Chemical Detection of Non-Volatile and Semi-Volatile Decomposition Markers from Clandestine Gravesites

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Abstract

Over 134,000 individuals went missing last year of which 1,340 were not found at all, the number of people who disappear due to a homicide is indeterminate as a victim's body is required to prove a homicide unequivocally. A variety of search methodologies are applied to locate clandestine graves ranging from victim recovery dogs to geophysics. It has been highlighted that the current search methodologies to locate clandestine gravesites are not always successful and require a significant amount of time and public funding. This study sought therefore to detect the non-volatile and semi-volatile decomposition products from soil and water samples which could aid the detection of clandestine gravesites and lead to the development of field based chemical tests to speed up the search process.

Three novel alternative analytical methodologies have been developed in order to allow for the detection of non-volatile and semi-volatile decomposition products in in soil and water samples from a simulated grave environment and actual casework samples. The first methodology utilised high performance liquid chromatography (HPLC), which indicated that over 100 decomposition specific chemicals were detected in the leachate samples. This highlighted the potential for using HPLC as an alternative method for the detection of non-volatile and semi-volatile decomposition products from soil-water samples. The second methodology developed utilised ion chromatography (IC) and has proven its capabilities for the analysis of forensic samples by differentiating between the soil samples provided and highlighting areas of interest. The third and final methodology developed utilised derivatisation gas chromatography (GC) for the targeted analysis of biogenic amines putrescine, cadaverine and methylamine. A highly specific methodology was developed for the analysis of primary amines in soil-water samples following simultaneous derivatisation of these amines using pentafluorobenazaldehyde. These amines were detected in the leachate samples from 28 to 669 days post burial, which far exceeded other longevity studies conducted within the discipline of forensic taphonomy. Putrescine was detected in the casework samples where the individual went missing more than 15 years ago and therefore highlights the suitability of the established methodology to aid in the search and recovery process of clandestine gravesites. Utilisation of these methodologies will lead to further identification of the key decomposition products produced during the human decomposition process and allows for the development of field-based chemical

tests. These field-based test would allow for easier and more rapid search procedures, to aid in the detection of clandestine graves and eliminate some of the disadvantages of the current search methods.

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Chapter 1 Introduction

1.1 Context

In the United Kingdom over 134,000 individuals went missing last year. Research by Tarling and Burrows found that out of the 1008 missing persons cases examined, 10 people were not found at all (Tarling & Burrows 2004). This indicates that in general 1% of the people that go missing are not found equating to about 1,340 people each year. The number of people who disappear due to a homicide is indeterminate as a victim's body is required to unequivocally determine a homicide. The detection of a decomposing human body buried in a clandestine (hidden) grave is an essential element to a missing person/murder investigation in order to secure a conviction. A variety of search methodologies are applied to locate clandestine graves ranging from victim recovery dogs to geophysics. The use of chemical analysis to locate human remains has found its way into the American juridical system, although the research and methodology were not ready at the time according to the defence lawyer. This indicates that chemistry has a potential to aid in the detection of human remains as current search techniques are not always successful and cost a significant amount of public funding as has been seen in the search for Madeline McCann, Keith Bennett and Ben Needham.

Previous research has attempted to identify decomposition markers (chemical, physical and biological) to aid in locating clandestine gravesites (See Armstrong et al. 2016; Bergmann et al. 2014; Carter & Tibbett 2003; von der Lühe et al. 2013; Olakanye et al. 2014, 2015; Stadler et al. 2014; Stefanuto et al. 2015a; Vass et al. 1992), although due to the complexity of this research none have been conclusively identified (Forbes et al. 2016). The identification of chemical decomposition markers has been primarily focused on the volatile organic compounds (VOC's) produced during the decomposition process, as seen in studies published by (Agapiou et al. 2015; Dubois et al. 2018; Knobel et al. 2018; Perrault et al. 2015a; Stefanuto et al. 2015b; Vass et al. 2004, 2008; Vass 2012). While the research herein utilised alternative analytical techniques such as HPLC and derivatisation GC to detect additional non-volatile/ semi-volatile chemicals in soil-water samples. When adopted, field-based tests would allow for easier and more rapid search procedures, to aid in the detection of clandestine gravesites. It could be used in combination with victim

recovery dogs to confirm or eliminate their alerts, or it can be used systematically to locate decomposition hotspots within a particular area and will eliminate some of the disadvantages of the current search methods.

1.2 Detection of Clandestine Graves

The digging of a grave permanently alters the subsurface soil stratification through aerating and mixing the soil which is lifted out from below the surface (Haglund & Sorg 1996; Pickering & Bachman 2009; Powell 2006). The soil is deposited on top of adjacent surroundings, damaging any neighbouring plant growth, and the vegetation is uprooted where the grave is dug (Powell 2006). After the body is placed within the grave, the mixed soil is returned to cover the body, the alteration of these soil layers continue to be detectable (Killam 2004). In addition to removing the vegetation and soil stratification when digging a grave, surface debris such as dry leaves, pots, dead flowers, twigs and branches will also be moved. This debris settles and compacts over time and thus can't be replaced in the same manner if removed. The recovery time for natural replacement of this surface debris is unknown but upcast (soil dug out of the ground and backfilled) is clearly visible without any surface debris present (Powell 2006). Hunter et al. (1996) noted that the area of upcast is directly proportional to the grave size and even a small grave has a much larger disturbed surface in comparison to the grave size. See Table 1 for a summary of the key principles, advantages and limitations of the different search methodologies discussed in this section.

Table 1 Summary of different search methodologies, their key principles, advantages and limitations

Method	Key principles	Advantages	Limitations
Ground search (visual)	• Differences between gravesite and surrounding environment[1] • Abrupt change in vegetation[2]	• Inexpensive if volunteers used[3] • Non-destructive[3] • Adaptable to any terrain[3]	• Searchers require training[3] • Not specific[3]
Probing	• Detection of soil compaction differences between the gravesite and surrounding environment through insertion of a rod at regular intervals[3]	• Thorough[3] • Inexpensive if volunteers used[3] • Adaptable to any terrain[3] • Can be performed in combination with ground searches[3]	• Relatively destructive[3] • Slow[3] • Regular breaks required[3] • Not suitable in all weather conditions or seasons[3] • Searchers require training[3] • Not specific[3]
Victim Recovery Dogs	• Locates the scent of decomposition and alerts the handler towards the origin	• Inexpensive[3] • Non-destructive[3,4] • Quick to cover large areas[3] • Adaptable to any terrain[3] • Can be utilised in many weather conditions[3] • Effective over water[3,4] • Very specific[3]	• Limited availability[3] • Cannot be utilised in severe weather conditions[3] • May not always indicate the scent source[3] • Dog might not be specifically trained for this purpose[4] • Regular breaks required[3]
Resistivity surveying	• Detecting structural and chemical anomalies through the soils' ability to conduct electricity	• Relatively inexpensive[3] • Provides lateral position and depth of anomalies[3]	• Relatively destructive[3] • Good weather only[3] • Not suitable for all terrain types[3] • Grave may show insufficient contrast[3] • Affected by groundwater[3] • Requires trained operator[3] • Requires data processing and expert interpretation[3] • Interference from metal and electrical sources[3] • Not specific[3]

Method	Key principles	Advantages	Limitations
Electro-magnetic surveying / metal detector	• Detects anomalies in the soil via electrical conductivity. • Detects metal objects within clandestine gravesite if present.	• Quick[3,4] • Can be used in densely vegetative areas[3] • Non-destructive[3,4] • Detects metal objects[3]	• Very expensive[3] • Difficult on rough terrain[3,4] • Interferences from metal or electrical sources[3,4] • Grave may show insufficient contrast[3] • Requires trained operator[3] • Data processing and expert interpretation required[3,4] • Interference from electrical storms[3,4] • Good weather only[3] • Not specific[3]
Ground penetrating radar **Ground penetrating radar (continued)**	• Detects non-metallic buried objects. • Detects air voids beneath the soil surface	• Results available in field[3,4] • Lateral position and depth of anomalies[3] • Sensitive to small anomalies[3] • Relatively non-destructive[3,4] • Adjustable to different conditions[3] • Can be used over water[3,4]	• Very expensive[3] • Slow[3] • Requires trained operator[3] • Expert interpretation required[3] • Flat terrain only[3] • Clear ground required[3] • Good weather only[3] • Not effective in clay and salt water[3] • Not specific[3]

[1] (Powell 2006), [2] (Rodriguez & Bass 1985), [3] (Killam 2004), [4] (France et al. 1996)

1.2.1 Visual Signs (Ground Search)

Differences in vegetation is one of the visual signs to detect clandestine graves and can be detected via ground searches, aerial photography or drones. Vegetation differences are caused by digging and soil profile changes through excavation and refilling of the soil. In addition, the placement of a body fertilises vegetation through decomposition in their root systems but also affects plant growth. A deep grave benefits plant growth by providing looser soil for root penetration and trapping moisture, whilst shallow graves restrict root penetration and thus restricts plant growth (Killam 2004). “Opportunistic” plants will repopulate the disturbed gravesite first, which may not be observed in undisturbed areas but the vegetation changes as the gravesite progresses through the stages of succession. Hence it is beneficial to locating gravesites if local knowledge of the areas’ vegetation is available. Disturbed areas can look different from the surrounding area for over five years (France et al. 1996).

Another visual sign is the difference between grave fill and surrounding soil. After refilling a hole, soil is always left over due to the inflation of soil with air. In addition, the placement of a body in the hole will leave a mound (Powell 2006). Over time, depressions can be created due to compaction of the soil, depending on soil type, moisture and time. Furthermore, secondary depressions may occur over the abdomen area of the body, which is an indicator of a shallow grave (Killam 2004). Differences in soil chemistry, colour, texture, compactness, moisture retention, volume, organic content and pH can also be observed between grave fill and surrounding soil through mixing of surface soil with subsoil layers (Killam 2004).

1.2.2 Probing

Ground searches are generally non-intrusive, although they can be very intrusive when combined with probing techniques and can potentially puncture buried remains and confuse post-mortem examination. Probing is the act of inserting a rod into the ground in a regular search pattern for which the operator tries to detect the softness of a gravesite in comparison to surrounding soil. It is most effective within short time periods after body deposition and gets less effective over time as soil starts to compact again (Killam 2004). Probing is a very thorough but slow procedure, it is very labour intensive and prone to false positives such as rotting tree stumps, rodent burrows and trash burial pits. It can be used in conjunction with

additional test in the probe holes like subsurface soil temperature measurements, soil pH, victim recovery dogs and combustible gas vapour detection (Killam 2004).

1.2.3 Victim Recovery Dogs

Victim recovery dogs (VRD) are specially trained to locate decomposition scent and locate its handler towards the origin (Rebmann et al. 2000). They can be utilised before, in conjunction with or after conventional foot searches but extensive training is required to become fully qualified (Killam 2004). A dogs' scent capacity is 1000 times more sensitive than that of humans, which makes them an important component in the forensic tool kit (Larson et al. 2011). Decomposition gasses are carried by the air current and tend to pool in sheltered areas, which could be alerted to by VRD as false positives (Killam 2004). VRD are able to work on difficult terrain, are quick to cover large search areas, however they require regular rest intervals otherwise they can suffer from nose fatigue. Success is dependent on a variety of factors such as time since death, burial depth, barometric pressure, temperature and wind conditions (France et al. 1996; Killam 2004; Larson et al. 2011). Optimal working conditions for a VRD is between 4°C and 16°C with a humidity above 20%, moist soil and a wind speed of at least 8km/h (France et al. 1996).

1.2.4 Geophysics

Resistivity surveying is one of the geophysical techniques available to assist in locating clandestine gravesites through the detection of structural and chemical anomalies. If the spread between the instrumental electrodes is known and the subsurface is homogenous, then the current is predictable. Changes in these resistivity currents provides tentative identification of changes in subsurface physical conditions like a burial site. The ability of soil to conduct electricity is dependent on soil porosity and ionic compounds present (salinity) in the pore-water. Soil porosity is dependent on soil compactness, and shape of the pores (Carr 1982; Killam 2004), whilst salinity of the pore-water is critical to measure resistivity (Dobrin 1976). Resistivity surveys' are unpredictable due to changing soil moisture conditions which makes the data relative, but a grave containing a body contains an organic richer area of disturbed soil and thus should provide a difference in conductivity. Resistivity surveying equipment does not operate properly in dry conditions and might require the soil to be moistened around the electrodes but it is neither favourable in very dry or very wet conditions (Killam 2004). Accuracy is affected due to burial depth, rough terrain and the vast variability of soil physical and

chemical properties but is extremely powerful in combination with other geophysical search techniques (Killam 2004). In addition, resistivity surveying is cheap, easy to use but requires expert data interpretation, relatively non-destructive and based on well-established physical principles.

Electromagnetic surveying is another geophysical technique utilised to assist in locating clandestine gravesites. It creates electromagnetic induced currents into magnetic conductors, which themselves create electromagnetic waves which can be instrumentally detected (Killam 2004). Thus, the electrical conductivity of the ground is measured or it can be used to find electrical conductors such as metal objects (Wait 2012). Electromagnetic surveying is dependent on the soils' magnetic permeability, electrical conductivity and dielectric constant (property that governs the propagation of electromagnetic waves). In comparison to resistivity surveying, electromagnetic surveying is faster, can be performed wherever a person is able to walk and is in particular suitable in dry soil or dense vegetative areas. In addition, it can be operated by a single person instead of two or more people for resistivity surveying, is less destructive but still requires expert interpretation (Killam 2004). Electromagnetic surveying is less sensitive in hilly areas and has shown difficulties in both low and high conductive areas. Analysis is severely affected by cultural and natural features of conducting nature such as electrical storms, even at a distance away and cannot be used within 10 meters of metal objects (Killam 2004).

Metal detectors are a type of electromagnetic surveying instruments specifically designed to detect conductive metals and minerals. It applies the same principles as electromagnetic surveying equipment and is very sensitive, reliable and lightweight. As it only detects small metal objects it does not require any expert interpretation but is also not able to simultaneously detect the soil's conductivity/resistivity and metal objects (Killam 2004). Thus, metal detectors are less likely to locate clandestine gravesites unless conductive metal objects have been buried with the victim.

Ground penetrating radar (GPR) was developed by the military to detect non-metallic buried objects and has since been applied to aid in locating clandestine gravesites (Killam 2004). GPR has the ability to detect small objects such as a one-inch pipe under 2.5 feet of soil and is able to detect air voids and disturbed soils

beneath the soil surface (areas that have been excavated and refilled) (Killam 2004). Clay, slit and salt water severely affect GPR penetration whilst metals or metal mesh completely negate penetration. GPR surveying is very slow (1mile/H), works best on flat ground under arid conditions in sandy soil. Furthermore, it is quite expensive, relatively easy to use (except to manoeuvre), non-intrusive and non-destructive (Killam 2004).

1.3 Decomposition Process

Human decomposition commences approximately four minutes after death (Dent et al. 2004; Vass 2001; Vass et al. 2002), but is dependent on environmental parameters such as temperature, moisture content, oxygen availability and soil type (Galloway 1996; Gunn 2006; Vass et al. 1992). Decomposition occurs in a predictable order and can be characterised into two main stages, pre- and post-skeletonisation (Vass et al. 1992). Pre-skeletonisation can be broken down into four subsequent stages; fresh, bloated, active decay and dry, as was first described by Reed (Reed Jr. 1958). All decomposing bodies will go through these stages, but variables such as temperature, moisture content and oxygen availability will determine the length of each stage and the rate of decomposition (Vass et al. 2002).

The initial stage of decomposition, the fresh stage, starts with a process called autolysis, during which rigor mortis (stiffening of muscles), livor mortis (pooling of blood in the body) and algor mortis (cooling of the body to ambient temperature) also may be observed (Gunn 2006; Vass et al. 2002). This decomposition stage is usually observed after a few days post mortem through the appearance of fluid filled blisters and skin slippage. During autolysis the cells of the body are deprived of oxygen which increases the carbon dioxide content, this decreases the pH and poisons the cells through an accumulation of waste. Concurrently, cellular enzymes (such as lipases, proteases, amylases, etc.) start to digest the cells from the inside out, causing the cells to rupture and release nutrient-rich fluids into the body. This process starts sooner and advances more rapidly in high enzyme content tissues, such as the liver but it affects all cells eventually (Vass et al. 2002).

Putrefaction starts in the second decomposition stage, the bloated stage, after enough cells have ruptured and nutrient-rich fluids have become available in the body (Vass et al. 2002). Putrefaction is the catabolism of carbohydrates, proteins

and lipids, present in the soft tissue, into gasses liquids and small molecules (Gill-King 1997; Vass et al. 2002). Microorganisms such as bacteria, fungi and protozoa, are responsible for this soft tissue breakdown in a mostly anaerobic environment (Vass et al. 2002). The presence of sulfhemoglobin settled in blood is usually the first visible sign of putrefaction through a greenish discoloration on the skin (Gunn 2006; Vass et al. 2002). The formation of various gasses such as ammonia, carbon dioxide, hydrogen sulphide and VOC's result in the distension of tissues, especially in the bowels (Vass et al. 2002). These gasses are responsible for the bloating of the carcass and a build-up of pressure results in purging from the body, often severe enough to tear the skin apart and cause post-mortem injuries (Gunn 2006).

After the skin has been broken in one or more places due to putrefactive purging, active decay begins (Vass et al. 2002). Active decay can be observed through deflation of the body as various gasses escape, progressive loss of skin and soft tissues (Gunn 2006). During active decay, amino acids present in the body, and produced from protein catabolism, decompose to form volatile fatty acids or biogenic amines dependent on the decomposition pathway (see Section 1.4.3 Decomposition of Proteins). Glycerols and phenolic compounds are produced from the decomposition of protein and lipids. Putrefaction still occurs during this stage, as chemical constituents are still degraded and released (Swann et al. 2010b; Vass et al. 2002). Aerobic and anaerobic bacteria, prominent insect activity and carnivore activity contribute significantly to the decline of tissue during this decomposition stage (Vass et al. 2002).

The fourth and final pre-skeletonisation stage of the decomposition process is the dry stage. During this stage any remaining moist skin and tissue is converted to a leathery-like sheet that adheres to the bone. Skeletonisation of the cadaver occurs and is characterised by the appearance of over 50% exposed bone, however erosion of the skeletal remains has not yet begun (Swann et al. 2010b). Skeletonisation proceeds until only the resistant bone, teeth and cartilage remains (Dent et al. 2004; Swann 2011). Chemical weathering of the remains continues during this decomposition stage but takes substantially longer than the previous decomposition stages. Diagenesis, the exchange of ionic species from the bones to

the surrounding environment, occurs during this stage, but the rate is affected by different soil environments (Gill-King 1997; Swann 2011).

Although the decomposition process is divided into the four stages described above, distinction between these stages can be difficult to identify (Swann 2011). These stages merge into each other and it is impossible to separate them into discrete entities. A body rarely decomposes in a uniform manner and often one part of the body is reduced to a skeleton while another part of the body still has fleshy tissue (Gunn 2006). In addition, differences in ecological parameters produces specific variations on the general decomposition pattern (Galloway 1996).

1.3.1 Factors Affecting Decomposition

The chemical and physical changes observed during decomposition are strongly influenced by environmental conditions such as temperature, humidity/rainfall and oxygen availability (Gunn 2006; Vass et al. 2002). Other factors that can contribute to decomposition rates are carnivore activity and soil pH (Mann et al. 1990). Variables affecting the decomposition rate which relate to the cadaver itself are referred to as intrinsic factors, whilst those relating to the decomposition environment are referred to as extrinsic factors (Breton 2013). Only the latter will be discussed in this section as they affect the decomposition rate more significantly (Casper 1861).

Temperature has been regarded as the most significant factor affecting the rate of decomposition (Gill-King 1997; Mann et al. 1990). This has been adopted from an entomological perspective where temperature significantly affects the development of blowfly larvae (Archer 2004). A study conducted by Carter et al. (2008) highlighted a positive relationship between temperature and the decomposition process from a taphonomic point of view. Bio-chemical reactions within our bodies and microorganisms occur most optimal around 37°C, cooling or heating of the body to a different ambient temperature slows or speeds cell metabolism by affecting the enzyme systems that regulate most reactions. Enzymes are subject to denaturation, coagulation and crystallisation at extreme temperatures affecting the catabolism of proteins and carbohydrates (Gill-King 1997; Vass et al. 1992). The physical principle known as Van't Hoff's rule, also called the 'rule of ten', has been applied to the

decomposition process and states that the rate of chemical reactions increases two or more times with each 10°C temperature increase (Gill-King 1997).

Water affects the decomposition process in multiple ways, it has a stabilising effect on temperature and acts as a buffer controlling the tissues and environmental pH. Water is also a source of hydrogen for biochemical reactions in all cells, in addition to its diluting effect on chemical concentrations inside and outside the cells. The rate of decomposition for a body submerged in water may be accelerated or retarded depended whether the water is salty or fresh, moving or still, or differences in pH (Gill-King 1997). A cadaver in a wet soil environment tends to result in reducing conditions and decreases the decomposition rate, whilst low moisture content promotes desiccation (Carter et al. 2010). However, water also affects the osmotic environment of cells leading to protrusion of the organs and rupture of the cells and can therefore increase the decomposition rate (Ayers 2010; Gill-King 1997). Moisture also affects the soil microbial activity as its availability controls microbial mobility, diffusion of nutrients and waste, and the acidity of extracellular enzymes and thus encourages the growth of mycota, bacteria and plants (Carter et al. 2010; Gill-King 1997). These effects are however altered by soil texture and structure as moisture availability is partially determined by adhesion between water and the soil particles (Carter et al. 2010). Hydrolase enzymes utilise water to break down carbohydrates, lipids and proteins into smaller molecules, however their activity is affected by concentration and pH, which is affected by the amount of water present. It is assumed that the ambient atmospheric humidity plays an important role in these reactions when the bodies are not immersed in water (Gill-King 1997).

Cadavers restricted from an oxygen supply such as those deeply buried, submerged, at high altitude (above 3km), or left in any hermetic environment will decompose more slowly (Gill-King 1997; Gunn 2006). A retardation of oxidative processes is observed affecting the decomposition rate due to the lack of available oxygen, lowering the redox potential (Gill-King 1997; Rodriguez & Bass 1985). Casper (1861) noted that decomposition in soil takes approximately eight times longer than aboveground decomposition. Whilst, decomposition under water has been reported to be twice as long as when exposed to air and would be even longer at lower oxygen levels and temperatures (Gunn 2006). Soils and water that contain decaying organic matter without the availability of oxygen become reducing

environments (i.e. acidic), whilst the opposite is observed for oxygen rich environments. Mammalian cells are quickly affected by anoxia, however the opposite is true for many soil-borne bacteria which decompose the bodies in the soil (Gill-King 1997). Depletion of the available oxygen, decreased diffusion rates, initiates the decomposition process due to an increase in carbon dioxide, which stimulates the activity of the soil-borne bacteria (Gill-King 1997). Sub-surface decomposition at least when buried 30 cm deep will not experience scavenging and invertebrate colonisation which significantly affects the decomposition process (Gunn 2006; Rodriguez & Bass 1985).

1.4 Thanatochemistry

During decomposition, soft tissue will be broken down by endogenous enzymes and micro-organisms such as bacteria, fungi and protozoa resulting in the production of inorganic gasses, organic gasses (also called volatile organic compounds) and liquids (leachate) (Statheropoulos et al. 2005; Paczkowski & Schütz 2011; Vass et al. 2002). These substances are intermediate decomposition products of large biological macromolecules such as carbohydrates, proteins and nucleic acids (Statheropoulos et al. 2005; Vass et al. 2002). The body consists of approximately 64% water, 20% protein, 10% fat, 1% carbohydrate and 5% minerals (Dent et al. 2004).

Proteins are enzymatically broken down to proteoses, peptones, polypeptides and amino acids, which are further broken down through deamination, decarboxylation or desulfhydration (Dent et al. 2004; Gill-King 1997). This in combination with bacterial metabolic processes produces a variety of VOC's such as aldehydes, alcohols, aromatics, carboxylic acids (volatile fatty acids) and sulphides (Boumba et al. 2008; Paczkowski & Schütz 2011). Carbohydrates are broken down into their monomers by various microorganisms and are further broken down to carbon dioxide, water, volatile fatty acids and alcohols depending on oxygen availability (Dent et al. 2004; Paczkowski & Schütz 2011). During the decomposition of lipids, triglycerides are hydrolysed to produce glycerol, saturated fatty acids and unsaturated fatty acids, which subsequently undergo hydrogenation or oxidation. Hydrogenation of unsaturated fatty acids produces saturated fatty acids, whilst oxidation converts these fatty acids into ketones and aldehydes and can decompose further to carbon dioxide and water (Dent et al. 2004).

1.4.1 Decomposition of Carbohydrates

Carbohydrates in the soft tissue degrade as a result of decomposition, microorganisms breakdown polysaccharides such as glycogen to its glucose monomers (Corry 1978; Forbes 2008). The resulting sugars are either completely oxidised to carbon dioxide and water or incompletely decomposed to form volatile fatty acids and alcohols (Forbes 2008). Pyruvate is predominantly produced from the breakdown of hexoses through either the Embden-Meyerhof-Parnas glycolytic pathway and the Entner-Doudorff pathway (Paczkowski & Schütz 2011). The *Pseudomonadaceae* family, which is abundant in soil, water and on the skin, utilises the Entner-Doudoroff pathway for their energy production (Paczkowski & Schütz 2011). Pyruvate is in turn fermented by anaerobic bacteria to yield acetic acid, butanoic acid and lactic acid, whilst citric acid, glucuronic acid and oxalic acid are produced by aerobic fungi (Forbes 2008; Paczkowski & Schütz 2011). Some additional breakdown products are pyruvic acid, propanoic acid, acetaldehyde, acetone, 1-propanol, 2-propanol, 1-butanol and 1,3-butanediol, see Table 2 (Boumba et al. 2008; Dent et al. 2004).

Table 2 Metabolic products of carbohydrate decomposition, adopted from Paczkowski & Schütz (2011)

Microbial Family	Location	Metabolic pathway	Metabolic products
Baccilaceae	Upper soil layers	Mixed acid fermentation	Lactic acid, succinic acid, acetic acid, formic acid and ethanol
Bifidobacteriaceae	Intestine and sexual organs	Bifidum pathway	Acetic acid and lactic acid
Clostridiaceae	Intestine, anaerobic soil layers	Pyruvate fermentation	Acetone, ethanol, 1-butanol, acetic acid, butanoic acid and 1,3-butanediol
Enterobacteriaceae	Intestine, soil, water	Mixed acid fermentation	Lactic acid, succinic acid, acetic acid, formic acid and ethanol
		2,3-Butanediol fermentation	2,3-Butanediol, lactic acid, acetic acid, formic acid and ethanol
Enterococcaceae	oral cavity, intestine, urethra and sexual organs	Mixed acid fermentation	Lactic acid, succinic acid, acetic acid, formic acid and ethanol
Lactobaccilaceae	Intestine	Lactic acid fermentation	Acetate, ethanol and lactic acid
Propionibacteriaceae	Skin and intestine	Propanoic acid fermentation	Acetic acid and proanoic acid
Streptococcaceae	Oral cavity	Lactic acid fermentation	Acetate, ethanol and lactic acid

Carbohydrates are metabolised by the *Bifidobactiraceae* species through the bifidum pathway to form acetic acid and lactic acid. These anaerobic bacteria are present in the intestine and sexual organs (Paczkowski & Schütz 2011). Many *Clostridiaceae* species ferment pyruvate through the pyruvate fermentation pathway to yield acetic acid, acetone, butanoic acid, 1-butanol, 1,3-butanediol and ethanol (Boumba et al. 2008; Paczkowski & Schütz 2011). *Clostridiaceae* are anaerobic bacteria that are present in the intestine and anaerobic soil layers, but can survive in aerobic conditions as they sporulate and such colonise a body from the inside and outside (Paczkowski & Schütz 2011). The *Bacillaceae*, *Enterobacteriaceae* and *Enterococcaceae* families are facultative anaerobic bacteria present in the intestine and ferment pyruvate to yield acetic acid, ethanol, formic acid, lactic acid and succinic acid via mixed acid fermentation (Boumba et al. 2008; Paczkowski & Schütz 2011). The *Klebsiella* genus of the *Enterobacteriaceae* is predominantly present in soil and water and produces acetic acid, 2,3-butanediol, ethanol, formic acid and lactic acid through 2,3-butanediol fermentation. Species of the *Lactobacillaceae* and *Streptococceae* family are facultative anaerobic bacteria that produce acetate, ethanol and lactic acid through the lactic acid fermentation pathway. Whilst the air tolerant *Propionibacteriaceae* family ferments carbohydrates or lactic acid to yield acetic acid and propanoic acid through propanoic acid fermentation pathway. Species of the *Propionibacteriaceae* family are anaerobic bacteria which grow relatively slowly on the skin and in the intestine (Paczkowski & Schütz 2011).

Yeasts contribute much less to vertebrate decomposition in comparison to bacteria (Corry 1978; Paczkowski & Schütz 2011). The *Candida* Genus has been observed in the intestine, oral cavity, sexual organs and between the fingers and toes of decomposing vertebrates. It is the constant metabolism of these aforementioned micro-organisms that leads to the formation of organic and inorganic gaseous compounds that cause bloating which results in rupturing the outer skin. Consequently, the inner fluids come into contact with oxygen and aerobic micro-organisms from the air and soil, which will colonise the decomposing body (Paczkowski & Schütz 2011).

1.4.2 Decomposition of Lipids

All cells contain lipids in their phospholipid membrane, whilst triglycerides are present in intramuscular fat (inside muscular tissue) and depot fat (under the skin). Triglycerides can be hydrolysed by microbial lipolytic enzymes to yield glycerol and fatty acids. Glycerol is degraded to produce ATP (Adenosine triphosphate), NADH (Nicotinamide Adenine Dinucleotide) and pyruvate, which is in turn decomposed to a range of products, see Section 1.4.1 Decomposition of Carbohydrates (Paczkowski & Schütz 2011). The fatty acids are further oxidised or hydrogenised to yield acetaldehyde, acetic acid, acetone, ethanol, 1-propanol, 2-propanol and propionic acid (Boumba et al. 2008; Paczkowski & Schütz 2011).

Following death, lipids are hydrolysed by intrinsic lipases to yield a mixture of fatty acids that can undergo hydrolysis or oxidation, depending on the surrounding environment (Forbes 2008). Oxidation will be favoured in aerobic environments, which converts the unsaturated fatty acids to aldehydes and ketones by bacteria, fungi and air. However, oxidation is less likely to occur than hydrolysis in a grave environment as the body is constantly exposed to reducing conditions (Forbes 2008; Hopkins et al. 2000). The saturated and unsaturated fatty acids will undergo further hydrolysis or hydrogenation in an oxygen deficient environment, which is enhanced by the presence of bacterial enzymes and moisture (Forbes 2008; Schotsmans et al. 2017).

Lipolytic enzymes which aid in the anaerobic hydrolysis and hydrogenation of lipids are produced by various *Clostridiaceae* species. Moisture is essential for the survival of bacteria and the hydrolysis of lipids, which is usually present in the tissues for these reactions to occur (Forbes 2008). If sufficient moisture and enzyme activity is present, hydrolysis of the fatty tissues will continue until all lipids are reduced to fatty acids, which in turn can form adipocere under the suitable conditions (Forbes 2008). Fungal lipoxygenase enzymes mainly produce C6 or C9 aldehydes and C8 alcohols or C8 ketones, in addition to aldehydes and ketones other chemical compounds that can be formed are acids, esters, epoxides and hydrocarbons (Boumba et al. 2008; Dent et al. 2004; Paczkowski & Schütz 2011).

1.4.3 Decomposition of Proteins

Amino acids are the major components of muscle tissue proteins, membrane proteins and free proteins. During decomposition, proteins are enzymatically degraded to peptones, polypeptides and amino acids through proteolysis (Forbes 2008). Microbial proteases and peptidases yield free amino acids from peptones and polypeptides, which can be further degraded to the formation of volatile products (Paczkowski & Schütz 2011). Neuronal and epithelial cells are usually the first cells to be affected, however the connective tissues and cartilage will eventually also decompose (Dent et al. 2004; Forbes 2008). The epidermis and muscle protein will resist decomposition for some period of time but would not survive as long as keratin and collagen. Keratin is a water insoluble protein found in the skin, hair and nails and is resistant to most proteolytic enzymes and is often found intact on skeletal remains (Forbes 2008).

