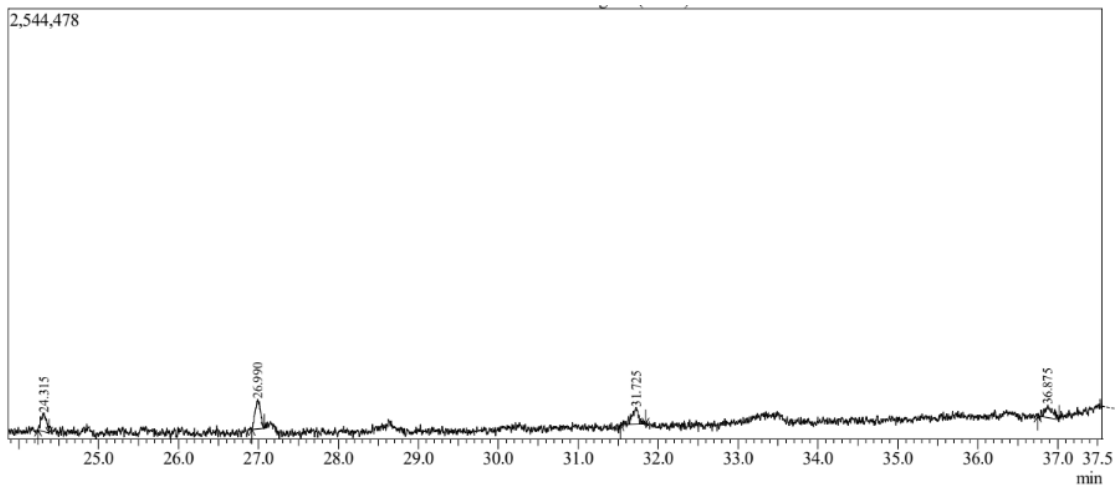

Figure 50: Chromatogram obtained in the Gas Chromatography Coupled to Mass Spectroscopy (GC-MS) analysis bile acids standard mixture (BA mix) when conducting the analysis of internal grave soil samples of exhumation 12.

Analysing the mass spectra obtained in the grave inner soil samples collected from exhumations number 7, 10 and 12 it was possible to verify that no bile acids were present in any of the samples since the reference ions were not found. Nonetheless, on a sample collected under the gluteal region of the individual from the exhumation 7 (7G10), at a retention time (RTm) of 22.340 and 29.583 min, the ions 215, 355 and 370 were identified. These ions are characteristic of coprostanol, epicholestanol and epicoprostanol, meaning that during the laboratorial procedure, the separation of the fraction containing sterols, stanols and stanones (neutral fraction) and the fraction containing bile acids (acidic fraction) may not have been well executed, resulting in the detection of sterols in the bile

acid fraction. In the grave inner soil samples analysed from exhumations10 and 12, some mass spectra had the presence of ions 255 and 355 but, since 370 was missing, it is not possible to claim the presence of CDCA. Besides this, in all the other samples it was not detected any relevant compound. The fact that no bile acids were identified, including the ones which were known to be present in the BA mix, could mean that their quantities in the samples were too low to be detected. Therefore, in future research, it would be better to increase the initial mass of sample, and also to increase the concentration of the stock solution of the BA mix standards.

V. Conclusions

Fatty acids and sterols can be detected in soil samples over the years following body deposition in soil, making them potentially useful post-mortem interval biomarkers, or to act as evidence of the presence of a decomposing body at a particular unknown time in the past. However, it is still not possible to define a precise timescale of a body decomposition process using biomarkers. The diversity of factors involved makes it difficult to predict the behaviour of the decomposition mechanism with any degree of confidence, and therefore it is important to develop further studies to analyse and test this phenomenon. The main aims of this work were to collect soil samples from graves (under corpses and from the grave walls) and also related control samples to characterise both the soil's inorganic parameters and organic constituents (fatty acid and steroids).

The adopted sampling method was efficient and the 25 centimetre length stainless steel augers were suitable for the purpose, as they allowed the collection of a solid core of soil enabling to split into different depth sections and also to visualise and evaluate the differences between the core depths. In addition, these collections should always be accompanied by the use of a 100 centimetre auger in order to see the different deeper and see soil horizons/profiles.

The inorganic characterisation and the organic matter content analysis of the soil samples were successfully performed in this study and showed relevant differences between the two cemeteries in terms of pH, moisture, and total organic matter. However, it is advisable to analyse more inorganic parameters in future, namely the texture of soil, to have more quantitative results to distinguish soils and to better discuss its influence in the human decomposition process.

Performing the organic characterisation was particularly challenging, and the results obtained were far from what was expected. Only fatty acids were successfully identified but not quantified, therefore not permitting firm conclusions to be made regarding the correlation between the time since death, and the body provenance. Therefore, it is recommended that further studies in this area are carried out using a greater initial mass of soil sample, being suggested a mass greater than 1.5 g dry weight, to extract lipids which may be present in small amounts. The poor results obtained could also be due to the fact that only 20-25 g of the collected soil was freeze-dried and sieved. Given that the soil was collected in blocks of 5 cm depth each (about 100-120 g per

sample), if the total amount of sample was treated, we might had been able to extract more organic compounds from soil. Also, it would have been interesting to perform organic characterisation on the samples collected from the top of each grave (topsoil samples). Since its organic characterisation could allow to discard some compounds that were found originated from plant activity. Additionally, new studies should focus more on sterols, stanols, stanones and bile acids since its study is not as broad as the fatty acid studies. Moreover, it is advisable to perform analysis on grave wall samples that due to lack of time were not analysed in this work. The grave wall samples can be a reference to the compounds found within the grave and may aid in the association between the organic compounds and the decomposition process.

As previously said, one of the things that was intended to be carried out and ended up not being performed was the quantification of the organic compounds. The quantification of the compounds present in graves is an important aspect of these studies since it enables to develop a correlation between the quantities of the compounds identified and the decomposition process to be tested and established. In addition, during the discussion of the results the individual variation (one of the intrinsic factors that affects the decomposition process) was one factor that was not taken into consideration given the lower number of individuals in the study. Given this, it would be interesting to broaden the study to more individuals and considerer factors such as sex and age at death. Also, when possible, researchers should consider gaining information through questioning the relatives to get the information regarding the cause of death given its influence in decomposition.

In conclusion, the analysis of fatty acids and steroids is a promising area of research. Yet, more studies in different soils and with bodies originating from different causes of dead and with different characteristics should be performed, testing different intrinsic and extrinsic factors. Ideally, studies should be conducted with human corpses, but due to ethical reasons, it may be challenging to develop studies that can ensure a control of all the variables involved in the decomposition process with adequate replication. Understanding which variables are involved and how they can affect decomposition is of upmost importance for the future safe use of this science in the criminal justice system.

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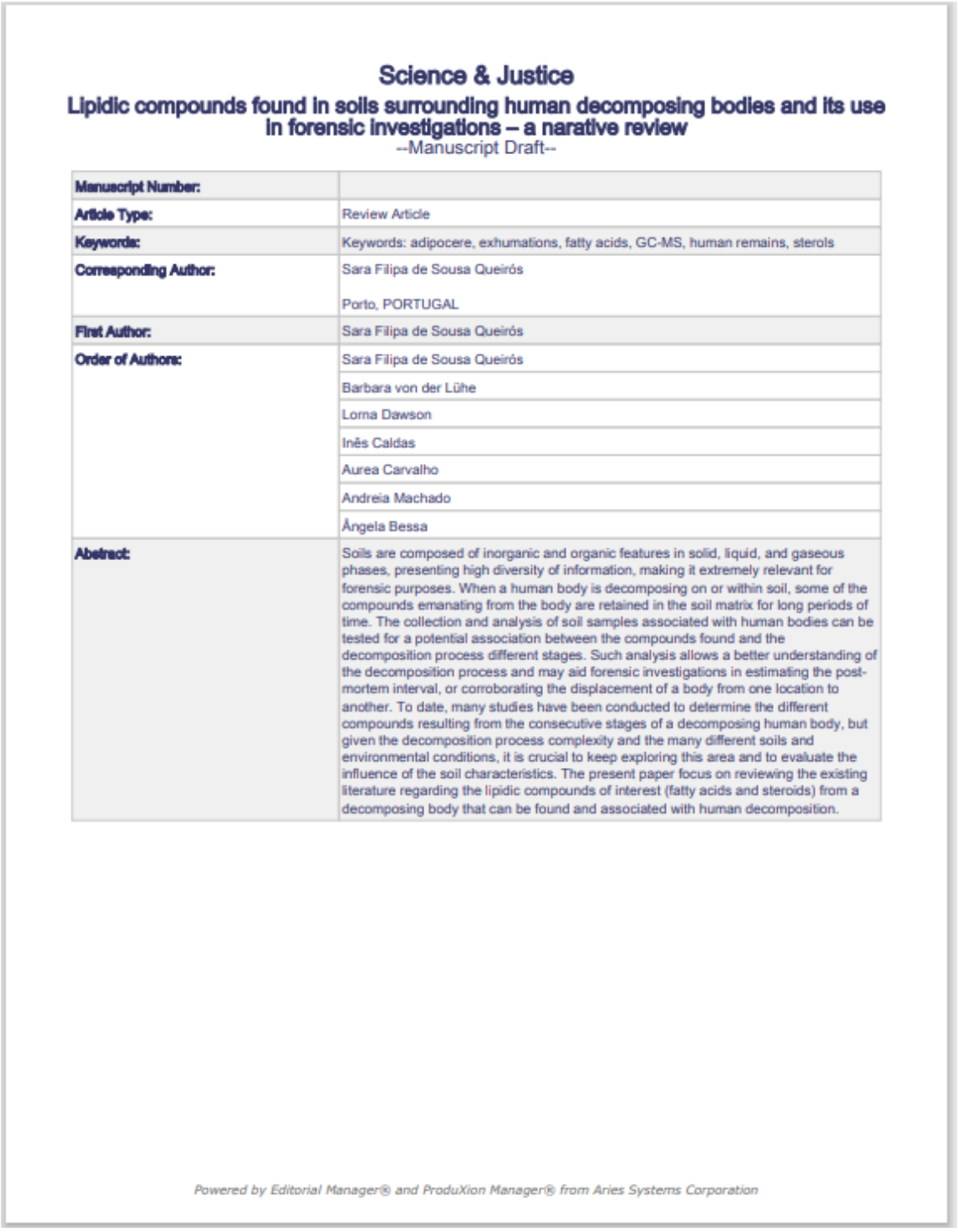
Appendixs

Appendix 1.

Review article entitled "Lipidic compounds found in soils surrounding human decomposition bodies and its use in forensic investigations – a review" submitted to Science and Justice.



Science & Justice

Lipidic compounds found in soils surrounding human decomposing bodies and its use in forensic investigations – a narative review

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Abstract:	Soils are composed of inorganic and organic features in solid, liquid, and gaseous phases, presenting high diversity of information, making it extremely relevant for forensic purposes. When a human body is decomposing on or within soil, some of the compounds emanating from the body are retained in the soil matrix for long periods of time. The collection and analysis of soil samples associated with human bodies can be tested for a potential association between the compounds found and the decomposition process different stages. Such analysis allows a better understanding of the decomposition process and may aid forensic investigations in estimating the post-mortem interval, or corroborating the displacement of a body from one location to another. To date, many studies have been conducted to determine the different compounds resulting from the consecutive stages of a decomposing human body, but given the decomposition process complexity and the many different soils and environmental conditions, it is crucial to keep exploring this area and to evaluate the influence of the soil characteristics. The present paper focus on reviewing the existing literature regarding the lipidic compounds of interest (fatty acids and steroids) from a decomposing body that can be found and associated with human decomposition.

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Novelty Statement (without Author Details)

None.

Highlights

- Soils can retain compounds resulting from human body decomposition processes
- Fatty acids and steroids can be associated to human body decomposition processes
- Chemical analysis of soil can provide important data for forensic investigations

Title Page (with Author Details)

Lipidic compounds found in soils surrounding human decomposing bodies and its use in forensic investigations – a narative review

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Abstract

Soils are composed of inorganic and organic features in solid, liquid, and gaseous phases, presenting high diversity of information, making it extremely relevant for forensic purposes. When a human body is decomposing on or within soil, some of the compounds emanating from the body are retained in the soil matrix for long periods of time. The collection and analysis of soil samples associated with human bodies can be tested for a potential association between the compounds found and the decomposition process different stages. Such analysis allows a better understanding of the decomposition process and may aid forensic investigations in estimating the post-mortem interval, or corroborating the displacement of a body from one location to another. To date, many studies have been conducted to determine the different compounds resulting from the consecutive stages of a decomposing human body, but given the decomposition process complexity and the many different soils and environmental conditions, it is crucial to keep exploring this area and to evaluate the influence of the soil characteristics. The present paper focus on reviewing the existing literature regarding the lipidic compounds of interest (fatty acids and steroids) from a decomposing body that can be found and associated with human decomposition.

Keywords: adipocere, exhumations, fatty acids, GC-MS, human remains, sterols

1. Introduction

Soil has been shown to be a very useful tool in forensic investigations answering questions to which other techniques fail, namely in cases involving the relocation of a body [1]. This matrix is largely used as evidence due to its ability to be transferred and to persist on clothes and objects (*e.g.*, footwear and vehicles), allowing to link a certain person to a particular place or context [2]. By analysing the characteristics of a soil sample (*e.g.*, mineral content, particle size distribution, colour, and pH), it is possible to distinguish and conclude whether or not two samples have the same geological origin. Additionally, due to its high capacity of retaining information, soil analysis may help answering questions related to the post-mortem interval (PMI). In forensic investigations, the estimation of the PMI and the inquiry of the circumstances involving death are highly important. PMI assessment is particularly relevant given that it can help authorities reduce the timeline of events that led to the individual's death.

It is estimated that body decomposition commences about four minutes after death and may take up to 15-25 years to reach full skeletonization [3]. However, decomposition process may not always follow a sequential path, and a body can simultaneously be mummified, in putrefaction, and saponified [4]. Human taphonomy depends on the extrinsic (*e.g.*, environmental factors and soil's characteristics) and intrinsic factors (*i.e.*, individual variation) that influence the decomposition process duration. The decomposition of a body is a continuous process that leads to the release of biochemical compounds due to the occurrence of the mortis triad, autolysis, putrefaction, decay, and skeletonization (Figure 1) [5].

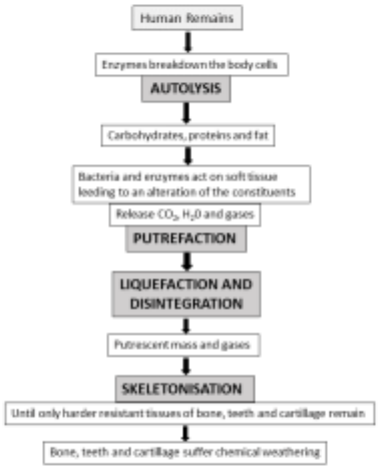

Figure 1: Summary of the human decomposition stages (Adapted from [5]).

Under anaerobic conditions, adipocere ("adipo" = fat and "cere" =wax) could be formed from soft tissues [6]. It forms as a result of the adipose tissue degradation, caused by the hydrolysis and hydration of the adipocytes. Although saturated free fatty acids such as myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids are adipocere major constituents [7], this substance constitution differs according to the body part where it is formed. Despite various studies that have been carried out through the years, the exact nature of adipocere is still being studied and determined [8]. Understanding its formation mechanism and conditions required in grave soils is of paramount importance for forensic investigators, given that the derived soil compounds can help determining the PMI.

Human body decomposition is a complex process in which several products are released into the soil as a consequence of the breakdown of cells. Studying these compounds and using effective techniques to identify and quantify them may aid forensic investigations. One way to achieve this is to extract the organic compounds such as fatty acids and sterols from grave soils and/or soil evidence [9, 10].

2. Lipids released from a decomposing body found in soil

2.1. Fatty acids

2.1.1. *Description of the compounds*

Fatty acids are carboxylic acids with hydrocarbon chains of variable sizes between four and thirty-six carbons (Figure 2). Unsaturated fatty acids have double bonds in the hydrocarbon chain while saturated fatty acids have no double bonds. These acids characteristics (*e.g.,* the melting point and solubility) depend on the number of carbons, the existence of double bonds, rings, hydroxyl groups, and/or branches of methyl groups [11].

Figure 2: Chemical structure of fatty acids (the carboxyl group and the hydrocarbon chain (R) are highlighted in red and black, respectively).

2.1.2. *Applicability in forensic investigations*

2.1.2.1. Identification of fatty acids in adipocere studies

Fatty acids can be found in soils due to human decomposition, being sometimes identified when adipocere is present. Currently, most studies are focused on adipocere constitution.

The first time adipocere was mentioned was in 1786, in a French study of the Holy Innocents' Cemetery, which was relocated (due to the fact that the original cemetery was placed in soil with high humidity content) and is now closed [6, 12].

In 1977, a study of adipocere constitution conducted on human remains found at sea, in Japan [13], allowed the identification of 10-hydroxystearic acid (10-OH-C18:0) and 10-hydroxyhexadecanoic acid (10-OH-C16:0), using Gas-Liquid Chromatography (GLC) and Gas Chromatographu Coupled to Mass Spectroscopy (GC-MS) techniques.

In 1983, Takatori et al. [14], using GC-MS, identified 10-hydroxy-12-stearic acid in human adipocere collected from different regions of human bodies found at sea, in Japan, with an estimated PMI of six to ten months.

In 1997, Adachi et al. [15] using GC-MS, discussed the relation between the lipid composition and the time of adipocere formation in submerged victims from one month to four years. They concluded that the samples collected from victims submerged during one to two months contained about 1% of hydroxy fatty acids, and that linoleic acid (C18:2) ranged between 13% to 15%. In adipocere samples from victims submerged during four months to four years, there was an increase in the percentage of hydroxy fatty acids (8%) and a decrease in the percentage of linoleic acid with the 10-ketostearic acid (10-Keto-C18:0) content ranging from 0.7% to 3.2%. Therefore, these results showed that with time the percentage of hydroxy fatty acids present in adipocere increases. Additionally, this study demonstrated that keto fatty acids were not detected in victims submerged for less than three months, which can be very useful in PMI estimation.

In 2002, Forbes et al. [16] published an article about the identification of adipocere in samples collected from cemetery graves, using GC-MS. The samples were taken from dry and wet environments with a wide range of burial times (five to fifty years). In addition to these, samples were also taken from several graves with unknown burial duration, and from cemetery sites with no proximity to the graves (control samples). It was found that the composition of adipocere varied according to the environment. Fatty acids were not found in the control samples, but they were detected in the remaining samples, namely C14:0, C16:0, C18:0, palmitoleic (C16:1), oleic (C18:1), and 10-hydroxy stearic acid (10-OH C18:0).

In 2003, an article describing the development of a new GC-MS method for quantitative analysis of adipocere samples collected from grave soils was published [7]. The authors

performed exhumations in Australian cemeteries and collected soil samples under the lower coffin board. Control samples were collected from the grave walls. The inhumation time spanned between six and twelve years, with one burial duration being unknown. Control samples did not present any fatty acids, thus confirming the relation between the fatty acids present in soil under the human remains and body decomposition. In the latter samples, C14:0, C16:0, and C18:0 were identified. Besides these acids, C18:1 and 10-OH C18:0 were also detected. However, in samples collected from the six years burial, the concentration of C18:1 was low. This suggests that the hydrogenation process was still occurring, and the conversion was not complete just like in the samples collected from twelve years burials. Additionally, the detection of 10-OH C18:0 only in some samples seemed to be reliant on the conditions of the decomposition environment.