Ammonia is one of the products produced from the degradation of amino acids and is produced through deamination of various amino acids (Dent et al. 2004). Decarboxylation, another degradation pathway for the degradation of amino acids yields biogenic amines such as putrescine and cadaverine from arginine/ornithine and lysine respectively (see Table 3). Section 1.5 Analysis of Biogenic Amines discusses the formation of biogenic amines such as putrescine and cadaverine in more detail (Paczkowski & Schütz 2011). Ammonia is present in soil as the ammonium ion at low pH and can be utilised by surrounding plants. The ammonium ions not utilised by plants can undergo nitrification (conversion of ammonia to nitrate) and denitrification (reduction of nitrate to nitrite, nitrogen gas and nitrous oxide) (Forbes 2008) which are detectable via ion chromatography. Two groups of micro-organisms are active in the nitrification process; the first group oxidises ammonia to nitrite (e.g. *Nitrosomonas* spp.), and the second group converts nitrite to nitrate (e.g. *Nitrobacter* spp) (Chapelle 2001; Forbes 2008). Nitrifying organisms are sensitive to environmental pH with *Nitrosomanas* spp working optimally between pH 7 and 9, and *Nitrobacter* spp working optimally between pH 5 and 8 (Forbes 2008). Nitrification occurs in an aerobic environment, conversely denitrification requires and anaerobic environment such as a gravesite by bacteria from the Achromobacter, *Bacillus*, *Micrococcus*, and *Pseudomonas* genus (Chapelle 2001; Forbes 2008). Accumulation of ammonia can occur in a grave environment as large quantities of ammonia can be produced from amino acids

under anaerobic conditions, and nitrification is inhibited under those conditions (Carter & Tibbett 2003; Forbes 2008).

Table 3 Metabolic products of amino acid decomposition, adopted from Paczkowski & Schütz (2011)

Amino acid	Metabolic pathway	Metabolic products
Arginine → Ornithine	Decarboxylation	Putrescine (1,4-diaminobutane)
Cysteine	Anaerobic	Sulphur, hydrogen sulphide, dimethyl sulphide, dimethyl disulphide, dimethyltrisulphide, dimethyltetrasulphide
	Desulfhydrase	Ammonia, hydrogen sulphide, pyruvate
Isoleucine	Ehrlich pathway, Anabolic biosynthetic pathway	1-Propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol
	Yeasts	1-Propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol
Leucine	Ehrlich pathway, Anabolic biosynthetic pathway	1-Propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol
	Moraxella phenylpyruvica, *Staphylococcus xylosus*, *Staphylococcus starnosus transforme*	3-Methyl-1-butanol, 3-methylbutanal, 3-methylbutanoic acid
	Yeasts	1-Propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol
Lysine	Decarboxylation	Cadaverine (1,5-diaminopentatne)
Methionine	Anaerobic	Hydrogen sulphide, dimethyl sulphide, dimethyl disulphide, dimethyltrisulphide, dimethyltetrasulphide, methanethiol
Methionine (continued)	Aerobic	Dimethylsulphide
	H. alvei, E. agglomeran, S. liquefaciens, A. putrefaciens, A. hydrophila	Dimethylsulphide, methanetiol
	Desulfhydrase	Ammonia, hydrogen sulphide, pyruvate
Threonine	Yeasts	1-Propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol
Tryptophan	*Bacteroides, Lactobacillus, Clostridium, Bifidobacterium, Peptostreptococcus*	Indole, Indonyl acetic acid, Indonyl prpanoic acid
Tyrosine	*S. albus, B. fragilis, Fusobacterium sp., Bifidobacterium spp., C. paraputrificum, C. butricum, C. sporogenes, C. septicum*	4-Methylpehnol, propanoic acid phenyl ester
	E. coli, Proteus sp., E. faecalis, S. albus	Phenol
Valine	Ehrlich pathway, anabolic biosynthetic pathway	1-Propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol
	Yeasts	1-Propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol

The sulphur-containing amino acids cysteine and methionine are broken down under anaerobic conditions to dimethyl sulphide, dimethyl disulphide, dimethyl trisulphide, dimethyl tetrasulphide, hydrogen sulphide and thiols (Dent et al. 2004; Gill-King 1997; Paczkowski & Schütz 2011). Methanethiol and dimethyl sulphide are produced during aerobic metabolism of methionine by *Hafnia alvei*, *Enterobacter agglomeran*, *Serratia liquefaciens*, *Alteromonas putrefaciens* and *Aeromonas hydrophila*, see Table 3 (Paczkowski & Schütz 2011). The anaerobic conditions in a grave environment favours the production of sulphides, which can transform to sulphurous acid, sulphur and sulphate under aerobic conditions (Dent et al. 2004; Forbes 2008; Paczkowski & Schütz 2011).

The amino acids isoleucine, leucine and valine are fermented through the Ehrlich pathway or through anabolic biosynthesis to produce 2-methylbutanol, 3-methylbutanol, propanol and 2-methylpropanol (Boumba et al. 2008; Paczkowski & Schütz 2011). Leucine can also decompose to 3-methylbutanal, 3-methylbutanoic acid and 3-methylbutanol by the *Moraxella phenylpyruvica, Staphylococcus xylosus and Staphylococcus starnosus transforme* bacteria. Decomposition of tryptophan by *Bacteroides*, *Lactobacilus*, *Clostridium*, *Bifidobacterium* and *Peptostreptococcus* yields indole, indoyl acetic acid and indoyl propionic acid (Paczkowski & Schütz 2011). Anaerobic tyrosine catabolism by a variety of bacteria (see Table 3) yields 4-methylphenol and propionic acid phenyl ester, whilst phenol is produced from tyrosine under facultative anaerobic conditions by *E. coli*, *Proteus sp*., *Enterococcus faecalis* and *S*. *albus* (Paczkowski & Schütz 2011).

Indolic and phenolic compounds are oxidised by mono- and di-oxygenases under aerobic conditions, but are fermented to acetic acid, butyric acid, hexanoic acid, hexanedioic acid, cyclohexanol, cyclohexanone, phenol, propionic acid, methane, CO_2 and H_2 gas under anaerobic conditions by methanogenic bacteria in the intestine (Paczkowski & Schütz 2011). In addition to bacterial catabolism, yeasts can catabolise the amino acids isoleucine, leucine, threonine and valine to yield 2-methylbutanol, 3-methylbutanol, pentanol and propanol (Boumba et al. 2008; Paczkowski & Schütz 2011).

1.5 Analysis of Biogenic Amines

Biogenic amines are nitrogen-containing compounds present in vegetable, microbial and animal cells and are mainly formed through decarboxylation of amino acids but can also be formed through amination of ketones and aldehydes (Innocente et al. 2006; Karovicova & Kohajdova 2005; Önal 2007; Pineda et al. 2012; Płotka-Wasylka et al. 2015; Teti et al. 2002). These amines are found in the aquatic environment, soil and air where they can be released by organisms while alive or during decomposition of animals and plants (Płotka-Wasylka et al. 2015). Oxidative decarboxylation of amino acids by bacteria for example the *Sherwanella spp* yields the formation of biogenic amines such as dimethylamine and trimethylamine (Paczkowski & Schütz 2011). By-products from the breakdown of proteins include biogenic amines such as histamine, tryptamine and phenylethylamine (Forbes 2008; Gill-King 1997; Paczkowski & Schütz 2011). Other reportedly commonly detected decomposition products are the biogenic amines putrescine and cadaverine, which are produced from the decarboxylation of ornithine (arginine hydrolysis yields ornithine) and lysine respectively, see Table 3 (Forbes 2008; Paczkowski & Schütz 2011). The decarboxylation products putrescine and cadaverine are reported in the literature as markers of decomposition and have been suggested to be key chemicals in locating human remains by VRD (Agapiou et al. 2015; DeGreeff & Furton 2011; Dekeirsschieter et al. 2009, 2012; Rebmann et al. 2000; Rosier et al. 2015; Statheropoulos et al. 2005, 2007, 2011; Vass et al. 2004). However, putrescine and cadaverine have not commonly been detected in decomposition related studies and is discussed in more detail below (Schotsmans et al. 2017).

Putrescine and cadaverine were first identified in 1885 by Ludwig Brieger through isolation from decomposing animal tissue (Lawrence 2004; Olle 1986). Putrescine (1,4-diaminobutane) is formed from decarboxylation of the amino acid ornithine and hydrolysis of the amino acid agmatine, which are both derived from the amino acid arginine. Cadaverine (1,5-diaminopentane) is formed through decarboxylation of the amino acid lysine (Lawrence 2004; Olle 1986). These biogenic amines can also rearrange upon heating to produce pyrroline and piperidine from putrescine and cadaverine respectively that can further react to form a variety of compounds including nitrosamines, see Figure 1 and Figure 2 (Callery & Geelhaar 1984; Cohen 1998; Glick 2009; Lundgren & Hankins 1978). Additionally, putrescine is also

a precursor for the production of spermine and spermidine (Cohen 1998; Olle 1986; Tabor & Tabor 1976).

Putrescine and cadaverine have regularly been cited as important biomarkers for decomposition and have been used in the training of victim recovery dogs (VRD) (Dent et al. 2004; Lorenzo et al. 2003; Stadler et al. 2012; Statheropoulos et al. 2007; Tipple et al. 2014; Vass et al. 2002). Several eminent researchers, however noted that these biogenic amines were not detected in their studies (Dekeirsschieter et al. 2009, 2012; Hoffman et al. 2009; Statheropoulos et al. 2005, 2007, 2011; Vass et al. 2004). These biogenic amines were also not reported or reported as absent in the majority of decomposition-related Volatile Organic Compound (VOC) characterisation studies (Brasseur et al. 2012; Cablk et al. 2012; Forbes & Perrault 2014; Forbes et al. 2014a, 2014b, 2016; DeGreeff & Furton 2011; DeGreeff et al. 2012; Kasper et al. 2012; Perrault et al. 2014, 2015b, Rosier et al. 2014, 2015; Stadler et al. 2013; Stefanuto et al. 2014, 2015a, 2015b; Paczkowski et al. 2015; Vass et al. 2008; Vass 2012). The only taphonomic studies which detected putrescine and/or cadaverine were by Bonte & Bleifuss (1977), Fiedler et al. (2004), Swann et al. (2012) and Vass et al. (2002).

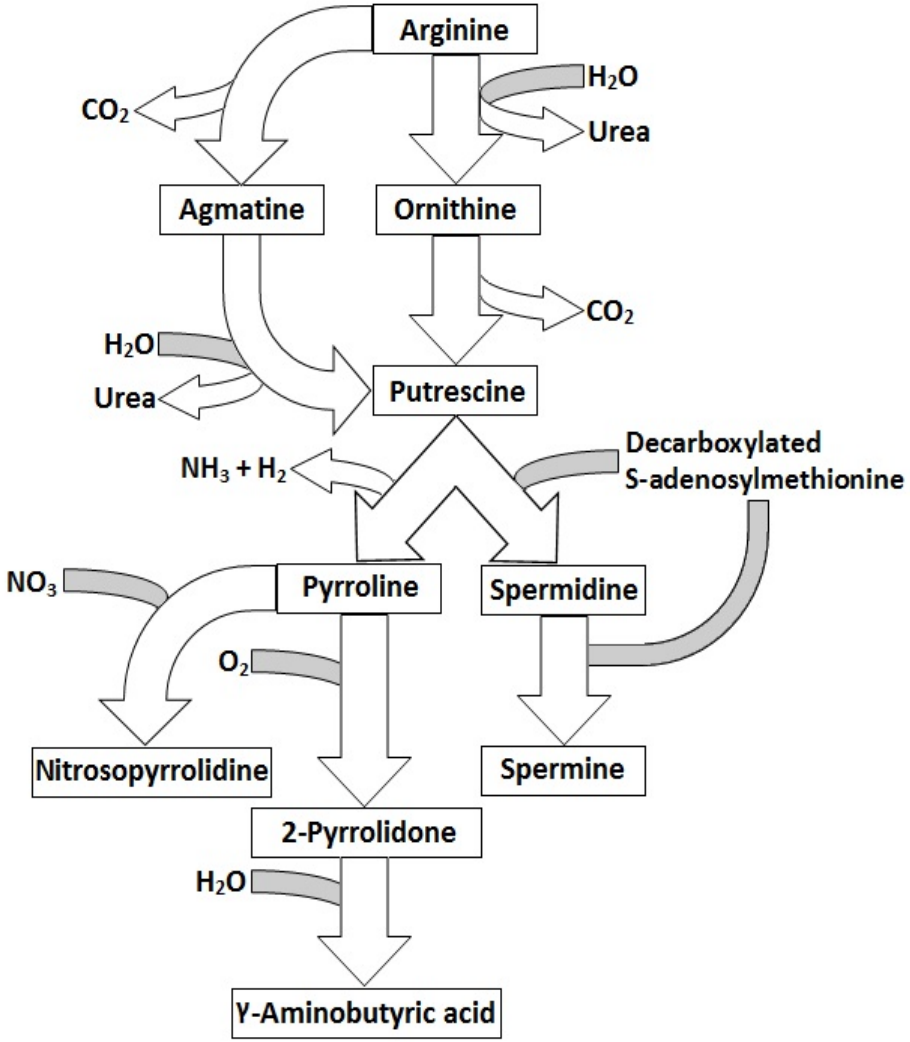

Figure 1 Formation and breakdown of putrescine

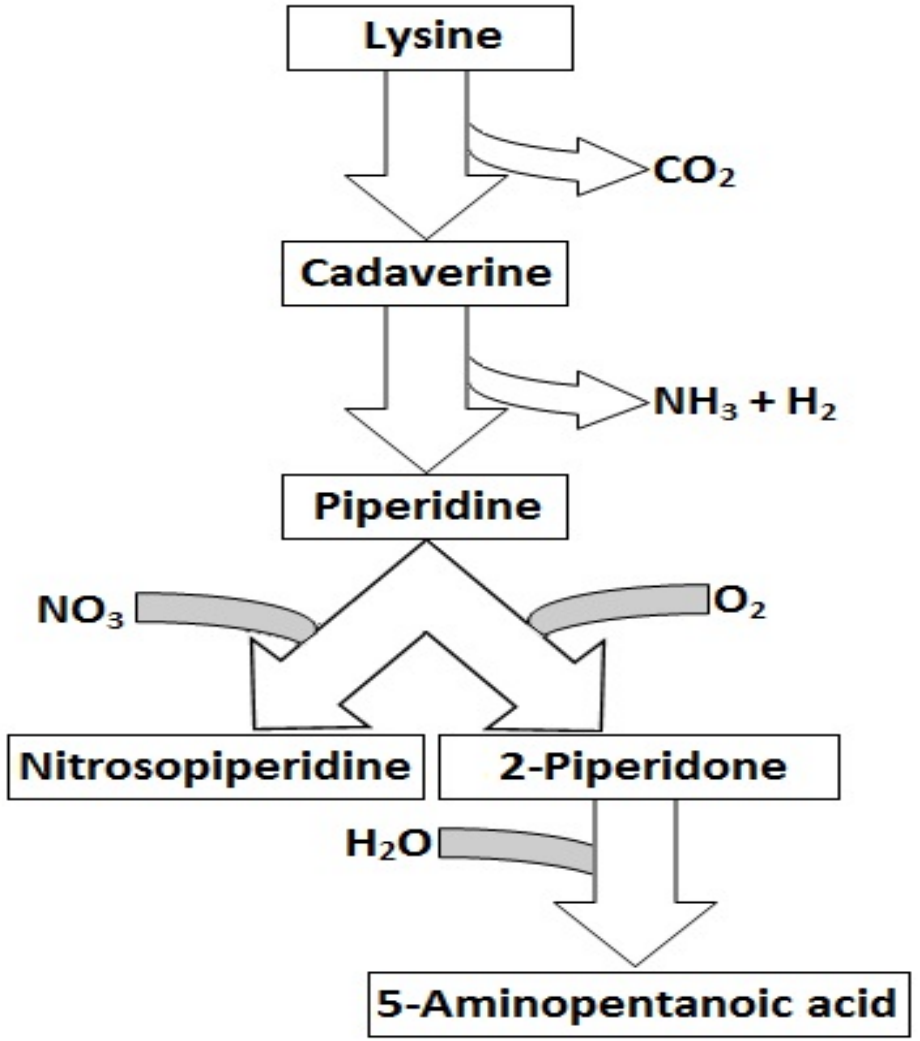

Figure 2 Formation and breakdown of cadaverine

Some explanations have been given in the literature as to why these biogenic amines were not detected during the decomposition of human remains. These explanations ranged from a lack of volatility to thermal lability, metabolism of putrescine and cadaverine and the formation of non-volatile salts (Lundgren & Hankins 1978; Lundgren & Fales 1980; Tipple et al. 2014; Vass et al. 2004). A study by Tipple et al. (2014) however demonstrated that putrescine and cadaverine were detected in commercially available VRD training aids, called pseudo scents, using liquid injection (direct injection) and in the headspace of a few millilitres (a significant quantity) neat putrescine and cadaverine in a sample container. They concluded incorrectly that there were no instrumental reasons why these amines would not be detected using gas chromatography (GC), although the concentration detected was unknown but was likely very high. Stadler et al. (2012) also reported the detection of putrescine and cadaverine including their breakdown products 2-pyrrolidone and 4-aminobutyric acid during the analysis of these pseudo scents by direct injection GC-MS. The levels of putrescine and cadaverine were not quantified, so the concentration of putrescine and cadaverine in the pseudo scents and their instrumental detection limits are still unknown. Tipple et al. (2014) stated that neither putrescine nor cadaverine were detected amongst the headspace volatiles in the pseudo scents, which highlights an issue regarding the current headspace detection mechanisms.

Putrescine and/or cadaverine have only been successfully detected in four taphonomic studies using various forms of chromatography and none of these studies utilised headspace detection. The first study was by Bonte & Bleifuss (1977) where the authors detected putrescine, cadaverine and additional biogenic amines in decomposing cattle blood and human liver homogenates using two-dimensional thin layer chromatography. They were the first to quantify putrescine and cadaverine, detecting concentrations of 20 mg L^{-1} in liver homogenates through the use of ninhydrin and a densitometer. Interestingly 4-aminobutyric acid, a breakdown product of putrescine, was detected from the start of their study whilst putrescine was only detected after approximately 25 days in untreated blood and liver homogenates (Bonte & Bleifuss 1977). The second study was by Vass et al. (2002), both putrescine and cadaverine were detected in addition to a variety of amino acids in decomposing human tissue samples using GC-MS after derivatisation with dimethylformamide dimethyl acetal. They concluded that putrescine and cadaverine were not useful biomarkers for decomposition due to their inconsistency between

cadavers but identified 4-aminobutyric acid, the breakdown product of putrescine, to be a critical marker for post-mortem interval calculation. Putrescine and cadaverine were detected in concentrations above 3 μg mg^{-1} tissue and thus could be very beneficial to detecting clandestine gravesites as it increases the probability of detecting these chemicals in the surrounding soil environment.

High performance liquid chromatography (HPLC) was utilised by Fiedler et al. (2004) in combination with an ultraviolet-visible (UV/VIS) spectrophotometer to detect putrescine and cadaverine, which is the third study to detect these amines. Cadaverine was detected along with 4-aminobutyric acid and tyramine in cemetery grave-soil following derivatisation with 9-fluorenylmethyl chloroformate. The methodology was adapted from Kirschbaum et al. (1994) and Fernandes & Ferreira (2000), both studies originated from the field of food chemistry for the determination of biogenic amines including putrescine and cadaverine. Fiedler et al. (2004) detected only cadaverine in soil samples near the lowest part of the coffin at concentrations between 11 and 40 μg kg^{-1} soil, cadaverine was also detected in the control soil up to a concentration of 35 μg kg^{-1}. It was assumed that leaching of cadaverine through the flow of ground water contaminated the control soil. It is unknown if putrescine was also present at similar concentrations to cadaverine as its detection limit was 50 μg kg^{-1}, whilst the detection limit for cadaverine was 10 μg kg^{-1}.

The final taphonomic study to detect putrescine and/or cadaverine was by Swann et al. (2012) where putrescine was detected as one of a total of nineteen biogenic amines from porcine decomposition fluid but cadaverine was not detected. HPLC-MS was utilised and thus did not require derivatisation to detect the chromophore lacking biogenic amines. A benefit of the mass spectrometer is that not all peaks need to be chromatographically resolved as they were filtered through multiple reaction monitoring and only thirteen of the nineteen peaks were actually observed in the total-ion-chromatogram indicating co-elution. The lack of derivatisation could however have disadvantages as it could lead to poor peak shape due to partial adsorption of the analyte with the stationary phase (see page 27). Swann et al. (2012) detected putrescine at m/z 89.2 $(M+H)^+$ although putrescine has two amino groups that could easily be ionised using electrospray ionisation resulting in $[M+2H]^{2+}$. These two positive charges on the putrescine molecule reduce its m/z value to approximately 45.1, which could result

in non-detection as m/z values below 50 will most likely be filtered out the mass spectrometer before it reaches its detection mechanism. Multiple ionisation could also have occurred for ornithine and lysine (the precursors for putrescine and cadaverine) and could be a reason why cadaverine was not detected in their study.

In order to determine why putrescine and cadaverine have been cited as important biomarkers for decomposition so regularly and been detected so scarcely, a review of the available literature was performed. It became apparent that putrescine and cadaverine became important markers due to incorrect citation, see Figure 3. Putrescine and cadaverine were in fact detected by Bonte & Bleifuss in 1977, however the interest in these compounds arose only after Gill-King suggested their importance as decomposition products due to their characteristic foul odour and detectability by VRD (Gill-King 1997). This was referenced from Killam (1990), however extensive review of this book showed no mention of putrescine and cadaverine. Rebmann et al. (2000) cited Gill-King and noted in “The Cadaver Dog Handbook” that putrescine and cadaverine, along with other inorganic gasses produced during putrefaction are detectable by VRD. Since then Gill-King (1997) and/or Rebmann et al. (2000) have been repeatedly cited in relation to the importance of putrescine and cadaverine as decomposition chemicals and key to detecting human remains by VRD. As previously discussed, eminent researchers such as Dekeirsschieter, Hoffman, Statheropoulos and Vass started looking for putrescine and cadaverine among other VOC’s and were unable to detect these biogenic amines. To date, the significance of putrescine and cadaverine within the field of taphonomy has been unclear due to a lack in detection (Schotsmans et al. 2017). This is due to a lack in understanding as putrescine and/or cadaverine have been detected in taphonomy related studies, indicating putrescine and cadaverine are produced during decomposition and could be key markers.

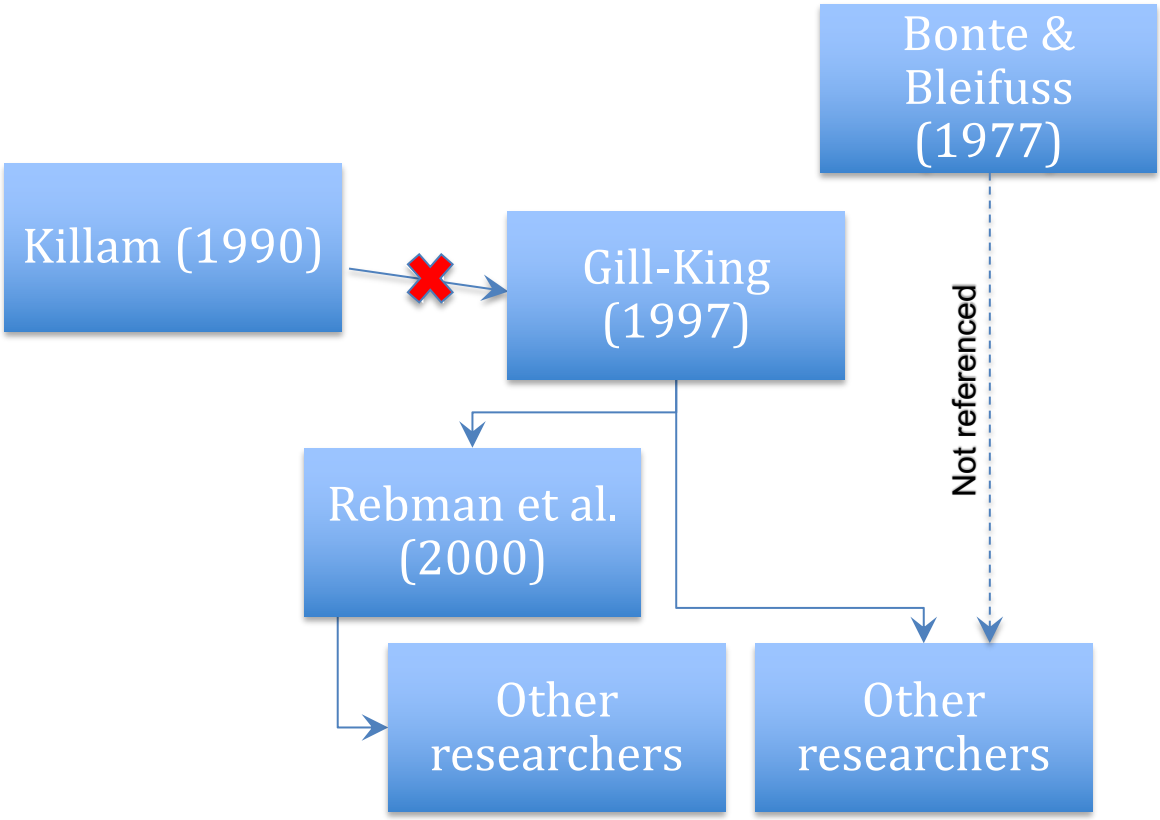

Figure 3 Overview of putrescine and cadaverine citations in taphonomic literature

One group that successfully detected cadaverine, Fiedler et al. (2004), adapted their methodology from the food industry, where putrescine and cadaverine have been of particular interest as indicators of quality and food spoilage (Awan et al. 2008; Ali Awan et al. 2008; Karpas et al. 2002; Önal 2007; Pineda et al. 2012; Saccani et al. 2005). The food industry is particularly interested in the histamine levels in food products, as low quantities can cause hypo- or hypertension, headache or anaphylactic shock. Diamines such as putrescine and cadaverine can synergistically increase the toxicological effects of histamine due to competitive inhibition of metabolising enzymes (Kirschbaum et al. 2000; Lange et al. 2002). According to Kumudally (2001) chromatographic methods are suitable for detecting and quantitating biogenic amines to monitor the freshness of food products. This further confirms that the non-detection of putrescine and cadaverine in most taphonomic studies is due to the use of unsuitable methodologies for the detection of these compounds. The detection and quantification of putrescine and cadaverine at low part per billion concentration from various complex matrices using GC has been widely published in food, wine, environmental and pathophysiological related studies (See Awan et al. 2008a; Cueva et al. 2012; Cunha et al. 2011; Fernandes & Ferreira 2000; Krzyzoaniak et al. 2011; Ngim et al. 2000; Pineda et al. 2012; Yamamoto et al. 1982) and derivatisation GC is therefore

suitable to analyse putrescine and cadaverine in taphonomy related studies. However GC analysis without derivatisation is often unsuitable for amine analysis due to the polarity of the amines, which makes them adsorb onto the column and exhibit excessive peak tailing (Ferreira et al. 2013; Płotka-Wasylka et al. 2015). In addition, many amines do not possess the structural features, such as a chromophore, to enable UV/VIS detection by HPLC (Płotka-Wasylka et al. 2015; Ferreira et al. 2013). This emphasises the need for derivatisation to enhance chromatographic detection of the amines and has been utilised by the studies discussed above.

Derivatisation increases the volatility, thermal stability and mass spectral detection of putrescine and cadaverine and enables accurate determination using GC-MS (Paczkowski & Schütz 2011; Płotka-Wasylka et al. 2015; Vass et al. 2002). Derivatisation removes issues associated with the instrumental analysis of amines, such as long elution times, chromatographic peak tailing and low reproducibility, as the alkalinity of the amine group introduces a large dipole onto the analyte (Avery & Junk 1985; Krzyzoaniak et al. 2011). This causes the amine group to interact with silanol groups and siloxane bridges present in the GC-column resulting in partial adsorption of the analyte making the analysis unreliable at low concentrations (Kataoka 1996; Krzyzoaniak et al. 2011; Nakovich 2003). Thus derivatisation of the analyte is often recommended as it increases the sensitivity, selectivity, analyte resolution and sample throughput (Ngim et al. 2000).

Many different derivatisation reactions are able to derivatise amines for GC analysis. Kataoka (1996) reported in his review eight different derivatisation mechanisms for the determination of amines by gas chromatography and the seven most important are displayed in Table 4. Other reaction mechanisms have also been reviewed by Kataoka (2005) and Ferreira et al. (2013) but are not commonly used in comparison to the reaction mechanisms described in Table 4 and are therefore not included. *In situ* derivatisation is preferred as the derivatisation occurs directly in the aqueous matrix so no prior solvent extraction is required therefore minimising sample preparation and reducing analysis time, associated errors and contamination (Ferreira et al. 2013; Pan et al. 1997). A disadvantage of *in situ* derivatisation is that many derivatisation reagents react or decompose in water thus requiring a suitable reagent for aqueous derivatisation. Specificity is, in this case, also very important as reactive matrix compounds could reduce the reaction yield (Ferreira et al. 2013).

Table 4 Amine derivatisation mechanisms for analysis by gas chromatography

Derivatisation mechanism	Aqueous derivatisation	Amine specific	Amine type	Derivatisation specific information
Acylation	No	No	1° & 2°	– Requires removal of excess reagent
Silylation	No	No	1° & 2°	– Requires catalyst – Unstable to moisture – Could derivatise primary amines twice
Dinitrophenylation	Yes	No	1° & 2°	– Easy to derivatise – Could sustain chromatographic peak tailing – Strong acid by-products
Alkylation (Permethylation)	Yes	No	1° & 2°	– Produces tertiary amines – Could sustain chromatographic peak tailing
Carbamate formation	Yes	No	1°, 2° & 3°	– Derivatives exhibit good GC-properties – Produces phosgene with water
Schiff base formation	Yes	Yes	1°	– Rapid derivatisation – Produces good yields – Very selective – Requires removal of excess reagent
Sulphonamide formation	Yes	Yes	1° & 2°	– Useful to separate and identify amine type through solubility

The most suitable derivatisation mechanism for the derivatisation of putrescine and cadaverine in Table 4 is through Schiff base-type derivatisation as it provides a rapid derivatisation with good yields, can be used in aqueous samples and is very selective towards primary amines (Ferreira et al. 2013; Kataoka 1996, 2005; Płotka-Wasylka et al. 2015). Acylation reactions are most frequently used to derivatise amines as it easily derivatises amino groups under mild conditions, but acylation reagents usually do not derivatise in aqueous environments and are not amine specific (Ferreira et al. 2013; Kataoka 1996, 2005; Płotka-Wasylka et al. 2015). Silylation reagents also do not derivatise samples in an aqueous environment and their derivatives are unstable to moisture (Ferreira et al. 2013; Kataoka 1996, 2005). In contrast to acylation and silylation reagents dinitrophenylation, permethylation (alkylation) and carbamate formation (acylation type derivatisation) reagents are capable of derivatising amines in an aqueous environment but their reagents are often not amine specific (see Table 4) and also derivatise other functional groups (Ferreira et al. 2013;

Kataoka 1996, 2005; Nakovich 2003; Płotka-Wasylka et al. 2015). In addition, dinitrophenylation derivatives (nitrosamines) are carcinogenic and the permethylation-type derivatisation creates tertiary amines that could still exhibit chromatographic complications (Ferreira et al. 2013; Kataoka 1996, 2005). The final derivatisation mechanism in Table 4 is a sulphonamide formation-type derivatisation, which can be utilised in an aqueous environment and is specific to primary and secondary amines but requires an additional clean-up step to separate primary and secondary amines (Ferreira et al. 2013; Kataoka 2005).

The Schiff base-type derivatisation reaction have been used to quantify amines in a variety of complex matrixes such as biological samples (Avery & Junk 1987; Jindal et al. 1980; Johansson & Vessman 1982), environmental samples (Avery & Junk 1985; Chia & Huang 2006; Deng et al. 2006; Lin et al. 2008; Llop et al. 2010a, 2010b, 2011; Pan et al. 1997) and food products (Ngim et al. 2000; Pendem et al. 2010). Pentafluorobenzaldehyde is often the reagent of choice and has been used to analyse low-molecular-mass amines as the reaction proceeds rapidly and is stable in water under the alkaline conditions required for the derivatisation (Ferreira et al. 2013; Kataoka 1996, 2005; Lin et al. 2008; Llop et al. 2010b). The reaction mechanism of pentafluorobenzaldehyde with a primary amine to form a pentafluorobenzylimine is illustrated in Figure 4. The reaction mechanism is divided into two parts, the first part is a base-catalysed addition to the carbonyl group and the second part of the reaction is a base-catalysed dehydration of the hydrate to form an imine (also called Schiff base). Pentafluorobenzaldehyde derivatives are able to be analysed at picogram sensitivity using GC-MS through single ion monitoring (SIM) of the characteristic α-cleavage product ion $(m/z\ 208)$ $[C_6F_5\text{-}CH{=}N\text{-}CH_2]^+$ (Kataoka 2005; Ngim et al. 2000). The by-product water does not undergo secondary reactions under the conditions required, however excess derivatisation reagent often needs to be removed which otherwise interferes with the analysis (Kataoka 1996, 2005).

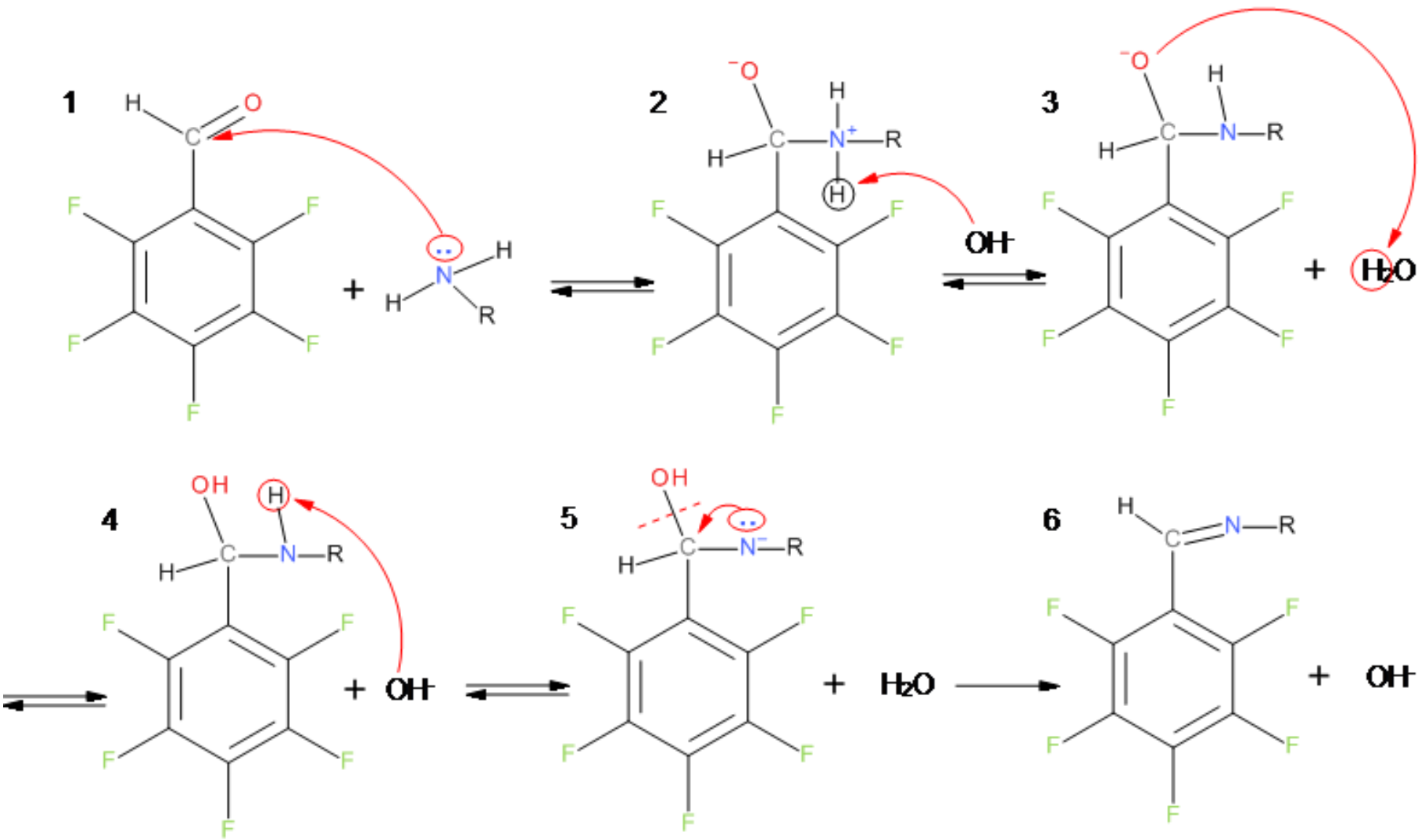

Figure 4 Schematic reaction mechanism of a primary amine with pentafluorobenzaldehyde

1.6 Analysis of Alternative Decomposition Products Using Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is excellent for the analysis of non-volatile and semi-volatile chemicals but has not been utilised frequently for the detection of decomposition related chemicals. It has been applied by Fiedler et al. (2004) and Swann et al. (2012) for the analysis of biogenic amines (including putrescine and cadaverine) using derivatisation HPLC and LC-MS respectively (See section 1.5 Analysis of Biogenic Amines). It has also been applied in laboratory scale decomposition studies for the determination of adipocere by Yan et al. (2001) and Durães et al. (2010). In the study by Yan et al. (2001) pig cadavers were submersed in distilled water, chlorinated water and saline water at controlled temperatures to allow adipocere formation. Water samples were collected and were analysed for the fatty acids; oleic, palmitic and stearic acid without derivatisation. This required the use of low detection wavelengths (210 nm) due to poor UV absorbance of the fatty acids, which could have limited the use of buffers and affected the sensitivity and detectability of the acids as can be observed from the baseline noise in Yan et al. (2001) chromatograms.

The research published by Durães et al. (2010) buried pork loin in four different types of soil (organic, sandy, gravel and clay-gravel) in plastic hermetic boxes. This experiment tried to simulate a body in a coffin, however the soil was directly in contact with the tissue which would not have been the situation in a coffin. Randomly selected soil samples (between 30 and 75 g) were extracted and HPLC-UV analysis was conducted for myristic, oleic, palmitic and stearic acid after derivatisation with 1-phenylethylamine at a similar low wavelength as reported by Yan et al. (2001), 215 nm. It may have been more suitable to analyse the derivatised fatty acids at a wavelength of 254 nm (corresponding to the phenyl group) instead of the reported 215 nm (Wade 2010). At a wavelength of 254 nm less interference would have been observed from the large quantities (90%) of methanol in the mobile phase. Algarra et al. (2010) applied this methodology to analyse soil samples collected from cemeteries using LC-MS and stated that the absorption maxima of the derivatised fatty acids is at 259 nm and made the interpretation of the chromatograms considerably easier. Analysis of adipocere has predominantly been performed using GC over HPLC as many publications, especially those from the Forbes group utilised GC-MS for the detection of adipocere

(Forbes et al. 2002, 2003, 2004, 2005a, 2005b, 2005c, 2005d; Cassar et al. 2011; Ueland et al. 2014). The utilised methodology can be tracked back to a publication from Takatori & Yamaoka (1977) where the authors analysed hydroxyl fatty acids in adipocere as TMS derivatives using GC-MS. This may explain the predominant usage of GC-MS over HPLC as the LC-MS only became commercially available during the late 1980's (Pullen 2010).

HPLC analysis has more regularly been utilised for the analysis of body fluid opposed to a grave environment within the field of thanatochemistry (chemistry of death) for the estimation of post-mortem interval. Researchers such as (James et al.1997; Madea et al. 1994; Muñoz Barús et al. 2002; 2006; Rognum et al. 1991) all utilised HPLC to determine hypoxanthine levels in vitreous humor for post-mortem interval estimation. Girela et al. (2008) also utilised HPLC in the research area of thanatochemistry to determine the cause of death as well as post-mortem interval through the quantification of free amino acids in both vitreous humor and cerebrospinal fluid. The use of HPLC along with other analytical techniques for the estimation of post-mortem interval using biochemical markers in blood has been extensively reviewed by Donaldson & Lamont (2014) and highlights the usefulness of HPLC for the detection of decomposition products within this matrix.

In the field of forensic science, HPLC analysis has shown potential as an alternative method for profiling forensic soil samples. In 1981, Reuland utilised HPLC analysis as an alternative technique to presumptively differentiate between soil samples through analysing the extractable organic components such as polycyclic aromatic hydrocarbons (Reuland & Trinler 1981). The methodology discriminated between soil samples taken from different environments but was not able to do so with samples taken within three meters from another. The work of Reuland and Trinler was adapted by Siegel & Precord (1985) and Reuland et al. (1992) to differentiate between close proximity soil samples but without any success. More recently, Bommarito et al. (2007) continued the work of Reuland and Siegel by extensively evaluating the discriminatory power of the HPLC and its ability to differentiate between close proximity soil samples. Aproximately 120 soil samples were analysed, however their conclusions were very similar to those reported by earlier research (Reuland & Trinler 1981; Reuland et al. 1992; Siegel & Precord 1985). The researchers stated that their methods were able to differentiate between soil

samples within a relatively small geographic area but no information was provided regarding the proximity. Nevertheless, HPLC analysis has demonstrated its capability to differentiate between soil samples and could be applied to the analysis decomposition fluids in a burial environment.

Liquid Chromatography-Mass Spectrometry (LC-MS) has proven to be invaluable in the field of forensic science and is extensively used for the analysis of highly polar, non-volatile and thermo-labile compounds (Wood et al. 2006). Since the introduction of electrospray ionisation and atmospheric pressure chemical ionisation, the popularity of LC-MS has increased. It evolved into a robust and reliable tool that offers versatility, specificity and sensitivity from a very infrequently used technique as an alternative to GC-MS for troublesome compounds (Wood et al. 2006). Furthermore, LC-MS has been recognised to be vital in a routine environmental laboratory carrying out monitoring of emerging contaminants. It is a complementary technique to GC-MS and became indispensable due to its advantage over GC-MS for environmental monitoring. The applications for which LC-MS has been utilised was reviewed by Wood et al. (2006) and highlighted its versatility and utility in forensic science. Even though, LC-MS is indispensable in many applications of analytical chemistry, it has only been used once in the field of forensic taphonomy and its capabilities are currently underused. Swann et al. (2012) reported the detection of nineteen amino acids and amines in porcine decomposition fluids and the use of LC has been reported to be under “current investigation” by Vass et al. (2004), but as far as the author is aware nothing has been published since regarding this.

Solid-phase extraction (SPE) is one of the sample preparation techniques available to an analyst to bridge the gap between sample collection and instrumental analysis. According to Simpson, it has been used for thousands of years, even though initially the science behind the process was unknown (Simpson 2000). The goal of SPE is to collect the compounds of interest, preferably by concentrating them from a sample and removing unwanted compounds. Sample pre-concentration is required to extract trace compounds in forensic, archaeological or environmental samples and SPE is the most widely used method to selectively concentrate analytes in aqueous samples (Notter et al. 2008; Dean 2009; How et al. 2014; Lindholm et al. 2014).

Other pre-concentration methods include solid-phase microextraction, single-drop microextraction, dispersive liquid-liquid microextraction and vortex assisted liquid-liquid microextraction. These methods require lower quantities of potentially harmful solvents but SPE is still used most widely because it removes interfering compounds from the sample matrix in addition to extracting the analytes (How et al. 2014; Lindholm et al. 2014). In comparison to traditional liquid-liquid extraction, SPE directly extracts the analyte onto the sorbent and thus only requires small volumes of solvent, has no emulsions, exhibits better recoveries, provides cleaner extracts and has the ability to remove interferences selectively (Budal 2013; Lindholm et al. 2014).

In the field of forensic taphonomy, SPE has been utilised as a means of sample clean-up and sample pre-concentration for the analysis of lipids. Notter et al. (2008) developed a methodology for the extraction of neutral lipids and free fatty acids from porcine samples using an aminopropyl extraction phase. The neutral lipids were extracted first using a 2:1 mixture of chloroform and 2-propanol and the free fatty acids were extracted through 2% acetic acid in diethyl ether. Good recovery and regression was observed for this method, which was likely due to the electrostatic interactions between the amine group on the SPE cartridge and the acids. Notter et al. (2008) used a multistage extraction methodology to provide more selective extracts through fractionation of different classes of chemicals.

1.7 Analysis of Inorganic Anionic Compounds Using Ion Chromatography

Taphonomic research conducted on human remains by Dr. Arpad Vass highlighted the use of ionic chemicals and Volatile Fatty Acids (VFA) to calculate post mortem interval (Vass et al. 1992). The ionic compounds believed to be prominent are sodium, chloride, ammonium, potassium, calcium, magnesium and sulphate, and VFA such as; formic acid, acetic acid, propionic acid and butyric acid were also detected due to their water solubility. These chemicals have been used to calculate post mortem interval confirming that these compounds are produced during the decomposition process and thus could be markers for decomposition.

Ion chromatography (IC) has not frequently been utilised for the detection of decomposition related ions within the field of forensic taphonomy. It has been utilised first in 1992 by Dr. Arpad Vass and colleagues to determine the cations and

anions that could be detected in the soil solution underneath a decomposing body for the estimation of time since death. Sixteen ions were analysed but only seven ions (including the anions chloride and sulphate) were determined to be useful according to Vass et al. (1992) due to their stability in the environment and reproducibility between bodies. A study performed by Aitkenhead-Peterson et al. (2012) also utilised ion chromatography for the analysis of soil samples underneath a decomposing body to map the spatial extend of a cadaver decomposition island and to determine the potential for movement of water soluble chemical constituents (leaching). A total of seven ions were determined along with DOC (dissolved organic carbon), TDN (total dissolved nitrogen) and orthophosphate using ion chromatography, TOC (total organic carbon analyser and colorimetric methods respectively. Of the three inorganic anions analysed slightly elevated levels of phosphate were present in the grave soil over the control soil and generally lower levels of sulphate were detected in the grave soil, whilst the chloride results were inconsistent between bodies. The author also suggested that migration of the decomposition products downslope was observed.

In 2007, research was published by Bommarito et al. analysing 120 soil samples using IC and HPLC to distinguish between soil samples in a forensic context. Quantitative determination was performed on a total of twelve anionic compounds of which the seven ions (nitrite, nitrate, phosphate, sulphate, perchlorate, bromide and thiosulphate were quantified and determined to be significant to distinguish between soil samples. Other ions detected were fluoride and chloride, and at least two additional signals were observed eluting between the fluoride and chloride peak that were not identified. Based on research performed at Staffordshire University the peak co-eluting with fluoride could have been acetate, whilst the other could have been formate as these have been observed to have a similar elution pattern and these acids have been reported to be abundant in nature (Vass et al. 1992).

Copious research has been conducted over the last 50 years to determine the effect of cemeteries on the groundwater chemistry using IC. A comprehensive review has been published by Zychowski in 2012 and reported that the first study on the impact of cemeteries on the surrounding environment was published in 1951 by van Haaren which revealed elevated concentrations of chlorides, sulphates and bicarbonates in nearby shallow groundwater. In the studies published by Trick et al. (2001, 2005), as reported by Zychowski (2012), relatively increased concentrations of chloride,

sulphate, sodium and calcium were detected in the lower part of a cemetery (indicating leaching of water soluble chemicals downhill), whilst nitrate, sulphate, bicarbonate, carbonate, potassium and magnesium ions were abundant in the middle part of the cemetery. Furthermore, high concentrations of sulphates, sodium and chlorides, in addition to high concentrations of VFA were detected in samples taken from piezometers placed in shallow groundwater near cemeteries. This information indicates that ionic compounds could indicate the presence of a decomposing body and highlighted the migration of decomposition products as had been suggested by Aitkenhead-Peterson et al. (2012).

1.8 Extraction of Decomposition Products from Soil Samples

In order to allow for the analysis of decomposition products from soil samples the literature has been reviewed to aid the development of a suitable solid-liquid extraction methodology that will allow analysis of the case samples using the techniques discussed previously.

Extraction is based on the transfer of mass from one substance to another, in this case from a solid to a liquid. Mass transfer of solutes is mainly caused by diffusion and if a fluid motion is present (agitation), convection also contributes to mass transfer. For the extraction from solid particles, depending on the extraction medium at least two different types of mass transfer happen; solvation of the analyte from the solid phase into the liquid phase and diffusion of the analyte through the liquid phase. If agitation is applied mass transfer could be speeded up through convection (movement of liquid). Efficient extraction between the sample matrix and extraction phase is mainly dependent on the distribution constant and kinetic factors such as diffusion coefficient and agitation conditions. Parameters such as temperature, pressure, pH, salt and organic concentration influence the distribution constant, whilst agitation, temperature and pressure affect extraction kinetics through speeding up movement and penetration of the extraction phase into the sample matrix (diffusion coefficient) (Pawliszyn et al. 2012). Grinding of the sample increases surface area and therefore increases extraction kinetics, reducing time required to reach equilibrium (Pawliszyn et al. 2012).

Commonly used extraction methods include shake flask, ultra-sonication, soxhlet, pressurised fluid (accelerated liquid), supercritical fluid extraction and microwave assisted extraction. The extraction principles can be classified into three different

groups; agitation, heat and heat & pressure. Shake flask extraction and ultrasound-assisted extraction fall under the first category, where the extraction kinetics are improved through introduction of convection to aid diffusion. Although heat can be produced during the ultrasound-assisted extraction either benefitting or hindering extraction efficiencies. Soxhlet extraction utilises only heat during the extraction procedure, which influences the distribution constant and diffusion coefficient (see paragraph above). In addition, Soxhlet extraction displaces the transfer equilibrium by constantly providing fresh extractant (Wang & Weller 2006; Luque de Castro & Priego-Capote 2010). Pressurised liquid extraction, supercritical fluid extraction and microwave assisted extraction all utilise both heat and pressure to extract analytes and therefore affect the distribution constant and diffusion coefficient. Table 5 provides an overview of the different extraction methods and their advantages and disadvantages, due to the high costs of supercritical fluid extraction and microwave assisted extraction they were not included in the review.

Table 5 Principles, advantages and disadvantages for the different solid-liquid extraction methodologies

Extraction Method	Shake flask (SF), Agitation by mixing	Ultra-sonication (USE), Agitation by sonication	Soxhlet (SE), Heat	Pressurised liquid (PLE), Heat & pressure
Extraction principles	• Sample is agitated or shaken for a specified time period[1,2]	• Creation of bubbles in the liquid which collapse and produce high-speed jets that impact the solid surface[3]	• Sample is repeatedly brought into contact with fresh extractant and facilitates displacement of transfer equilibrium[3,4]	• Extraction is carried out under pressure to keep solvent in liquid state and is forced into solid matrix[3,5] • Increased temperature improves extraction kinetics and diffusivity[3] • Increased temperature increases solubility and decreases viscosity allowing better penetration[5]
Advantages	• Less solvent required[1] • Less time required[1]	• USEPA approved • Intimate contact • Less solvent required[1] • Less time required[1] • Better for thermolabile compounds[3] • Ultrasound frequency improves extraction yield and kinetics[3] • Quicker than SF and SE[1,2]	• Can analyse extracts directly[3,4,6] • USEPA approved[6] • Intimate contact • Displacement of transfer equilibrium[3,4] • Better reproducibility and efficiency[2,3]	• USEPA approved • Less solvent required[3] • Less time required[3,5] • Intimate contact • Could be more effective and selective[5]
Disadvantages	• Not USEPA approved[1] • Requires three subsequent extractions[1] • Filtration required afterwards[2] • May need sample conentration[2]	• Not always leads to increased extraction efficiencies[3,6] • Requires three subsequent extractions[2] • Filtration required afterwards[2] • Not as rigorous as other USEPA methods • Excess sonication can damage quality of extracts[3] • May need sample conentration[2]	• Long extraction times[3,4,6] • Sample requires conentration[3,4,6] • Possibility of thermal decomposition[3,4] • No agitation to speed up reaction[2,3,4] • Diffusion might be limited by matrix[3] • Requires large quantities of sample[2]	• Possibility for thermal decomposition[3] • May need sample concentration • May need filtering
When used	• High concentration compounds or not associated with soil components[6]	• Often using aqueous solutions[6] • For phenols/ amines/ PAH's[6] • Non-volatile and semi-volatiles	• Relatively low concentration compounds[6] • Non-volatile and semi-volatiles	• Water insoluble and semi-soluble compounds

[1] (Dean & Xiong 2000), [2] (Dean 1998), [3] (Wang & Weller 2006), [4] (Luque de Castro & Priego-Capote 2010), [5] (Péres et al. 2006), [6] (Conklin 2013)

Based on the information provided in Table 5, the most suitable method to extract both the anions and amines was through the use of ultrasound-assisted extraction. It is a simple and inexpensive method to use, allows for a wide variety of solvents and requires less solvent and time over shake flask and soxhlet extraction (Dean & Xiong 2000; Wang & Weller 2006). Furthermore, ultrasound-assisted extraction is better for thermolabile compounds, more suitable for anions associated with soil components in comparison to shake flask extraction and is USEPA approved as a solid-liquid extraction procedure (Wang & Weller 2006; Conklin 2013). Unfortunately, ultrasound-assisted extraction is not as rigorous as other USEPA approved methods such as soxhlet and pressurised liquid extraction and requires three subsequent extractions (for low concentration samples) for quantitative analysis but is much better for the analysis of multiple samples simultaneously (Dean 1998). Shake flask extraction also requires three subsequent extractions and it is not USEPA approved, therefore making the extraction technique less reliable but see Stanisic in the paragraph below (Dean & Xiong 2000). Soxhlet extraction has been used for many years due to its high recovery and reproducibility, however it requires long extraction times, is prone to thermally decompose analytes and requires large quantities of soil (Dean 1998; Luque de Castro & Priego-Capote 2010; Wang & Weller 2006). Pressurised liquid extraction has been reported to be more effective than shake flask extraction and ultrasound assisted extraction and requires less solvent and time due to intimate contact with the sample (Péres et al. 2006). However, it may also thermally decompose extractable analytes and will only extract samples sequentially, therefore increasing the overall extraction time required (Wang & Weller 2006).

Ultrasound-assisted extraction utilises sound waves with frequencies higher than 20 kHz to create mechanical vibrations in a solid, liquid or gas (Dean 2009; Luque-García & Luque De Castro 2003; Suslick 1989; Wang & Weller 2006). These vibrations, sound waves, travel through matter and experience expansion and compression cycles whilst traveling through the medium. The expansion cycle creates negative pressure forming microscopic cavities which will grow and collapse (implode). The cavity collapse is asymmetrical close to solid boundaries, creating high-speed jets of liquid that strongly impacts solid surfaces and takes place within 400 μs (Luque-García & Luque De Castro 2003; Suslick 1989; Wang & Weller 2006). This collapse creates rapid adiabatic compression of vapours in the cavities producing extremely high temperatures and pressures, which have

been estimated to be about 5000°C and roughly 1000 atm. The size of the cavities is very small relative to the total liquid volume and the heat produced is rapidly displaced with little to no changes to the environmental conditions (Luque-García & Luque De Castro 2003; Suslick 1989).

The use of ultrasound-assisted extraction has been utilised by researchers several times and would therefore be suitable to be applied for the extraction of the case samples. In 2007, Bommarito et al. published research that utilised sonication to aid the extraction of inorganic anions using water for the comparison of forensic soil samples. Stanisic et al. (2011) compared the shake flask, ultrasound-assisted and microwave extraction to determine the efficiency of extracting inorganic anions from soil. To no surprise, it was concluded that that the microwave assisted extraction was very quick, efficient and reliable but would not have been able to be used in this study. It was also concluded that the ultrasound-assisted extraction was less reproducible and less efficient than the shake flask extraction, however it was much quicker and less quantities of solvent was required. This would allow for three subsequent extraction as has been suggested by Dean (1998), which was not conducted by Stanisic et al. (2011) and could explain the lack in reproducibility and efficiency. Fiedler et al. (2004) used a soxhlet extractor for the extraction of putrescine and cadaverine from soil but this would not have been viable using the quantity of samples provided. The use of ultrasound-assisted extraction is still used within the field of geoforensics as can be observed in the paper recently published by McCulloch et al. (2017).

In summary, a wide range of search methodologies are utilised for the detection of clandestine gravesites that encompass many scientific disciplines ranging from victim recovery dogs to geophysics. Most of these search techniques, with the exception of VRD, locate anomalies in the environment and therefore are very prone to false positive and false negative indications. In addition, the ability of a VRD is highly dependent on many variables and thus their performance can vary day by day (Killam 2004). The use of chemistry adds more robustness to the search and recovery operation. As noted previously, a wide variety of chemicals are detected during mammalian decomposition (Schotsmans et al. 2017), which is likely to be only a fraction of the chemicals produced during the decomposition process. As previous research mainly focused on the detection of the VOC’s produced during the decomposition process because these compounds could be directly related to

VRD indications. Analysis of soil or soil-water samples for the detection of non-volatile and semi-volatile decomposition products has not been attempted with the exception for the analysis of inorganic anions by Aitkenhead-Peterson et al. (2012) and Vass et al. (1992), and amines by Fiedler et al. (2004).

1.9 Research Aims

It has been highlighted that the current search methodologies to locate clandestine gravesites are not always successful and require a significant amount of time and public funding. This study sought therefore to detect the non-volatile and semi-volatile decomposition products from soil and water samples which could aid the detection of clandestine gravesites and lead to the development of field based chemical tests to speed up the search process. The overall aim of this study was to determine if the detection of non-volatile and semi-volatile decomposition products, is a viable alternative to the current search methodologies available. The objectives to achieve this aim were:

- To develop a highly specific and sensitive methodology for the detection of putrescine and cadaverine in aqueous samples and determine if biogenic amines such as putrescine and cadaverine could be detected in mammalian decomposition.
- To explore why most of the researchers in the field of taphonomy were unable to detect the decomposition markers putrescine and cadaverine in their studies.
- To determine the usefulness of ion chromatography as a tool to analyse mammalian decomposition products.
- To determine the effectiveness of high performance liquid chromatography and liquid chromatography mass spectrometry as a tool to analyse products of mammalian decomposition.
- To determine if the developed analytical methodologies (gas chromatography and ion chromatography) can aid in the intelligence gathering process for locating clandestine gravesites.
- To develop an extraction methodology in order to allow for the analysis of soil samples as well as water samples.

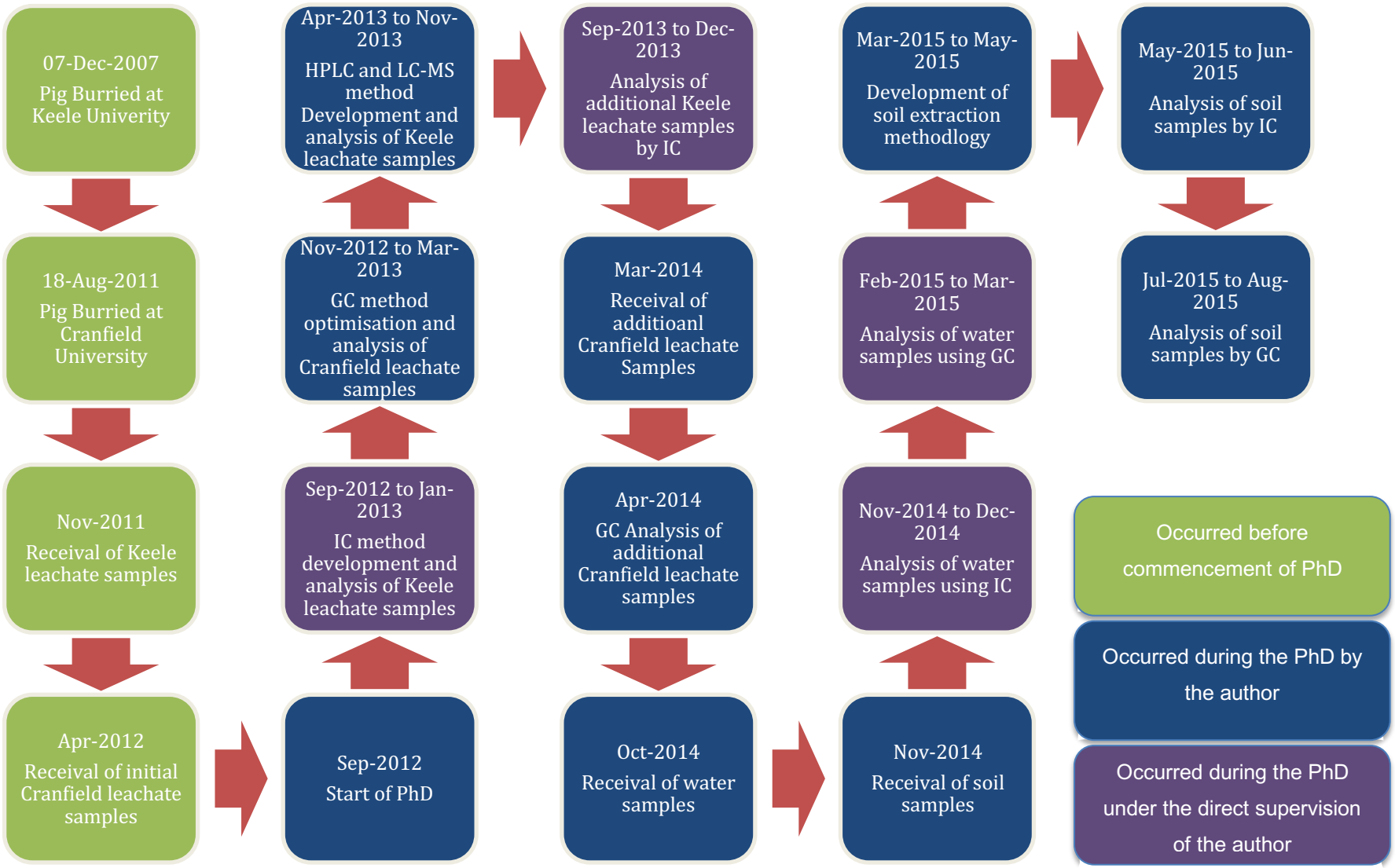

Figure 5 Chronology of the research process and its relationship to sample chronologies

Chapter 2 Methodology

2.1 Sample Collection

2.1.1 Leachate Samples

Gravesite and control leachate samples (labelled as Keele leachate samples) were collected from a simulated gravesite at Keele University (Figure 6) by Dr. Jamie Pringle following the procedure described in Pringle et al. (2010). In summary, a pig (*Sus Scrofa*) was buried with a porous end cap 1900 soilwater lysimeter, which was placed under vacuum so soilwater was drawn from the surrounding soil into the lysimeter. The control lysimeter was placed far enough away and upslope to avoid potential contamination from the gravesite Pringle et al. (2015). Another set of leachate samples (labelled as Cranfield leachate samples) were collected from a simulated gravesite at Cranfield University (Figure 7) by Dr. Anna Williams following the procedure described above. All the leachate samples were stored in the freezer at approximately -19°C until required for analysis and were defrosted for a minimum of three hours, or until completely defrosted, prior to sample preparation and analysis. Table 6 and Table 7 display the leachate samples analysed during the PhD including information such as when samples were obtained, received at Staffordshire University and when analysed.