Another study was conducted in 2005, to determine the effect of the burial method on the adipocere formation, using GC-MS [17]. Unlike the 2002 referred study, the authors did not take samples from human exhumations since it was intended to control the environment as much as possible, to reduce the number of variables and obtain more reliable results [16, 18]. Domestic pigs (*Sus scrofa domesticus*) were used as human proxies given that both species have monogastric digestive systems. However, a study conducted in 2017 [19] showed that fundamental differences exists between the two species and different structural and functional gastrointestinal tract may affect rate, timing, and trajectory of decomposition. In this study, a section of adipose tissue from the abdominal region of pigs subjected to the same diet was cut and buried in controlled environments for one year. Some samples were buried in plastic bags to understand the behaviour of adipocere formation in situations where a body is wrapped in plastic. It was verified that C16:0 was present in all samples, reaching higher values than other fatty acids, followed by C18:0. There was also a significant presence of 10-OH-C18:0 in the coffin samples, which is strong evidence of adipocere formation. It was also possible to verify that saturated fatty acids were found in greater quantities than unsaturated fatty acids. However, in samples taken from plastic bags, the amount of unsaturated fatty acids was greater than in other samples. Thus, it seems possible to verify that adipocere does not occur in cases where the body is submitted to these conditions. Moreover, results obtained in this experiment confirm the previous written observations about adipocere forming more quickly in bodies buried directly in soil, showing that this matrix has a strong interference in the formation of this substance and consequently in the

decomposition of a body. These results and conclusions provide us new insights regarding adipocere formation. However, the study was conducted in pigs and, as mentioned before, differences between pigs and humans must be considered when conducting body composition studies..

2.1.2.2. Identification of fatty acids in soils

Fatty acids can be detected due to the emanation of decomposition fluid into the soil. However, soils are complex and require suitable analysis. Throughout the years, forensic scientists have studied soil's composition and characteristics [21]. Fatty acids have been detected in decomposition fluid [22] and also been successfully extracted from textiles in decomposing bodies [23]. However, studies regarding the detection of fatty acids extracted from soils where a body is decomposing are scarce.

Studies conducted in 2008 and 2009 on domestic pig carcasses (*Sus scrofa domesticus*) were performed to analyse the physical and chemical characteristics of soil surface during decomposition [9]. The authors evaluated inorganic characteristics such as pH, and fatty acid content of soil samples. The equipment used for the analysis was the GC-MS. The trimethylsilyl fatty acid esters identified were C14:0, C16:0, C16:1, C18:0 and C18:1. These compounds were detected in the soil due to decomposition fluid that emanate from the pig carcasses. Being C18:1 the most abundant fatty acid in adipose tissue [24], it was expected that this was the compound found in higher quantities. However, the most abundant fatty acid detected was C16:0 followed by C18:0. This can be explained by the occurrence of anaerobic putrefactive process within the pig carcasses. This study also evaluated soil intervals throughout the decomposition process. For this, several soil samples were collected from 0 cm up to 50 cm from the soil-carcass boundary. As expected, the higher quantities of fatty acids were detected at 0 cm, since it was the region closest to the body.

In 2017, von der Lühe et al. [15] studied the changes of fatty acids from soil samples collected underneath a decomposing human body. One of the most relevant conclusions of this study was the demonstration that human derived fatty acids were still preserved in the soil one year after the body's removal. Besides this, it has been proved that fatty acids derived from the human adipose tissue can be used as indicators of the presence of decomposition fluid.

2.2. Steroids

2.2.1. *Description of the compound*

Sterols are structural lipids present in the membranes of most eukaryotic cells and are composed of four joined carbon rings (Figure 3). These compounds, in addition to structural functions, are also the origin of products with relevant and specific biological activity. Cholesterol is known to be the main sterol in animal tissues, and it is a precursor of a wide range of steroids, such as bile acids, sexual hormones, and adrenocorticoids. This molecule has a structure formed by a polycyclic ring skeleton with 27 carbon atoms. Intestinal cholesterol can either be absorbed by the individual or suffer conversion to other metabolites by the microbiota. One of the main metabolites of that conversion is coprostanol which is unabsorbable and therefore is excreted in the faeces. In vertebrates, most of the cholesterol synthesis occurs in the liver. From this organ a fluid is exported designated as bile, which is mainly composed of bile acids and their salts. Bile acids are particularly important in the digestive process as they help to convert large fat particles into smaller structures [11].

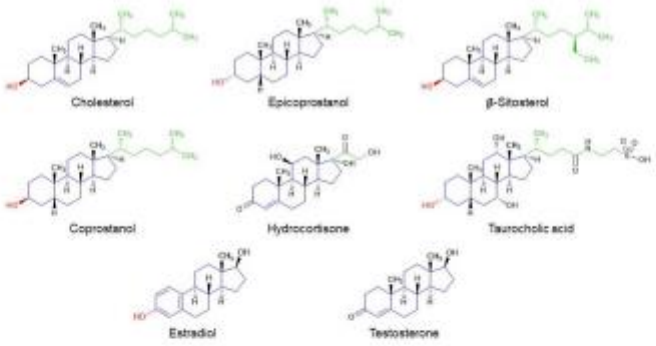

Figure 3: Chemical structure of sterols (the hydroxyl group, steroid nucleus, and hydrocarbon side chain are highlighted in red, blue, and green, respectively).

2.2.2. *Applicability in forensic investigations*

2.2.2.1. Identification of steroids in adipocere studies

In a study conducted in 1997, and described earlier, the authors analysed the adipocere samples using GC-MS [15]. From this analysis, they were able to identify and quantify

lipids present in samples, documenting for the first time the presence of epicoprostanol in adipocere which is a coprostanol isomer (Figure 4).

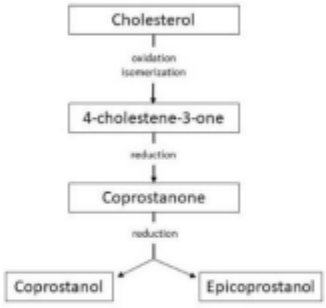

Figure 4: Scheme of the formation of epicoprostanol [15].

The first step of the conversion of cholesterol to coprostanol is the oxidation of cholesterol to 4-cholestene-3-one which then suffers reduction reactions, being converted to coprostanone. Coprostanone reduction leads to the formation of coprostanol and its isomer, epicoprostanol [16]. Adachi et al. [15] discovery is particularly interesting since epicoprostanol is not present in the normal adipose tissue. Once this was the only metabolite of cholesterol found in samples, this could mean that epicoprostanol is more easily produced in adipocere. The ratio of epicoprostanol:cholesterol increased with immersion time, showing that epicoprostanol analysis can be useful to determine PMI. However, since epicoprostanol is present in the intestinal content of humans, its presence in the seawater samples could be explained by sewage-contaminated sediments.

2.2.2.2. Identification of steroids in soils

In 2013, a research was conducted in order to analyse typical animal sterols from grave soil and identify potential biomarkers of decomposition fluids [25]. The authors collected soil samples below four buried domestic pig carcasses (*Sus scrofa domesticus*) from shallow graves. Two of the carcasses were exhumed after three months, and the other two were exhumed six months after the former. Sample collection was performed beneath the head, torso, and rear of the pig. Wall samples from the grave were also collected. After soil characterisation, sterols were extracted from samples. The results obtained showed that higher concentrations of cholesterol and coprostanol were detected in the graves of the first two exhumed pigs. This can be explained with putrefaction beginning after three months of burial: the decomposition phase in which the body releases higher amounts of

carbohydrates, lipids, and proteins. Given that cholesterol is present in human and non-human animal cells, its detection in soil can be related to body decomposition and used as a biomarker for the detection of burial sites. Similarly, coprostanol may also be a reliable biomarker; however, the fact that coprostanol can be also present in animal faeces needs to be considered. The presence of β-sitosterol (one of the main plant sterols) was also detected. Its high concentrations can be due to the dietary behaviour of pigs, since this compound was found in samples collected beneath them, possibly coming from gut content. Notwithstanding, this compound could also have come from roots or plant litter. Authors referred the importance of performing new studies and suggested the analysis of soil properties and its association with the body decomposition.

A more recent study was developed in 2018 [10], in a real forensic case, where concentrations of tissue and faecal steroids in soil beneath a human decomposing body were determined. Samples were collected immediately after the cadaver's removal and one year later. Given that steroids can derive from various sources besides the human activity, reference samples were also collected using them to distinguish the compounds source [26]. Steroids were extracted and then analysed by GC-MS. Despite the translocation and transformation influence on the steroids' quantity, one year after the removal of the corpse they continued high. This study demonstrated that if the analysis of steroids is performed and all the variables are considered, these types of compounds can be a relevant biomarker to forensic soil analysis, since its presence can be indicative of decomposition.

In 2020, von der Lühe et al. [27] published an article in which they presented the study of soil samples recovered from temporary mass graves of World War II. This study reenforced the idea of the utility of using steroids as biomarkers for human decomposition fluids. For this, the authors did a geological and historical study of the place where the graves were located and used GC-MS to analyse soil samples. However, given the human impact for sixty-seven years, the natural distribution of the steroids was disturbed. The presence of human decomposition fluids was confirmed with the detection of coprostanol and epicoprostanol.

Other class of compounds with increasing interest in forensic context are bile acids. These are a group of C24, C27 and C28 steroidal acids produced by the digestive system of animals [26]. Bile acids, and its respective salts, are synthetised in the liver and derived from cholesterol. These hydrophilic compounds act in the intestine converting big fat

particles into small micelles, increasing the interaction surface with the digestive enzymes [11]. In mammals, these lipids present a C24 structure which means they have twenty-four carbon atoms forming a steroid nucleus and a five-carbon side chain with a carboxyl group [28]. These compounds are usually used in study of soils with pollution detection purposes but given its origin, it may be relevant for forensic investigations to keep studying this type of lipids in forensic contexts in order to provide helpful information to investigations [29, 30]. In 2012, Birk et al. [29] quantified faecal sterols, stanols, stanones, and bile acids in terrestrial sediments. In their work, the authors were able to purify the compounds and develop a method, using the GC-MS technique. In 2020, von der Lühe et al. [27] detected bile acids - litholic acid (LCA) and deoxycholic acid (DCA) - in high concentrations on samples from mass graves. However, they were also detected in reference samples probably due to animal activity. Bile acids can be a very useful biomarker in soils given that these compounds can only be produced by vertebrates and are more stable in soils than stanols and sterols. Notwithstanding, more studies need to be performed since the data regarding its natural distribution is scarce.

With the combined information on these compounds, fatty acids, and/or sterols, investigators can answer several relevant questions regarding the presence of decomposition fluid. Besides that, analysing compounds like sterols, stanols, stanones, and bile acids can also be relevant to study the diet of the individual by analysing the faecal material [30].

3. Conclusion and future perspectives

Given that corpses can be (and often are) exposed on soil or buried within it, the study of this geological matrix is of utmost importance.

Fatty acids and sterols can be detected in soil samples over the years following deposition in soil, making them possibly useful PMI biomarkers, or to act as evidence of the presence of a decomposing body at one time in the past. However, it is still not possible to define a precise timescale of a body decomposition process using biomarkers. The diversity of factors involved makes it difficult to predict the behaviour of the decomposition mechanism with any degree of confidence, and therefore it is important to develop new studies to analyse and test this phenomenon. More studies regarding the presence of sterols and bile acids in soils must be conducted since its origin may be faecal and not exclusively from human decomposition. Besides more studies regarding the compounds

present in soil during decomposition, it is also of upmost importance to improve the study of soil by widening the investigation of soil type and features in several areas of the countries.

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Appendix 2.

Queirós S. S., et al. 2022. Poster communication at the V Congresso da Associação Portuguesa de Ciências Forenses/ XIV Jornadas Científicas de Ciências do Instituto Universitário de Ciências da Saúde/ I Congresso ToxRun.

POSTER

IDENTIFICATION AND QUANTIFICATION OF ADIPOCERE FROM HUMAN GRAVE SOILS AND ITS FORENSIC APPLICATION

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Introduction: Human body degradation on soil leads to the release and accumulation of a wide series of decomposition products. Their analysis allows to establish a temporal relationship between compounds found in soil and different stages of decomposition, helping determine the *post-mortem* interval [1]. In addition, the identification of these components in soil may be a strong indicator that a human body had been buried in a particular location [1, 2].

Aims: As such, we herein aimed to review the main organic compounds deriving from human decomposition, already identified in soils.

Materials and Methods: The terms "soil chemistry", "human decomposition" and "adipocere" were searched in PubMed (US National Library of Medicine), without time limitation.

Results: The autolysis processes occurring during cadaveric decomposition hydrolyse adipose tissue; if the environment is humid and hot, adipocere, a mixture of saturated (myristic, palmitic and stearic) and unsaturated fatty acids (oleic and palmitoleic) and triglycerides, can be formed [3]. Identifying and quantifying these lipidic compounds, enables an association with the different decomposition stages. The most frequently used identification and quantification technique of adipocere is Gas Chromatography coupled

with Mass Spectrometry [3]. Normally, in advanced human decomposition stages, adipocere is present in greater abundances. Different body parts have different predominance of adipocere, with the abdominal area showing a higher lipid concentration, justified by the greater volume of adipocytes in this region [4]. The knowledge of adipocere compounds present in soil can also make possible to test the possible movement of a body, since the state of decomposition must agree with the existing compounds in the place where it is found. Additionally, adipocere may also contribute to the preservation of evidence, possibly helping ascertain the cause of death [4]. Other compounds such as steroids can also be identified and quantified in soils, aiding forensic investigations [1]. Moreover, intrinsic and extrinsic factors affecting decomposition should be considered [5].

Conclusion: The existence of adipocere in soil may contribute to establish the *post-mortem* interval, simultaneously indicating that a certain soil was possibly in contact with a decomposing body. However, the exact composition of adipocere remains to be studied.

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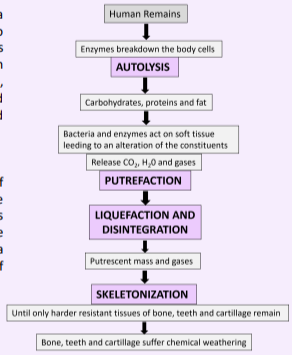
INTRODUCTION

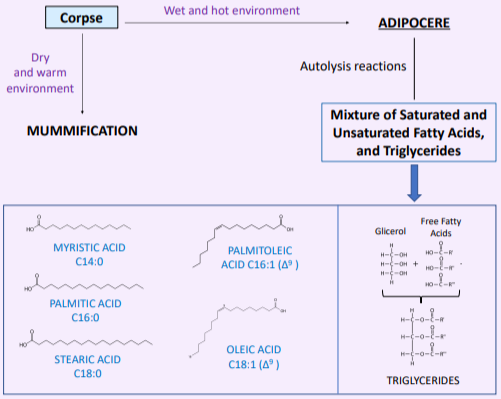
Soils are structurally complex, with organic and inorganic compounds. Due to their porous matrix, soils retain information of high scientific interest, namely in the area of Forensic Sciences. When a body is decomposing in soil, the products resulting from the putrefaction process are released. By analysing soil from a place where there is a decomposing body, it is possible to identify and quantify that products. The identification of these compounds may allow establishing temporal relationships between the compounds found, their concentration and the stages of the deterioration process [1]. In addition, the compounds found might be relevant in cases of suspected body´s transfer from one place to another, as they may be indicative of whether or not a decomposing body was in that soil. Soil is, therefore, a matrix with high forensic interest that can help criminal investigations.

BODY DECOMPOSITION AND SOIL

Cadaveric decomposition is a continuous process that leads to the phased release of various compounds resultant from processes of autolysis, putrefaction, liquefaction and disintegration (scheme adapted from [2]).



Within specific conditions of temperature and humidity, the body can undergo changes leading to mummification or the formation of adipocere, as a result of the degradation of adipose tissue [3].



Fatty acids (in blue) that are usually present in greater amounts in soil where a decomposing human body is present [4]. From the displayed fatty acids, the one found in greater quantity is Palmitic Acid, followed by Stearic and Myristic.

FACTORS AFFECTING DECOMPOSITION [2]

Intrinsic	Extrinsic
• Body size	• Mineralogy and soil's texture
• Age	• Soil's microbial activity
• Cause of death	• Temperature
• Presence of wounds/traumas	• Soil's pH
	• Soil's moisture content
	• Coffin's material
	• Clothing

IDENTIFYING AND QUANTIFYING BODY RELEASED COMPOUNDS

Decomposition involving adipocere can lead to the deposition of substances in soil, which can be preserved for several years. By collecting soil for analysis, it is possible to identify and quantify these compounds, as well as to associate them with decomposition.
In addition to the fatty acids that are strong indicators of adipocere (in blue), other compounds can also be studied and analysed. Sterols are a class of compounds that can be identified in soil and associated with cadaveric degradation processes. However, the possibility that they may come from animal or plant activity must be considered.
In 2018 [1], a study showed that the found steroids are suitable for associating cadaveric decomposition with a given location.

How can we identify and quantify compounds from the soil?

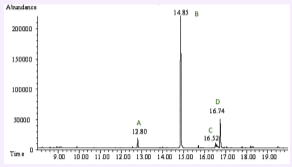
- There are several techniques that are used to analyse soils, such as Infrared Spectroscopy (FTIR) and Gas Chromatography Coupled with Mass Spectrometry (GC-MS). However, we must consider that, in this type of studies, sample amounts are often vestigial and, as such, a FTIR analysis may not prove to be efficient [5]. Thus, currently, the most used technique is **GC-MS**.

Soil sample (properly treated) is injected
⇩
The chromatography column separates the compounds
⇩
Mass spectrometry generates a characteristic spectrum of each substance allowing its identification and quantification

Spectrum example [5] obtained from GC-MS:

The following compounds were identified:

A – Myristic Acid
B – Palmitoleic Acid
C – Oleic Acid
D – Stearic Acid



CONCLUSIONS

The presence of adipocere in soils can be a strong indicator of cadaveric decomposition and, by determining and quantifying the compounds from the decomposition process, it is possible to establish a temporal relationship. In addition, by identifying the lipid profile of the soil, it is also possible to assess whether a certain questioned sample comes from a place with a decomposing body or not. However, the behaviour and exact composition of adipocere remain to be studied. It is necessary to continue to analyse the compounds that result from different phases of cadaveric degradation, as well as their distribution through the soil and their concentration over time. In addition, it is advisable to consider all factors that can increase or decrease the rate of decomposition of a body so that it is possible, in the future, to more accurately assist criminal investigation.

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CERTIFICADO **COMUNICAÇÃO EM POSTER**

Sara Sousa Queirós, Barbara von der Lühe, Inês Morais Caldas, Lorna Dawson, Áurea Madureira-Carvalho

Apresentou o trabalho intitulado: *IDENTIFICATION AND QUANTIFICATION OF ADIPOCERE FROM HUMAN GRAVE SOILS AND ITS FORENSIC APPLICATION*

I CONGRESSO INTERNACIONAL DA **TOXRUN**

V CONGRESSO DA **ASSOCIAÇÃO PORTUGUESA DE CIÊNCIAS FORENSES**

XIV JORNADAS CIENTÍFICAS DE CIÊNCIAS DO **INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE**

7 e 8 de abril de 2022

Pela organização

Ricardo Dinis-Oliveira

CESPU INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE

TOXRUN TOXICOLOGY RESEARCH UNIT

APCF ASSOCIAÇÃO PORTUGUESA DE CIÊNCIAS FORENSES

Appendix 3.

Favourable endorsement from the Ethics Committee of the Instituto Universitário de Ciências da Saúde (IUCS) to the research project entitled "Characterisation of Organic and Inorganic Constituents in Cemetery soils and its Application as Forensic Evidence".

CESPU
INSTITUTO UNIVERSITÁRIO
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Comissão de Ética

Exma. Senhora Investigadora
Prof. Doutora Áurea Marília Madureira e Carvalho

N/Ref.ª: CE/IUCS/CESPU-03/21 **Data:** 2021/abril/09

Assunto: - **Parecer relativo ao Projeto de Investigação:** 3/CE-IUCS/2021
- **Título do Projeto:** *"Characterisation of Organic and Inorganic Constituents in Cemetery soils and its Application as Forensic Evidence"*
- **Investigador responsável:** Prof Doutora Áurea Marília Madureira e Carvalho

Exma. Senhora,

Informo V. Exa. que o projeto supracitado foi analisado na reunião da Comissão de Ética do IUCS, da CESPU, CrI, no dia 08/04/2021.

A Comissão de Ética emitiu um parecer **favorável** à realização do projeto tal como apresentado.

Com os melhores cumprimentos.