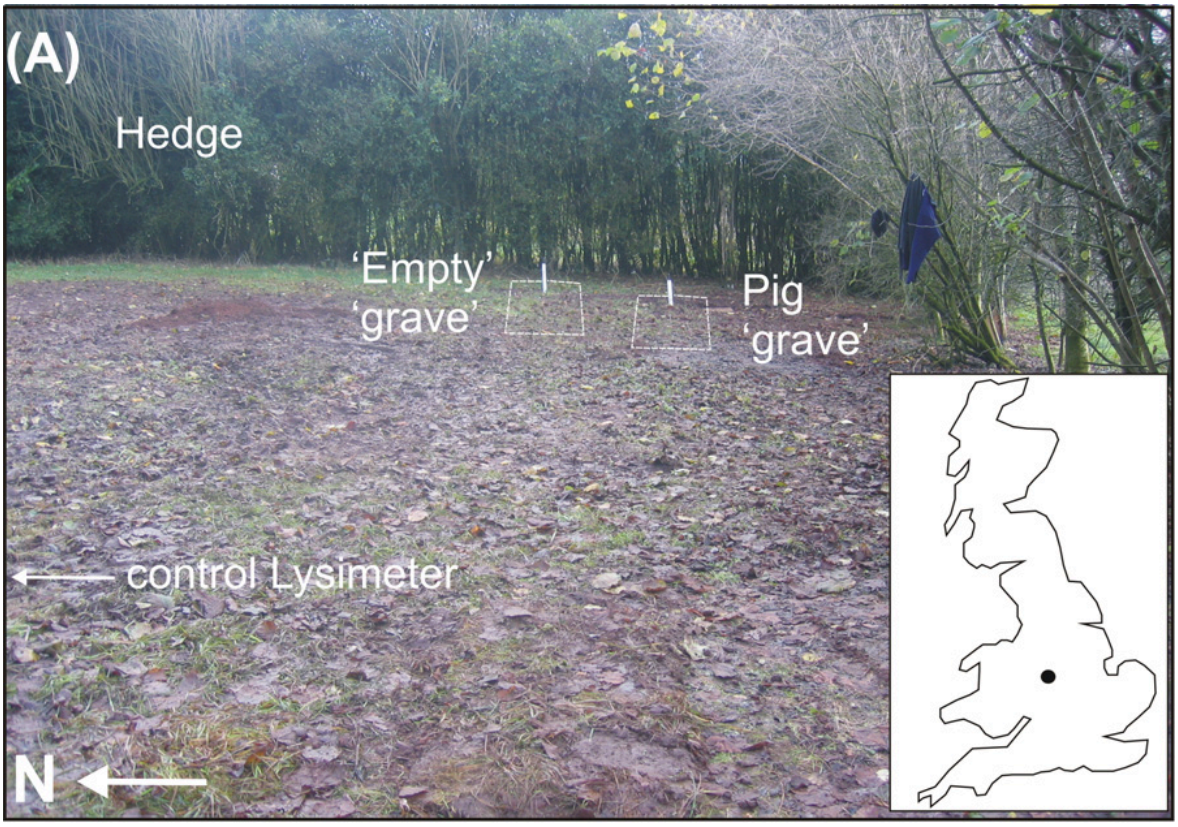

Figure 6 Photograph of Keele University test site. Taken from Pringle et al. (2010)

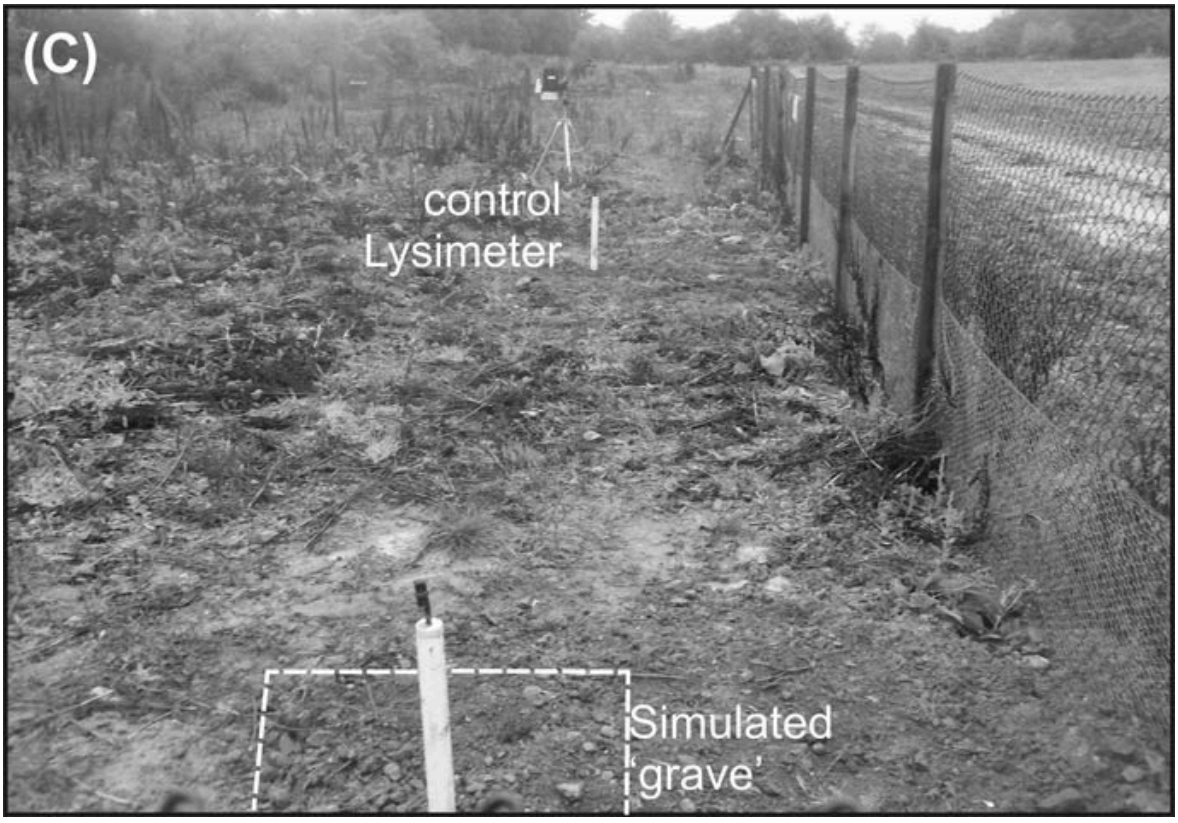

Figure 7 Photograph of Cranfield University test site. Taken from Pringle et al. (2015)

Table 6 Displaying the Keele leachate samples used and displaying information such as when obtained, when analysed and what type of analysis

Sampling date	Post burial interval (weeks)	Date Samples received	Date of Analysis	Type of Analysis
19/12/2007	2	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
10/01/2008	5	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
17/01/2008	6	Nov 2011	Nov 2012-Jan 2013	IC
14/02/2008	10	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
28/02/2008	12	Nov 2011	Nov-Dec 2013	IC
14/03/2008	14	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
27/03/2008	16	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
24/04/2008	20	Nov 2011	Nov-Dec 2013	IC
08/05/2008	22	Nov 2011	Nov 2012-Jan 2013	IC
22/05/2008	24	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
05/06/2008	26	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
19/06/2008	28	Nov 2011	Nov 2012-Jan 2013	IC
17/07/2008	32	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
14/08/2008	36	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
11/09/2008	40	Nov 2011	Nov 2012-Jan 2013	IC
09/10/2008	44	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
06/11/2008	48	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
18/06/2009	80	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
03/12/2009	104	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
28/01/2010	112	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
26/03/2010	120	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
26/04/2010	125	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov 2012-Jan 2013	IC
27/05/2010	129	Nov 2011	Nov-Dec 2013	IC

25/06/2010	133	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
01/10/2010	147	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
29/10/2010	151	Nov 2011	Nov-Dec 2013	IC
11/02/2011	166	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
11/03/2011	170	Nov 2011	Nov-Dec 2013	IC
18/04/2011	176	Nov 2011	May-Jun2013/ Nov-2013	HPLC/ LC-MS
			Nov-Dec 2013	IC
23/05/2011	181	Nov 2011	Nov-Dec 2013	IC

Table 7 Displaying the Cranfield leachate samples used and displaying information such as when obtained, when analysed and what type of analysis

Sampling date	Post burial interval (weeks)	Date Samples received	Date of Analysis	Type of Analysis
15/09/2011	4	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
19/09/2011	5	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
26/09/2011	6	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
29/09/2011	6	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
05/10/2011	7	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
12/10/2011	8	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
28/10/2011	10	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
13/12/2011	17	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
22/02/2012	27	Apr 2012	Mar 2013	GC
		Mar 2014	Apr 2014	GC
08/05/2012	38	Mar 2014	Apr 2014	GC
30/05/2013	93	Mar 2014	Apr 2014	GC
17/06/2013	96	Mar 2014	Apr 2014	GC

2.1.2 Casework Samples

Three 1.0 L water samples were collected from a small lake in the Republic of Ireland suspected to conceal human remains (over 25 years post burial) and two 50 mL water samples from a control lake nearby were provided by Dr. Alastair Ruffell to identify a potential gravesite. Prior to analysis the samples were stored in the cold room in a sealed container, were analysed using ion chromatography (IC) following the procedure described in Section 2.4 Analysis of Inorganic Anionic Compounds using Ion Chromatography and for the analysis of putrescine, cadaverine and methylamine using gas chromatography mass spectrometry (GC-MS), Section 2.5 Analysis of Biogenic Amines Using Gas Chromatography, following derivatisation using pentafluorobenzaldehyde, Section 2.2 Sample Extraction and Derivatisation.

Twenty-four soil samples were provided by Dr. Laurance Donnelly taken at the location of an active murder enquiry (over fifteen years post burial). Samples were taken from twelve different locations (including control site) and taken from the top (AU) and bottom part (AL) of the auger, see Figure 8 and
Table 8 (Donnelly et al. 2018). Prior to analysis the samples were stored in the cold room in a sealed container and then were extracted, derivatised and prepared for IC and GC analysis following the procedures described in Sections 2.2 Sample Extraction and Derivatisation, 2.4 Analysis of Inorganic Anionic Compounds using Ion Chromatography and 2.5 Analysis of Biogenic Amines Using Gas Chromatography.

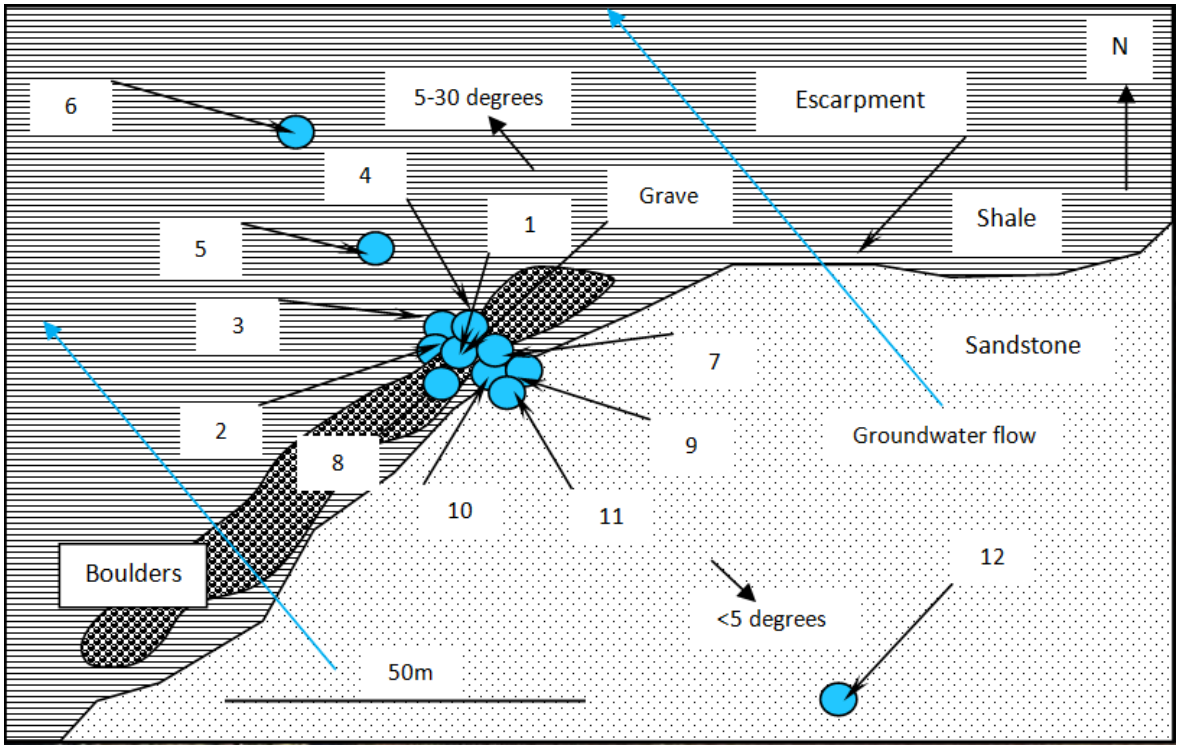

Figure 8 Schematic drawing of sampling location, blue arrows show general flow of surface and groundwater. Taken from Donnelly et al. (2018)

Table 8 Displaying the sample depth and locations. Taken from Donnelly et al. (2018)

Sample Number	Depth (mmbgl)	Location	Soil Type
1A upper **1A lower**	0-80 10-110	Centre of grave	Organic and granular grave backfill
2A upper **2A lower**	10-110 240-310	West of grave	Brown fibrous peat Black fibrous peat, sand
3A upper **3A lower**	30-170 400-510	East of grave	Brown fibrous peat Black fibrous peat, sand
4A upper **4A lower**	10-120 240-320	North of grave	Brown fibrous peat Black peat, sand
5A upper	10-80 260-350	North of grave	Brown fibrous peat Orange sand, organic clay

5A lower			
6A upper **6A lower**	10-160 290-350	100m north of grave	Black peat White-grey clay
7A upper **7A lower**	10-120 460-540	South of grave	Black peat Orange sand and peat
8A upper **8A lower**	10-90 280-320	South of grave	Black fibrous peat Orange sand and peat
9A upper **9A lower**	10-90 690-760	South of grave	Black fibrous peat Orange sand and peat
10A upper **10A lower**	10-90 290-360	South of grave	Peaty sand White-grey clay and sand
11A upper **11A lower**	10-90 480-520	South of grave	Black fibrous peat Brown sand
12A upper **12A lower**	10-70 280-350	200m south of grave (control)	Black peat, sand, clay Sandy clay

2.2 Sample Extraction and Derivatisation

For the analysis of the soil samples an extraction methodology had to be developed. Due to limited time and equipment availability it had been decided to develop an extraction methodology using an ultrasonic bath for the extraction of anions and amines. For the analysis by ion chromatography, a portion of soil was accurately weighed into 20 mL labelled glass vials (unlidded) and dried in the oven at 60°C for 15 hours. After drying the soil was re-weighed (to determine the moisture content) then ground and sieved and a 3.0 g portion of each sample was transferred into a centrifuge tube and 15 mL of deionised water was added. The samples were then sonicated for 15 minutes and centrifuged at 2500 rpm for 20 minutes after which the

aqueous layer was collected and filtered, similar to the procedure used by (Bommarito et al. 2007). The extraction procedure was repeated twice so every sample was extracted three times and the filtrates were combined. The amines were extracted following the same procedure described above, however a 1.0 g portion of soil was dried using 1.0 g anhydrous sodium sulphate to prevent the amines from volatilising. The filtrates were derivatised and analysed following the procedure described below.

The recovery of the extraction procedure was determined through spiking 7.0 g of control soil taken from a previous study with 14 mL 25 ppm fluoride, chloride, nitrite, phosphate, and sulphate and 50 ppm nitrate mixed anion solution before extraction. Another 7.0 g portion of the same soil was spiked with 14 mL deionised water to act as a negative control. The recovery of the amine extraction was determined through spiking control soil with 1.0 mL 1.0 mmol L^{-1} mixed amine solution prior extraction, the same soil was utilised as a negative control sample.

The method used to derivatise the leachate and control samples was based on Ngim et al. (2000) and Blom (2012). The samples were derivatised by pipetting 1.0 mL of each solution into 4.0 mL vials and the pH of the solutions was adjusted to 11 using 1.0 mol L^{-1} sodium hydroxide, 0.1 mol L^{-1} sodium hydroxide and 0.1 mol L^{-1} hydrochloric acid as appropriate. Next 0.5 mL of 10 mg mL^{-1} pentafluorobenzaldehyde in acetonitrile was added to the vials, aluminium foil and plastic caps were used to seal the tops, the vials were shaken and placed into an oven to incubate for one hour at 60°C. After incubation the vials were placed in an ice bath for 1-2 minutes then 1.0 mL of a 0.5% undecane in hexane solution, 100 mg sodium sulphate and 1.0 mL 0.1 mol L^{-1} sodium hydroxide solution were added. The vials were resealed, vortexed for 10-15 seconds and the top layer was pipetted into 2.0 mL auto sampler vials.

2.3 Analysis of Alternative Decomposition Products Using Liquid Chromatography

Table 9 Chemicals and reagents

Chemical	Purity	CAS	Supplier
Acetic acid	>99%	64-19-7	Fisher Scientific
Acetonitrile	HPLC grade	75-05-8	Fisher Scientific
Ammonium acetate	>98%	631-61-8	Acros Organics

Formic acid	>95%	64-18-6	Sigma Aldrich
Isopropanol	HPLC grade	67-63-0	Fisher Scientific
Methanol	HPLC grade	67-56-1	Fisher Scientific
Phosphoric acid	>85%	7664-38-2	Acros Organics
Sodium formate	>98%	141-53-7	Acros Organics
Sodium phosphate	>99%	7558-79-4	Acros Organics
Trifluoroacetic acid	>99%	76-05-1	Alfa Aesar
Tetrahydrofuran	>99.5%	109-99-9	Acros Organics

2.3.1 High Performance Liquid Chromatography with Diode Array Detector

Three Keele grave leachate samples 10-01-08, 26-04-10 and 11-03-11, and their controls were used for UV-Vis analysis using a Thermo Scientific Evolution 201 UV-Vis spectrophotometer. The samples were filtered using a Whatman 0.45 μm PVDF syringe filter before analysis and were analysed in quartz cuvettes.

For HPLC analysis all leachate samples were filtered using a Whatman 0.45 μm PVDF syringe filter prior to analysis on a Perkin Elmer 200 series HPLC-DAD. A range of different stationary and mobile phases were used to optimise the separation of the compounds within the leachate samples. The stationary phases used on the HPLC-DAD were a Phenomenex HyperClone 5 μm 250 x 4.60 mm ODS (C18) column and a modified silica column used for hydrophilic interaction liquid chromatography (hilic). The column used for the modification was a Phenomenex SphereClone 5 μm 250 x 4.60 mm silica column.

During the method optimisation for the C18 column various combinations of mobile phases were used, see Appendix III. The first run was carried out using an isocratic eluent of 40:60 acetonitrile:water with a flow rate of 1 mL min^{-1}. Due to unsatisfactory separation the mobile phase combination was changed to isocratic elution using 30:70 methanol:water, the retention and separation of the compounds was still not sufficient so the mobile phase was changed to a gradient elution starting with 10:90 methanol:water increasing to 50:50 methanol:water after 10 minutes.

For hilic, the silica column was modified by running 100% isopropanol through the column at a flow rate of 0.5 mL min^{-1} for one hour, followed by 100% acetonitrile for another hour. Finally a small fraction of distilled water was introduced to the mobile phase to create a ratio of 90:10 acetonitrile:water (Based on guidance from

Phenomenex). The mobile phase was initially an isocratic elution of 90:10 acetonitrile:water at a flow rate of 1 mL min^{-1}, however this was changed to 95:5 acetonitrile:water to improve the retention of early eluting compounds. In order to gain even more retention the mobile phase ratio was changed to 98:2 acetonitrile:water and a clean-up step was included after each run to elute well-retained compounds.

The addition of buffers to the aqueous portion of the mobile phase was studied, as ionisation was key to the separation and retention of chemicals (Dong 2006; Bayne & Carlin 2010). The buffers used on the C18 column were a 50 mmol L^{-1} formate buffer (pH 3.2), 50 mmol L^{-1} phosphate buffer (pH 7.0) and a 50 mmol L^{-1} phosphate buffer (pH 3.0). For the silica column a 50 mmol L^{-1} acetate buffer (pH 5.8) and a 50 mmol L^{-1} formate buffer (pH 3.2) were tested. The effect of changing the flow rate to 0.5 mL min^{-1} and 2 mL min^{-1} was tested for their effect on peak separation as McCally (2007) stated that the flow rate affects the plate height and thus indirectly influenced separation. The wavelengths of the DAD (diode array detector) were initially set at 260 and 280 nm, lower wavelengths of 200 and 220 nm were also studied and the use of an UV-Vis detector in combination with a fluorescence detector was also tested. Small amounts of trifluoroacetic acid (TFA), 0.005%, was added to the mobile phase to determine its effect on the shape of the peaks in the chromatogram as small quantities of TFA could greatly improve the shape of eluting peaks.

2.3.2 Solid Phase Extraction

Solid-phase extraction was carried out using three different types of SPE cartridges supplied by Machery-Nagel; Chromabond 200 mg C18, Chromabond 200 mg Drug I (C8 combined strong cation exchange) and Chromabond 200 mg Drug II (C8 combined strong anion exchange), see Table 10 for extraction procedure. The samples were subsequently analysed on the HPLC-DAD.

Table 10 Solid-phase extraction procedures

SPE Steps	C18	C8 SCX[1]	C8 SAX[2]
Sample pre-treatment	None	Add 1.4 mL 0.1 mmol L^{-1} KH_2PO_4 (pH 6) to 0.1 mL sample	Add 1.4 mL 0.1 mmol L^{-1} KH_2PO_4 (pH 7) to 0.1 mL sample
Column conditioning	3 mL MeOH	3 mL MeOH	3 mL MeOH

Column equilibration	3 mL H_2O	3 mL H_2O 3 mL 0.1 mmol L^{-1} KH_2PO_4 (pH 6)	3 mL H_2O 3 mL 0.1 mmol L^{-1} KH_2PO_4 (pH 7)
Elution	1 mL H_2O 1 mL H_2O 1 mL ACN 1 mL ACN 1 mL THF 1 mL THF	1 mL 0.1 mmol L^{-1} CH_3COOH 1 mL MeOH 1 mL H_2O + 5% NH_3 1 mL H_2O + 10% NH_3 1 mL MeOH + 5% NH_3 1 mL MeOH + 10% NH_3	1 mL 0.1 mmol L^{-1} NH_4OH 1 mL MeOH 1 mL H_2O + 10% COOH 1 mL MeOH + 10% COOH

[1] (Weinmann 1998), [2] (Machery-Nagel 2006)

2.3.3 High Performance Liquid Chromatography Mass Spectrometry

Twenty Keele leachate samples were analysed at Avans University in the Netherlands on a HPLC coupled to an Agilent 6320 Ion trap Mass Spectrometer and were prepared the same way as the leachate samples analysed on the HPLC-DAD as sample preparation using SPE was discontinued. The initial analysis and method optimisation was conducted on a Scherzo SM-C18 3 μm 100 x 2.0 mm cation and anion exchange column. The mobile phase gradient started with 5:90:5 methanol:water:0.1 mmol L^{-1} formate buffer (pH 3.2) for five minutes and gradually changed to 90:5:5 methanol:water:0.1 mmol L^{-1} formate buffer (pH 3.2) over a 20 minute period. Additional experiments were performed using 10% formate buffer instead of 5%, a Pursuit pentafluoropropyl (PFP) stationary phase 5 μm 100 x 2.00 mm and a Zorbax Eclipse C18 stationary phase 3.5 μm 100 x 3.00 mm. The samples (N=40), with dates ranging over three and a half years post burial were selected for analysis using LC-MS. The samples selected were collected at approximately one-month intervals in the first year and a three to six-month intervals from the second year onwards, See Table 6 for the leachate samples analysed. The MS^n function of the Ion trap was used to collect fragmentation data of certain compounds and produce neutral loss spectra to aid compound identification.

2.4 Analysis of Inorganic Anionic Compounds using Ion Chromatography

Table 11 Chemicals and reagents

Chemical	Purity	CAS	Supplier
Sodium acetate trihydrate	99%	6131-90-4	Fisher Scientific
Sodium bromate	>99%	7789-38-0	Acros Organics
Sodium bromide	99%	7647-15-6	BDH Chemicals
Sodium carbonate	99.9%	497-19-8	BDH Chemicals

Sodium chlorate	98%	7775-09-9	BDH Chemicals
Sodium chloride	>99%	7647-14-5	Sigma Aldrich
Sodium fluoride	99%	7681-49-4	Fisher Scientific
Sodium formate	98%	141-53-7	BDH Chemicals
Sodium hydroxide	97%	1310-73-2	Fisher Scientific
Sodium nitrate	>99%	631-99-4	Sigma Aldrich
Sodium nitrite	98%	7631-99-4	BDH Chemicals
Sodium oxalate	99.5%	62-76-0	BDH Chemicals
Sodium perchlorate	98%	7601-89-0	BDH Chemicals
Sodium phosphate dibasic	99.5%	7558-79-4	Sigma Aldrich
Sodium sulphate	99%	7757-82-6	Acros Organics
Sodium thiosulphate	99.0%	7772-98-7	Sigma Aldrich

Analysis of Standards, System Suitability Samples and (Unknown) Samples

A standard solution containing five anions (fluoride, chlorate, nitrate, phosphate and sulphate) was prepared to a concentration of 10 mg L^{-1} in a 100 mL volumetric flask and was injected twice a day to determine the instrument performance and system suitability. For compound identification, salts listed in Table 11 were prepared in deionised water (resistance 18.2MΩ per cm). Detection and quantification limits of the ions detected in the leachate samples were determined by preparation of calibration standards through dilutions of the 100 mg L^{-1} stock solution.

Grave and control leachate samples collected at Keele University were provided by Dr. Jamie Pringle, see Section 2.1.1 Leachate Samples. Whilst the leachate samples were defrosting prior to sample preparation and analysis, in the meantime, mixed calibration standards were analysed. After the leachate samples were completely defrosted and reached room temperature 1.0 mL sample was transferred into a 10 mL volumetric flask and diluted using deionised water. Although, the casework samples, described in Section 2.1.2 Casework Samples were not diluted. All samples were injected in triplicate using a 1.0 mL BD Plastipack syringe and Whatmann 0,45 µm PVDF syringe filters and analysis was performed on a Dionex ICS-900 ion chromatograph, see Table 12 for the analysis parameters.

Table 12 Dionex ICS-900 instrumental parameters.

Parameter	**Conditions**
Injection volume	0.5 mL

Eluent	4.5 mmol L^{-1} Na_2CO_3 + 1.4 mmol L^{-1} $NaHCO_3$
Flow	0.3 mL min^{-1}
Regenerant	3.6 mmol L^{-1} H_2SO_4
Guard column	Dionex RFIC IonPac® AG22 Guard (2 x 50 mm)
Separation column	Dionex RFIC IonPac® AS22 Column (2 x 250 mm)
Suppressor	2 mm AMMS-300
Detector	DS5 Conductivity Detector
Run time	20 min

2.5 Analysis of Biogenic Amines Using Gas Chromatography

Table 13 Chemicals and reagents

Chemical	Purity	CAS	Supplier
Cadaverine	>95%	462-94-2	Acros Organics
Hydrochloric acid	>37%	7647-01-0	Fisher Scientific
Methylamine hydrochloride	>99%	593-51-1	Acros Organics
Pentaflurorbenzaldehyde	>98%	653-37-2	Acros Organics
Putrescine	>99%	110-60-1	Sigma Aldrich
Sodium hydroxide	>95%	1310-73-2	Fisher Scientific
Sodium sulphate anhydrous	>99%	7757-82-6	BDH Chemicals
Undecane	>97%	1120-21-4	Fluka Analytical

Table 14 Instrumental parameters for GC-FID and GC-MS analysis

Parameter	Conditions	
Injector temperature:	250°C	
Injection type:	Splitless	
Injection volume:	3 µL	
	FID	**MS**
Carrier gas (helium)	8,0 psi	1,5 mL min^{-1}
Air flow	400 mL min^{-1}	
Hydrogen flow	40 mL min^{-1}	
Detector temperature	250°C	200°C
Transfer line temperature		300°C
Ionisation mode		EI positive mode
Electron energy		70ev
Range (m/z)		TIC: 30-500 m/z
Quantification (m/z)		Putrescine: 181, 208, 249 Cadaverine: 181, 222, 263 Methylamine: 117, 208, 209
Scan time		0,2 msec
Scan delay		0,05 msec
Temperature program	A: $45°C_{4min} \xrightarrow{15°C\ min^{-1}} 280°C_{15min}$	A: $45°C_{4min} \xrightarrow{15°C\ min^{-1}} 280°C_{15min}$ B: $45°C_{15min} \xrightarrow{15°C\ min^{-1}} 280°C_{15min}$
Run time	A: 34.67 min	A: 34.67 min B: 45.67 min

2.5.1 Analysis of Putrescine, Cadaverine and Methylamine

0.1 mol L^{-1} standard solutions of putrescine, cadaverine and methylamine (this was included due to its significant presence in the initial Cranfield leachate samples) were prepared in deionised water and diluted to a 1.0 mmol L^{-1} mixed amine solution. This solution was used as a positive control and deionised water was used as a negative control for the derivatisation of the leachate samples. Unless stated otherwise, the derivatised samples were analysed on a Clarus 500 GC-MS using a Supelco SLB-5MS 30 m x 0.32 mm 0.25 μm column. The instrumental settings are provided in Table 14.

2.5.2 Optimisation of Incubation Time

The incubation time was optimised using a 1 mmol L^{-1} solution of methylamine, isopropylamine, butylamine and phenylethylamine. Three 4.0 mL vials containing 1.0 mL 1.0 mmol L^{-1} mixed amine solution and one vial containing 1.0 mL distilled water (negative control) were derivatised using a one, two and three hours incubation time and were analysed on a Clarus 500 Gas Chromatograph Flame Ionisation Detector (GC-FID) using a Supelco SLB-5MS 30 m x 0.32 mm 0.25 μm column, see Table 14 for instrumental settings.

2.5.3 Analysis of Cranfield Leachate and Casework Samples

Cranfield grave and control samples listed in Table 7 were derivatised, following the procedure described in Section 2.2 Sample Extraction and Derivatisation, and analysed twice (n=36). As highlighted in Table 7, the first set was of samples were sent to Staffordshire University in April 2012 and analysed in March 2013, whilst the second set was stored at Cranfield University and was received at Staffordshire University in March 2014 and analysed in April 2014. The casework samples, described in Section 2.1.2 Casework Samples were extracted and derivatised following the procedure described in Section 2.2 Sample Extraction and Derivatisation.

2.6 Storage Conditions and GC Method Validation

Method validation was performed on the GC method described in Section 2.5 Analysis of Biogenic Amines Using Gas Chromatography following derivatisation of putrescine, cadaverine and methylamine described in Section 2.2 Sample Extraction and Derivatisation and the procedure is described below.

2.6.1 Method Validation

The selectivity of the GC method was determined by calculating the resolution between putrescine, cadaverine and methylamine and their closest eluting peaks in the positive control sample and leachate samples. The mass spectra of putrescine, cadaverine and methylamine in the leachate samples were visually compared to the mass spectra of their reference compounds to determine if any other chemicals were co-eluting.

The linearity, limit of detection (LOD) and limit of quantification (LOQ) of the derivatisation was calculated through the preparation and derivatisation of nine mixed amine standards containing putrescine, cadaverine and methylamine at concentrations ranging from 0.1 µmol L^{-1} to 1000 µmol L^{-1}. The correlation coefficient, linearity and best-fit regression line (least squares method) were calculated (see Appendix I). The limit of detection and limit of quantification were determined using the standard deviation of the intercept and residual plots were created to check for outliers.

The accuracy of the analytical procedure was determined through interpretation of the coefficient of determination (indicating how well the data fits the linear model) and through calculating the average error in recovery over the calibration range. Whilst the reproducibility was determined through calculating the RSD from the positive control samples (n=4) containing 1000 µmol L^{-1} concentrations of putrescine, cadaverine and methylamine.