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Presidente da Comissão de Ética do IUCS

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(ANTERIOR INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE – NORTE)
DENOMINAÇÃO E RECONHECIMENTO DE INTERESSE PÚBLICO ALTERADOS PELO DECRETO-LEI Nº 57/2015, DE 20-04
RUA CENTRAL DE GANDRA, 1317 . 4585 116 . GANDRA PRD . T.:+351 224 157 100 . F.:+351 224 157 101
CESPU – COOPERATIVA DE ENSINO SUPERIOR, POLITÉCNICO E UNIVERSITÁRIO, CRL
CONTR: 501 577 840 . CAP. SOCIAL 1.250.000.00 EUR . MAT.CONS. R. C. PORTO Nº 216 . WWW.CESPU.PT

Appendix 4.

Soil samples aimed to be collected during all exhumations, under the corpse and at the top, right, bottom and left grave walls, that were not laboratory analysed (cm = centimetres; CMA = Monte D'Arcos Cemetery; CCM = Castelo de Mértola Cemetery; CPR = Prado do Repouso Cemetery; H = head; T = thorax; A = abdomen; G = gluteal region; RT =right thigh; LT =left thigh; F =feet; W= wall; TG = top grave; RG = right grave; BG = bottom grave; LG = left grave).

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
2	CMA	Head	0-5	2H5	Yes
			5-10	2H10	Yes
			10-15	2H15	Yes
			15-20	2H20	Yes
		Thorax	0-5	2T5	Yes
			5-10	2T10	Yes
			10-15	2T15	Yes
			15-20	2T20	Yes
		Abdomen	0-5	2A5	Yes
			5-10	2A10	Yes
			10-15	2A15	Yes
			15-20	2A20	Yes
		Gluteal Region	0-5	2G5	Yes
			5-10	2G10	Yes
			10-15	2G15	Yes
			15-20	2G20	No
		Right Thigh	0-5	2RT5	Yes
			5-10	2RT10	Yes
			10-15	2RT15	Yes
			15-20	2RT20	Yes
		Left Thigh	0-5	2LT5	Yes
			5-10	2LT10	Yes
			10-15	2LT15	Yes
			15-20	2LT	Yes
		Feet	0-5	2F5	Yes
			5-10	2F10	Yes
			10-15	2F15	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			15-20	2F20	Yes
		Top Wall	0-5	W2TG5	Yes
			5-10	W2TG10	No
			10-15	W2TG15	No
			15-20	W2TG20	No
		Bottom Wall	0-5	W2BG5	Yes
			5-10	W2BG10	Yes
			10-15	W2BG15	No
			15-20	W2BG20	No
		Left Wall	0-5	W2LG5	Yes
			5-10	W2LG10	No
			10-15	W2LG15	No
			15-20	W2LG20	No
		Right Wall	0-5	W2RG5	Yes
			5-10	W2RG10	No
			10-15	W2RG15	No
			15-20	W2RG20	No
3	CCM	Head	10-15	3H15	No
			15-20	3H20	No
		Thorax	10-15	3T15	Yes
			15-20	3T20	Yes
		Abdomen	10-15	3A15	No
			15-20	3A20	No
		Gluteal Region	10-15	3G15	Yes
			15-20	3G20	No
		Right Thigh	10-15	3RT15	No
			15-20	3RT20	No
		Top Wall	0-5	W3TG5	Yes
			5-10	W3TG10	Yes
			10-15	W3TG15	No
			15-20	W3TG20	No
		Bottom Wall	0-5	W3BG5	Yes
			5-10	W3BG10	Yes
			10-15	W3BG15	No
			15-20	W3BG20	No

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
		Left Wall	0-5	W3LG5	Yes
			5-10	W3LG10	Yes
			10-15	W3LG15	No
			15-20	W3LG20	No
		Right Wall	0-5	W3RG5	Yes
			5-10	W3RG10	Yes
			10-15	W3RG15	No
			15-20	W3RG20	No
4	CCM	Head	0-5	4H5	Yes
			5-10	4H10	Yes
			10-15	4H15	Yes
			15-20	4H20	No
		Thorax	0-5	4T5	Yes
			5-10	4T10	Yes
			10-15	4T15	Yes
			15-20	4T20	No
		Abdomen	0-5	4A5	Yes
			5-10	4A10	Yes
			10-15	4A15	Yes
			15-20	4A20	No
		Gluteal Region	0-5	4G5	Yes
			5-10	4G10	No
			10-15	4G15	No
			15-20	4G20	No
		Right Thigh	0-5	4RT5	Yes
			5-10	4RT10	Yes
			10-15	4RT15	Yes
			15-20	4RT20	No
		Left Thigh	0-5	4LT5	Yes
			5-10	4LT10	Yes
			10-15	4LT15	Yes
			15-20	4LT20	No
		Feet	0-5	4F5	Yes
			5-10	4F10	Yes
			10-15	4F15	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			15-20	4F20	Yes
		Top Wall	0-5	W4TG5	Yes
			5-10	W4TG10	Yes
			10-15	W4TG15	Yes
			15-20	W4TG20	Yes
		Bottom Wall	0-5	W4BG5	Yes
			5-10	W4BG10	No
			10-15	W4BG15	No
			15-20	W4BG20	No
		Left Wall	0-5	W4LG5	Yes
			5-10	W4LG10	Yes
			10-15	W4LG15	Yes
			15-20	W4LG20	No
		Right Wall	0-5	W4RG5	Yes
			5-10	W4RG10	Yes
			10-15	W4RG15	Yes
			15-20	W4RG20	No
5	CPR	Head	0-5	5H5	Yes
			5-10	5H10	Yes
			10-15	5H15	Yes
			15-20	5H20	No
		Thorax	0-5	5T5	Yes
			5-10	5T10	Yes
			10-15	5T15	No
			15-20	5T20	No
		Abdomen	0-5	5A5	Yes
			5-10	5A10	Yes
			10-15	5A15	Yes
			15-20	5A20	No
		Gluteal Region	0-5	5G5	Yes
			5-10	5G10	Yes
			10-15	5G15	No
			15-20	5G20	No
		Right Thigh	0-5	5RT5	Yes
			5-10	5RT10	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			10-15	5RT15	No
			15-20	5RT20	No
		Left Thigh	0-5	5LT5	Yes
			5-10	5LT10	Yes
			10-15	5LT15	No
			15-20	5LT	No
		Feet	0-5	5F5	Yes
			5-10	5F10	Yes
			10-15	5F15	No
			15-20	5F20	No
		Top Wall	0-5	W5TG5	No
			5-10	W5TG10	No
			10-15	W5TG15	No
			15-20	W5TG20	No
		Bottom Wall	0-5	W5BG5	No
			5-10	W5BG10	No
			10-15	W5BG15	No
			15-20	W5BG20	No
		Left Wall	0-5	W5LG5	Yes
			5-10	W5LG10	Yes
			10-15	W5LG15	No
			15-20	W5LG20	No
		Right Wall	0-5	W5RG5	Yes
			5-10	W5RG10	No
			10-15	W5RG15	No
			15-20	W5RG20	No
6	CMA	Head	0-5	6H5	Yes
			5-10	6H10	Yes
			10-15	6H15	Yes
			15-20	6H20	No
		Thorax	0-5	6T5	Yes
			5-10	6T10	Yes
			10-15	6T15	Yes
			15-20	6T20	No
		Abdomen	0-5	6A5	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			5-10	6A10	Yes
			10-15	6A15	Yes
			15-20	6A20	No
		Gluteal Region	0-5	6G5	Yes
			5-10	6G10	Yes
			10-15	6G15	Yes
			15-20	6G20	Yes
		Right Thigh	0-5	6RT5	Yes
			5-10	6RT10	Yes
			10-15	6RT15	Yes
			15-20	6RT20	No
		Left Thigh	0-5	6LT5	Yes
			5-10	6LT10	Yes
			10-15	6LT15	Yes
			15-20	6LT	Yes
		Feet	0-5	6F5	Yes
			5-10	6F10	Yes
			10-15	6F15	Yes
			15-20	6F20	Yes
		Top Wall	0-5	W6TG5	Yes
			5-10	W6TG10	Yes
			10-15	W6TG15	No
			15-20	W6TG20	No
		Bottom Wall	0-5	W6BG5	Yes
			5-10	W6BG10	Yes
			10-15	W6BG15	No
			15-20	W6BG20	No
		Left Wall	0-5	W6LG5	Yes
			5-10	W6LG10	No
			10-15	W6LG15	No
			15-20	W6LG20	No
		Right Wall	0-5	W6RG5	Yes
			5-10	W6RG10	Yes
			10-15	W6RG15	No
			15-20	W6RG20	No

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
7	CMA	Head	10-15	7H15	Yes
			15-20	7H20	Yes
		Thorax	10-15	7T15	Yes
			15-20	7T20	No
		Abdomen	10-15	7A15	Yes
			15-20	7A20	Yes
		Gluteal Region	10-15	7G15	Yes
			15-20	7G20	Yes
		Right Thigh	10-15	7RT15	Yes
			15-20	7RT20	Yes
		Left Thigh	0-5	7LT5	Yes
			5-10	7LT10	Yes
			10-15	7LT15	Yes
			15-20	7LT20	Yes
		Feet	0-5	7F5	Yes
			5-10	7F10	Yes
			10-15	7F5	Yes
			15-20	7F20	Yes
		Top Wall	0-5	W7TG5	Yes
			5-10	W7TG10	Yes
			10-15	W7TG15	Yes
			15-20	W7TG20	Yes
		Bottom Wall	0-5	W7BG5	Yes
			5-10	W7BG10	Yes
			10-15	W7BG15	Yes
			15-20	W7BG20	No
		Left Wall	0-5	W7LG5	Yes
			5-10	W7LG10	Yes
			10-15	W7LG15	Yes
			15-20	W7LG20	Yes
		Right Wall	0-5	W7RG5	Yes
			5-10	W7RG10	Yes
			10-15	W7RG15	Yes
			15-20	W7RG20	No
8	CMA	Head	0-5	8H5	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			5-10	8H10	Yes
			10-15	8H15	Yes
			15-20	8H20	No
		Thorax	0-5	8T5	Yes
			5-10	8T10	Yes
			10-15	8T15	Yes
			15-20	8T20	Yes
		Abdomen	0-5	8A5	Yes
			5-10	8A10	Yes
			10-15	8A15	Yes
			15-20	8A20	Yes
		Gluteal Region	0-5	8G5	Yes
			5-10	8G10	Yes
			10-15	8G15	Yes
			15-20	8G20	No
		Right Thigh	0-5	8RT5	Yes
			5-10	8RT10	Yes
			10-15	8RT15	Yes
			15-20	8RT20	No
		Left Thigh	0-5	8LT5	Yes
			5-10	8LT10	Yes
			10-15	8LT15	Yes
			15-20	8LT20	No
		Feet	0-5	8F5	Yes
			5-10	8F10	Yes
			10-15	8F15	Yes
			15-20	8F20	Yes
		Top Wall	0-5	W8TG5	Yes
			5-10	W8TG10	Yes
			10-15	W8TG15	Yes
			15-20	W8TG20	Yes
		Bottom Wall	0-5	W8BG5	Yes
			5-10	W8BG10	Yes
			10-15	W8BG15	Yes
			15-20	W8BG20	No

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
		Left Wall	0-5	W8LG5	Yes
			5-10	W8LG10	Yes
			10-15	W8LG15	Yes
			15-20	W8LG20	No
		Right Wall	0-5	W8RG5	Yes
			5-10	W8RG10	Yes
			10-15	W8RG15	Yes
			15-20	W8RG20	Yes
9	CMA	Head	0-5	9H5	Yes
			5-10	9H10	Yes
			10-15	9H15	Yes
			15-20	9H20	Yes
		Thorax	0-5	9T5	Yes
			5-10	9T10	Yes
			10-15	9T15	Yes
			15-20	9T20	Yes
		Abdomen	0-5	9A5	Yes
			5-10	9A10	Yes
			10-15	9A15	Yes
			15-20	9A20	Yes
		Gluteal Region	0-5	9G5	Yes
			5-10	9G10	Yes
			10-15	9G15	Yes
			15-20	9G20	Yes
		Right Thigh	0-5	9RT5	Yes
			5-10	9RT10	Yes
			10-15	9RT15	Yes
			15-20	9RT20	Yes
		Left Thigh	0-5	9LT5	Yes
			5-10	9LT10	Yes
			10-15	9LT15	Yes
			15-20	9LT20	Yes
		Feet	0-5	9F5	Yes
			5-10	9F10	Yes
			10-15	9F15	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			15-20	9F20	No
		Top Wall	0-5	W9TG5	Yes
			5-10	W9TG10	Yes
			10-15	W9TG15	Yes
			15-20	W9TG20	No
		Bottom Wall	0-5	W9BG5	Yes
			5-10	W9BG10	Yes
			10-15	W9BG15	Yes
			15-20	W9BG20	Yes
		Left Wall	0-5	W9LG5	Yes
			5-10	W9LG10	Yes
			10-15	W9LG15	Yes
			15-20	W9LG20	No
		Right Wall	0-5	W9RG5	Yes
			5-10	W9RG10	Yes
			10-15	W9RG15	Yes
			15-20	W9RG20	Yes
10	CMA	Head	10-15	10H15	Yes
			15-20	10H20	Yes
		Thorax	10-15	10T15	Yes
			15-20	10T20	Yes
		Abdomen	10-15	10A15	Yes
			15-20	10A20	Yes
		Gluteal Region	10-15	10G15	Yes
			15-20	10G20	Yes
		Right Thigh	10-15	10RT15	Yes
			15-20	10RT20	Yes
		Left Thigh	0-5	10LT5	Yes
			5-10	10LT10	Yes
			10-15	10LT15	Yes
			15-20	10LT	Yes
		Feet	0-5	10F5	Yes
			5-10	10F10	Yes
			10-15	10F15	Yes
			15-20	10F20	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
		Top Wall	0-5	W10TG5	Yes
			5-10	W10TG10	Yes
			10-15	W10TG15	Yes
			15-20	W10TG20	Yes
		Bottom Wall	0-5	W10BG5	Yes
			5-10	W10BG10	Yes
			10-15	W10BG15	Yes
			15-20	W10BG20	Yes
		Left Wall	0-5	W10LG5	Yes
			5-10	W10LG10	Yes
			10-15	W10LG15	Yes
			15-20	W10LG20	No
		Right Wall	0-5	W10RG5	Yes
			5-10	W10RG10	Yes
			10-15	W10RG15	Yes
			15-20	W10RG20	No
11	CPR	Head	0-5	11H5	Yes
			5-10	11H10	Yes
			10-15	11H15	No
			15-20	11H20	No
		Thorax	0-5	11T5	Yes
			5-10	11T10	Yes
			10-15	11T15	Yes
			15-20	11T20	Yes
		Abdomen	0-5	11A5	Yes
			5-10	11A10	Yes
			10-15	11A15	Yes
			15-20	11A20	Yes
		Gluteal Region	0-5	11G5	Yes
			5-10	11G10	Yes
			10-15	11G15	Yes
			15-20	11G20	No
		Right Thigh	0-5	11RT5	Yes
			5-10	11RT10	Yes
			10-15	11RT15	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
			15-20	11RT20	Yes
		Left Thigh	0-5	11LT5	Yes
			5-10	11LT10	Yes
			10-15	11LT15	Yes
			15-20	11LT	Yes
		Feet	0-5	11F5	Yes
			5-10	11F10	Yes
			10-15	11F15	Yes
			15-20	11F20	Yes
		Top Wall	0-5	W11TG5	Yes
			5-10	W11TG10	No
			10-15	W11TG15	No
			15-20	W11TG20	No
		Bottom Wall	0-5	W11BG5	Yes
			5-10	W11BG10	No
			10-15	W11BG15	No
			15-20	W11BG20	No
		Left Wall	0-5	W11LG5	Yes
			5-10	W11LG10	No
			10-15	W11LG15	No
			15-20	W11LG20	No
		Right Wall	0-5	W11RG5	Yes
			5-10	W11RG10	No
			10-15	W11RG15	No
			15-20	W11RG20	No
12	CMA	Head	10-15	12H15	Yes
			15-20	12H20	Yes
		Thorax	10-15	12T15	Yes
			15-20	12T20	Yes
		Abdomen	10-15	12A15	Yes
			15-20	12A20	Yes
		Gluteal Region	10-15	12G15	Yes
			15-20	12G20	Yes
		Right Thigh	10-15	12RT15	Yes
			15-20	12RT20	Yes

(Appendix 4 Continued)

Exhumation number	Cemetery	Sample origin	Depth (cm)	Sample labelling	Collection performed
		Left Thigh	0-5	12LT5	Yes
			5-10	12LT10	Yes
			10-15	12LT15	Yes
			15-20	12LT	Yes
		Feet	0-5	12F5	Yes
			5-10	12F10	Yes
			10-15	12F15	Yes
			15-20	12F20	Yes
		Top Wall	0-5	W12TG5	Yes
			5-10	W12TG10	Yes
			10-15	W12TG15	Yes
			15-20	W12TG20	No
		Bottom Wall	0-5	W12BG5	Yes
			5-10	W12BG10	Yes
			10-15	W12BG15	Yes
			15-20	W12BG20	Yes
		Left Wall	0-5	W12LG5	Yes
			5-10	W12LG10	Yes
			10-15	W12LG15	Yes
			15-20	W12LG20	No
		Right Wall	0-5	W12RG5	Yes
			5-10	W12RG10	Yes
			15-20	W12RG15	Yes
			20	W12RG20	No

Appendix 5.

Control soil samples from Monte D'Arcos Cemetery (CMA) and Castelo de Mértola Cemetery (CCM) (DD = day; MM = month; YYYY = year; m = metre; cm = centimetre; L = local; C = control).

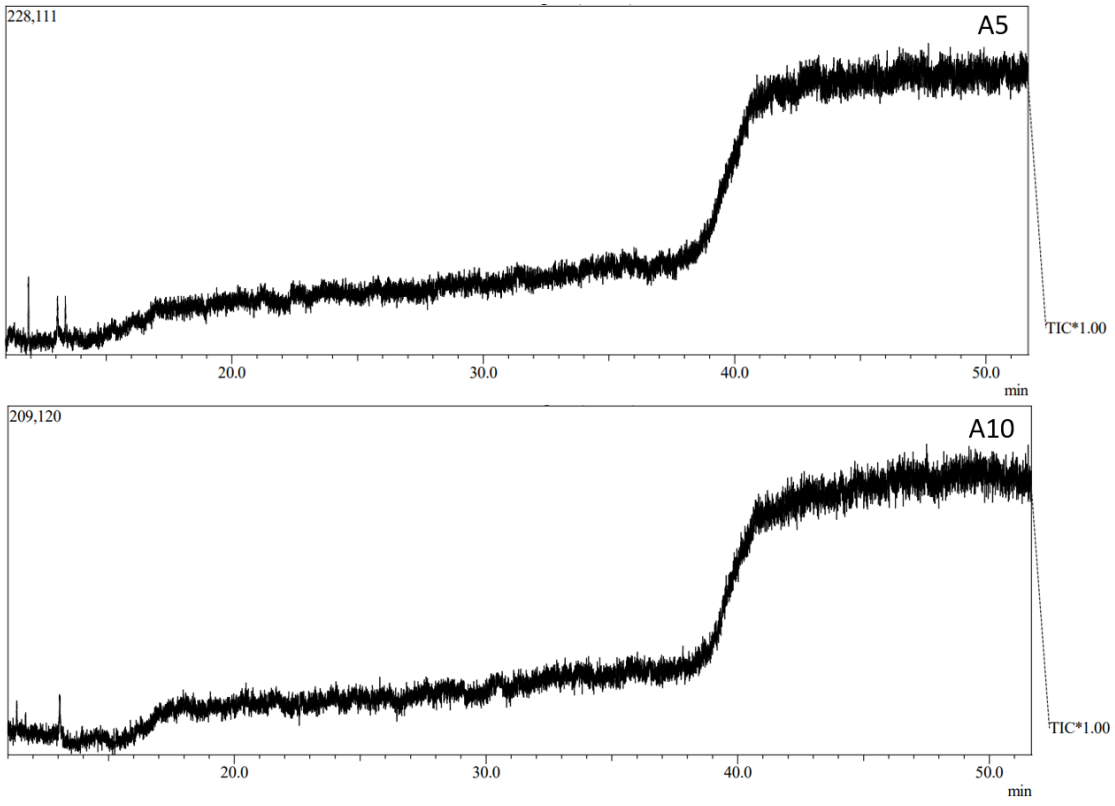
Cemetery	Collection date (DD/MM/YYYY)	Local	Distance to the nearest grave (m)	Depth (cm)	Sample labelling	Sample collecion
CMA	09/03/2021	1	15	5	L1/C2/15/5	Yes
				10	L1/C2/15/10	Yes
				15	L1/C2/15/15	Yes
				20	L1/C2/15/20	No
		2	15	5	L2/C2/15/5	Yes
				10	L2/C2/15/10	Yes
				15	L2/C2/15/15	Yes
				20	L2/C2/15/20	Yes
		3	15	5	L3/C2/15/5	Yes
				10	L3/C2/15/10	Yes
				15	L3/C2/15/15	No
				20	L3/C2/15/20	No
		4	15	5	L4/C2/15/5	Yes
				10	L4/C2/15/10	Yes
				15	L4/C2/15/15	No
				20	L4/C2/15/20	No
CCM	22/03/2021	1	1	5	L1/C4/1/5	Yes
				10	L1/C4/1/10	Yes
				15	L1/C4/1/15	No
				20	L1/C4/1/20	No
		2	5.5	5	L2/C4/5.5/5	Yes
				10	L2/C4/5.5/10	Yes
				15	L2/C4/5.5/15	No
				20	L2/C4/5.5/20	No
		3	8	5	L3/C4/8/5	Yes
				10	L3/C4/8/10	Yes
				15	L3/C4/8/15	Yes
				20	L3/C4/8/20	No

Appendix 6.

Chromatograms obtained from the analysis of the FAME fraction (Fatty acid methyl ester) of the soil samples collected from exhumation 12 where no compounds were identified (abdomen, gluteal region and right thigh, all from 0-5 centimetre and 5-10 centimetre).

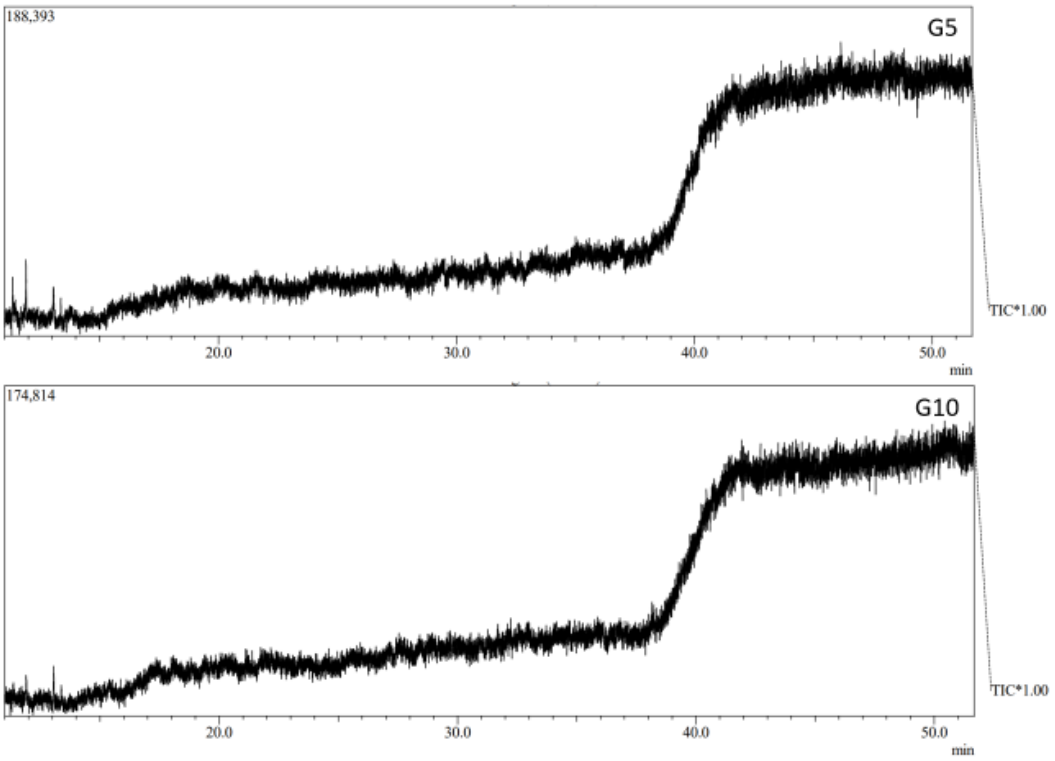
6.1

Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the abdomen of the individual from the exhumation 12 (Monte D'Arcos Cemetery), at 0-5 cm (A5) and 5-10 cm (A10) soil depth.



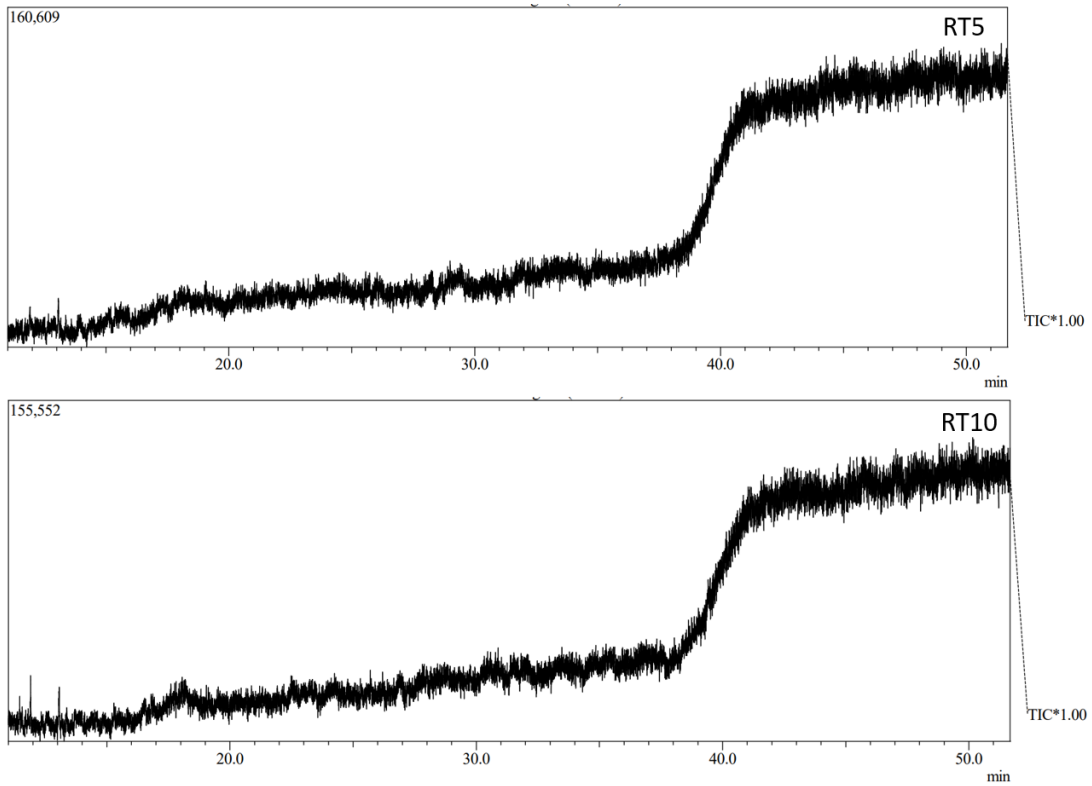
6.2

Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the gluteal region of the individual from the exhumation 12 (Monte D'Arcos Cemetery), at 0-5 cm (G5) and 5-10 cm (G10) soil depth.



6.3

Fatty Acid Methyl Ester (FAME) gas chromatogram coupled with mass spectroscopy, obtained from the grave inner soil samples collected under the right thigh of the individual from the exhumation 10 (Monte D'Arcos Cemetery), at 0-5 cm (RT5) and 5-10 cm (RT10) soil depth.

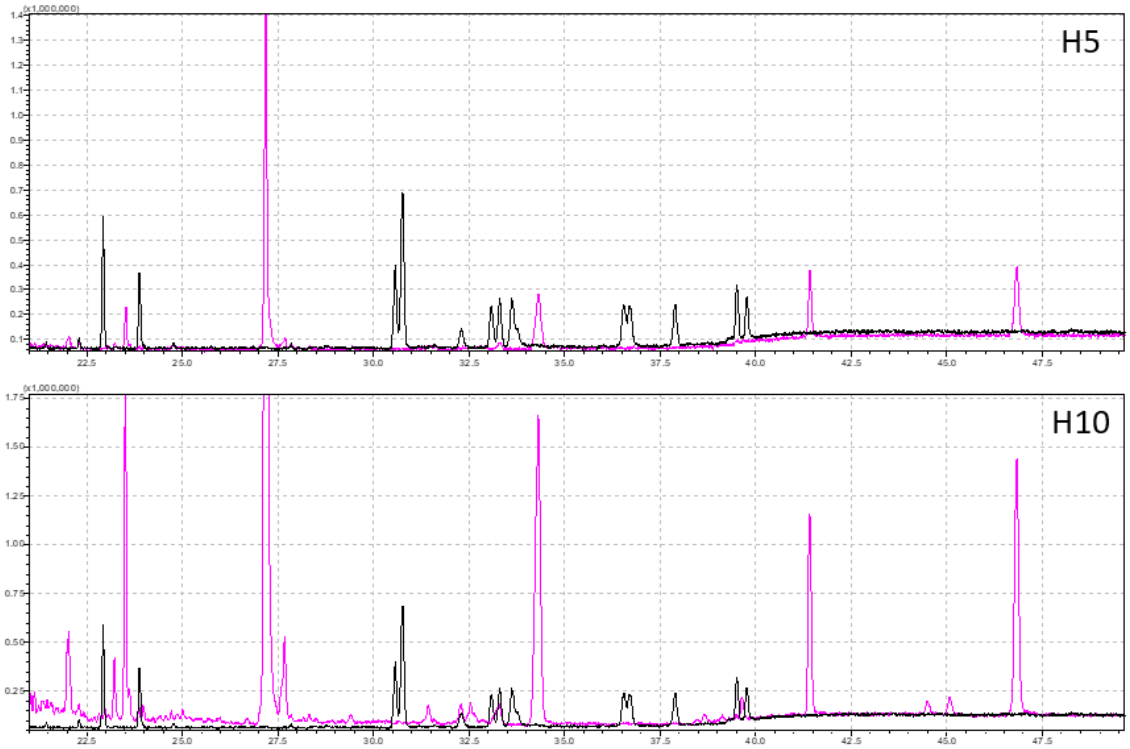


Appendix 7.

Chromatograms obtained from the analysis of the ALC fraction (fraction containing sterols, stanols and stanones) of the soil samples collected from exhumation 7 where no sterols, stanols or stanones were identified, compared to the respective chromatograms obtained in the analysis of the sterols, stanols and stanones standard mix.

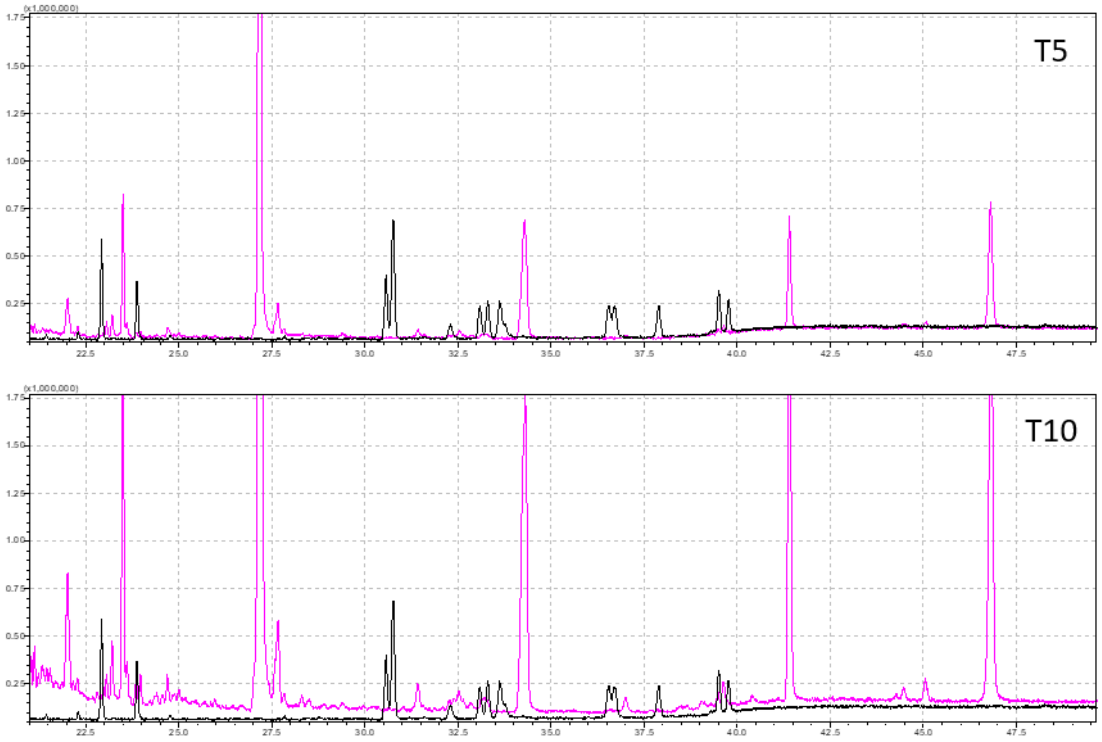
7.1

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the head at 0-5 cm (H5) and 5-10 cm (H10) (both in pink) of the internal grave soil samples collected in exhumation 7.



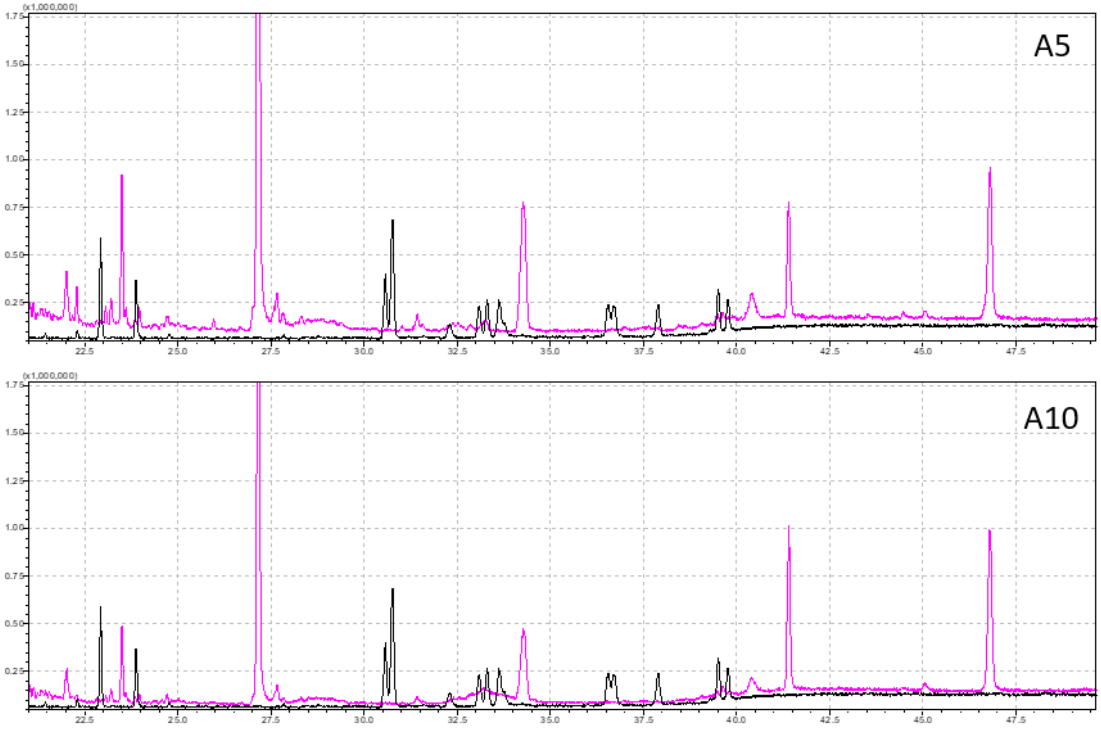
7.2

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the thorax at 0-5 cm (T5) and 5-10 cm (T10) (both in pink) of the internal grave soil samples collected in exhumation 7.



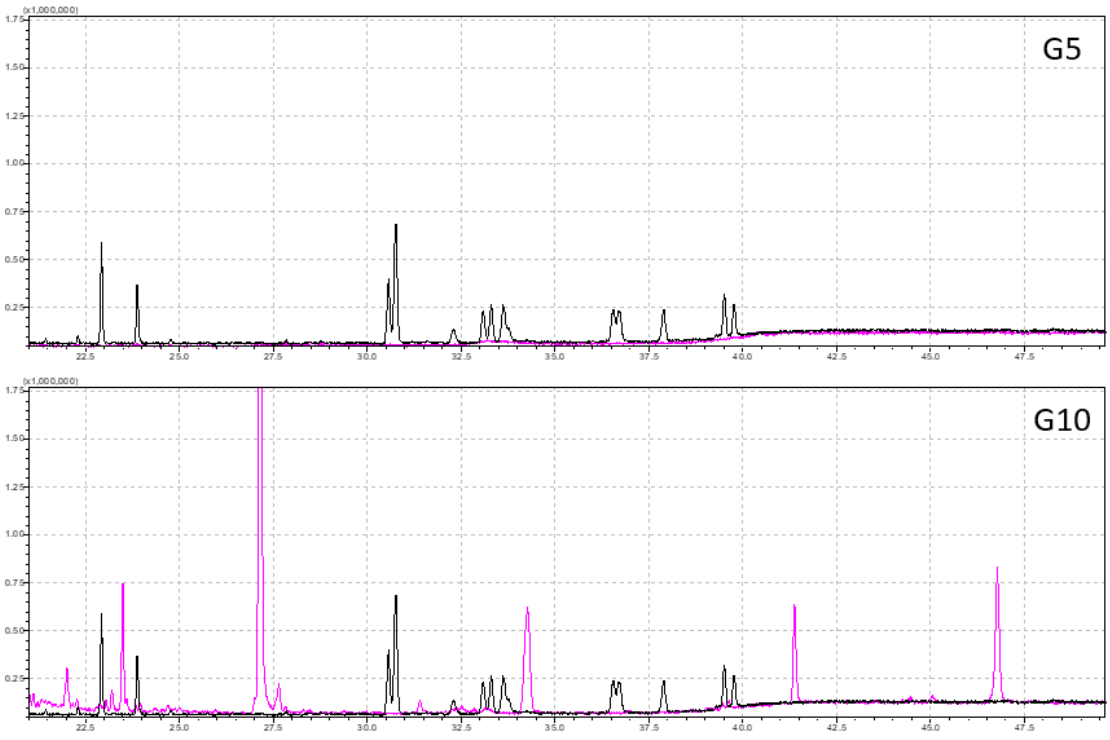
7.3

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the abdomen at 0-5 cm (A5) and 5-10 cm (A10) (both in pink) of the internal grave soil samples collected in exhumation 7.