2.6.2 Storage Experiment

Analysis of the Cranfield leachate samples indicated variability in the relative detection of putrescine, cadaverine and methylamine between duplicate samples. A storage experiment was carried out to investigate the stability of these compounds under various storage conditions. Standard solutions of putrescine, cadaverine and methylamine at a concentration of 0.1 mol L^{-1} were prepared in deionised water and were diluted to create a 1.0 mmol L^{-1} mixed amine solution. Next 1.0 mL mixed amine solution was pipetted into fifteen 4.0 mL vials (five sets of three samples), one set containing three vials was derivatised immediately to act as the initial starting point of this storage experiment. The other four sets were stored for three months before derivatisation; one set was stored at room temperature at approximately 21°C, another set was stored in the fridge at approximately 6°C, the

next set was stored in the freezer at approximately -19°C. The last set was also stored in the freezer, however these samples were taken out of the freezer repeatedly to defrost simulating usage of the samples. All samples were derivatised and analysed following the procedure described in Sections 2.2 Sample Extraction and Derivatisation and 2.5 Analysis of Biogenic Amines Using Gas Chromatography.

To determine the effects of storing the water casework samples prior to analysis and their sample matrix, 0.1 mol L^{-1} standard solutions of putrescine, cadaverine and methylamine were prepared in distilled water and diluted to create a 20 mmol L^{-1} and 1.0 mmol L^{-1} mixed amine solutions. 100 mL 1.0 mmol L^{-1} spiked case samples were created for solutions A, B, and C and 25 mL 1.0 mmol L^{-1} spiked control was also created (the difference in volumes was due to sample availability). Each sample was transferred into a container and stored in the cold room, the remaining solutions were used as the initial starting point of the storage experiment and to determine the matrix effects through derivatisation and analysis following the procedures described in Sections 2.2 Sample Extraction and Derivatisation and 2.5 Analysis of Biogenic Amines Using Gas Chromatography.

Chapter 3 Analysis of Alternative Decomposition Products Using Liquid Chromatography

3.1 High Performance Liquid Chromatography Diode Array Detector

The most suitable wavelengths for the analysis of leachate samples using HPLC were determined to be 260nm and 280nm, this would allow for the detection of aromatic compounds such as conjugated and substituted phenyl compounds. The wavelength was chosen based on analysis of the leachate samples using UV/Vis spectroscopy, Figure 9, which demonstrated high absorption in the low UV region.

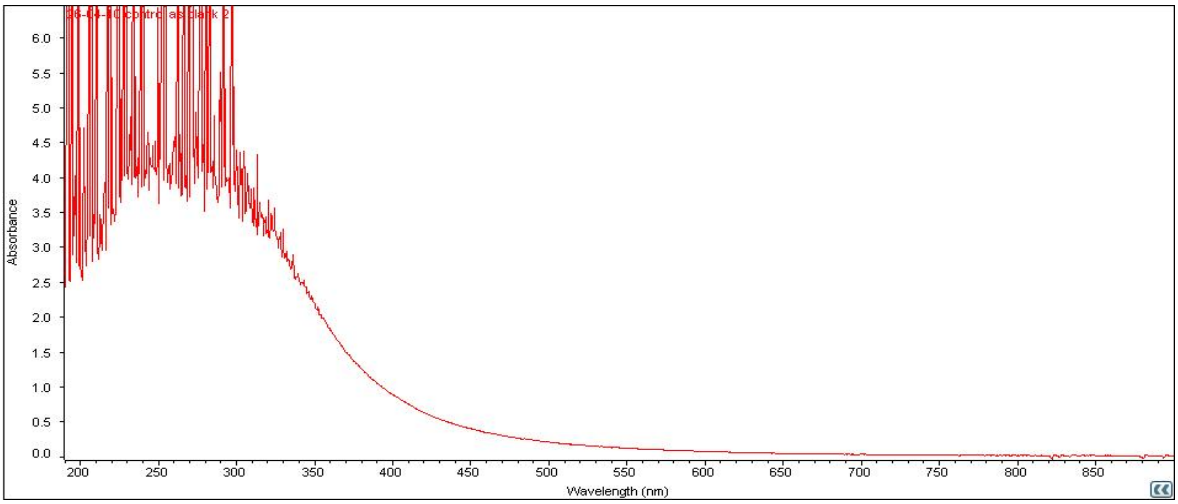

Figure 9 UV-Vis spectrum of Keele grave leachate sample 26-04-10 using its corresponding control as blank

Following analysis of grave leachate sample 03-12-09 and the corresponding control sample using a C18 column clear differences were visible between both samples, see Figure 10. This implied that these samples had a dissimilar chemical composition and that the grave samples contained decomposition products. Using the C18 column (Figure 10B) the compounds eluted near the void time, which indicated that the chemicals in the leachate were very polar with a greater affinity towards the polar mobile phase (10:90 MeOH:H_2O) than the non-polar stationary phase. During the hilic analysis, Figure 11, the peaks were poorly separated, exhibited poor shapes and most compounds eluted near the void time, whilst others retained strongly, therefore another run was required to elute these well-retained chemicals. Further optimisation failed to improve the hilic results and was therefore discontinued. When comparing the C18 and hilic data it was observed that the different stationary phase interactions produced very different chromatograms and

reversed phase chromatography is more suitable to analyse decomposition products, see Figure 10B and Figure 11.

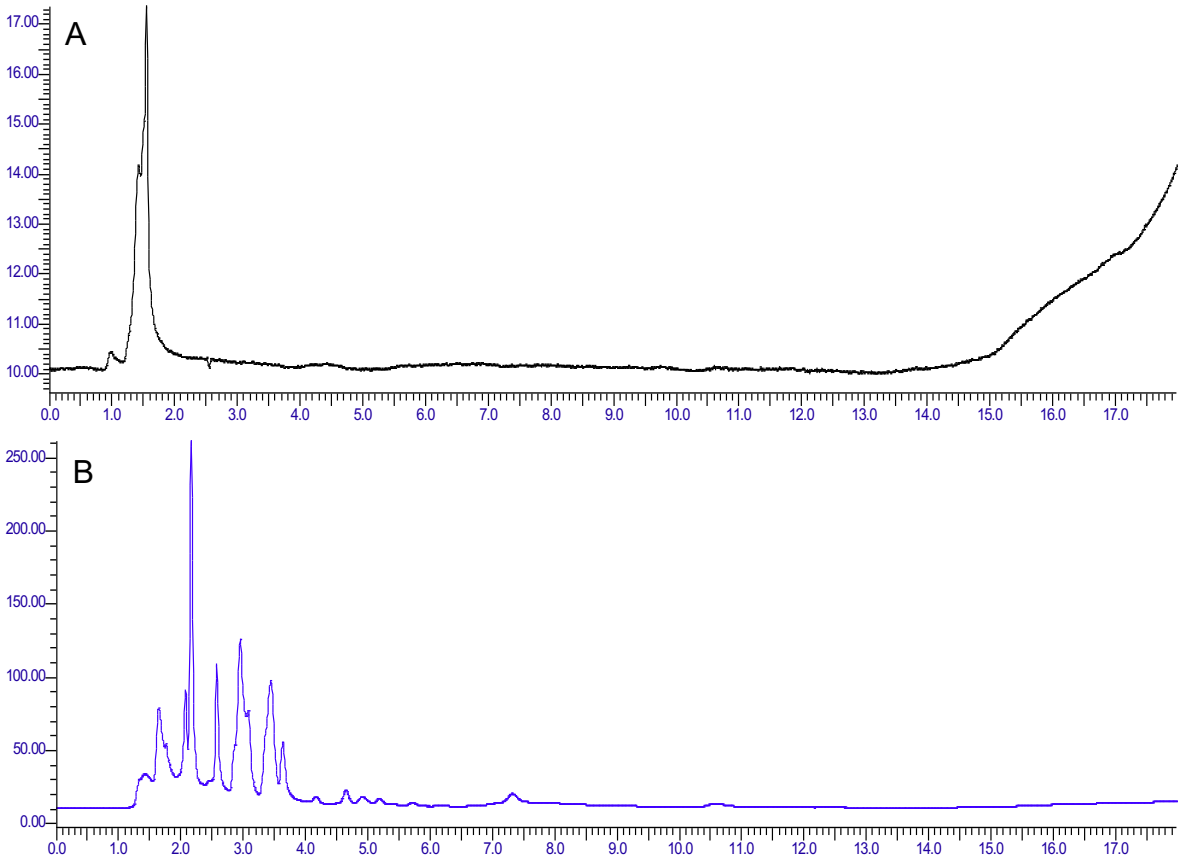

Figure 10 Analysis of Keele 03-12-09 on HPLC-DAD using a C18 column: Control leachate sample (A); Grave leachate sample (B)

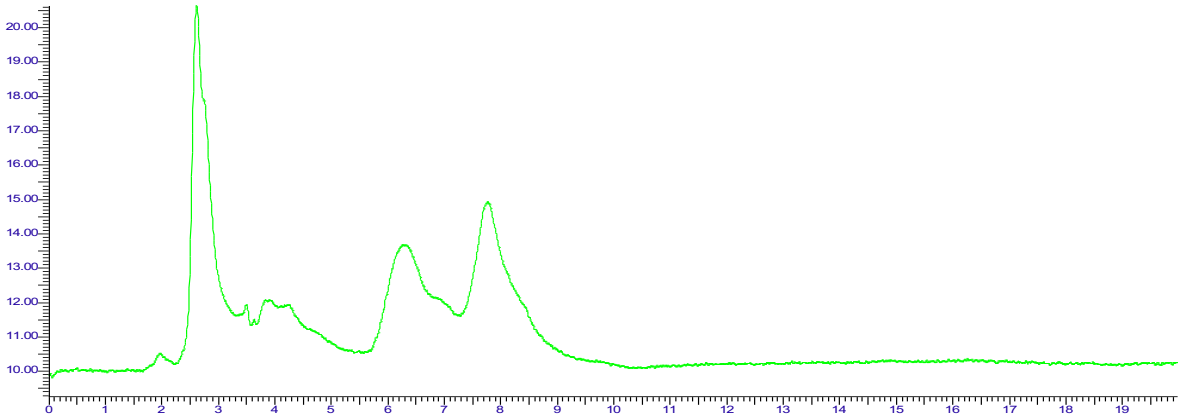

Figure 11 Analysis of Keele grave leachate sample 03-12-09 on HPLC-DAD using a hilic column

Different mobile phase combinations were tested to further retain the early eluting compounds and improve resolution, see Figure 12, and needed to be performed without the chemicals within the leachate being identified. Resolution between two chromatographic peaks, retention and the number of peaks present in a chromatogram were the main qualifiers to identify a suitable mobile phase to analyse the leachate samples. Clear differences were visible regarding the resolution, retention and the number of peaks present between the chromatograms

in Figure 12, which displayed the chromatograms of leachate sample 26-04-10 under different mobile phase conditions. Chromatogram D) using method 4 [10:90 ramp to 50:50 methanol:water + optimised injection delay and stabilisation time], was determined most suitable out of these four chromatograms due to the number of peaks present, indicating better separation. Additionally, chromatogram D) used an optimised injection delay and stabilisation time, which increased the retention of most compounds and thus fewer compounds eluted at the void time.

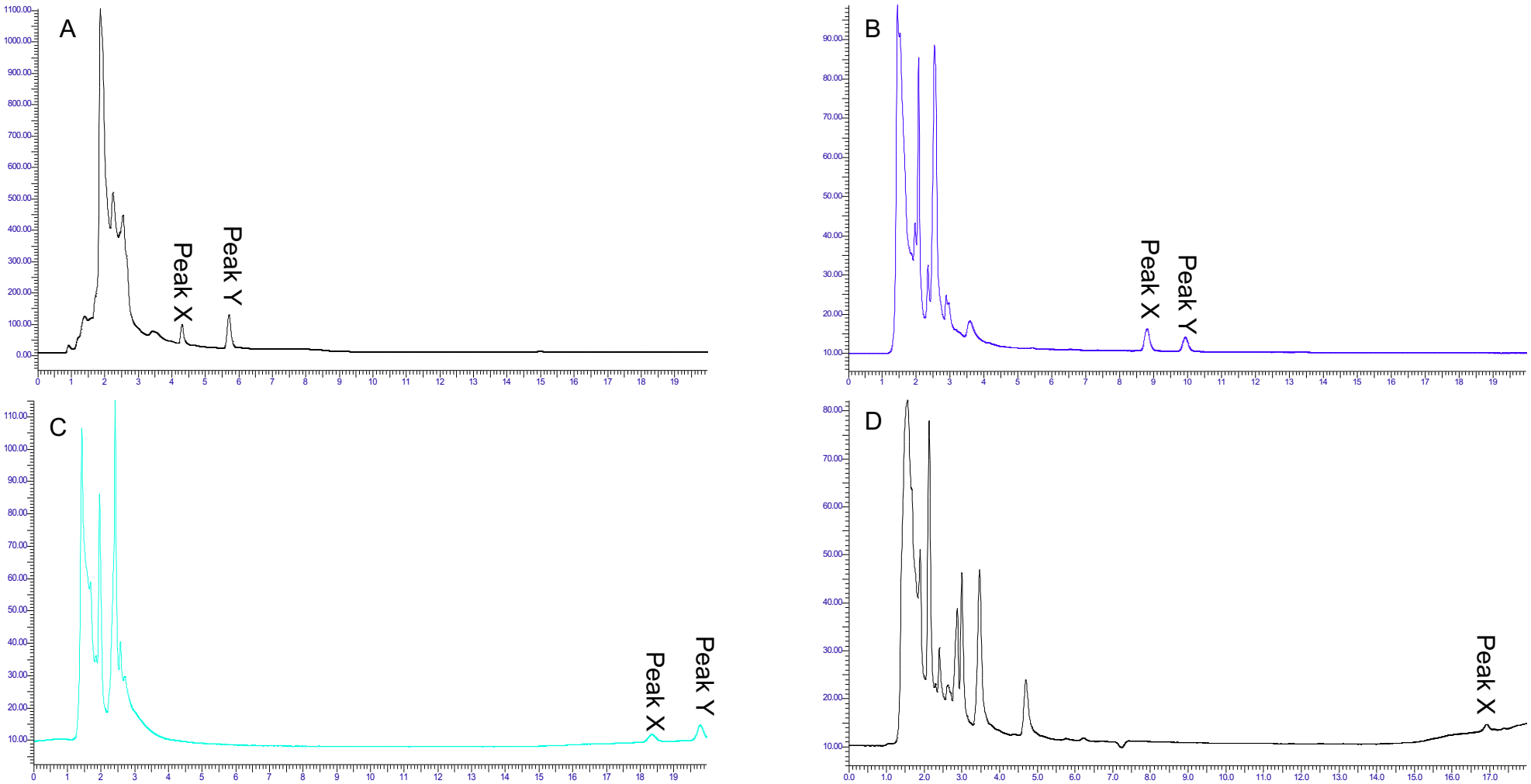

Figure 12 Chromatogram of leachate sample 26-04-10 analysed on HPLC-DAD using mobile phase: Method 1 (40:60 MeCN:H_2O) (A); Method 2 (30:70 MeOH:H_2O) (B); Method 3 (10:90 slow ramp to 50:50 MeOH:H_2O) (C); Method 4 (10:90 medium ramp to 50:50 MeOH:H_2O + optimised injection delay and stabilisation time) (D)

Initial HPLC analysis of the leachate samples followed an isocratic elution of 40:60 acetonitrile:water, this resulted in poor separation (Figure 12A) so the mobile phase was made more polar by substituting acetonitrile with methanol (Figure 12B). As visible in Table 15, methanol is more polar in comparison to acetonitrile although it also has a better capability of accepting and donating hydrogen bonds as seen in Figure 13 (Dolan 2010). This altered the selectivity of the eluent in multiple ways through changing the mobile phase polarity and its capabilities of accepting and donating hydrogen bonds. Using this eluent, some compounds assumed to be non-polar and slightly non-polar were retained slightly longer (see peaks X and Y) but many compounds remained poorly retained as was observed in Figure 12B. A more polar mobile phase was created by using a gradient eluent with an initial mixture of 10:90 methanol:water which changed to 50:50 methanol:water after ten minutes. Peaks X and Y eluted at the end of the chromatogram while the other (polar) chemicals were not affected significantly. The slope of the gradient was increased for chromatogram D and a five-minute injection delay plus a five-minute mobile phase equilibration time was included to equilibrate the mobile phase before analysis which resulted in better separation of the polar (slightly retained) compounds. It appeared that peak Y eluted after the twenty-minute detection window so a larger percentage of organic solvent was required to elute this compound within the twenty minute detection window.

Table 15 Solvent characteristics

Polarity	Solvent	Water miscibility
Non-polar	Hexane	No
	Chloroform	No
	Tetrahydrofuran	Yes
	Acetone	Yes
	Acetonitrile	Yes
	Isopropanol	Yes
	Methanol	Yes
	Water	Yes
Polar	Acetic acid	Yes

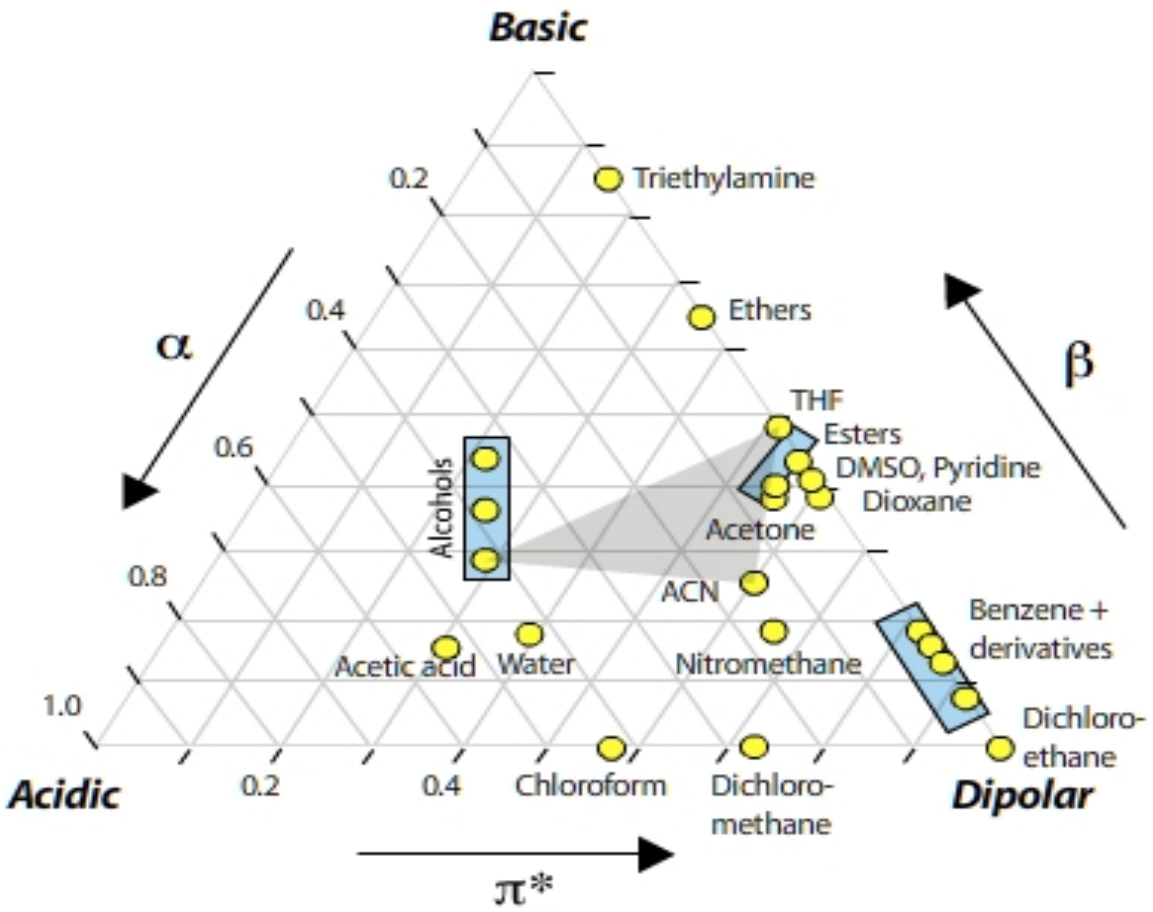

Figure 13 Solvent selectivity triangle. Taken from (LCGC's Chromacadamy n.d.)

The mobile phase can be altered in two different ways; changing the organic portion of the mobile phase, *i.e.* changing acetonitrile to methanol (as seen above), the aqueous fraction of the mobile phase can also be altered by the addition of buffers such as formate and phosphate. Buffers alter and control the mobile phase pH and therefore affect the retention of ionisable compounds. Due to limited sample availability during the method optimisation, further experiments comparing different mobile phase conditions utilised leachate sample 03-12-09, Figure 14. This sample was analysed using the method utilised in Figure 12D and is displayed in Figure 14A. For the other chromatograms, the aqueous portion of the mobile phase was replaced by a phosphate buffer pH 7.0 (Figure 14B), formate buffer pH 3.2 (Figure 14C) and phosphate buffer pH 3.0 (Figure 14D). The formate buffer (Figure 14C) was determined to be most suitable out of these four chromatograms due to compounds eluting over the entire retention window and the lowest quantity of peaks eluting at the void time. As further optimisation, 30% acetonitrile was added to the gradient after ten minutes to ensure that well-retained compounds eluted within the twenty minute detection window, see Figure 15. The addition of 0.005% TFA was

tested and determined to have limited effect on the resolution and retention and was therefore excluded from the method.

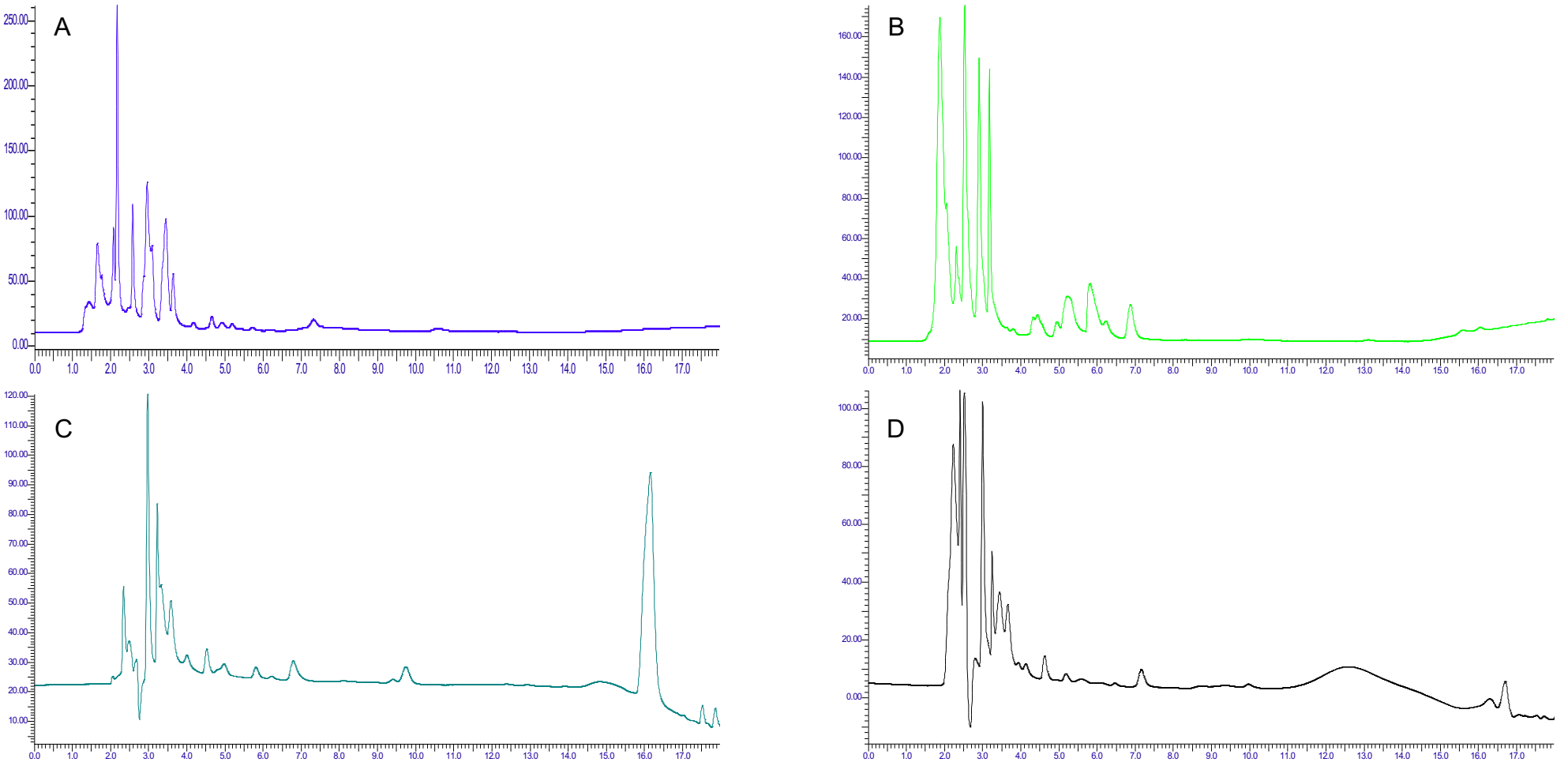

Figure 14 Chromatogram of leachate sample 03-12-09 analysed on HPLC-DAD using mobile phase using optimised injection and stabilisation time: Method 4 (10:90 medium ramp to 50:50 MeOH:H2O) (A); Method 6 (10:90 medium ramp to 50:50 MeOH:H_2PO4^- pH 7) (B); Method 7 (10:90 medium ramp to 50:50 MeOH:HCOO- pH 3.2) (C); Method 8 (10:90 medium ramp to 50:50 MeOH:HPO_4^{2-} pH 3) (D)

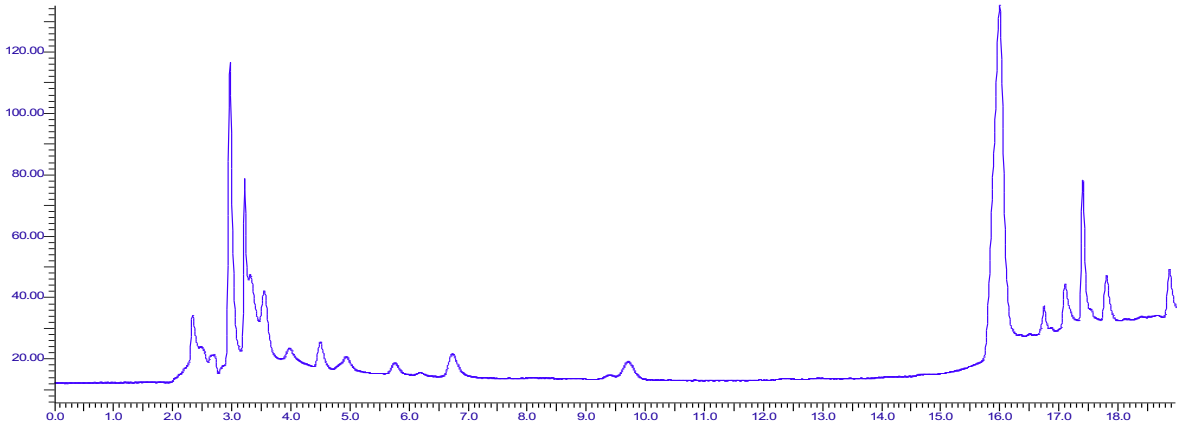

Figure 15 Chromatogram of leachate sample 03-12-09 optimised (method 9)

The chromatograms in Figure 14A and Figure 14B, a buffered and non-buffered mobile phase at a similar pH (pH 7), exhibited a significant difference between both chromatograms. This could be due to a change in the mobile phase selectivity as addition of a buffer stabilises and supresses small changes in the mobile phase pH and increases mobile phase polarity (Bayne & Carlin 2010). A buffer maintains analyte ionisation, depending on pH, throughout the analysis and therefore could enhance peak shapes (see Figure 16), which was partially observed in Figure 14B.

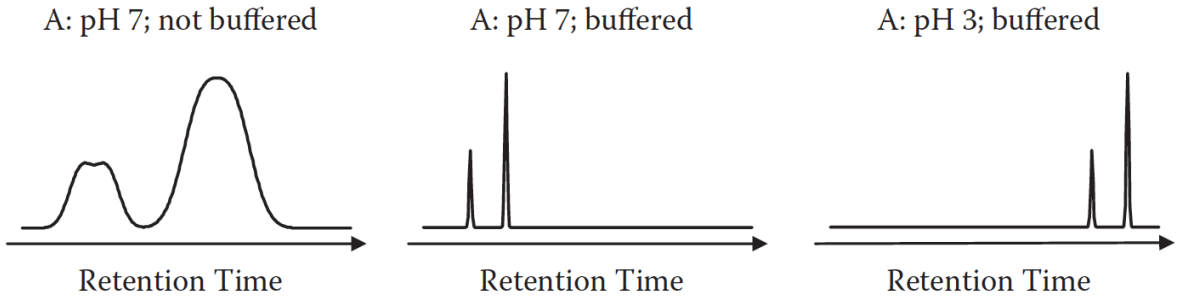

Figure 16 Effect of buffering compound on the separation of acidic compounds. Taken from Bayne & Carlin (2010)

Different chromatograms were produced using the phosphate buffer (pH 7.0), formate buffer (pH 3.2) and phosphate buffer (pH 3.0), see Figure 14. The formate buffer, pH 3.2, (Figure 14C) had a lower pH than the phosphate buffer, pH 7.0, (Figure 14B), which kept acidic compounds non-ionised (less polar) and resulted in longer retention times for acidic compounds. Differences between the formate buffer, pH 3.2, and phosphate buffer, pH 3.0, were most likely not due to changes in the mobile phase pH but due to the differences in mobile phase selectivity such as ionic strength (Bayne & Carlin 2010). Figure 13 highlights the slight differences in selectivity between different alcohols (Dolan 2010), this could also have been the case for these different buffers.

The final method for the analysis of the leachate samples utilised a formate buffer at pH 3.2 and had an addition of 30% acetonitrile after ten minutes, see Figure 15. The formate buffer enhanced the retention and resolution due to ionisation suppression of acidic compounds. Furthermore, the formate buffer is more suitable for LC-MS analysis as it is volatile. TFA was discontinued as it did not improve the retention, separation or peak shapes and also has negative effects on the ionisation of chemicals during LC-MS analysis (Dong 2006; McMaster 2007).

3.2 Solid Phase Extraction

Solid-phase extraction was trialled with the aim of separating the polar and non-polar chemicals, to further enhance the resolution and detection of the chemicals within the leachate as discussed by Buszewski & Noga (2012).

The C18 (mainly dispersive interactions) SPE cartridge was able to separate the polar and non-polar compounds using water and acetonitrile. However, the resolution was not enhanced sufficiently as only one compound was retained on the SPE cartridge and the others eluted during the water (polar) elution step, see Figure 17. This further confirmed the hypothesis that most of the chemicals in the leachate were polar. The remaining compound was eluted in the acetonitrile (relatively non-polar) elution step and no chemicals were eluted during the THF (non-polar) elution, which was the least polar water miscible solvent (see Table 15). This compound had a Rt. of 17.9 minutes during the HPLC analysis and further confirmed that this compound was less polar. In addition, the detector response was low due to sample dilution (due to availability of small sample volumes) during the extraction as was observed when the chromatograms were compared to Figure 15, which displayed the chromatogram of the same sample before extraction; this could result in a failure to detect trace level compounds.