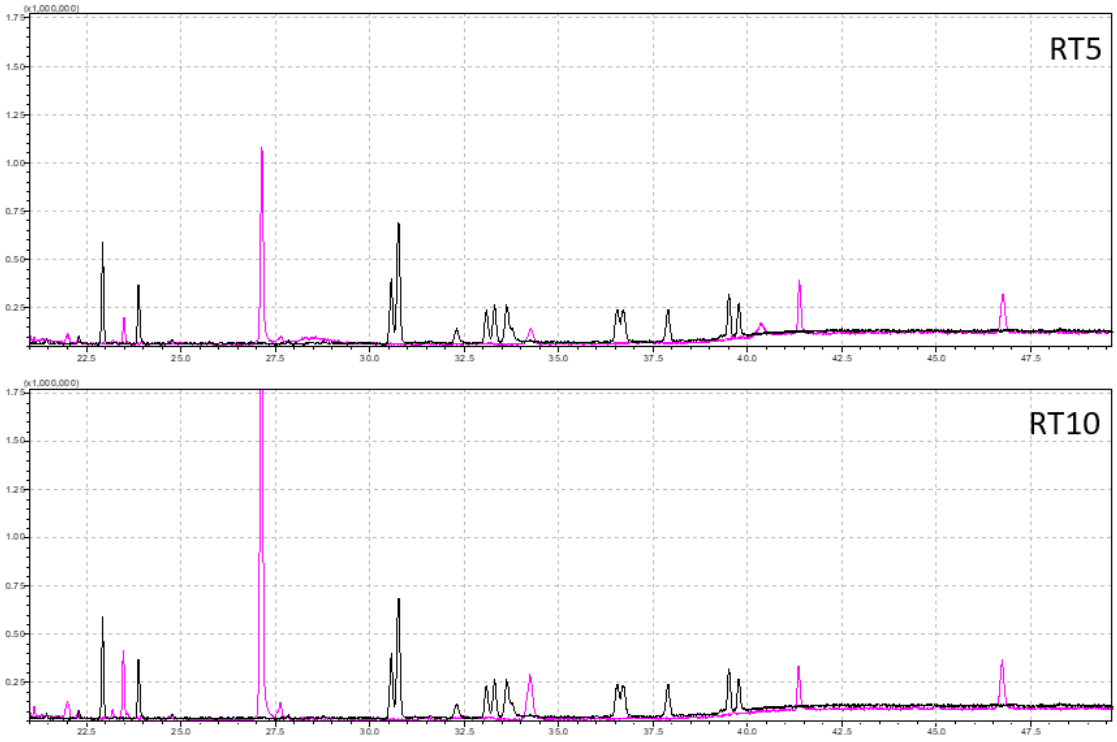
7.4

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the gluteal region at 0-5 cm (G5) and 5-10 cm (G10) (both in pink) of the internal grave soil samples collected in exhumation 7.



7.5

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the right thigh at 0-5 cm (RT5) and 5-10 cm (RT10) (both in pink) of the internal grave soil samples collected in exhumation 7.

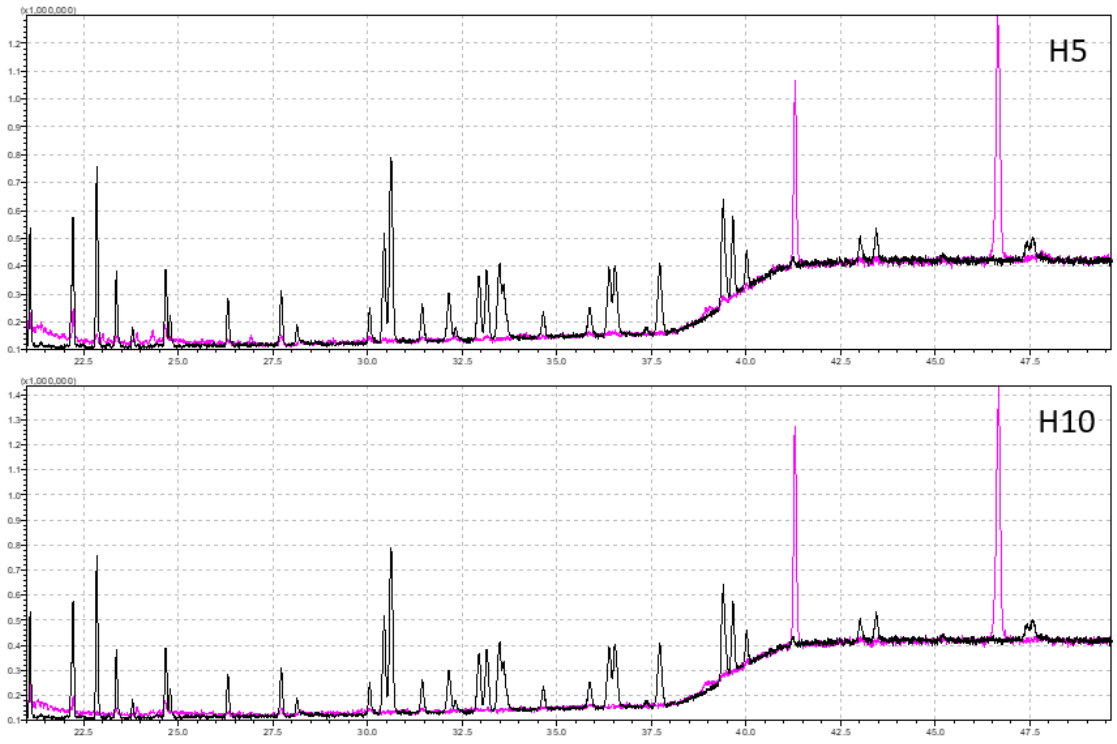


Appendix 8.

Chromatograms obtained from the analysis of the ALC fraction (fraction containing sterols, stanols and stanones) of the soil samples collected from exhumation 10 where no sterols, stanols or stanones were identified, compared to the respective chromatograms obtained in the analysis of the sterols, stanols and stanones standard mix.

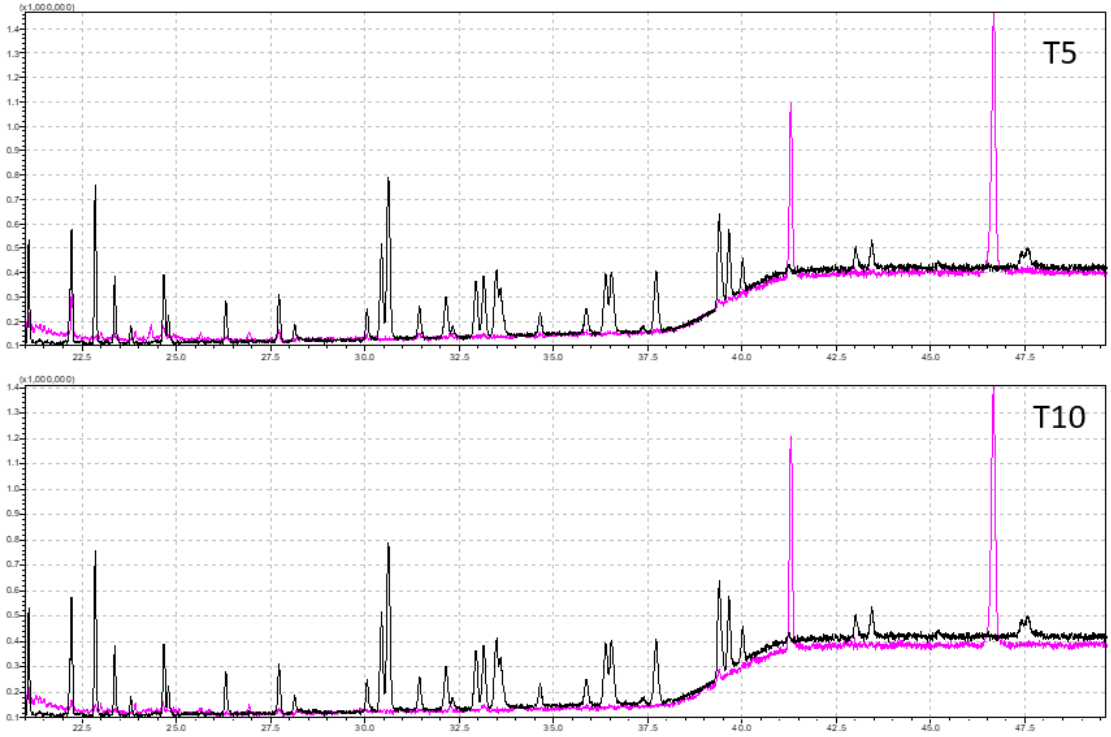
8.1

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the head at 0-5 cm (H5) and 5-10 cm (H10) (both in pink) of the internal grave soil samples collected in exhumation 10.



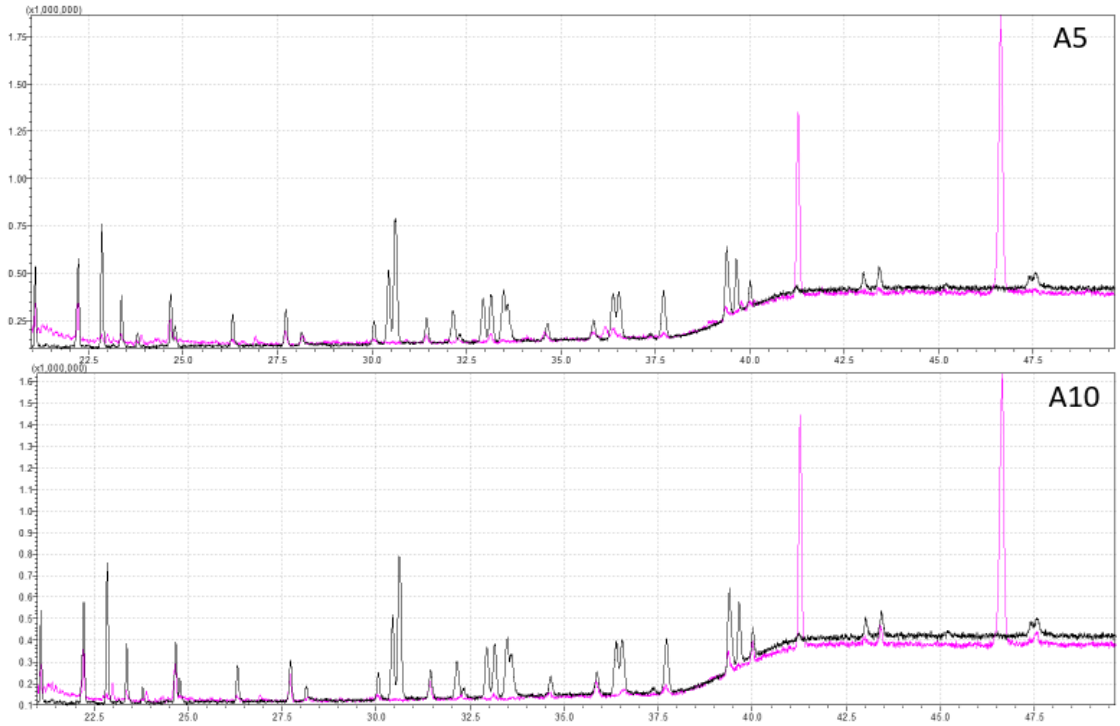
8.2

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the thorax at 0-5 cm (T5) and 5-10 cm (T10) (both in pink) of the internal grave soil samples collected in exhumation 10.



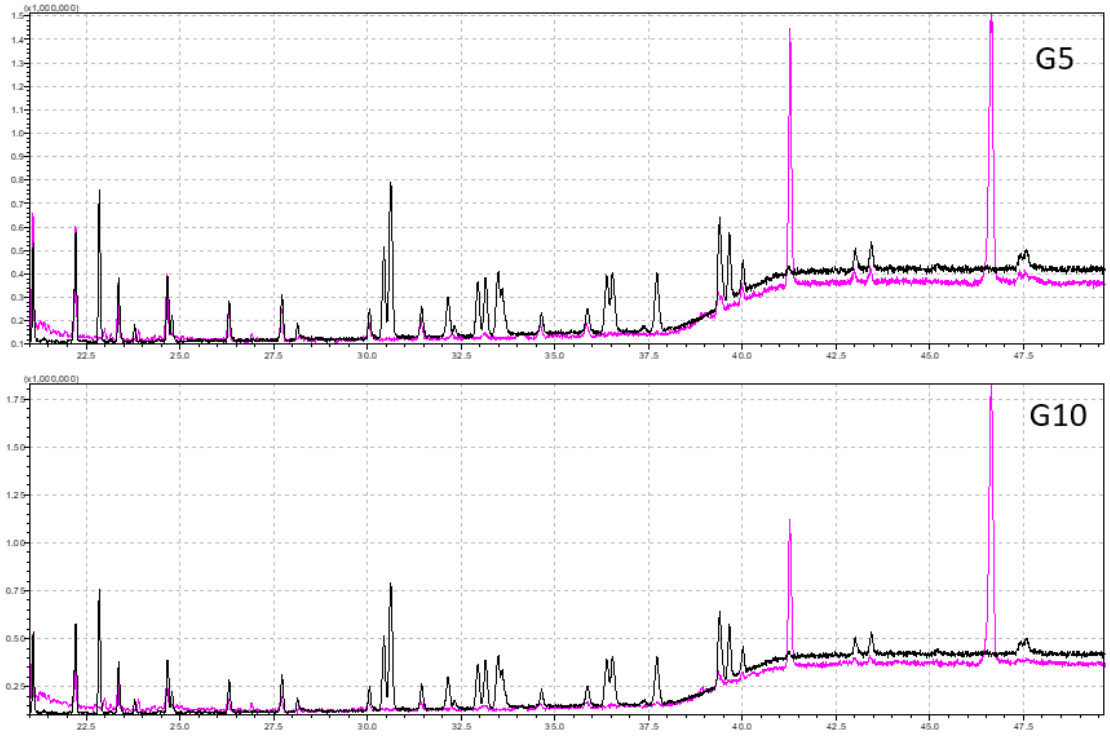
8.3

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the abdomen at 0-5 cm (A5) and 5-10 cm (A10) (both in pink) of the internal grave soil samples collected in exhumation 10.



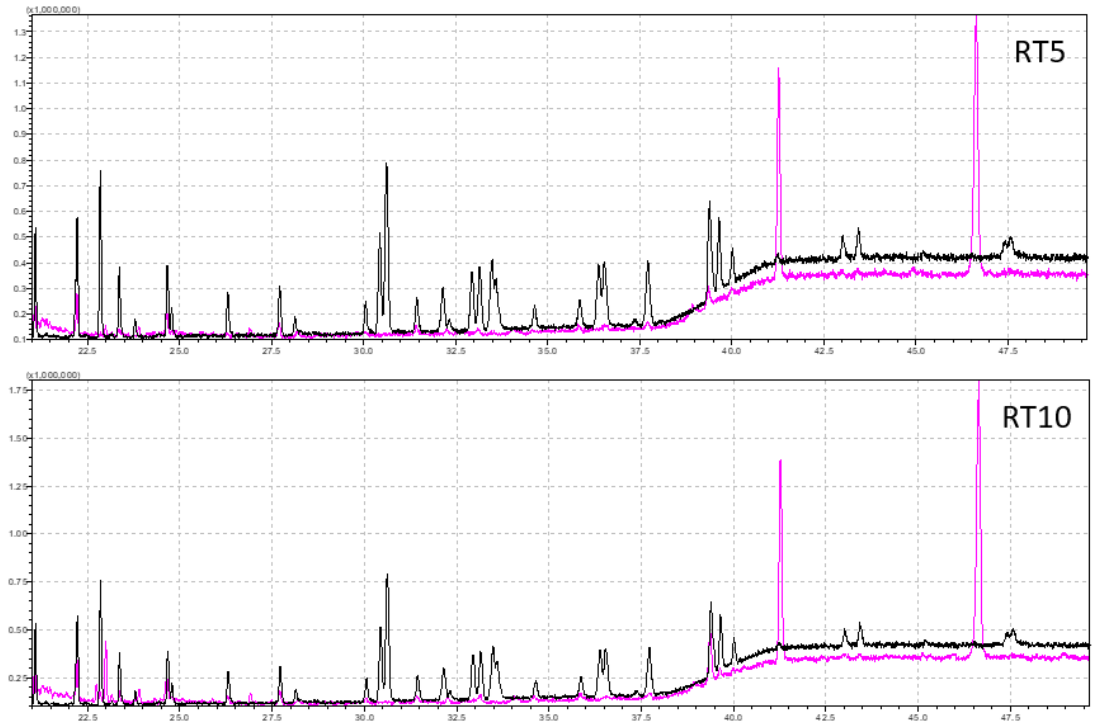
8.4

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the gluteal region at 0-5 cm (G5) and 5-10 cm (G10) (both in pink) of the internal grave soil samples collected in exhumation 10.



8.5

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the right thigh at 0-5 cm (RT5) and 5-10 cm (RT10) (both in pink) of the internal grave soil samples collected in exhumation 10.

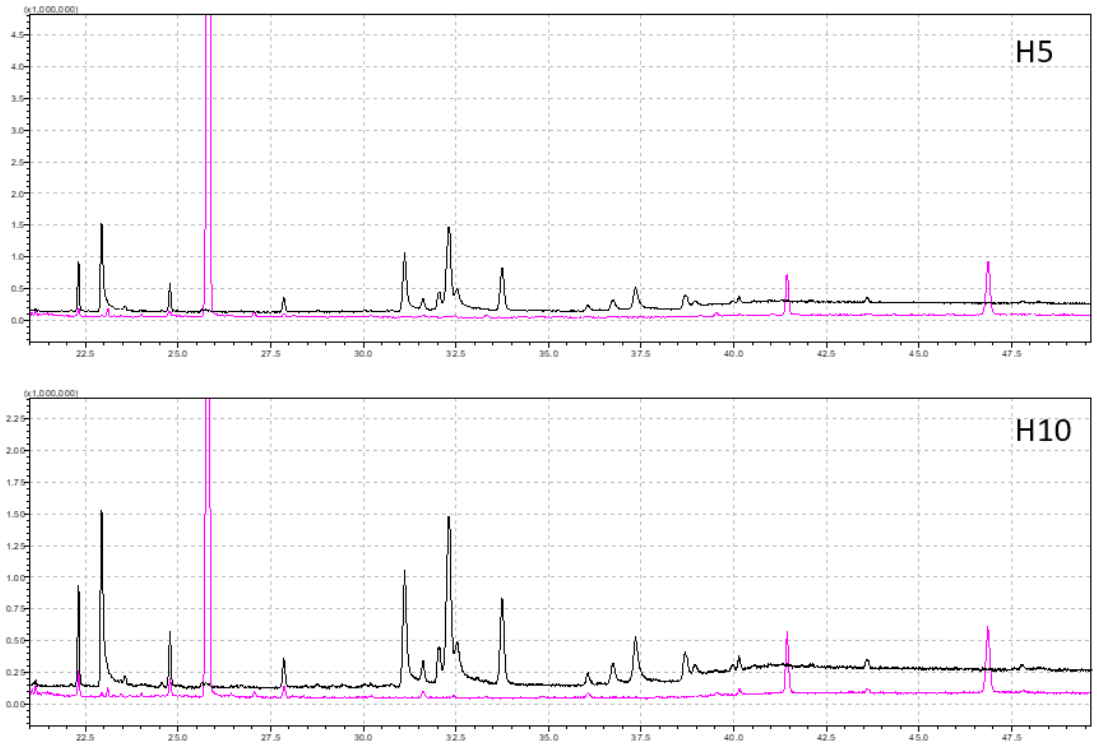


Appendix 9.

Chromatograms obtained from the analysis of the ALC fraction (fraction containing sterols, stanols and stanones) of the soil samples collected from exhumation 12 where no sterols, stanols or stanones were identified, compared to the respective chromatograms obtained in the analysis of the sterols, stanols and stanones standard mix.

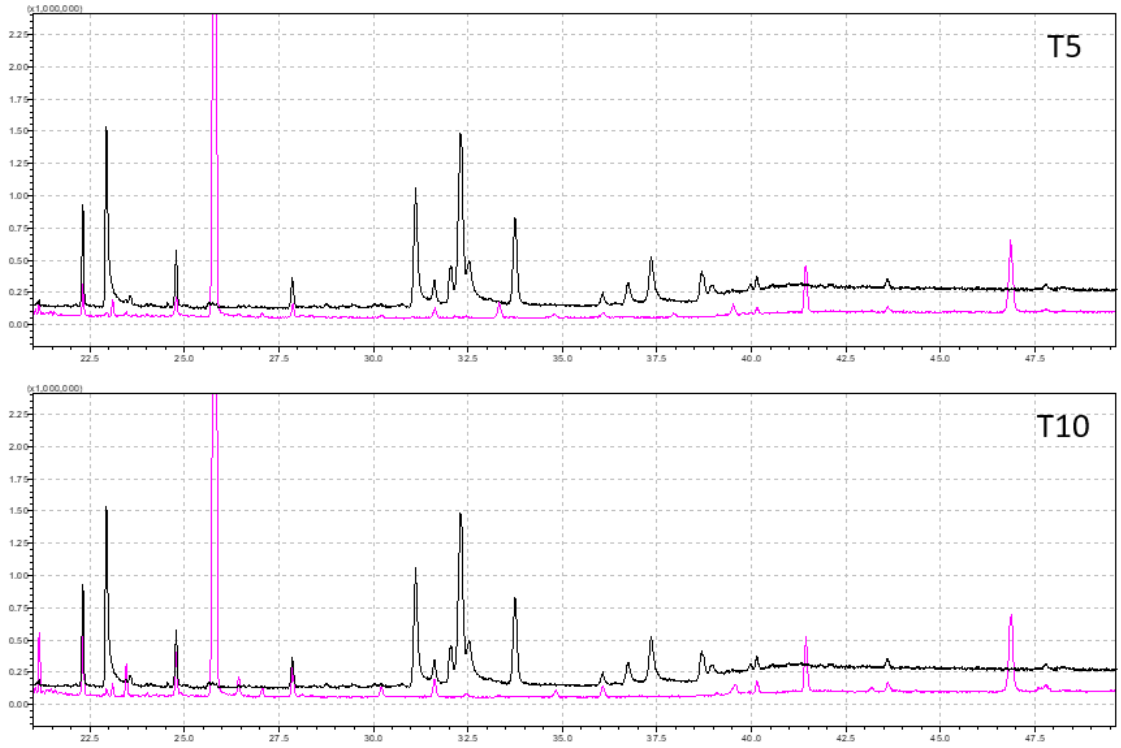
9.1

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the head at 0-5 cm (H5) and 5-10 cm (H10) (both in pink) of the internal grave soil samples collected in exhumation 12.



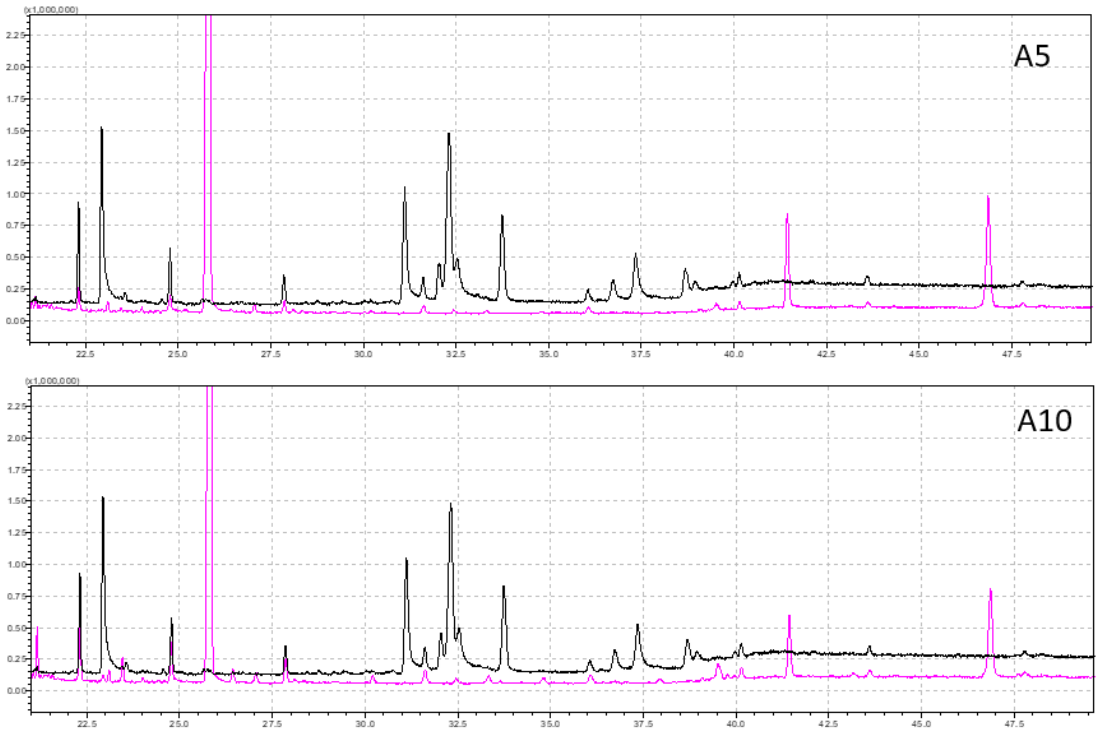
9.2

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the thorax at 0-5 cm (T5) and 5-10 cm (T10) (both in pink) of the internal grave soil samples collected in exhumation 12.



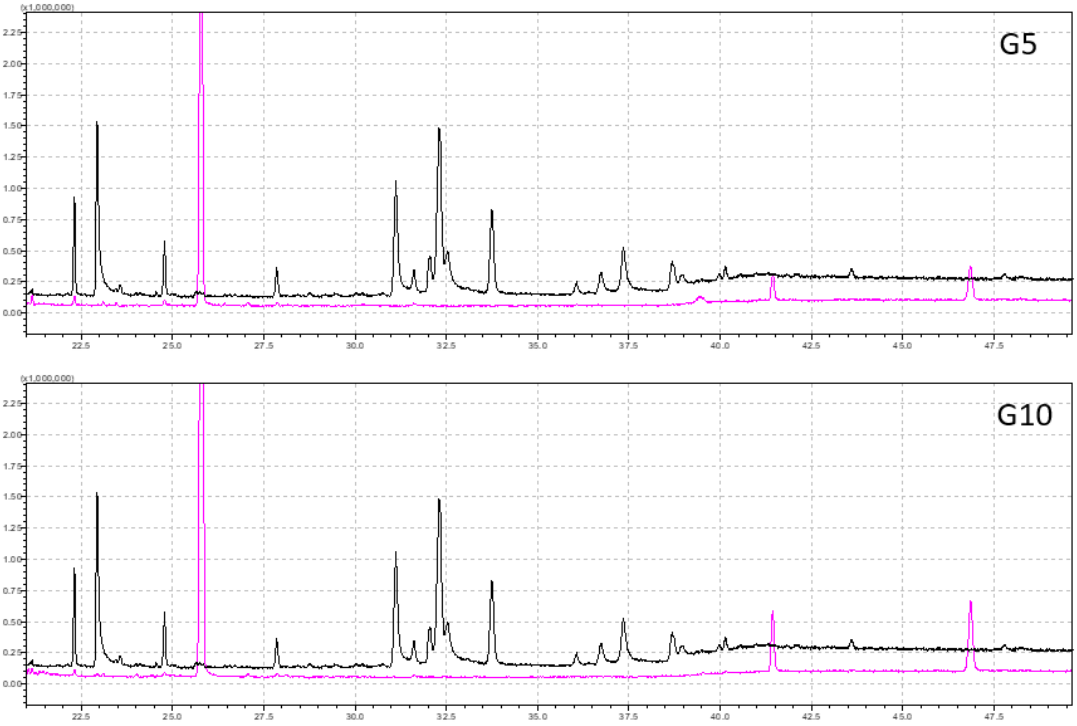
9.3

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the abdomen at 0-5 cm (A5) and 5-10 cm (A10) (both in pink) of the internal grave soil samples collected in exhumation 12.



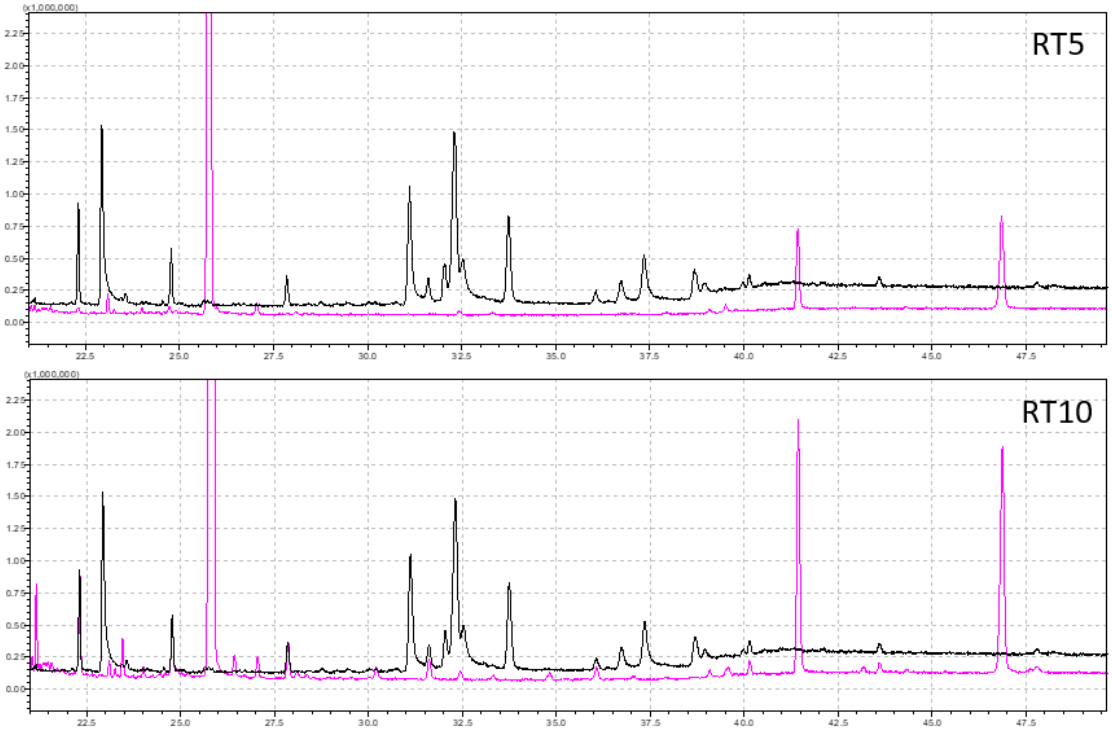
9.4

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the gluteal region at 0-5 cm (G5) and 5-10 cm (G10) (both in pink) of the internal grave soil samples collected in exhumation 12.



9.5

Comparison between the sterol, stanol and stanone standard mix (SSS mix) chromatogram (in black) and the chromatograms obtained in the analysis of the samples collected from the right thigh at 0-5 cm (RT5) and 5-10 cm (RT10) (both in pink) of the internal grave soil samples collected in exhumation 12.



Appendix 10.

Chromatograms obtained in the analysis of Bile Acid Methyl Ester (BAME) fraction from individuals 7 (Appendix 10.1), 10 (Appendix 10.2) and 12 (Appendix 10.3).

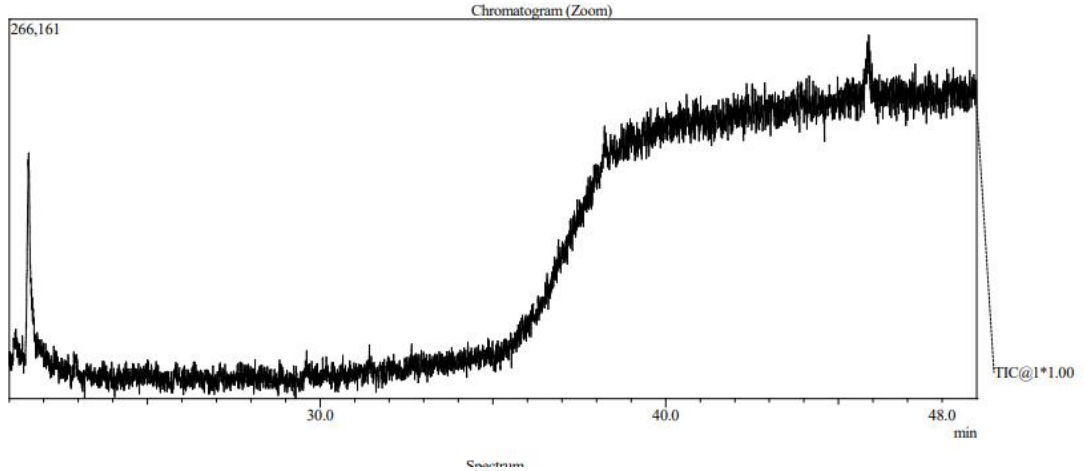
10.1

Chromatograms obtained from the analysis of the BAME fraction of exhumation 7.

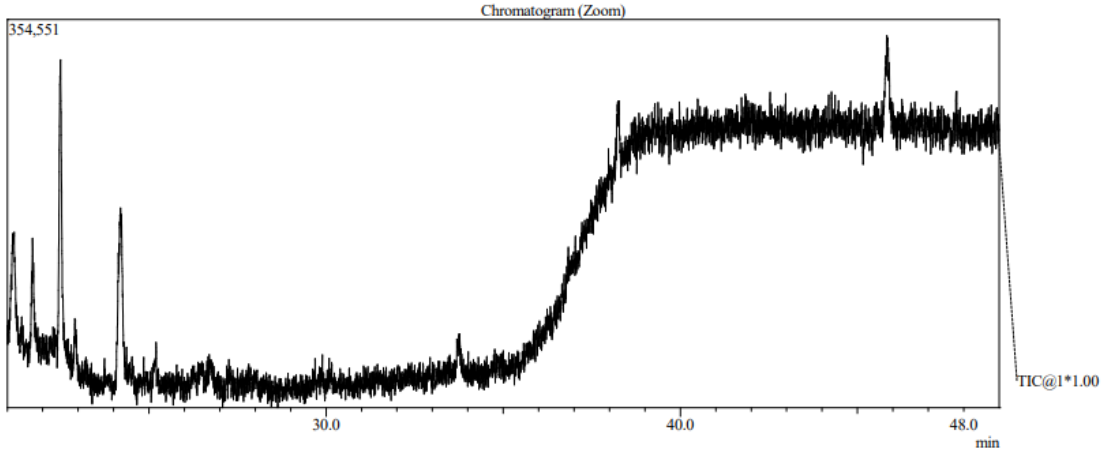
10.1.1

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the head (at 0-5 cm and 5-10 cm) of the individual from exhumation 7.

H5:



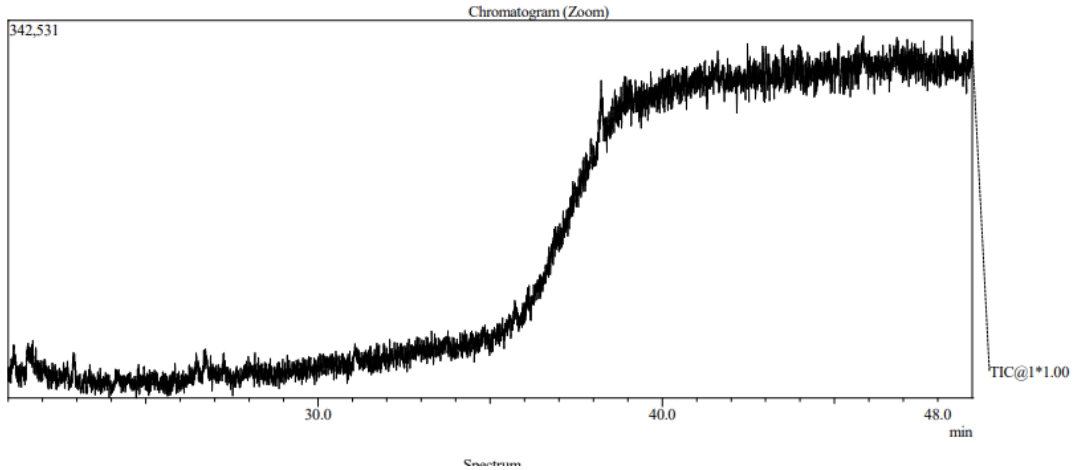
H10:



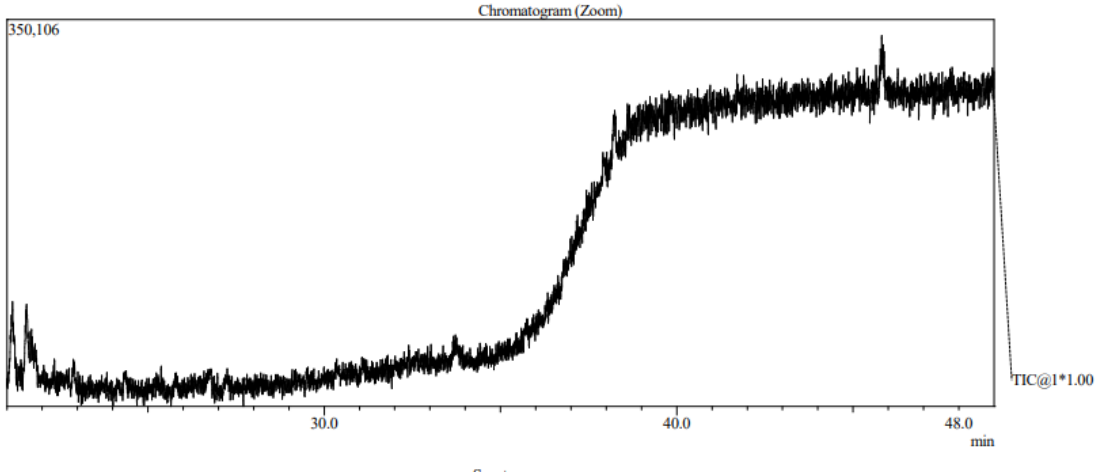
10.1.2

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the thorax (at 0-5 cm and 5-10 cm) of the individual from exhumation 7.

T5:



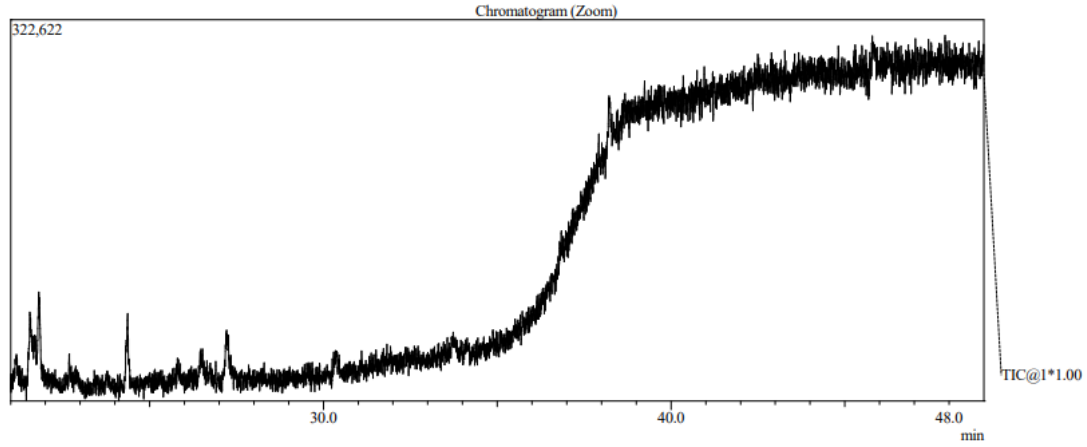
T10:



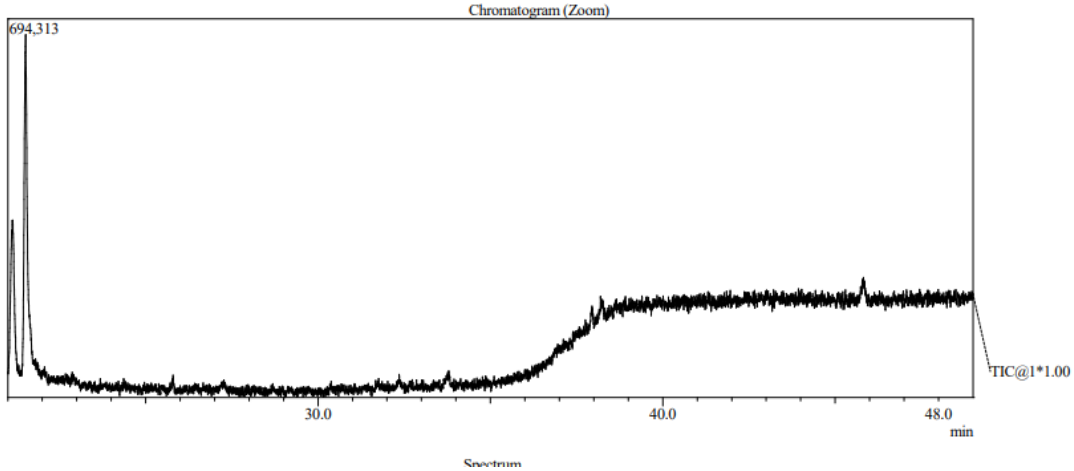
10.1.3

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the abdomen (at 0-5 cm and 5-10 cm) of the individual from exhumation 7.