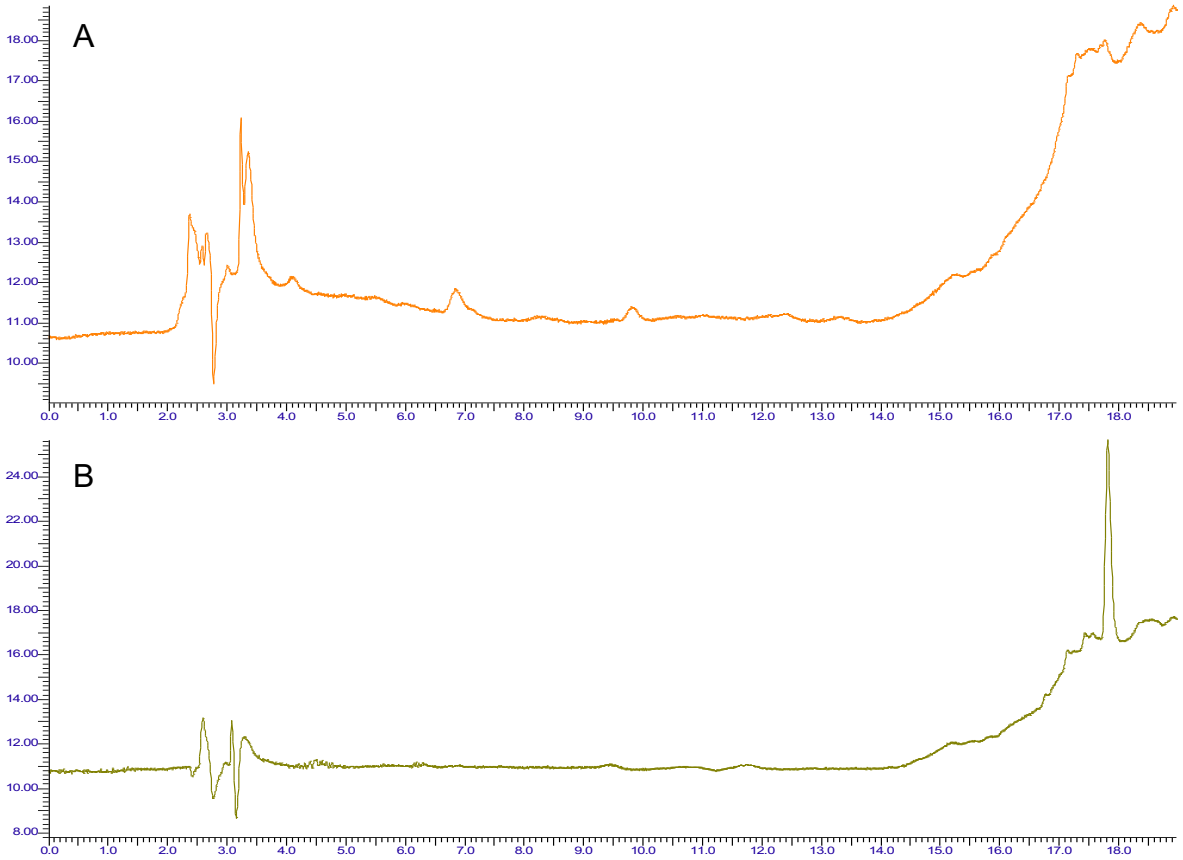

Figure 17 Analysis of Keele grave leachate sample 03-12-09 on HPLC-DAD after C18 SPE: H_2O elution (A); MeCN elution (B)

The C8/cation exchange (weaker dispersive, polar and cationic interactions) cartridge was also able to separate the compounds in the leachate, however the addition of chemicals such as acetic acid for the extraction procedure could interfere with the analysis, see Figure 18. Despite the addition of acetic acid to the first elution step, most of the chemicals in the leachate were not retained on the cartridge and eluted during this step. The addition of acetic acid would have supressed ionisation of acidic compounds and maintained ionisation of alkaline compounds, which would retain on the SPE cartridge as it retained alkaline (cationic) and semi-polar acidic compounds. Most of the chemicals eluted during this extraction step and again supported the theory of polar chemicals being present in the leachate. The peak at Rt. 16.3 minutes was detected in both the acetic acid and methanol elution which indicated that this compound was not retained properly during the first elution step. The remaining compounds (non-polar neutral and non-polar acidic) except for one compound eluted in the methanol extraction step, whilst the last compound (cationic) eluted during the ion exchange extraction see Figure 18C. It was therefore concluded that the ion exchange mechanism did not contribute significantly to the extraction.

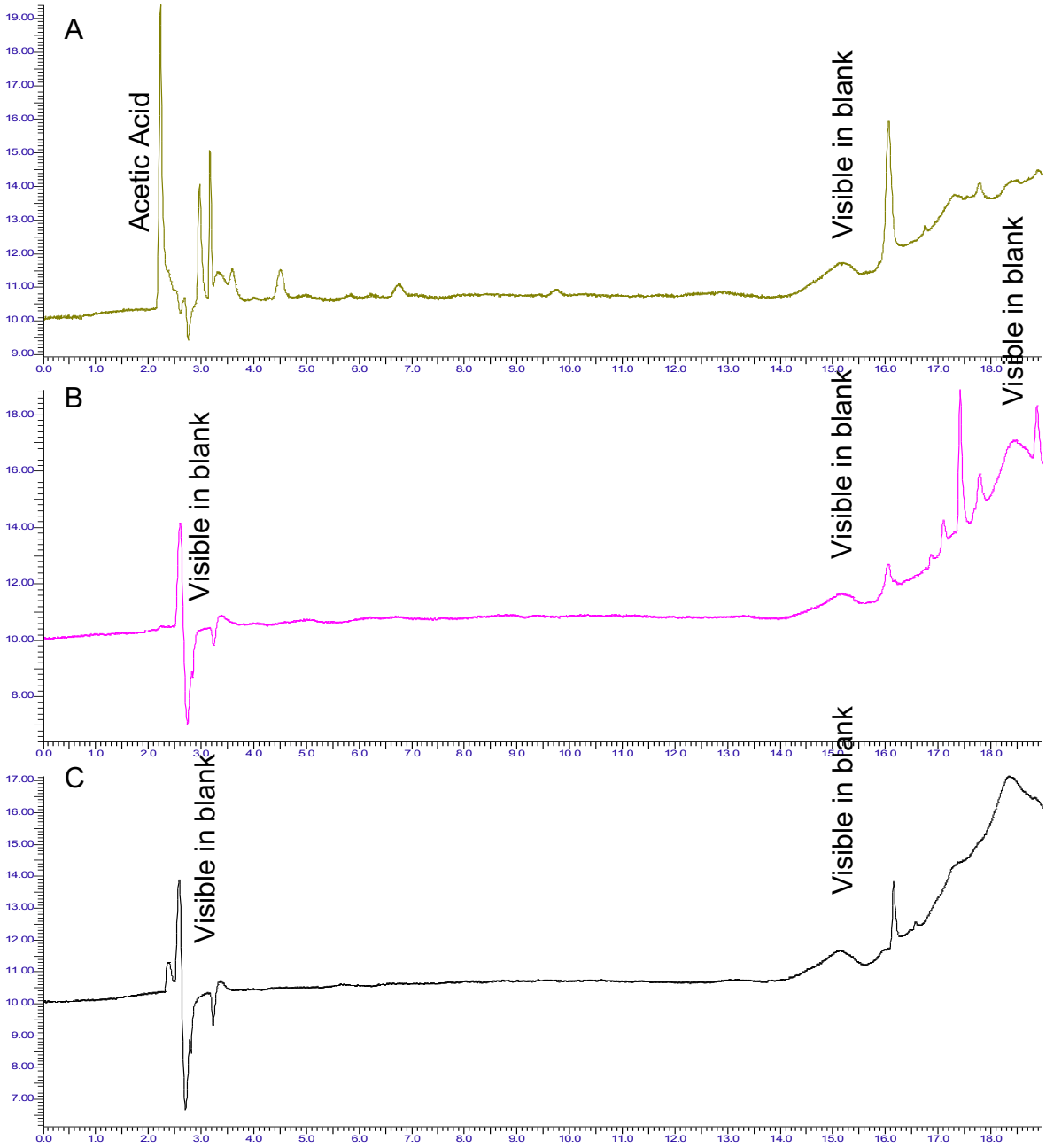

Figure 18 Analysis of Keele grave leachate sample 03-12-09 on HPLC-DAD after C8/cation exchange SPE: Acetic acid elution (A); MeOH elution (B); 5% NH_3 in MeOH elution (C)

The results from the C8/anion exchange (weaker dispersive, polar and anionic interactions) cartridge should be opposite in terms of ionisation and retention of the ionic compounds, thus acidic compounds should be ionised and ionisation of alkaline compounds should be supressed. The similarities between the cation and anion exchange SPE cartridges indicated that ionic interactions did not play a major part in the extraction mechanism as only a few compounds were affected by these differences. The C8/anion exchange cartridge was able to separate the polar and non-polar compounds, however additional compounds were included for the extraction procedure and might interfere with the analysis as visible in Figure 19. No compounds eluted during the ion exchange extraction (Figure 19C), whilst most of

the polar chemicals eluted during the first extraction step and the less polar compounds eluted during the second extraction step. More compounds were retained during the first elution step (ammonium hydroxide, Figure 19A) in comparison to the cation exchange cartridge (acetic acid, Figure 18A). Peaks at Rt. 6.7 minutes and Rt. 17.8 minutes were visible in Figure 18A whilst they did not appear in Figure 19A, but both peaks appeared in Figure 19B. Peak Rt. 16.0 minutes only eluted during the ammonium hydroxide elution (Figure 19A).

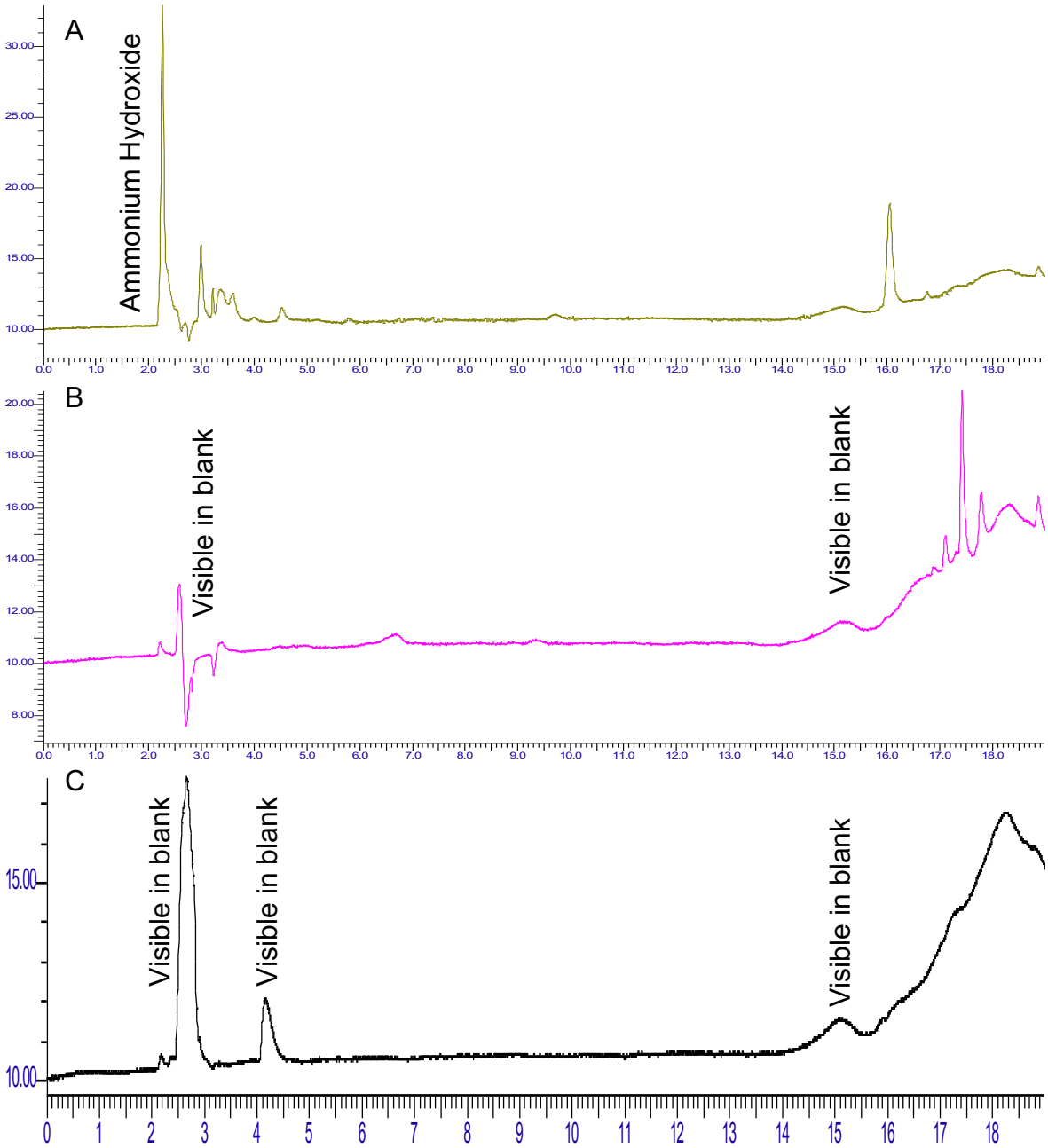

Figure 19 Analysis of Keele grave leachate sample 03-12-09 on HPLC-DAD after C8/anion exchange SPE: NH_4OH elution (A); MeOH elution (B); 10% HCOOH in MeOH elution (C)

The ion exchange mechanism of both SPE cartridges did not contribute significantly to the extraction as only one compound was detected during this extraction step (Figure 18C) and nothing in Figure 19C. This indicated that the chemicals in the

leachate were not ionic or that they adsorbed strongly to the SPE cartridge and therefore did not elute from the SPE cartridge. The first possibility was most likely due to the minor differences between both SPE cartridges, which suggested that the use of ion exchange cartridges was potentially not the most suitable mechanism for preparation of the leachate samples. However, weak ion exchange cartridges could be tested to see if any strong ionic chemicals would elute from the cartridge (Telepchak et al. 2004). Additionally, SPE could be more effective if larger quantities of sample would have been used during the extraction process and SPE was able to concentrate samples instead of diluting them, as was the case due to limited sample availability.

3.3 High Performance Liquid Chromatography-Mass Spectrometry

Mass spectrometry was more capable than the DAD to detect the range of chemicals in the leachate, see Figure 20. The two chromatograms labelled A) show UV/Vis data from the grave sample (blue line) and its corresponding control sample (red line), chromatograms B) showed mass spectral data in the positive ionisation mode whilst chromatograms C) displayed mass spectral data in the negative ionisation mode. The compounds detected using UV/Vis spectrometry appeared to be satisfactorily ionised using either the positive or negative ionisation mode, though not all peaks detected using the MS were detected using the DAD. This confirms that the MS was able to detect more compounds in the leachate as a DAD only detects chemicals containing a chromophore, as seen in Figure 20.

The compounds detected using the DAD were better separated and more sensitive for certain compounds than the MS. The increased number of peaks in the chromatogram and extensive tailing from peak Rt. 1.3 minutes (highlighted in Figure 20B) resulted in poorer resolution of the MS results as the mass spectrum of peak Rt. 1.3 minutes showed the detection of multiple m/z peaks in its spectrum (Figure 21). Most or even all of those peaks were different compounds instead of being multiple fragments from a single compound as electrospray ionisation (ESI) was a soft ionisation technique and hardly fragments chemicals (Bayne & Carlin 2010).

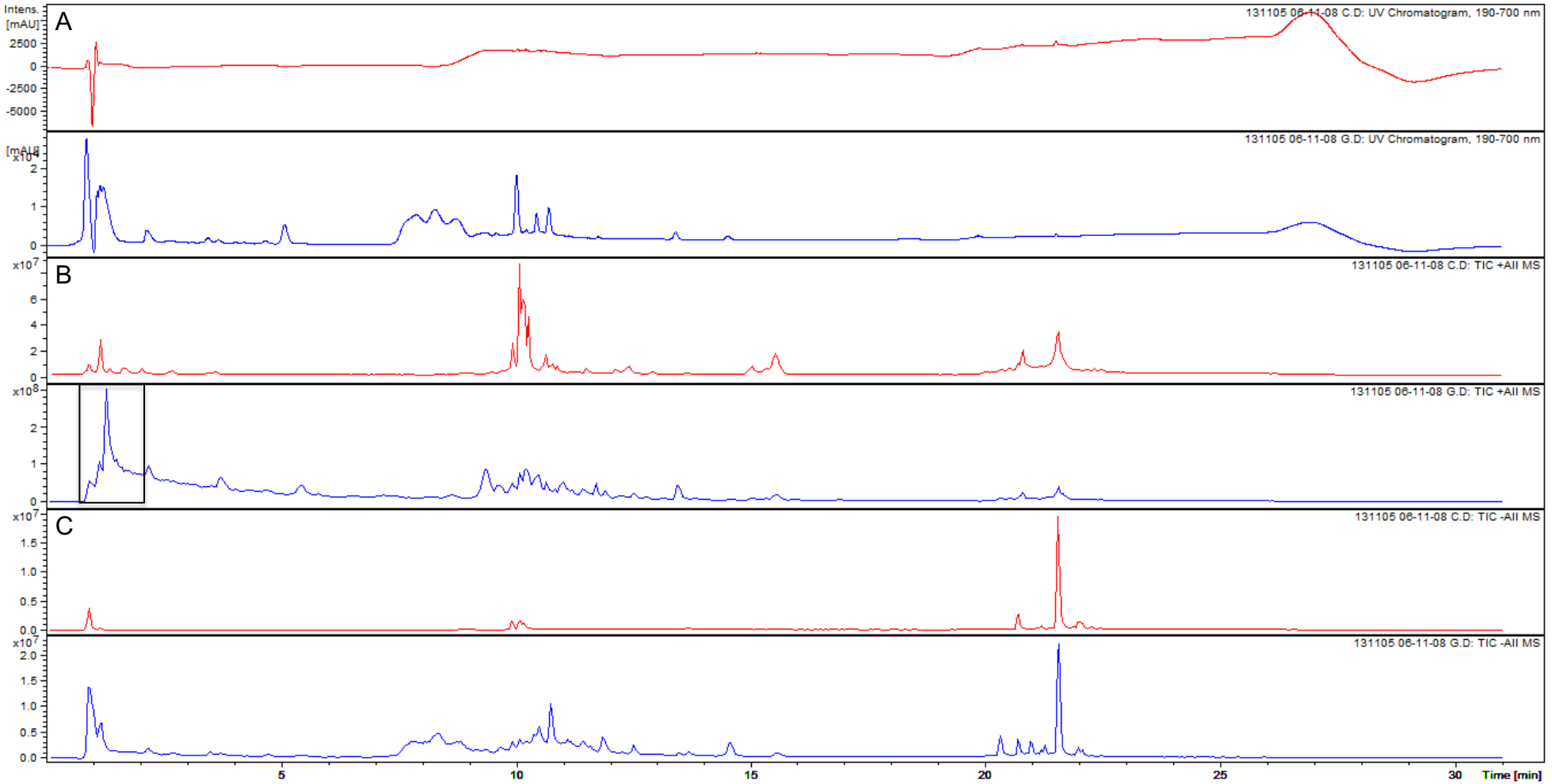

Figure 20 Analysis of Keele grave leachate sample 06-11-08 (blue line) and its correspondent control (red line) on LC-MS: UV/Vis spectrometry 190-700nm (A); Mass spectrometry total ion current positive ionisation mode (B); Mass spectrometry total ion current (C)

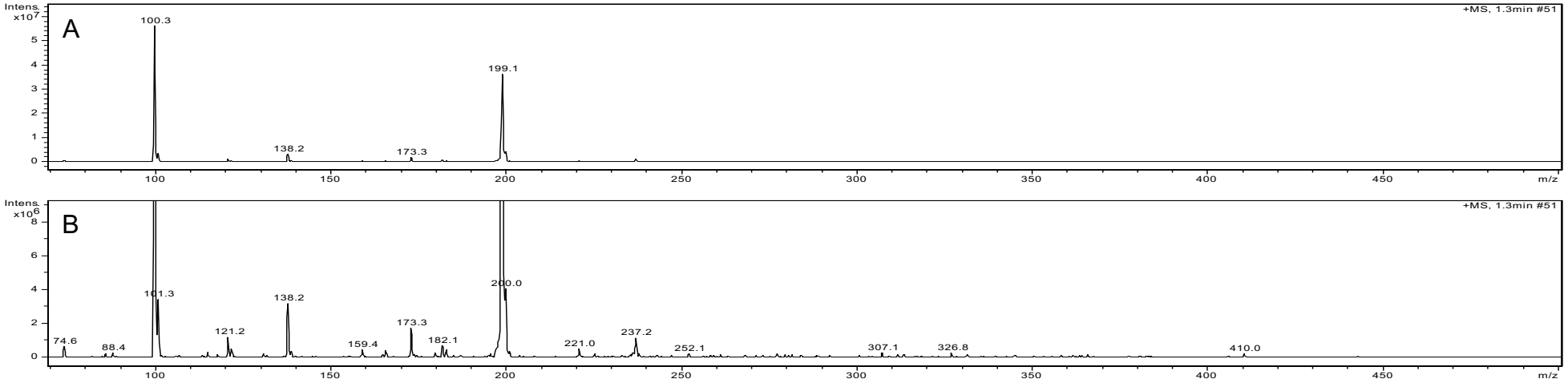

Figure 21 Mass spectrum of Keele grave leachate sample 06-11-08 at 1.3 minutes using LC-MS positive ionisation mode: Normal view (A); Baseline focused view (B)

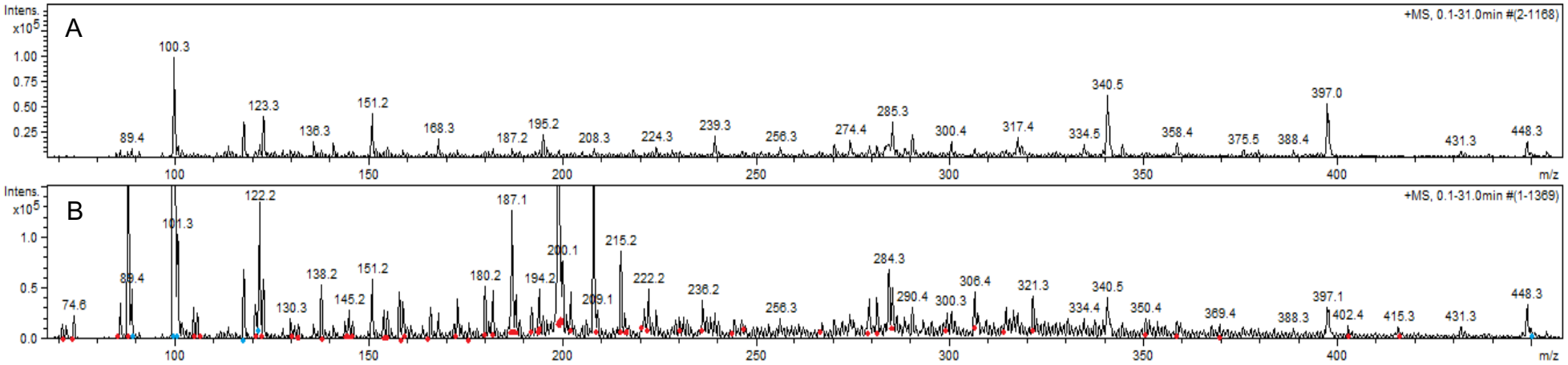

Figure 22 Mass spectrum of Keele grave leachate sample 06-11-08 and its correspondent control using LC-MS: Positive ionisation mode mass spectra control sample (A); Positive ionisation mode mass spectra grave sample (B)

In order to further illustrate the differences between the grave and control samples a mass spectrum of the positive ionisation was taken over the entire chromatogram, which resulted in a mass spectrum containing all m/z spectra of the chromatogram in a single spectrum, Figure 22. This clearly showed distinctive differences between the grave and control sample. The m/z peaks present in the grave sample but not in the control were given a red mark and the peaks present in both samples but much more abundant in the grave sample were given a blue mark. The peaks marked red were then plotted in Figure 23, and the ten largest peaks, in yellow, were classed as main compounds of interest whilst the others were left to explore at a later stage. The mass spectral data provided in Figure 22 indicated the presence of more than 100 decomposition specific chemicals in the leachate. The chemicals with a blue mark in Figure 22, chemicals with the same m/z values but different retention times and chemicals detected in the negative ionisation mode were not included in Figure 23.

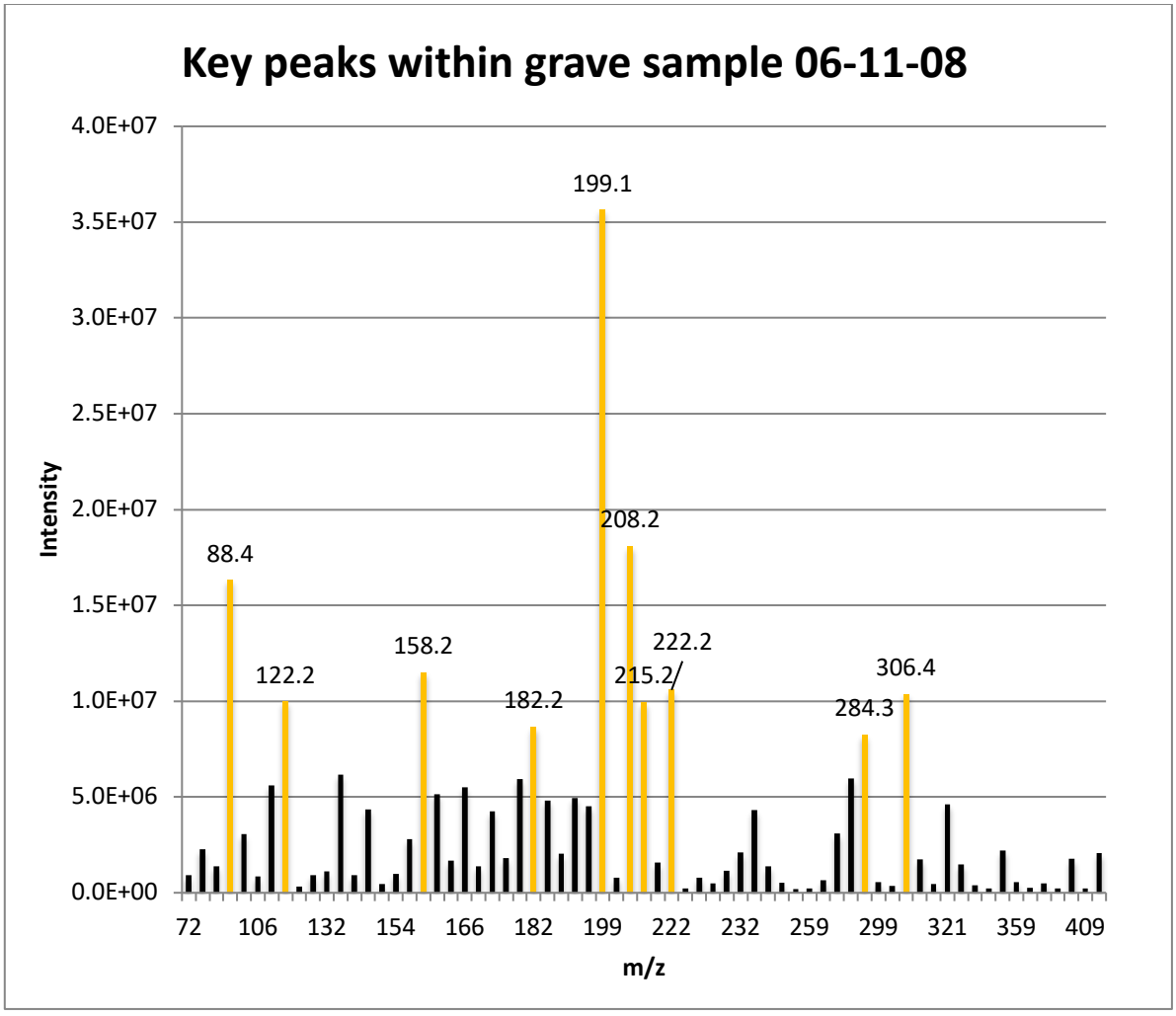

Figure 23 Bar chart of the key peaks within grave sample 06-11-08 positive ionisation

The lack of resolution and accuracy of the Ion-trap-MS made compound identification very difficult due to the possibility for multiple compounds having the same nominal mass (Kienzl-Wagner & Brandacher 2014). MS instruments having higher resolution than the Ion-trap such as a Time-of-Flight Mass Spectrometer could be able to differentiate between compounds with the same nominal mass. A library is usually required for qualification of unknown compounds, however a LC-MS does not have an internal library such as seen with GC-MS. Online LC-MS databases such as Massbank and HMDB (Human Metabolome Data Base) were available to compare the spectra but the lack in resolution of the MS resulted in over 100 different possible matches for the ions investigated.

Structure interpretation was possible through manual calculation of the isotope ratio within a mass spectrum, which provided possible molecular formulae (McLafferty 1973). This was done by calculating the ratio of "A+1"[1] and "A+2"[2] peaks in regards to the most abundant isotope, which enabled the calculation of the number of carbon atoms and eventually the assignment of a molecular formula. This has been done for the first peak of interest plotted in Figure 23 having a m/z of 88.4 (Rt 1.4 minutes). Its molecular formula would be either $C_5H_{13}N$ or C_4H_9NO (see Appendix IV for calculations), however it was still uncertain that one of those two molecular formulae corresponds to this particular m/z peak due to the lack in mass spectral resolution. The masses corresponding to putrescine+H^+ (89.2) and cadaverine+H^+ (103.2) have been detected but it is likely that the detection of putrescine and cadaverine has been compromised due to the possibility multiple ionisation and therefore would not have been detected (also see table below).

The data was also compared to decomposition related compounds detected in decomposition fluids by other researchers, see Table 16, Table 17 and Table 18. When the data was compared to amino acids and amines (Table 16) many compounds appeared to have also been detected in the Keele leachate sample 06-11-08. Due to the instrumentation used and the lack of standards these

[1] Elements with multiple natural isotopes, the second one being one mass unit heavier than the most abundant isotope (A).

[2] Elements with multiple natural isotopes, the second one being two mass units heavier than the most abundant isotope (A).

compounds were not conclusively identified in the leachate, instead for the amino acids the presence of a ($M+H^+$) and a ($M-H^-$) ion at the same retention time indicated the presence of that amino acid. Furthermore, isotope pattern calculations either further confirmed the findings or disproved them. After calculating the number of carbon atoms present in each peak only lysine, tyramine, phenylalanine, indole and tryptamine appeared to be present in the leachate samples, highlighted in Table 16. Identification of the volatile fatty acids and other compounds was less accurate as these compounds did not have a ($M+H^+$) and a ($M-H^-$) ion eluting at the same retention time. After determining the number of carbon atoms in each peak only phenylpropanoic acid, palmitic acid, oleic acid, linoleic acid, β-sitosterol and piperidone appeared to be present in the leachate samples, see Table 17 and Table 18.

Table 16 Comparison of results with amino acids and amines detected by other researchers in decomposition fluids

Compound	*Formula*	*Monoisotopic mass (Da)*	*Instrument*	*Molecular ion detected in leachate samples*	*Comments/ Isotope calculations*
Putrescine[1]	$C_4H_{12}N_2$	88.100044	HPLC	Yes	2 carbon atoms
Cadaverine[2]	$C_5H_{14}N_2$	102.115700	HPLC	Yes	6-8 carbon atoms
Serine[1]	$C_3H_7NO_3$	105.042595	HPLC	No	
Histamine[1]	$C_5H_9N_3$	111.079643	HPLC	Yes	4 carbon atoms
Proline[1]	$C_5H_9NO_2$	115.063332	HPLC	No	
Indole[1,3]	C_8H_7N	117.057846	HPLC/ GC	Yes	7-8 carbon atoms
Valine[1]	$C_5H_{11}NO_2$	117.078979	HPLC	Yes	6-7 carbon atoms
Threonine[1]	$C_4H_9NO_3$	119.058243	HPLC	No	
Iso-Leucine[1]	$C_6H_{13}NO_2$	131.094635	HPLC	Yes	8-10 carbon atoms
Leusine[1]	$C_6H_{13}NO_2$	131.094635	HPLC	Yes	7-9 carbon atoms
Asparagine[1]	$C_4H_8N_2O_3$	132.053497	HPLC	Yes	7-8 carbon atoms
Tyramine[1,4]	$C_8H_{11}NO$	137.084061	HPLC/ CE	Yes	8-10 carbon atoms
Lysine[1]	$C_6H_{14}N_2O_2$	146.105530	HPLC	Yes	6-7 carbon atoms
Glutamic acid[1]	$C_5H_9NO_4$	147.053162	HPLC	Yes	6-7 carbon atoms
Methionine[1]	$C_5H_{11}NO_2S$	149.051056	HPLC	No	
Histidine[1]	$C_6H_9N_3O_2$	155.069473	HPLC	No	
Tryptamine[4]	$C_{10}H_{12}N_2$	160.100052	CE	Yes	10-12 carbon atoms
Phenylalanine[1,4]	$C_9H_{11}NO_2$	165.078979	HPLC/ CE	Yes	9-10 carbon atoms
Arginine[1]	$C_6H_{14}N_4O_2$	174.111679	HPLC	No	
Tyrosine[1,4]	$C_9H_{11}NO_3$	181.073898	HPLC/ CE	Yes	10-12 carbon atoms
Tryptophan[1,4]	$C_{11}H_{12}N_2O_2$	204.089874	HPLC/ CE	Yes	12-15 carbon atoms

Table 17 Comparison of results with volatile fatty acids detected by other researchers in decomposition fluids

Compound	*Formula*	*Monoisotopic mass (Da)*	*Instrument*	*Molecular ion detected in leachate samples*	*Comments/ Isotope calculations*
Formic acid[5]	CH_2O_2	46.005478	GC	No	
Acetic acid[3,5,6]	$C_2H_4O_2$	60.021130	GC	No	
n-Propionic acid[3,5,6]	$C_3H_6O_2$	74.036781	GC	Yes	4 carbon atoms
Iso-Butyric acid[3,5]	$C_4H_8O_2$	88.052429	GC	Yes	5 carbon atoms
n-Butyric acid[3,5,6]	$C_4H_8O_2$	88.052429	GC	Yes	5 carbon atoms
Iso-Valeric acid[3,5,6]	$C_5H_{10}O_2$	102.068077	GC	Yes	
n-Valeric acid[3,5]	$C_5H_{10}O_2$	102.068077	GC	Yes	
Methyl-Valeric acid[3]	$C_6H_{12}O_2$	116.083733	GC	No	
Iso-Caproic acid[6]	$C_6H_{12}O_2$	116.083733	GC	No	
n-Caproic acid[3,5]	$C_6H_{12}O_2$	116.083733	GC	No	
n-Heptanoic acid[5]	$C_7H_{14}O_2$	130.099380	GC		
Phenylacetic acid[3,6]	C_8H_8O	136.052429	GC	Yes	
Phenylpropionic acid[3,6]	$C_9H_{10}O_2$	150.068085	GC	Yes	9 carbon atoms
Myristic acid[6]	$C_{14}H_{28}O_2$	228.208923	GC	No	
Palmitoleic acid[6]	$C_{16}H_{30}O_2$	254.224579	GC	No	
Palmitic acid[6]	$C_{16}H_{32}O_2$	256.240234	GC	Yes	14-17 carbon atoms
Linoleic acid[6]	$C_{18}H_{32}O_2$	280.240234	GC	Yes	18-22 carbon atoms
Oleic acid[3,6]	$C_{18}H_{34}O_2$	282.255890	GC	Yes	15-19 carbon atoms
Stearic acid[6]	$C_{18}H_{36}O_2$	284.271515	GC	No	

Table 18 Comparison of results with additional decomposition compounds detected by other researchers in decomposition fluids

Compound	*Formula*	*Monoisotopic mass (Da)*	*Instrument*	*Molecular ion detected in leachate samples*	*Comments/ Isotope calculations*
Phenol[3]	C_6H_6O	94.041862	GC	No	
Piperidone[3,6]	C_5H_9NO	99.068413	GC	Yes	
Cholesterol[7]	$C_{27}H_{46}O$	386.354858	GC	No	
Coprostanol[7]	$C_{27}H_{48}O$	388.370514	GC	No	
β-sitosterol[7]	$C_{29}H_{50}O$	414.386169	GC	Yes	24-29 carbon atoms

[1] (Swann et al. 2012), [2] (Fiedler et al. 2004), [3] (Swann et al. 2010b), [4] (Swann et al. 2010c), [5] (Vass et al. 1992), [6] (Swann et al. 2010a), [7] (von der Lühe et al. 2013)

The response for the four most abundant (unidentified) mass signals from Figure 23 were plotted in separate bar charts over time-since-burial and illustrated similar patterns of an increase in abundance up to 12 months post-burial, followed by a gradual decrease over a longer period of time, see Figure 24. This indicated the longevity of the detection of these chemicals, especially chart D) as this compound was detected up to at least 30 months post burial.

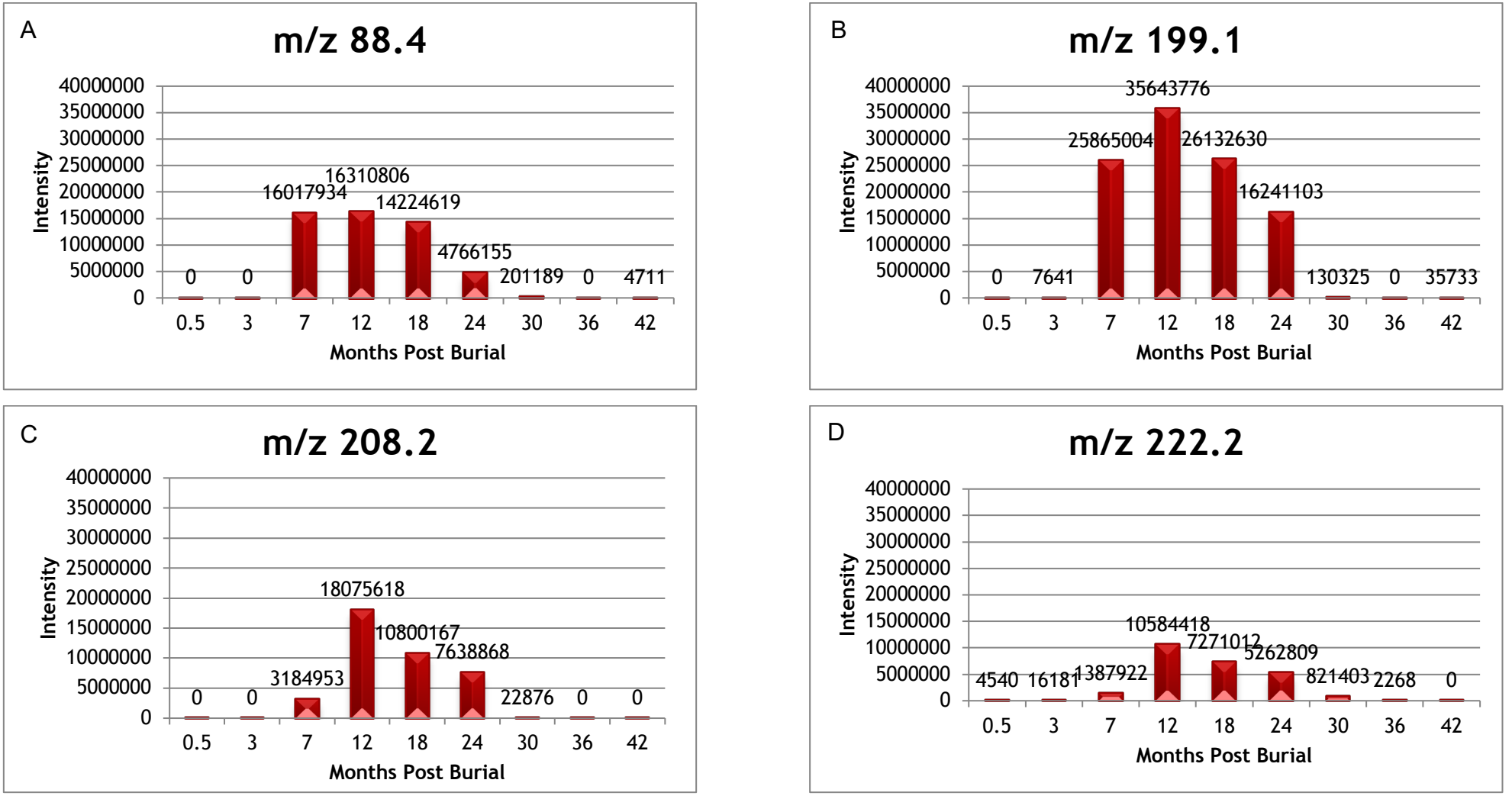

Figure 24 Bar chart of the key peaks within grave sample 06-11-08

Chapter 4 Analysis of Inorganic Anionic Compounds Using Ion Chromatography

The research described in this chapter has been performed by Avans University students Max Krens and Stepanie van Rens under the direct supervision of the author as partial fulfilment of their internship project at Staffordshire University.

4.1 Analysis of Standards and System Suitability Samples

Daily system performance was determined through evaluation of pump pressure, absolute conductivity, retention times and peak areas of the anion standard solution injections. The anion standard solution contains fluoride, chlorate, nitrite, phosphate and sulphate and a chromatogram is displayed in Figure 25. The relative standard deviations (RSD) of each of the aforementioned criteria were required to be below 5% to pass the daily system suitability criteria.

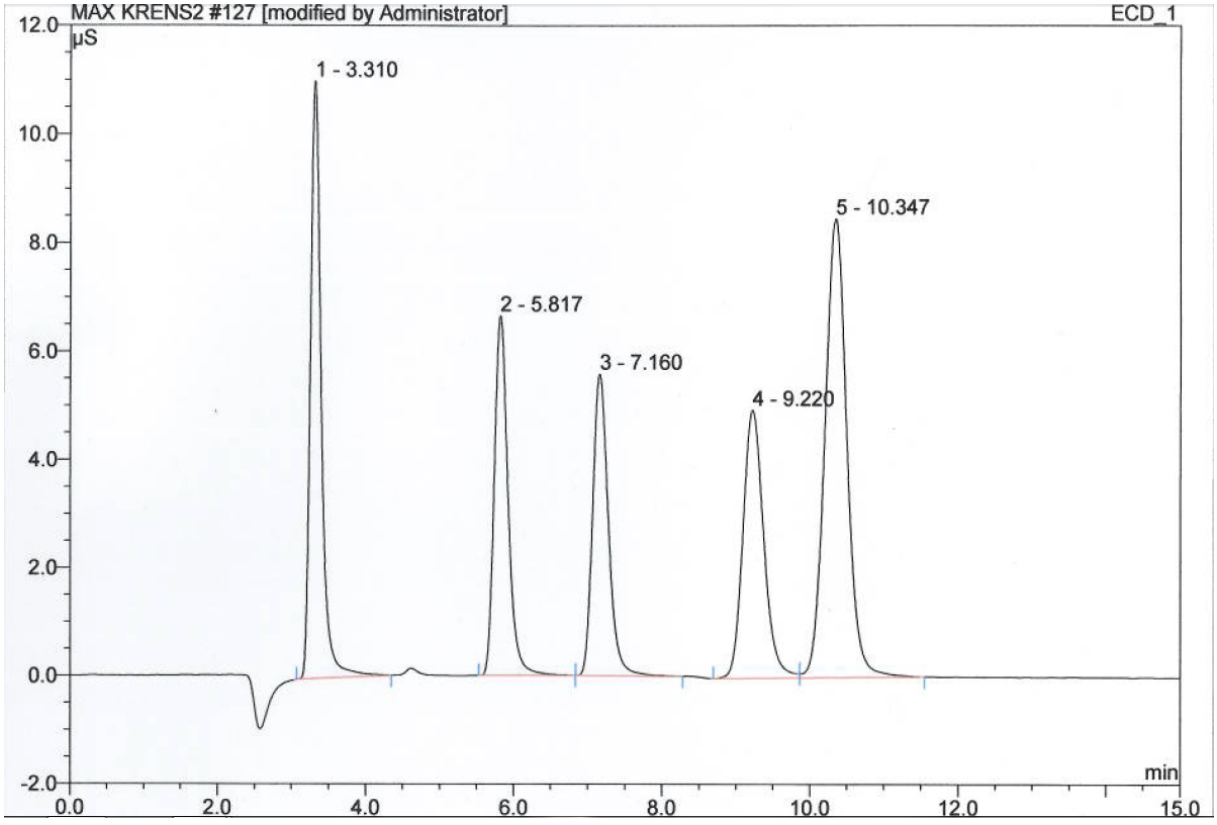

Figure 25 Chromatogram of the prepared mixed anion standard. The shown peaks from left to right are identified as fluoride, chlorate, nitrate, phosphate and sulphate.

The anion standards displayed in Table 11 were analysed for the purpose of retention time confirmation and compound identification, which was performed through comparison of retention times and spiking of the sample with standard solutions. When an increase in peak area was observed and the peak shape did not indicate any obvious patterns of co-elution, positive identification was made. For the

determination of the detection and quantification limits of the identified ions peak heights of diluted standard solutions were compared to the baseline noise which was determined to be 0.02 μS following blank injections (n=10). Thus the detection and quantification limits were determined at three and ten times the baseline noise, see Table 19 for the detection and quantification limits determined for the anions present in the leachate.

Table 19 Detection and quantification limits for the identified anions

Ion	Detection limit (ppm)	Quantification limit (ppm)
Acetate	5.9	19.8
Bromate	1.6	5.2
Chloride	1.8	6.1
Nitrite	0.6	1.8
Nitrate	0.5	1.8
Phosphate	1.4	4.8
Sulphate	0.1	0.2

4.2 Analysis of Leachate Samples

When analysing leachate sample 129 weeks post burial and its corresponding control sample, Figure 26, significant differences were observed between the two chromatograms. This highlights the capability of ion chromatography to differentiate between decomposition related samples and their corresponding control samples. These chromatograms also highlight the longevity for the detection of decomposition related anions as they are still able to be detected after approximately two and a half years post burial. The signals observed in the control sample (Figure 26B) correspond to chloride, nitrite, phosphate and sulphate respectively. These ions are also detected in the grave sample but in significantly larger quantities and additional ions are detected in the gravesite sample (Figure 26A).

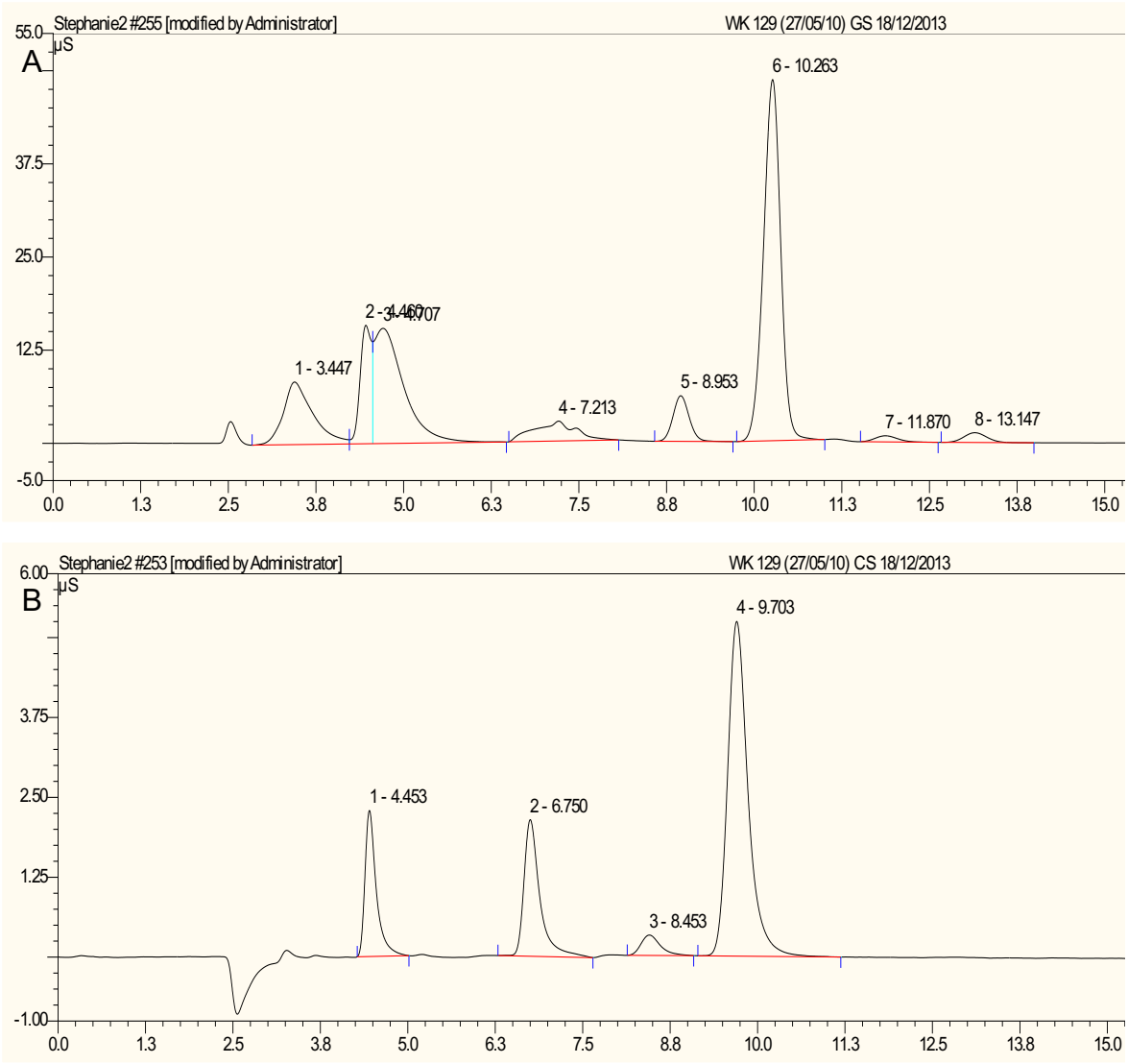

Figure 26 Chromatogram of leachate sample wk 129 grave sample (A) and its corresponding control sample (B)

The ions detected in the leachate samples collected within the first two months post burial were compared with their control samples (3 injections per sample) to determine after which time point post burial differences between grave and control samples could be observed, see Table 20. Clear differences were observed for chloride, nitrate and sulphate after five weeks post burial, whilst acetate appeared after six weeks post burial. Relatively large concentrations of chloride 0.87 mg mL^{-1} were detected, however the concentrations of acetate were detected up to 20 mg mL^{-1}. As has been discussed in Section 1.4 Thanatochemistry, acetic acid the free acid of acetate, has been noted as a significant decomposition product through decomposition of carbohydrates, lipids and proteins. Vass et al. (1992) stated that the use of formic and acetic acid are abundant in nature and their levels are too variable to be used for time since death determination but these results highlight that it should not be excluded as a potential marker to locate clandestine

gravesites. The levels of chloride detected in this study exceeded the largest quantity of chloride detected by Vass et al. (1992) which was below 250 ppm g^{-1} dry soil. But this could be inherent to differences between the samples and soil type.

Table 20 Earliest detection of anions and largest concentration detected in leachate samples

Compound	*First detected (weeks post burial)*	*Highest concentration detected (mg mL^{-1})*
Chloride	5	0.87
Nitrate	5	0.15
Sulphate	5	0.14
Acetate	6	19.99

The relative abundance of the ions detected in the leachate samples between 26 and 40 weeks post burial were visually compared, see Figure 27. A clear pattern is visible between acetate, chloride and nitrate. The relative abundance of acetate and nitrate increase over time since burial, whilst the relative abundance of chloride decreases. Both acetate and nitrate have been reported to be products of mammalian decomposition, see Section 1.4 Thanatochemistry. When inspecting Figure 28 it can be observed that the absolute concentration of chloride is stable over time, which is not the situation for acetate and nitrate, hence the change in relative ion abundance. Aitkenhead-Peterson et al. (2012) observed a decrease in the levels of sulphate in comparison to the control samples, however the data in Figure 28 indicate a general increase of the sulphate concentration (produced from cysteine and methionine, see Section 1.4 Thanatochemistry) up to 120 weeks post burial. Phosphate has not been detected in many grave samples (only in samples 5, 120, 125 and 129 weeks post burial) which could have been due to the formation of water insoluble phosphate salts such as calcium or magnesium salts and could have prevented leaching of phosphate into the lysimeter. Carbonate, one of the main inorganic constituents of bone (Schultz et al. 1997), has been detected up to concentrations of 15 mg mL^{-1} and follows as similar pattern to the detection of acetate over time and thus could also become a decomposition marker but is difficult to analyse using the IC parameters used.

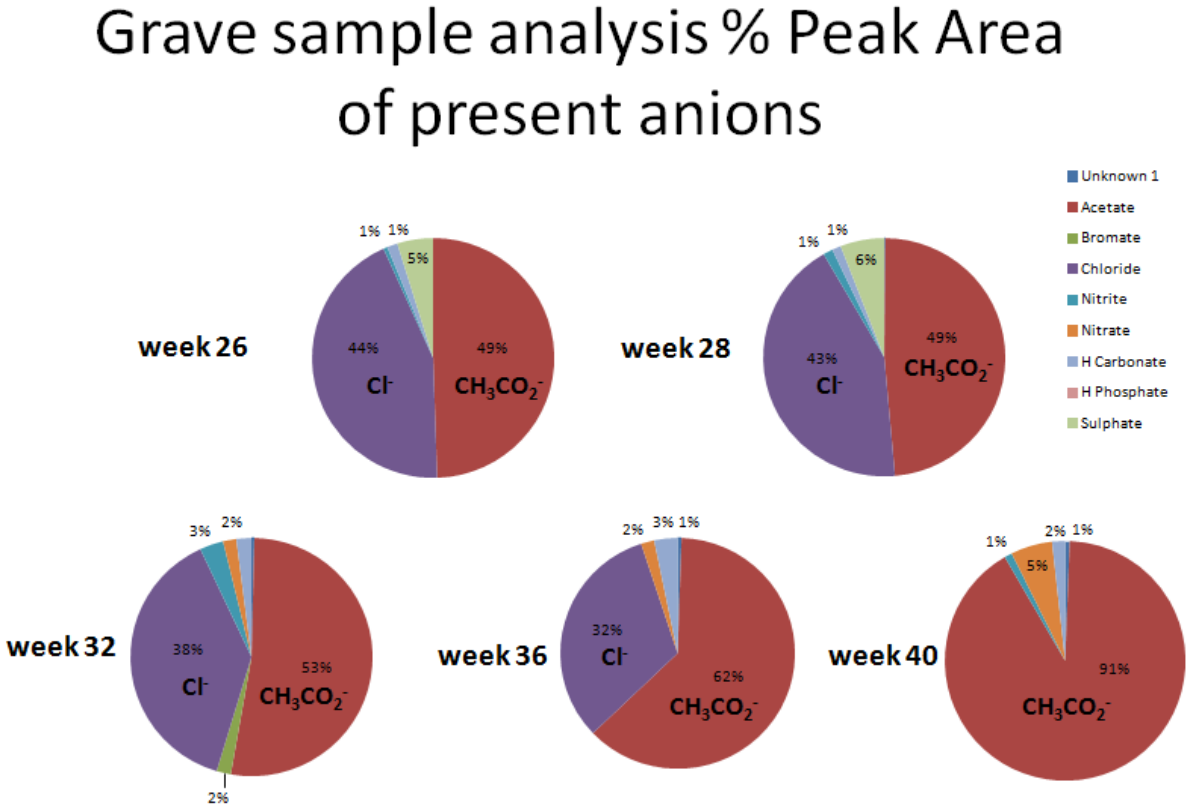

Figure 27 Pie chart displaying the difference in relative peak area of the anions detected in the leachate, see Figure 28 for the absolute concentartions

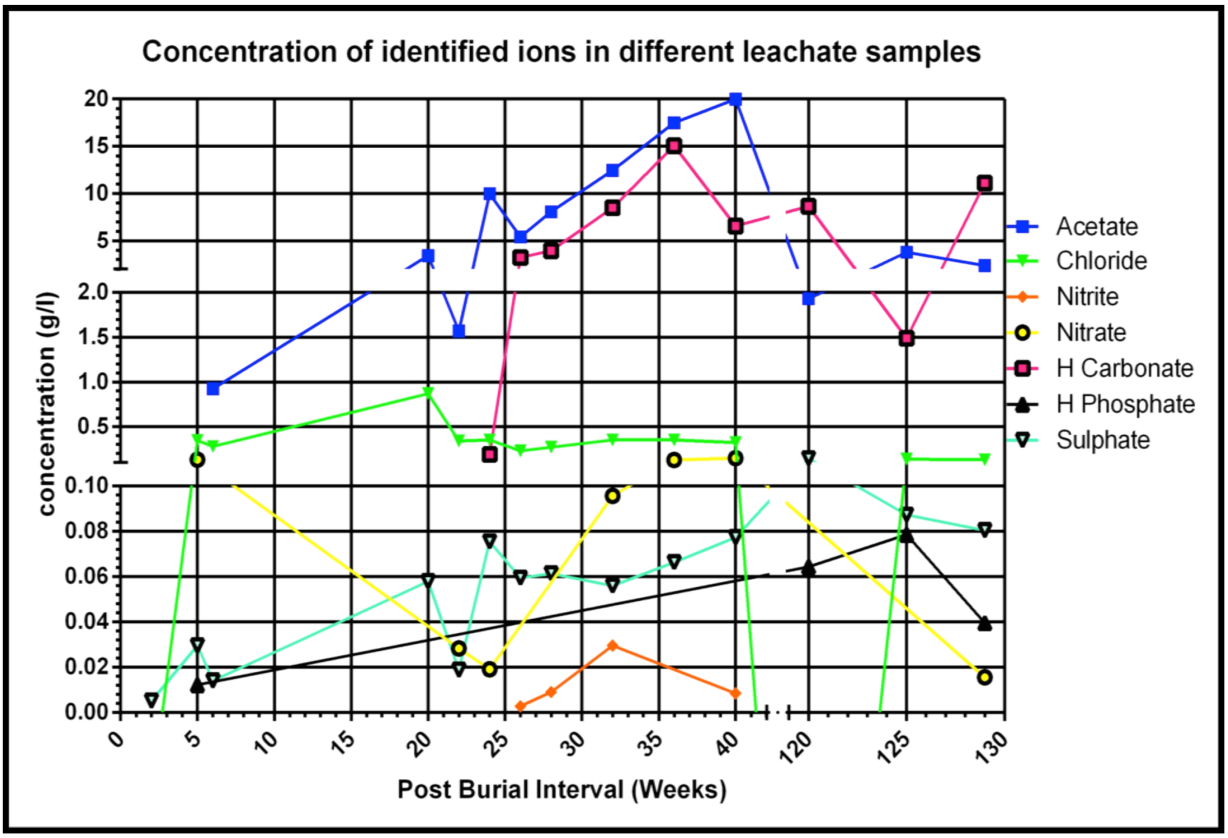

Figure 28 Chart displaying the absolute concentrations of anions detected in the leachate samples over time since burial

Following repeated analysis of the standard solutions, significant degradation of the ions was observed after three weeks storage at room temperature, the peak height degraded by approximately 50% and additional peaks appeared in the chromatograms. It is however unknown if this was only the situation for the standard solutions or also for the leachate samples. Pringle et al. (2010) highlighted a decrease in conductivity of approximately 40% after analysing defrosted leachate samples and thus degradation could have occurred before initial IC analysis commenced. The addition of preservatives, such as ethylene diamine has been suggested by the USEPA analysis method for the determination of anionic compounds in drinking water and should inhibit degradation of the ions in the standard solutions (Hautman & Munch 1997). The physical properties of ethylene diamine are very similar to those of putrescine and cadaverine and thus these amines could have possibly preserved the ions in the sample solutions.

4.3 Analysis of Water Casework Samples

Analysis of the water samples was performed by Avans University student Vincent Voorwerk under the direct supervision of the author as partial fulfilment of his internship project at Staffordshire University. The stability and the matrix effects study on the water samples was performed by the author.

The water samples were analysed using ion chromatography and the data is displayed in Figure 29. Chloride, phosphate, sulphate and thiosulphate were the only ions detected in the samples analysed and were identified through spiking of the sample solution with appropriate standard solutions. The data in Figure 29 highlighted that chloride had been detected in all samples, including the control samples. The data obtained from the analysis of the leachate samples highlighted that chloride was detected in both grave and control samples, however significant differences were observed in the concentration of chloride in the associated gravesite samples. In addition, chloride has been reported as a significant decomposition product by Vass et al. (1992) but is not conclusive for the samples provided, see Table 21. Phosphate has been reported as a significant decomposition product by Aitkenhead-Peterson et al. (2012) and has also been detected in the suspected gravesite samples but also in one of the control samples. Again no observable differences were noted between either of the suspected grave or control samples. Sulphate was detected in suspected grave samples A and C but also in control sample 2. The leachate data highlighted a significant increase in

sulphate during decomposition but it did not distinguish between any of the samples analysed here. Thiosulphate was the only anion detected in the suspected grave samples only and was detected in samples A and C. The significance of the detection of thiosulphate is however unknown as the detection of thiosulphate has not been reported in the literature as a mammalian decomposition product, neither has it been detected in the leachate samples. Thiosulphate was not readily detected in any of the 120 soil samples analysed (only once) by Bommarito et al. (2007) and therefore could indicate some significance as it has not been commonly detected in the environment.

Table 21 Concentration of chloride detected in the water samples (courtesy of Vincent Voorwerk)

Sample	Concentration chloride detected (ppm)
Sample A	8.74
Sample B	7.53
Sample C	8.34
Control sample 1	12.69
Control sample 2	12.90

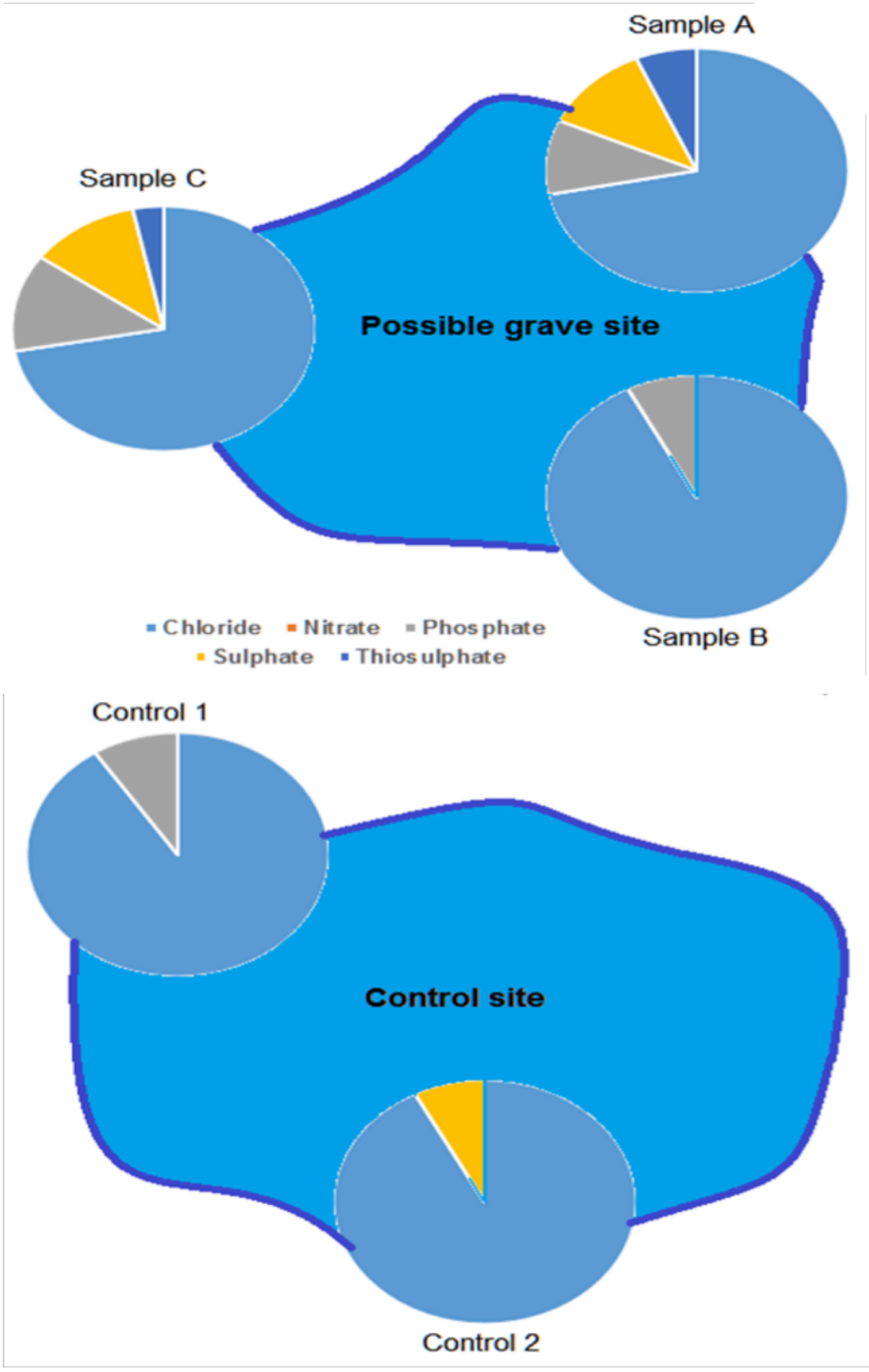

Figure 29 Pie charts displaying the relative quantities of anions present in the water samples (courtesy of Vincent Voorwerk)

4.4 Analysis of Soil Casework Samples

A chromatogram of a spiked soil sample is displayed in Figure 30, the spiked quantities are visible in the second column of Table 22 and the recovery values and their RSD's are displayed in the final two columns. The recovery of the developed methodology was evaluated through spiking of soil samples with standard solutions. It had been observed that under the current extraction methodology the recovery

was very poor reaching a recovery of around 20% for the anions analysed. A similar extraction procedure, using ultrasonic bath and deionised water had been used by Bommarito et al. (2007) but no recovery data was provided and thus these poor recoveries might have been inherent to the extraction methodology. Nevertheless, this extraction procedure has been applied to the analysis of the case samples, see Figure 31, as it was intended to keep an aqueous matrix as that had been used for the analysis of the leachate samples. Furthermore, the author was unsure how long the samples could be stored without degradation. Despite the low recovery, the extraction methodology was reproducible having a relative standard deviation below 10% and less than 5% for fluoride and chloride.