A5:



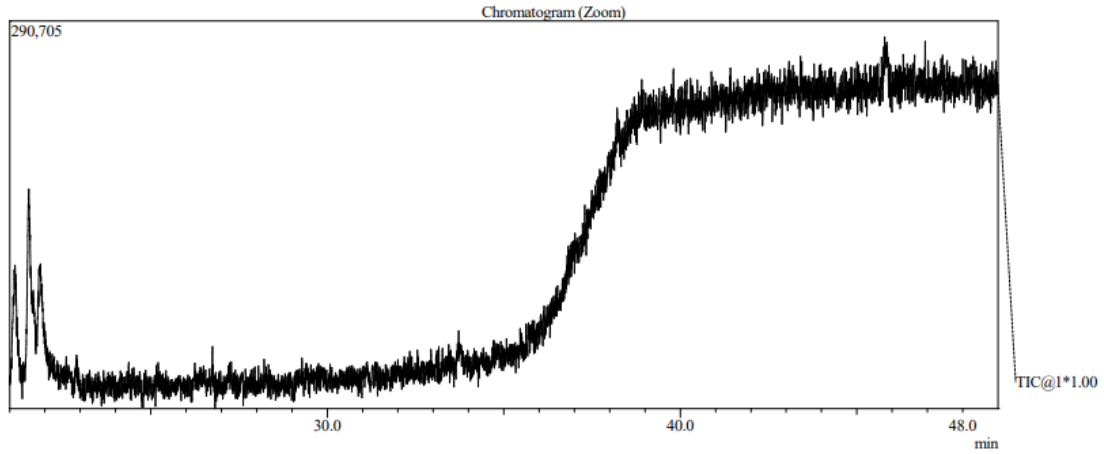
A10:



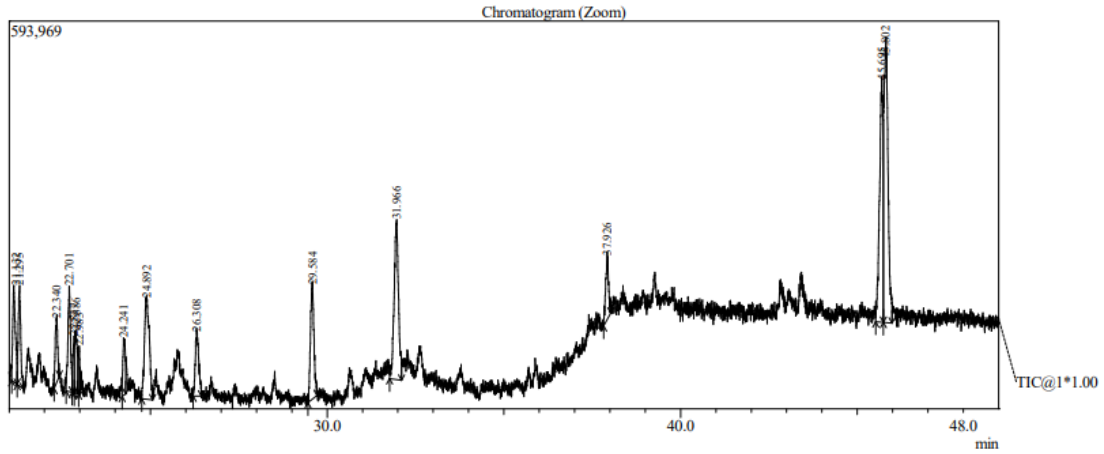
10.1.4

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the gluteal region (at 0-5 cm and 5-10 cm) of the individual from exhumation 7.

G5:



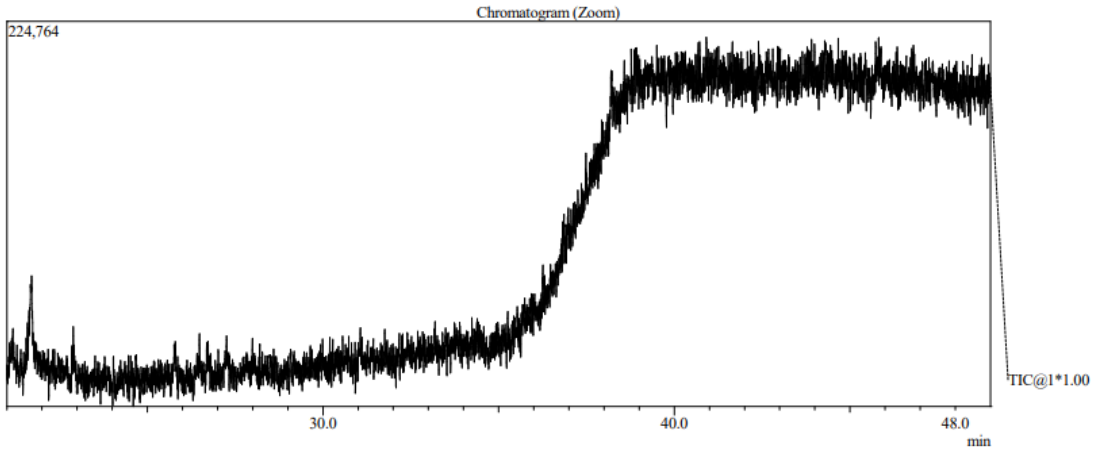
G10:



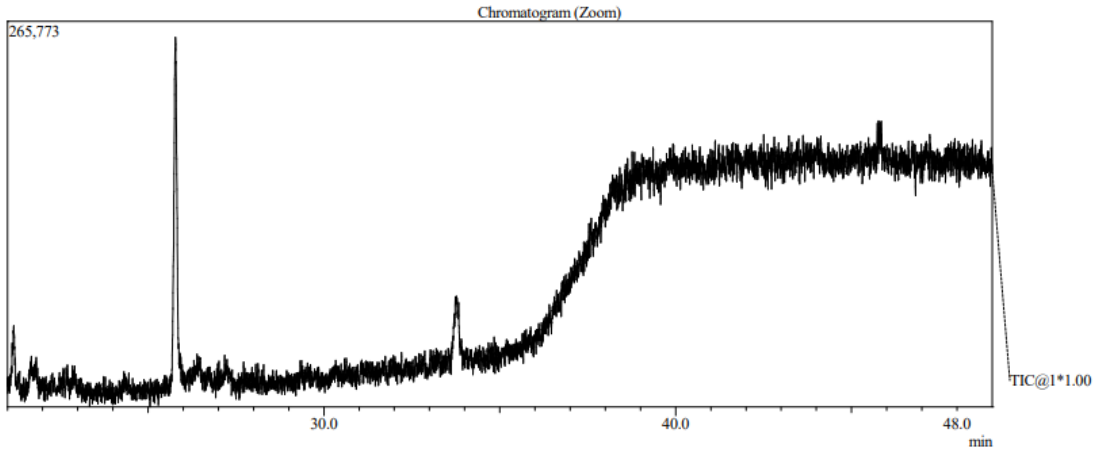
10.1.5

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the right thigh (at 0-5 cm and 5-10 cm) of the individual from exhumation 7.

RT5:



RT10:



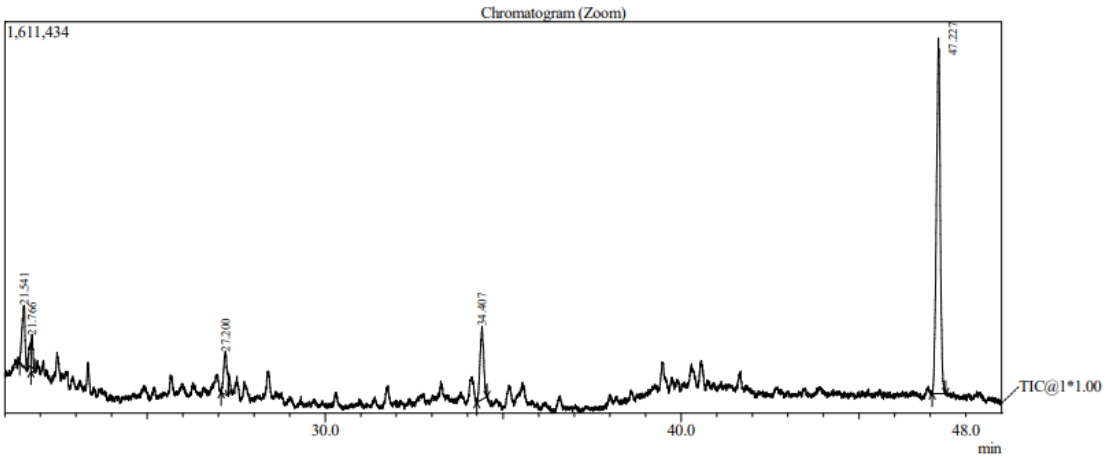
10.2

Chromatograms obtained from the analysis of the BAME fraction of exhumation 10.

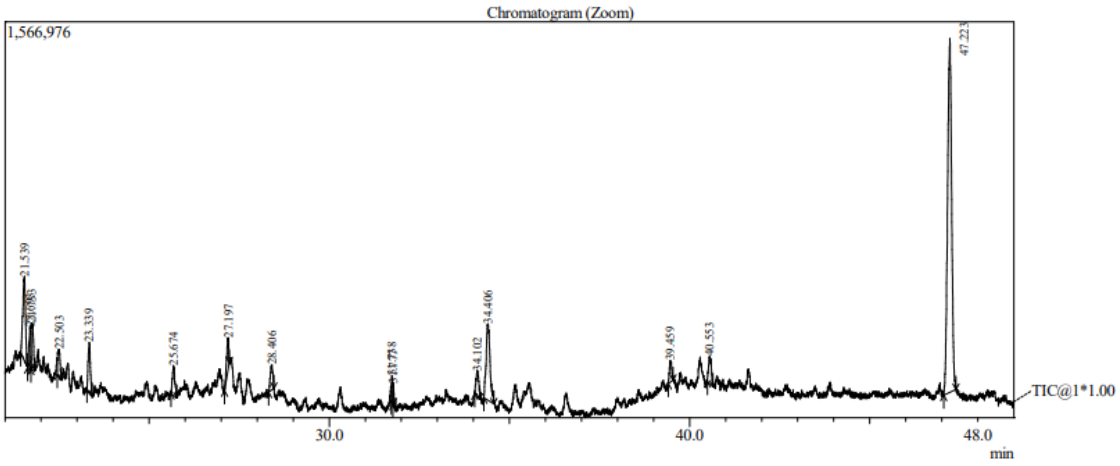
10.2.1

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the head (at 0-5 cm and 5-10 cm) of the individual from exhumation 10.

H5:



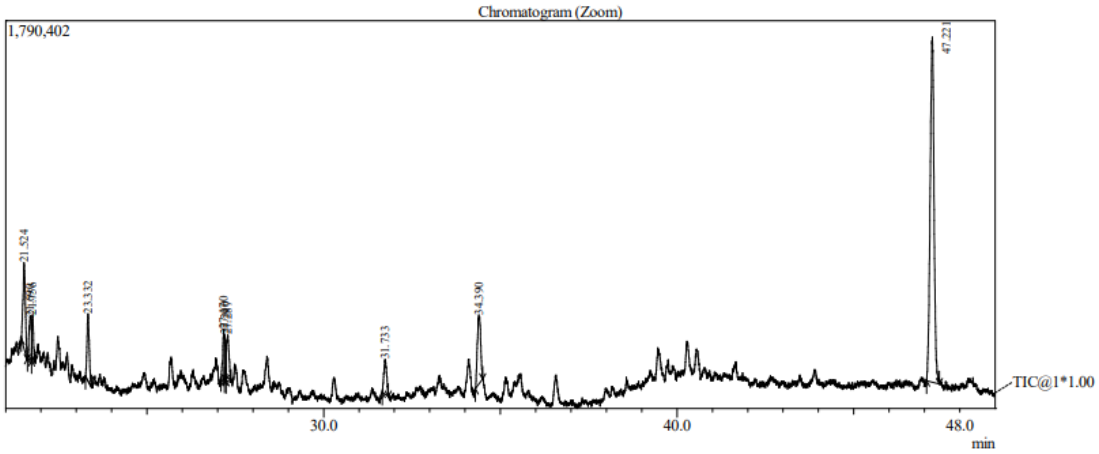
H10:



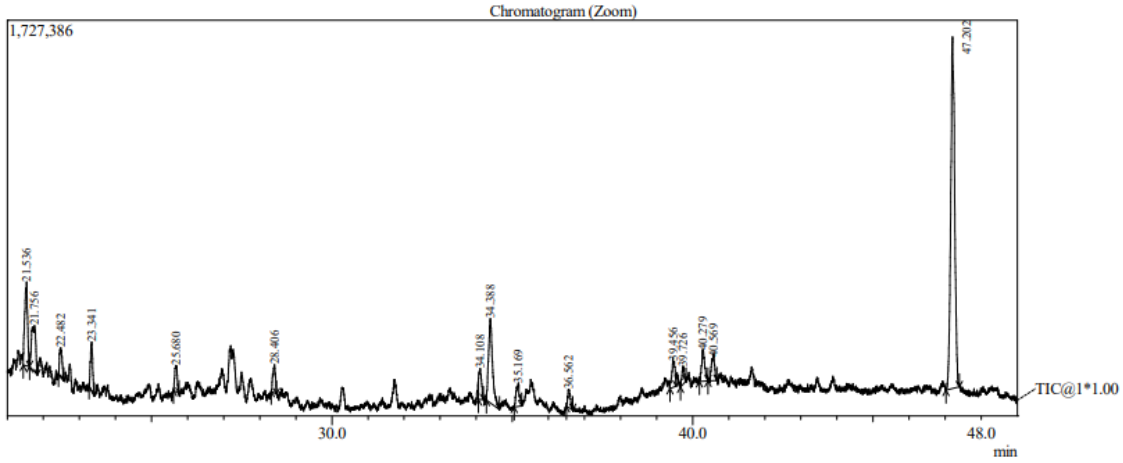
10.2.2

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the thorax (at 0-5 cm and 5-10 cm) of the individual from exhumation 10.

T5:



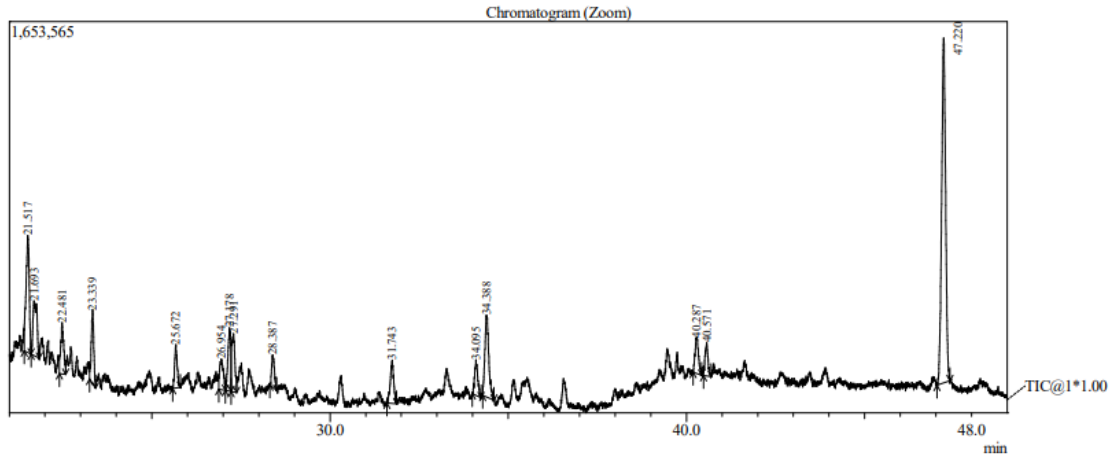
T10:



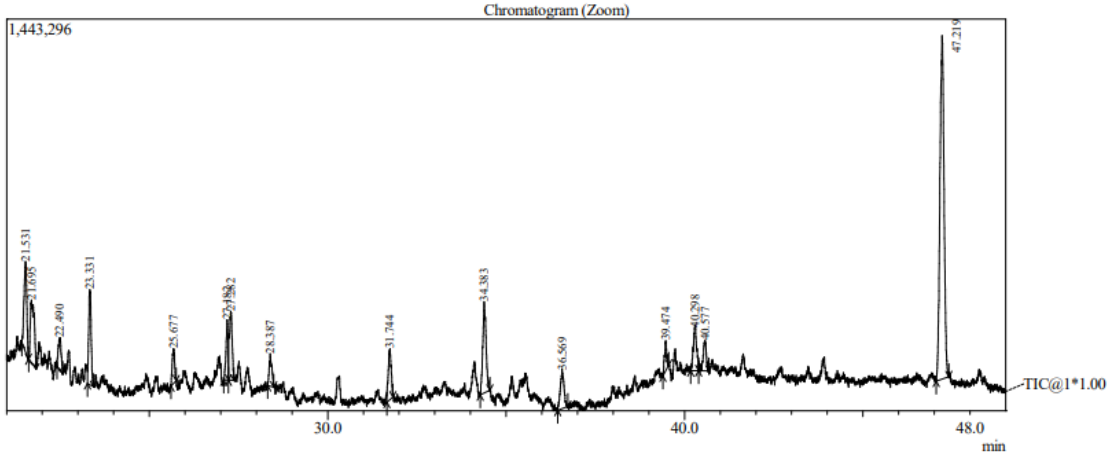
10.2.3

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the abdomen (at 0-5 cm and 5-10 cm) of the individual from exhumation 10.

A5:



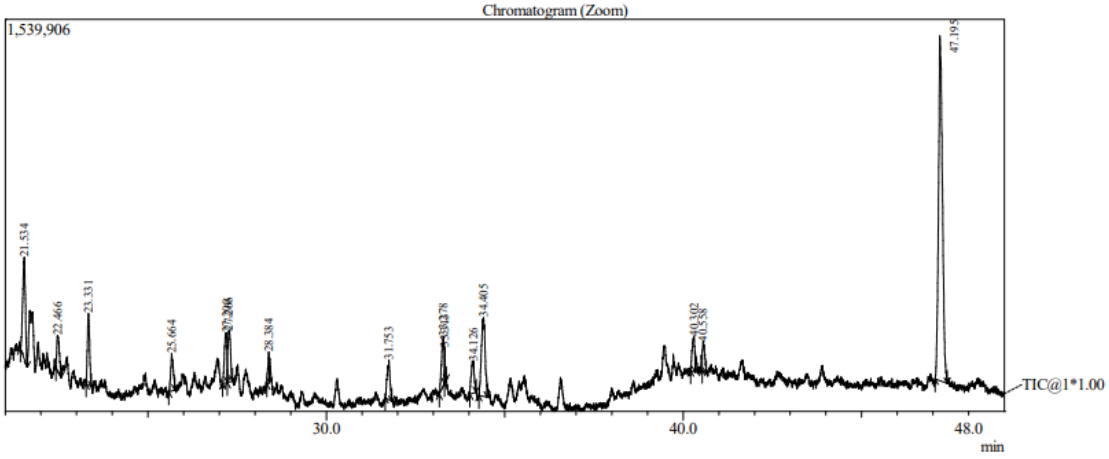
A10:



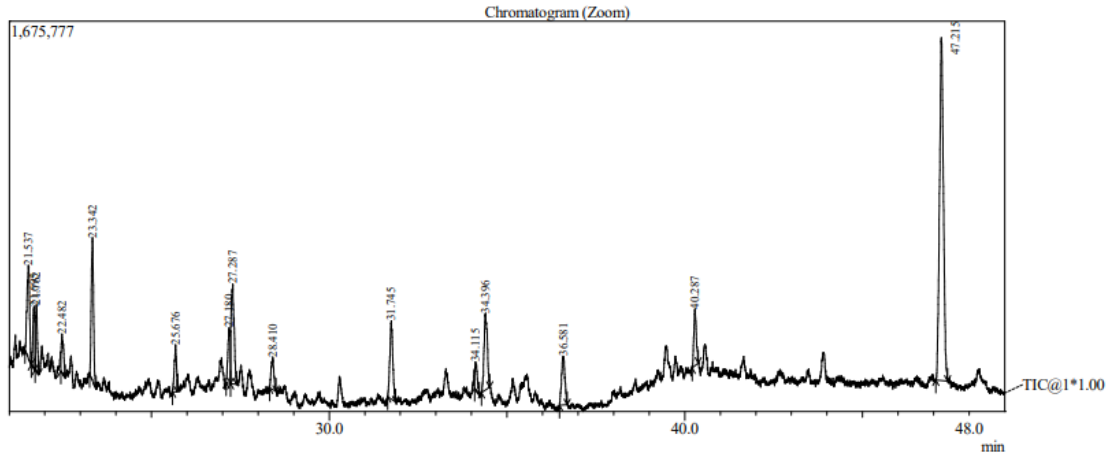
10.2.4

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the gluteal region (at 0-5 cm and 5-10 cm) of the individual from exhumation 10.