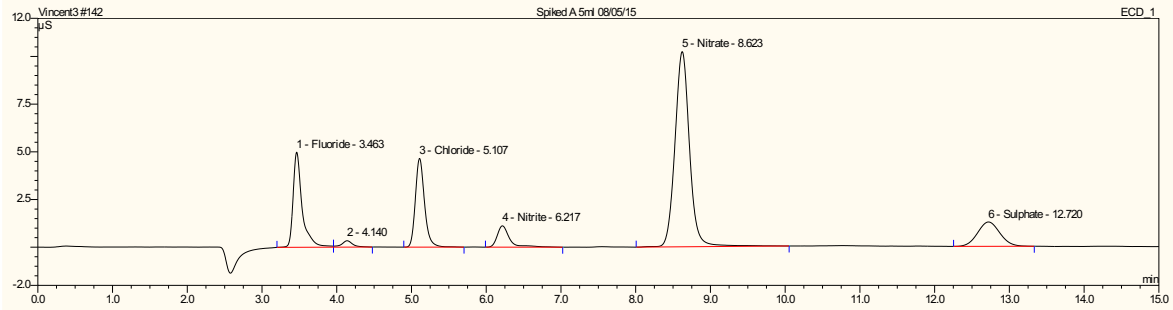

Figure 30 Chromatogram of spiked soil sample (courtesy of Vincent Voorwerk)

Table 22 Displays the recovery of the extraction methodology for the different anions (courtesy of Vincent Voorwerk)

Anion	Spiked ($mg \cdot L^{-1}$)	Recovered ($mg \cdot L^{-1}$)	Control ($mg \cdot L^{-1}$)	Recovery (%)	RSD (%)
Fluoride	3.3245	0.9571	0.3005	19.75	3.0
Chloride	3.3245	0.9270	0.2457	20.49	2.4
Nitrite	3.3245	0.9419	0.4165	15.80	9.1
Nitrate	6.6490	5.9045	4.0984	27.16	7.9
Sulphate	3.3245	1.1875	0.4931	20.89	9.2

The samples provided have been extracted for IC analysis and the data is displayed in Figure 31. The gravesite (sample 1) generally showed lower levels of soluble anions in comparison to the samples taken in the immediate surrounding area, possibly due to improved drainage of the soil following excavation. However, slightly elevated levels of nitrate were detected in the lower levels of the grave soil compared to samples in the immediate area and elevated levels of phosphate were found in the sample taken from the upper layer of soil immediately West of the grave (sample 2). In addition, elevated levels of phosphate were detected in sample 8 that was at or near a dog indication upslope from the grave. Elevated levels of phosphate have been reported to be associated to human decomposition

(Aitkenhead-Peterson et al. 2012). Samples 6 and 8 showed the largest variety and abundance of ions present in all the soil samples provided, as samples 11 and 12 only showed large quantities of nitrate.

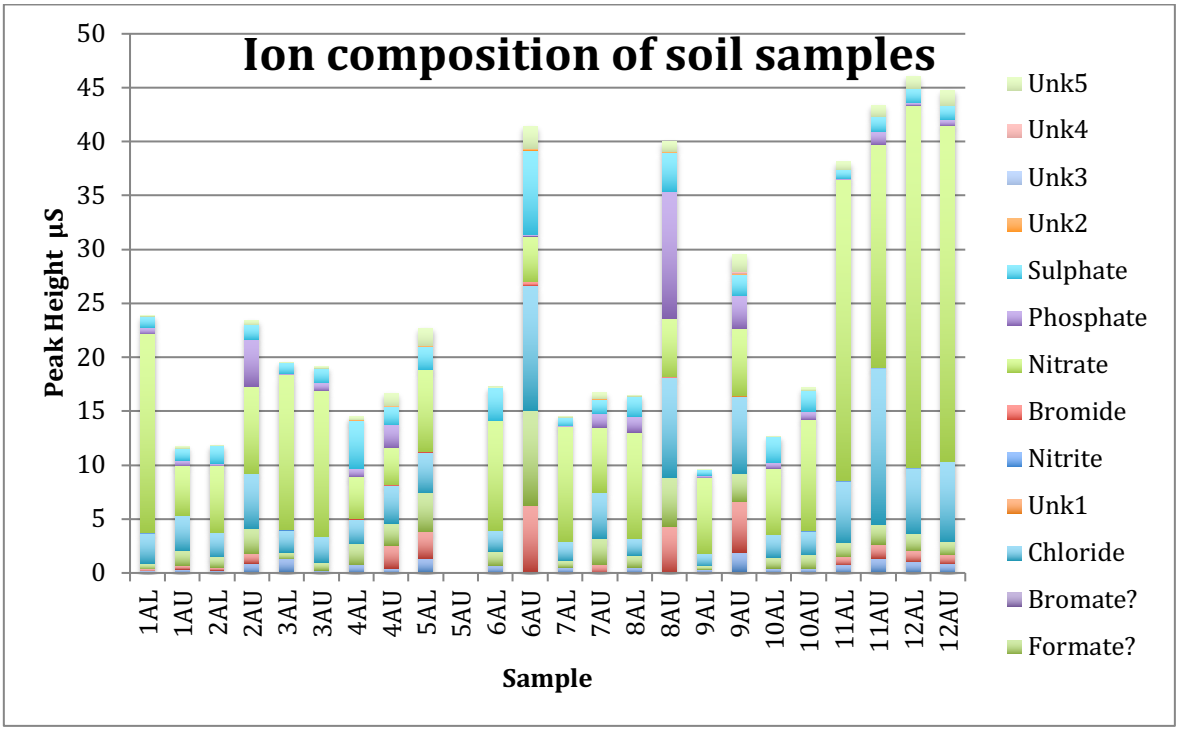

Figure 31 Bar chart displaying the quantities of ions detected in each sample

Even though only a small sample size was available, principal component analysis (PCA) was used to filter the data and select a subset of samples for GC analysis, see Figure 32. As visible in Figure 32, most samples cluster together around the control sample (sample 12). Sample 6 (approximately 100m downhill) and sample 8 (near VRD indication) showed the most variance and were thus selected for GC-MS analysis alongside samples 4 (directly downslope behind a boulder of sandstone), 1 (location where victim was buried) and 12 (control sample, over 200m upslope). The variation was also observed in Figure 31, where samples 6 and 8 showed the most significant difference between the samples analysed (see previous paragraph).

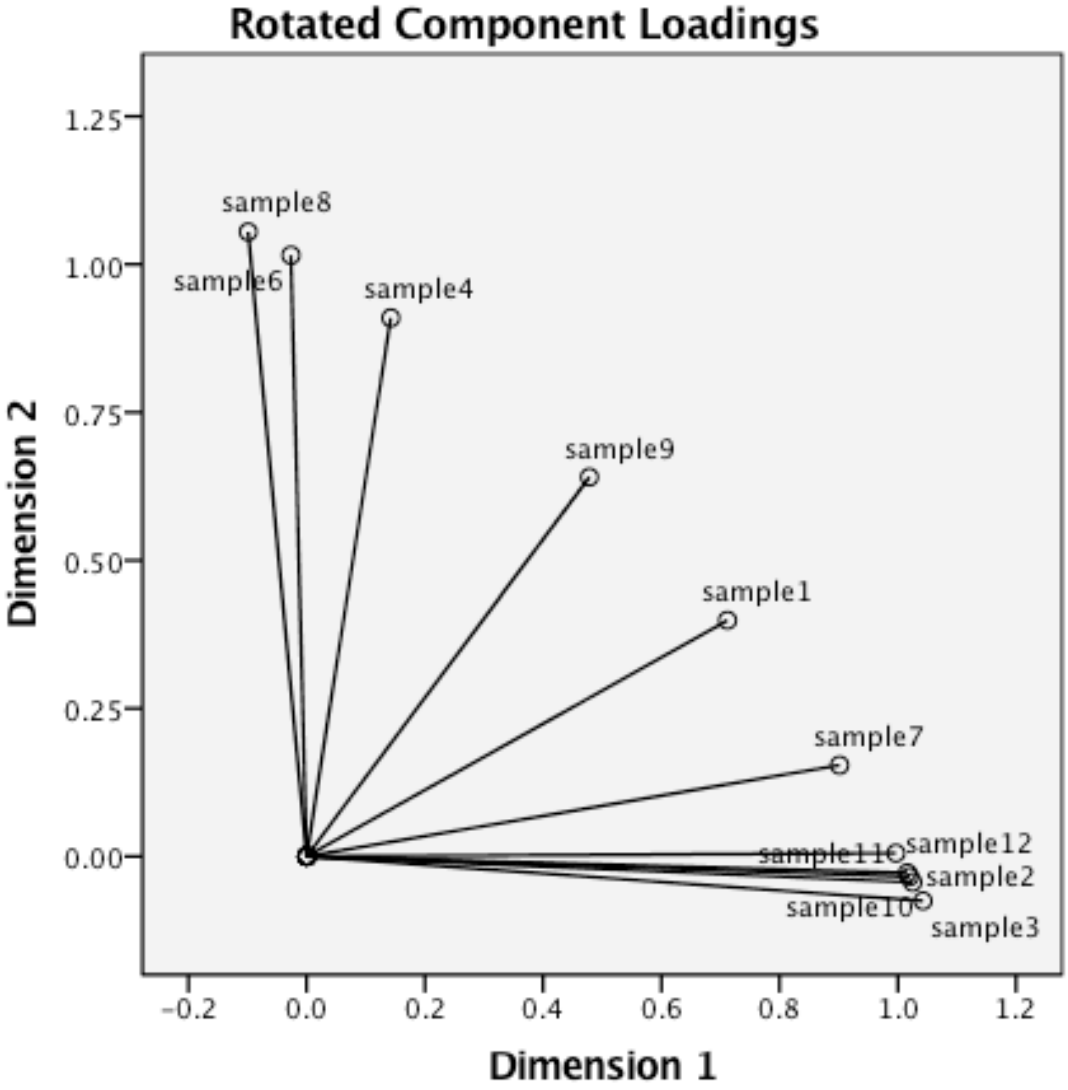

Figure 32 PCA plot of the IC data for each sample

Chapter 5 Detection of Putrescine, Cadaverine and Methylamine in Mammalian Decomposition Fluids

5.1 Analysis of Putrescine, Cadaverine and Methylamine

The GC-MS analysis of a derivatised standard solution of putrescine (Rt. 16.99 minutes), cadaverine (Rt. 17.53 minutes) and methylamine (Rt. 7.67 minutes) is displayed in Figure 33. This chromatogram demonstrates the detection of these amines using GC-MS following derivatisation and indicates that derivatisation of these amines occurred and is stable under the current operating conditions (Hoshika 1977). Blom (2012) used this method to detect putrescine and cadaverine in Keele leachate samples, where most of the compound identification and method optimisation was performed. The derivatisation reaction with pentafluorobenzaldehyde was very selective as only primary amines react with pentafluorobenzaldehyde (Hoshika 1977; Knapp 1979) but it produced geometrical (*cis/trans*) isomers, which lead to the detection of multiple peaks for putrescine and cadaverine, Figure 33.

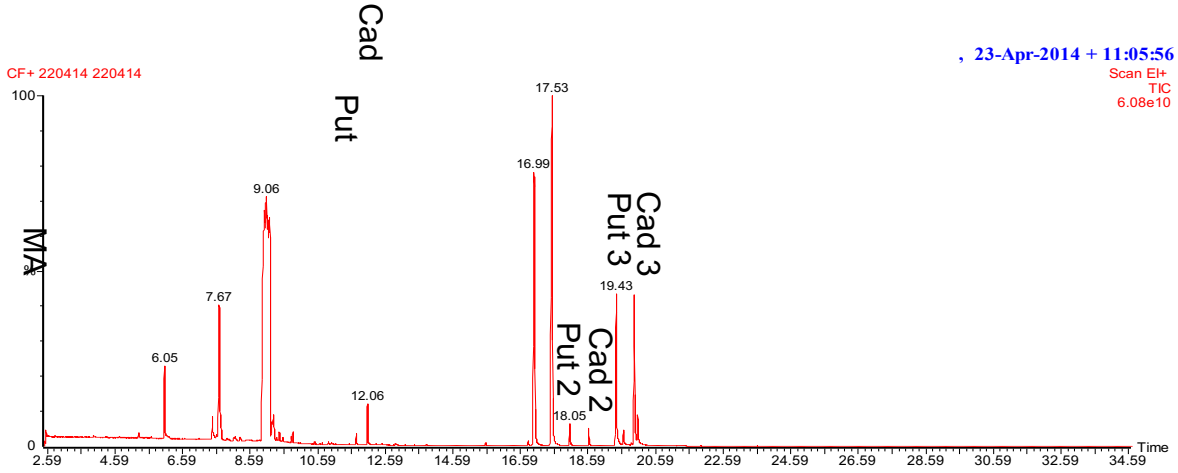

Figure 33 Analysis of derivatised putrescine (Put), cadaverine (Cad) and methylamine (MA) using GC-MS

Putrescine, cadaverine and methylamine were identified by individually analysing standards to confirm their retention time and mass spectra. The most abundant m/z peaks in each mass spectra are displayed in Table 23 and were compared to the peaks published by Ngim et al. (2000). As illustrated in Table 23 the m/z peaks for each compound are similar to the m/z peaks described by Ngim et al. (2000), however the relative intensities of these peaks differed. As all mass spectra were produced by 70eV electron impact ionisation, this variation was likely due to differences in instrumentation, calibration of the instrument or its internal settings (McMaster 2011). Table 24 compares the most abundant m/z peaks of

methylamine from this study to those reported by Avery & Junk (1985) and Ngim et al. (2000), and illustrates clear differences in the relative abundance of the m/z peaks between all three studies, further confirming the previous statement. Positive identification of putrescine, cadaverine and methylamine was observed.

Table 23 Comparison of mass spectral data for derivatised putrescine, cadaverine and methylamine to reference article Ngim et al. (2000)

Methylamine (m/z)		Putrescine (m/z)		Cadaverine (m/z)	
Ngim	Sample	Ngim	Sample	Ngim	Sample
208 (100)	209 (100)	249 (100)	222 (100)	181 (100)	222 (100)
209 (88)	208 (97)	181 (77)	230 (86)	208 (83)	263 (78)
117 (26)	181 (36)	208 (55)	180 (80)	263 (78)	190 (73)
161 (19)	161 (14)	194 (54)	249 (61)	244 (61)	181 (72)
	117 (11)	221 (50)	208 (60)	190 (54)	250 (71)
		230 (31)	202 (51)	222 (39)	208 (64)

Table 24 Comparison of mass spectral data for derivatised methylamine to Ngim et al. (2000) and Avery & Junk (1985)

Avery 1985	Ngim 2000	This study
208 (100)	208 (100)	209 (100)
209 (85)	209 (88)	208 (97)
181 (29)	117 (26)	181 (36)
117 (18)	161 (19)	161 (14)
161 (15)		117 (11)

The detection of multiple peaks corresponding to putrescine and cadaverine from the pentafluorobenzaldehyde derivatives (Figure 33), was expected to be due to the formation of geometrical (*cis/trans*) isomers during the derivatisation, see Figure 34 and Figure 35. Geometrical isomers, a sub-group of diastereomers may be separated using chromatography due to differences in their physical properties (Wade 2010). Derivatisation of methylamine was assumed to also produce a geometrical isomer but has not been observed which was most likely due to negligible differences in its geometrical configuration leading to minor changes in physical properties, visible in Figure 35. The production of isomers has not been reported by Ngim et al. (2000), Avery & Junk (1985, 1987) or many other researchers using Schiff base (imine) producing derivatisation agents. However, Dai et al. (1999) stated that two geometrical isomers are produced during the formation of imines and that the *trans*-isomer is usually preferred by stereo-chemical configuration. Putrescine and cadaverine, comprised of two amine groups therefore

produced three different isomers while only two isomers would have been formed for methylamine, visible in Figure 34 and Figure 35. This indicated that judging on relative peak heights, Put and Cad in Figure 33 were the *trans-trans*-isomer, Put 2 and Cad 2 are the *cis-cis*-isomer and Put 3 and Cad 3 were the *cis-trans*-isomer, although no further experiments have been conducted to confirm this.

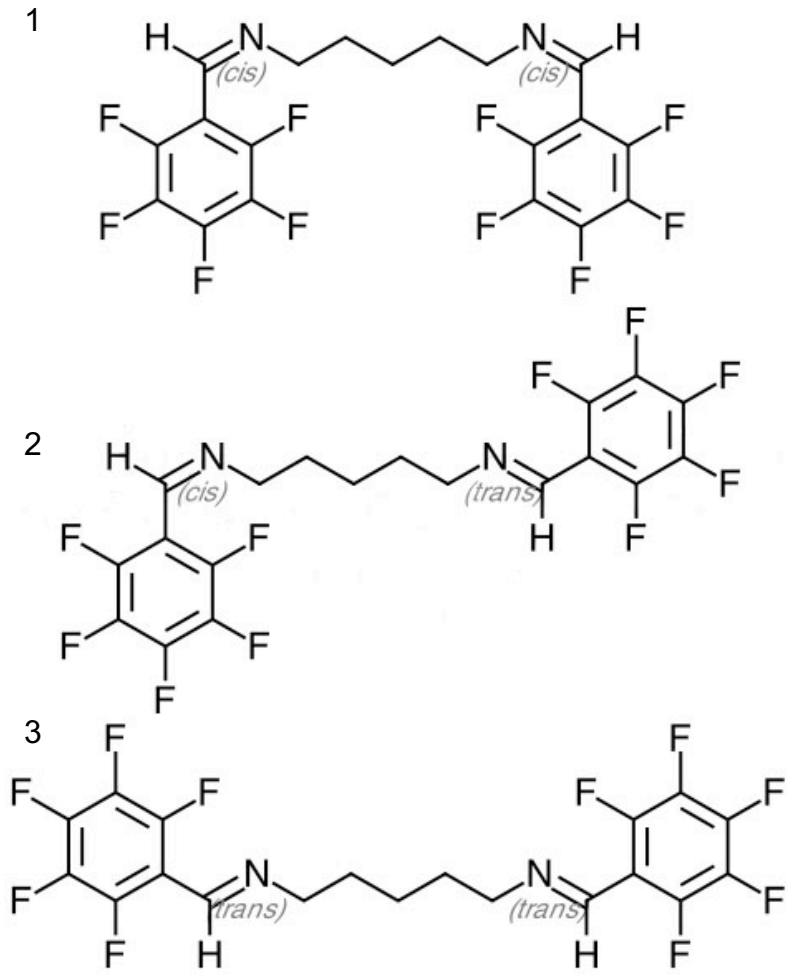

Figure 34 Geometrical isomers of pentafluorobenzaldehyde derivatised cadaverine

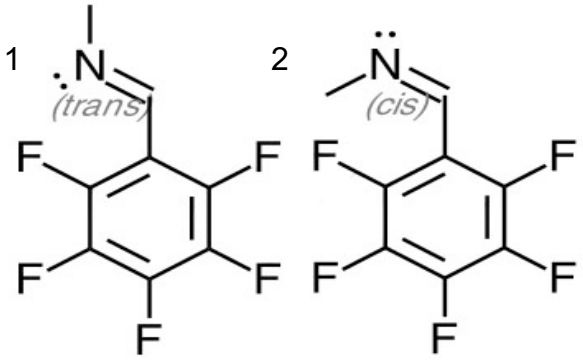

Figure 35 Geometrical isomers of pentafluorobenzaldehyde derivatised methylamine

5.2 Optimisation of Incubation Time

The incubation time was optimised to obtain the most suitable reaction efficiency of the derivatisation for several primary amines analysed using GC-FID by relative

peak area (Figure 36). Additionally, its effect on the reproducibility of the derivatisation is shown in Table 25. Putrescine and cadaverine were not included in this optimisation as the purpose was to optimise the derivatisation reaction to detect and identify other primary amines, whilst the derivatisation reaction for putrescine and cadaverine was already optimised by Blom (2012).

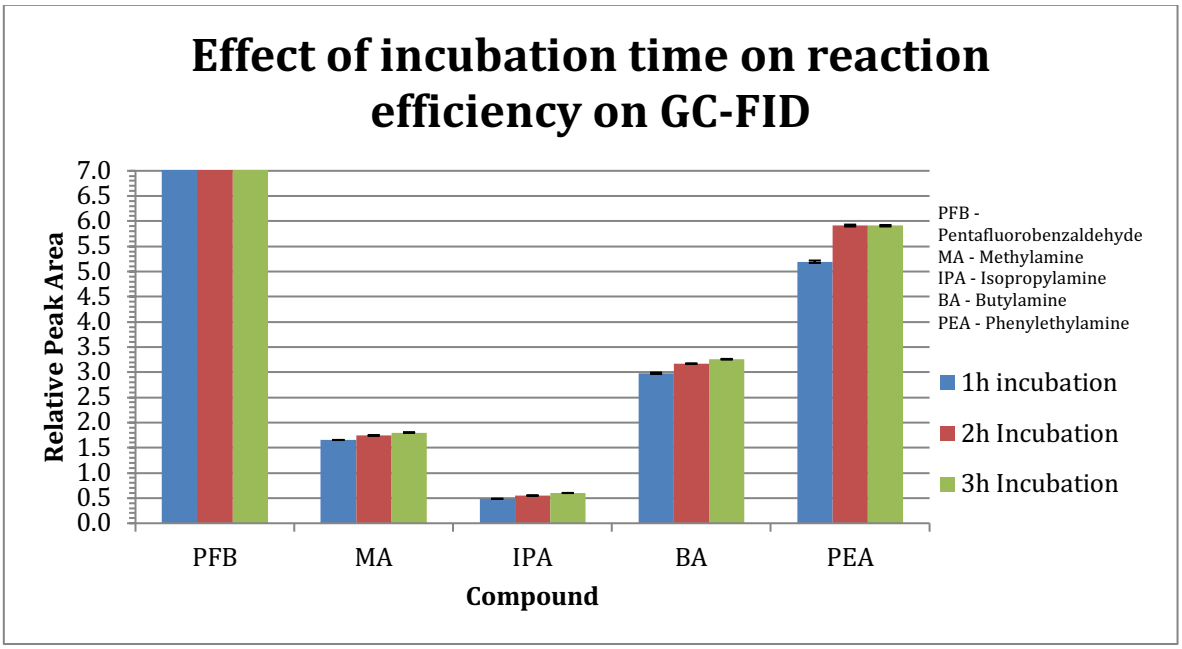

Figure 36 Bar chart for the effect of incubation time on the derivatisation efficiency of several primary amines

Table 25 Comparison of derivatisation reproducibility on primary amines using different incubation times

Compound	1h incubation (% RSD)	2h incubation (% RSD)	3h incubation (% RSD)
Methylamine	0.20	0.26	0.34
Isopropylamine	1.36	0.95	0.62
Butylamine	1.14	0.51	0.50
Phenylethylamine	1.04	0.71	0.57

Even though a larger quantity of amines were derivatised using longer incubation times, an incubation time of one hour was determined to be most suitable to derivatise the leachate samples. This was concluded as longer incubation times increased the overall sample preparation time required and was not necessary as the one hour incubation time proved to be reproducible RSD <2%. In addition, the increase in recovery between one hour and three hours was below 10% for methylamine and butylamine, below 15% for penylethylamine and 24% for isopropylamine, although the latter is most likely not expected in the decomposition process. Ngim et al. (2000) reported similar findings and also stated that shorter

incubation times *e.g.* 30 minutes would hinder the derivatisation efficiency of putrescine and cadaverine, Hoshika (1977) and Moffat & Horning (1970) also used a one hour incubation time.

The incubation temperature for the derivatisation was concluded to remain at 60°C as Ngim et al. (2000) reported putrescine and cadaverine required at least this temperature to derivatise both amine groups, whilst lower temperatures were more suitable for methylamine. Hoshika (1977) and Moffat & Horning (1970) also derivatised their amines at 60°C for one hour to derivatise short chain aliphatic primary amines, although Hoshika (1977) used a different derivatisation agent but still using the same reaction mechanism as seen with pentafluorobenzaldehyde. Ngim et al. (2000) determined the optimum pH to be 12 but a previous study by Blom (2012) determined the optimum pH to be 11 for putrescine and cadaverine.

5.3 Method Validation

The first aspect of the method validation was to determine the specificity by determining if co-elution occurred between the analyte peaks of interest and any interfering chemicals. Co-elution should be minimised as it produces bias if it is not detected and complicates the quantification procedure. To determine this, peak asymmetry and resolution were calculated using only the first peak of putrescine, cadaverine and methylamine in the Cranfield leachate samples to determine if the amines were resolved (Res > 1.5) from interfering compounds in the sample matrix and thus could be quantitated. Peak asymmetry values were required to determine the resolution as Dolan (2002) stated that peak tailing could greatly affect the resolution. It also acted as a useful indicator to determine if co-elution occurred through affecting the peak shape. Peak asymmetry values between 0.9 and 1.5 are usually deemed acceptable although an asymmetry factor of 2.0 is considered to be acceptable in certain situations depending on the separation and the resolution of the peaks (Dolan 2002; Harris 2010). Peak asymmetry values outside the 0.9 - 2.0 region (visible in bold in Table 26) and resolution values below 1.5 (visible in bold in Table 26) indicate potential concerns regarding co-elution that could affect quantification. In addition, mass spectra of the leachate samples were compared to reference samples to inspect if co-elution occurred, see Figure 37, Figure 38, Figure 39 and Table 26.

Table 26 Peak assymetry and peak resolution values for putrescine, cadaverine and methylamine

Leachate sample date	Putrescine			Cadaverine			Methylamine		
	P_{as}	R_{es}	Co-elution	P_{as}	R_{es}	Co-elution	P_{as}	R_{es}	Co-elution
15-09-11	1.94	NC[1]	No	1.33	NC[1]	No	**3.45**	**1.02**	Yes
19-09-11	2.00	NC[1]	No	**0.56**	NC[1]	No	1.43	**1.24**	Yes
26-09-11	1.30	NC[1]	No	1.20	NC[1]	No	1.34	NC[1]	Yes
29-09-11	NQ[2]	NQ[2]		**4.20**	1.70	Yes	1.38	**1.30**	Yes
05-10-11	2.00	2.22	No	**4.56**	2.70	Yes	**0.73**	**1.37**	Yes
12-10-11	ND[3]	ND[3]		NQ[2]	NQ[2]		**0.65**	**0.86**	Yes
28-10-11	1.29	NC[1]	No	**2.90**	3.15	Yes	**0.79**	NC[1]	Yes
13-12-11	**0.86**	NC[1]	Yes	1.44	**1.23**	Yes	**0.36**	NC[1]	Yes
22-02-12	1.00	NC[1]	Yes	2.00	**1.22**	Yes	1.11	NC[1]	Yes
08-05-12	0.95	NC[1]	Yes	1.00	**1.14**	Yes	1.33	NC[1]	Yes
30-05-13	**3.33**	**0.85**	Yes	**0.74**	**1.23**	Yes	**3.10**	**1.04**	Yes
17-06-13	0.55	**0.50**	Yes	0.91	**1.18**	Yes	**2.42**	1.79	Yes

P_{as} = peak asymmetry, R_{es} = resolution.
[1] Not calculated as the chromatographic peaks were visibly resolved.
[2] Not quantitated as analyte was below the quantification limit.
[3] Not detected as analyte was below the detection limit.

Peak asymmetry was observed for putrescine, cadaverine and methylamine in the leachate samples but most were within the acceptable limits (between 0.9 and 2.0). The asymmetry values that were not within the acceptable limits, in bold, were inspected for co-elution and all samples except cadaverine in sample 19-09-11 did experience co-elution through either inadequate resolution or the presence of additional fragments in their mass spectrum (see paragraph below). The resolution was only calculated in the samples where it was difficult to determine if the chromatographic peaks were adequately resolved. A resolution value below 1.5, in bold, indicated that the peaks were not adequately resolved, as shown in Table 26 the resolution of most peaks was above the threshold. Although, the resolution for putrescine in leachate samples 30-05-13 and 17-06-13 was below 1.5 and the same was observed for cadaverine from leachate sample 13-12-11 onwards. This is most likely due to progression of the decomposition process, which created a wider variety of decomposition chemicals along with higher concentrations in comparison to earlier samples. The resolution for methylamine was below 1.5 for the majority of samples and Table 26 also indicates that methylamine co-eluted with another chemical in all leachate samples (see paragraph below). Co-elution was observed

for putrescine from sample 13-12-11 onwards, whilst cadaverine co-eluted in all samples except the first three.

Due to potential co-elution the mass spectra for the putrescine, cadaverine and methylamine signals in the Cranfield leachate samples were compared to the positive control taken along with its derivatisation and a positive control using a different temperature program (program B), and it was observed that co-elution occurred in the leachate samples (see Figure 37, Figure 38 and Figure 39). When observing Figure 37 no obvious differences were observed between the mass spectra of methylamine in the leachate sample and its positive control (Figure 37A and Figure 37B), however when the mass spectra of the two positive control samples were compared (see Figure 37B and Figure 37C) differences were observed. Several m/z fragments such as 32, 43, 57, 71 and 85 were present in Figure 37B but not in Figure 37C, indicating that another compound was co-eluting with methylamine in the leachate and positive control samples. This happened to be the case for all the leachate samples, hence the co-elution observed in Table 26. After further inspection the co-eluting compound turned out to be decane which originated from the internal standard and thus was also present in the control samples. When comparing the three different mass spectra for putrescine (Figure 38) and cadaverine (Figure 39) no differences were observed but differences were observed in the mass spectra of leachate samples 13-12-11 onwards for putrescine and 29-09-11 onwards for cadaverine.

The previous sections highlighted an issue regarding quantification as in certain samples the chromatographic peaks were not resolved and co-eluted with other compounds. These issues could be solved via either a change in the GC temperature program (as seen in Figure 37, Figure 38 and Figure 39) or the creation of a more specific method so no additional chemicals were detected. However, the derivatisation procedure is very specific as only primary amines were derivatised. The change in temperature program worked well to separate the methylamine and decane peak but also increased the sample analysis time by another 15 minutes (total run time per sample 50 minutes, excluding cool down time). This was not ideal due to the quantity of samples needed to be analysed, so the desired solution was to make the analysis process more specific using the GC-MS. It was decided to quantify the amines using three specific fragments (see Figure 27) of each amine, instead of the total ion chromatogram as highlighted in Table 14.

Avery & Junk (1987) and Ngim et al. (2000) quantified their derivatised amines using a single fragment, m/z 208, which had high sensitivity due to α-cleavage, however the use of three specific m/z values was preferred in this instance as it was determined to be most reproducible. As this method was less prone to interferences it has been applied to the analysis of all leachate samples, calibration data and other samples used for further analysis.

Table 27 m/z fragments used to quantify putrescine, cadaverine and methylamine

Analyte	m/z value
Methylamine	117, 208 and 209
Internal standard	Total Ion Current
Putrescine	181, 208 and 249
Cadaverine	181, 222 and 263