G5:



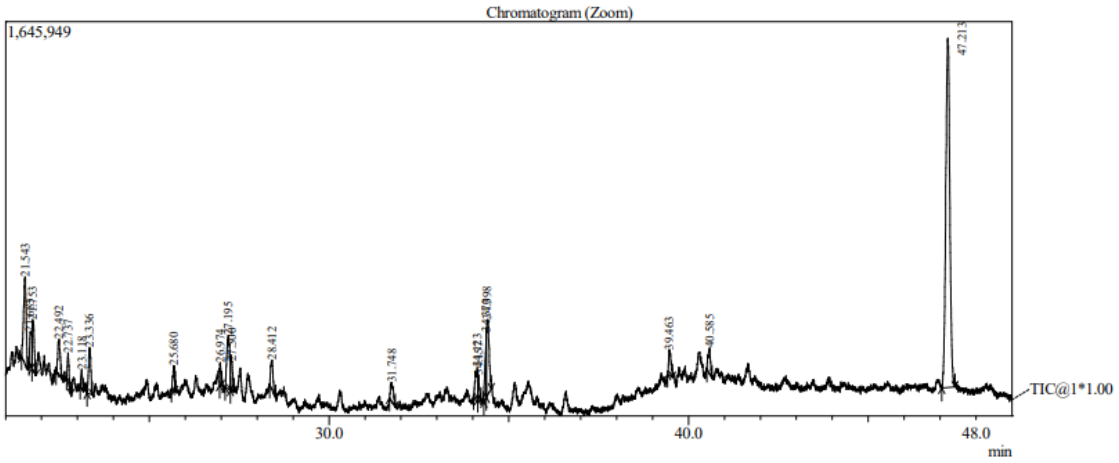
G10:



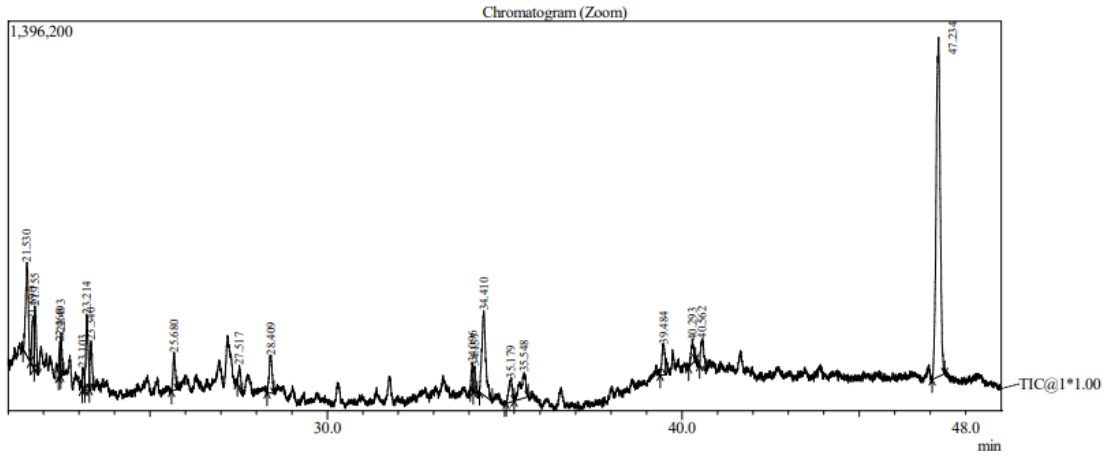
10.2.5

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the right thigh (at 0-5 cm and 5-10 cm) of the individual from exhumation 10.

RT5:



RT10:



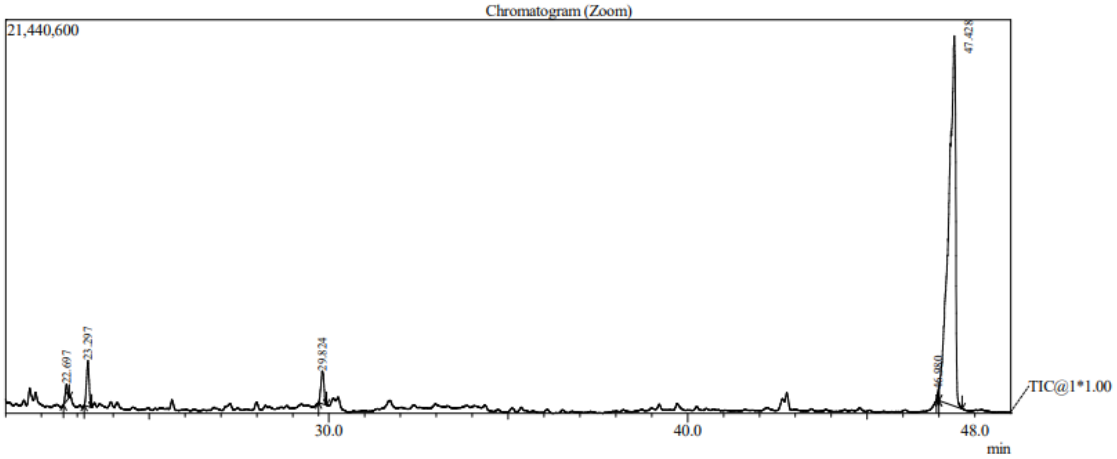
10.3

Chromatograms obtained from the analysis of the BAME fraction of exhumation 12.

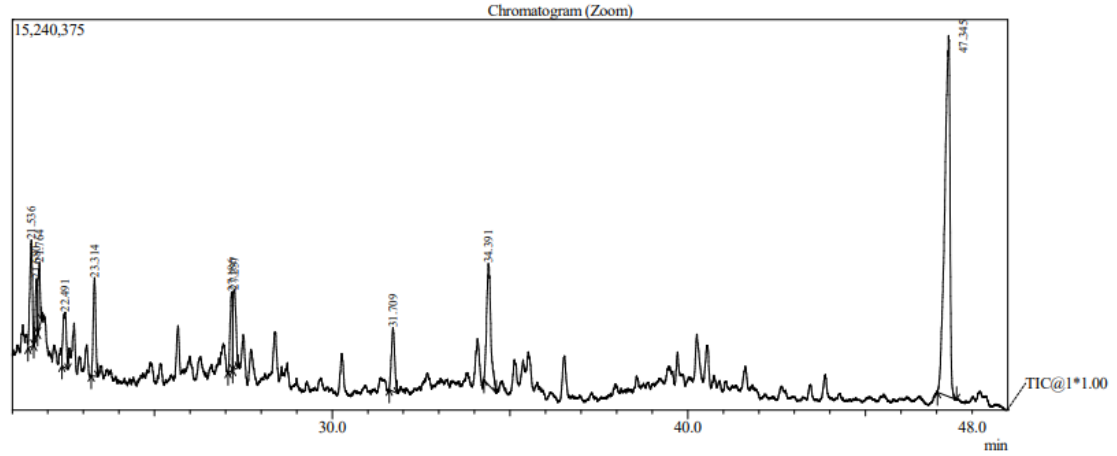
10.3.1

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the head (at 0-5 cm and 5-10 cm) of the individual from exhumation 12.

H5:



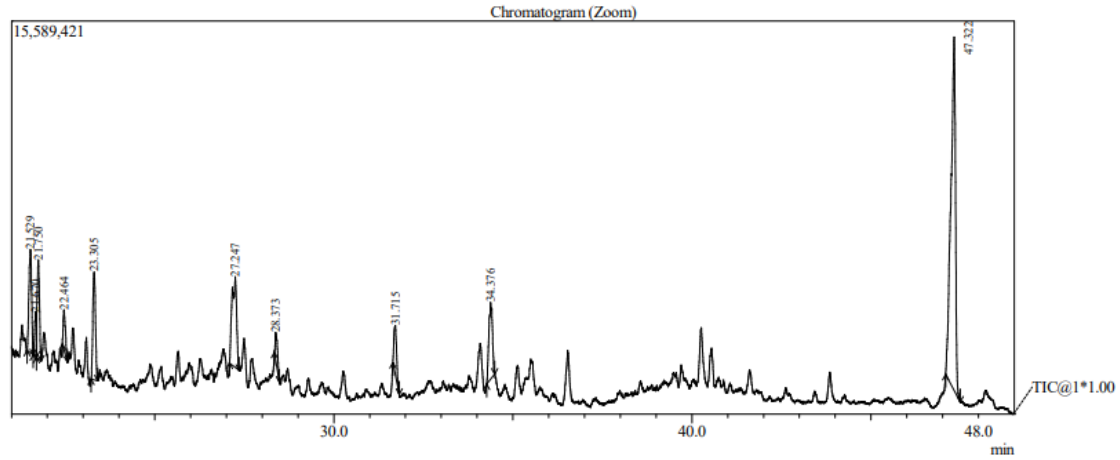
H10:



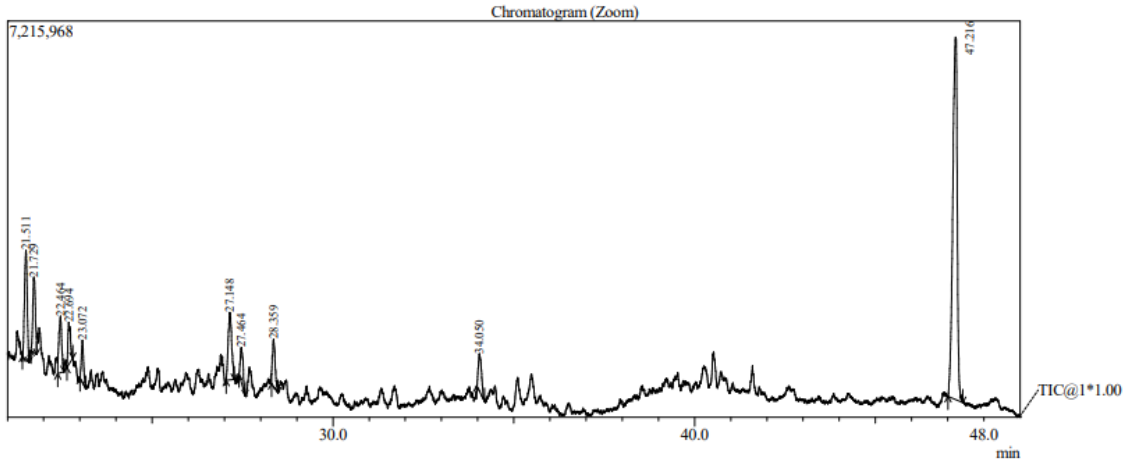
10.3.2

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the thorax (at 0-5 cm and 5-10 cm) of the individual from exhumation 12.

T5:



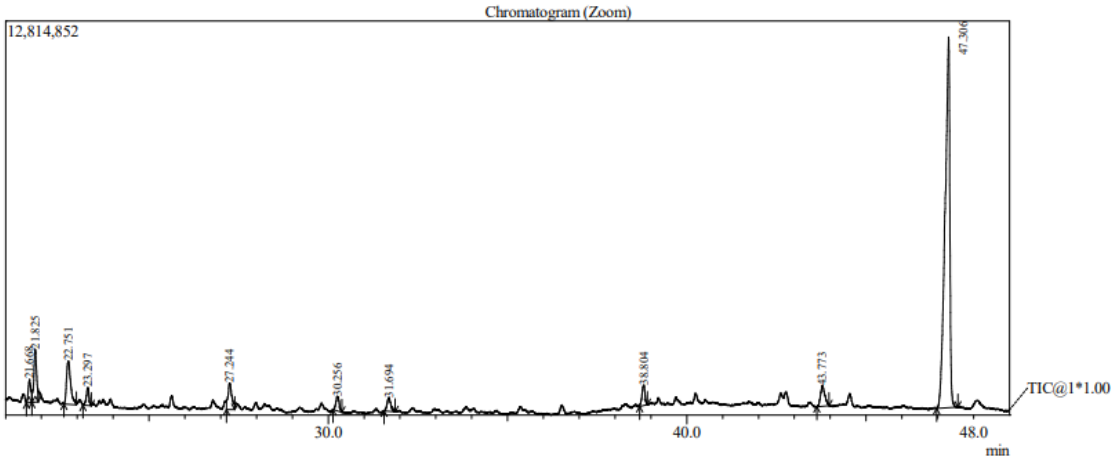
T10:



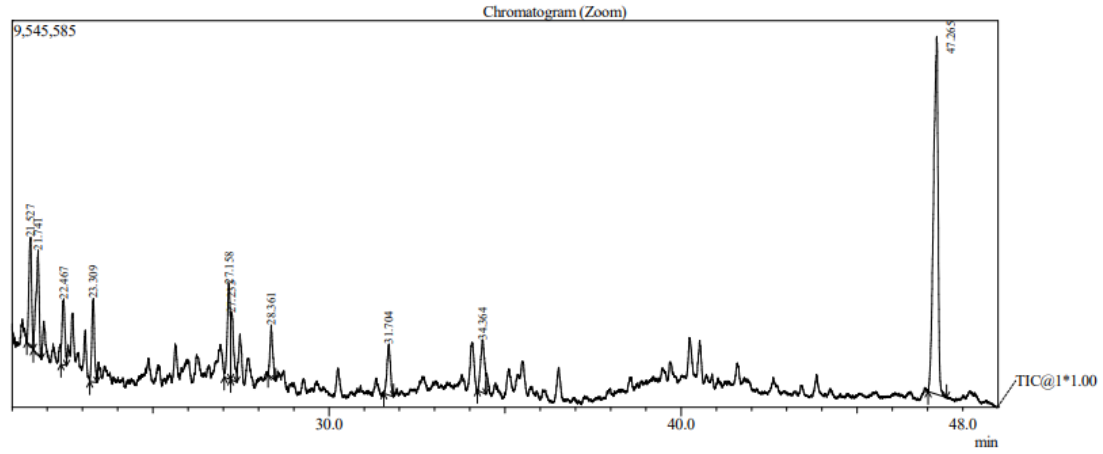
10.3.3

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the abdomen (at 0-5 cm and 5-10 cm) of the individual from exhumation 12.

A5:



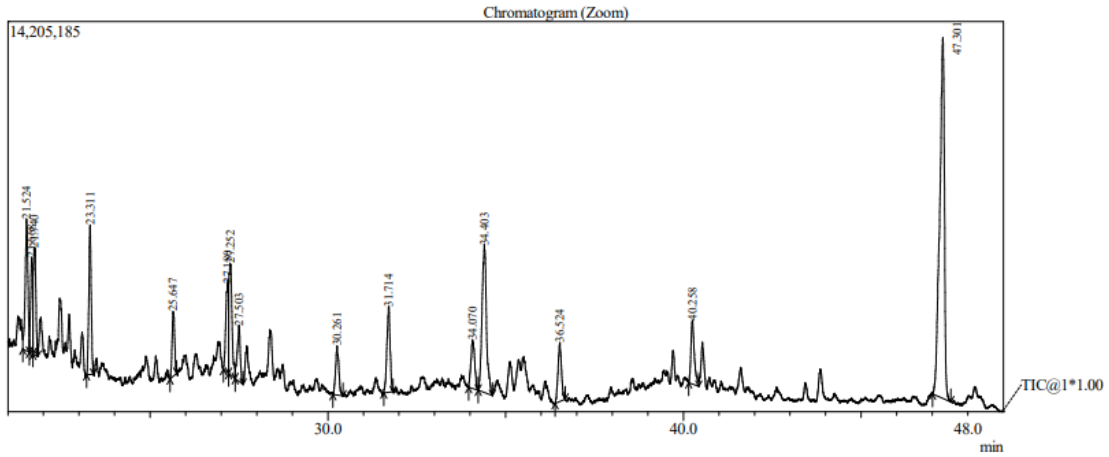
A10:



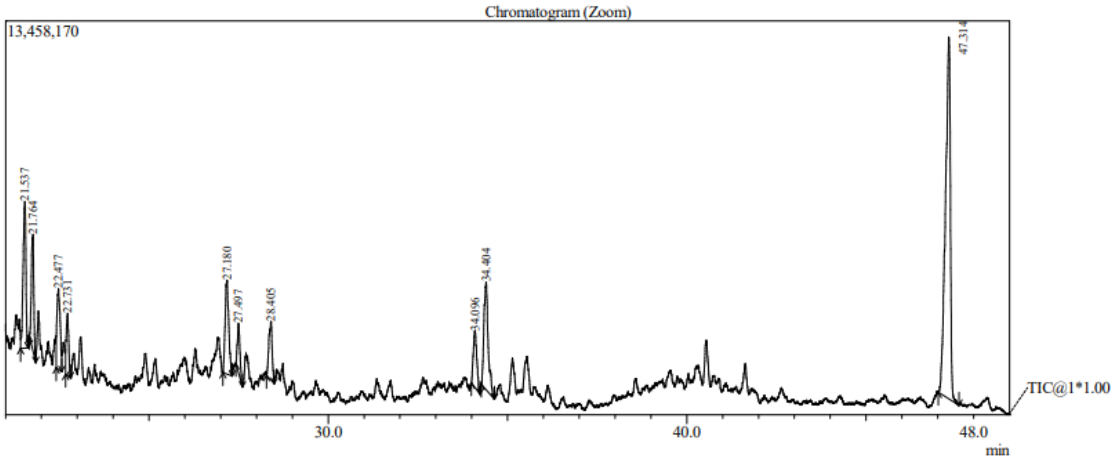
10.3.4

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the gluteal region (at 0-5 cm and 5-10 cm) of the individual from exhumation 12.

G5:



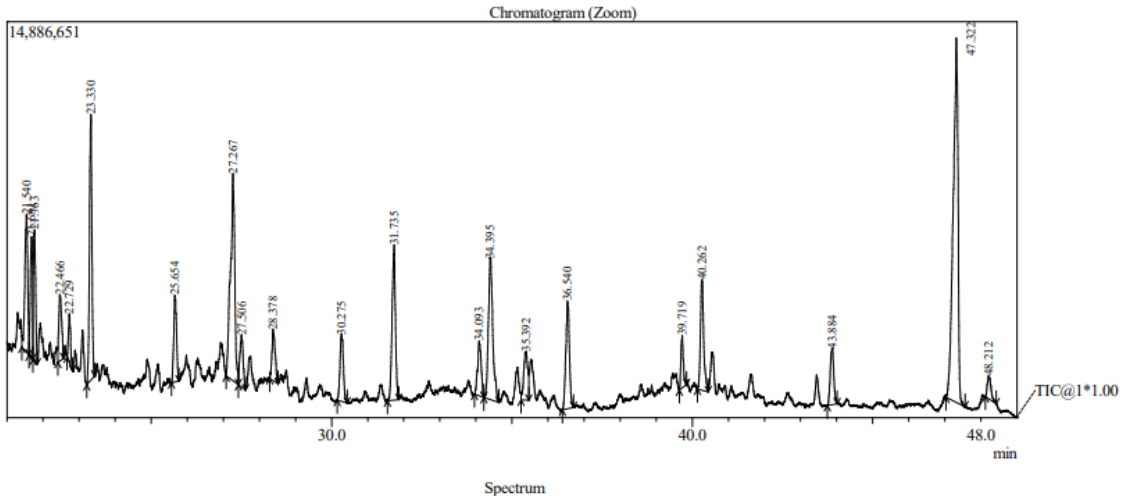
G10:



10.3.5

Chromatograms obtained from the analysis of the BAME fraction of the soil samples collected under the right thigh (at 0-5 cm and 5-10 cm) of the individual from exhumation 12.

RT5:



RT10: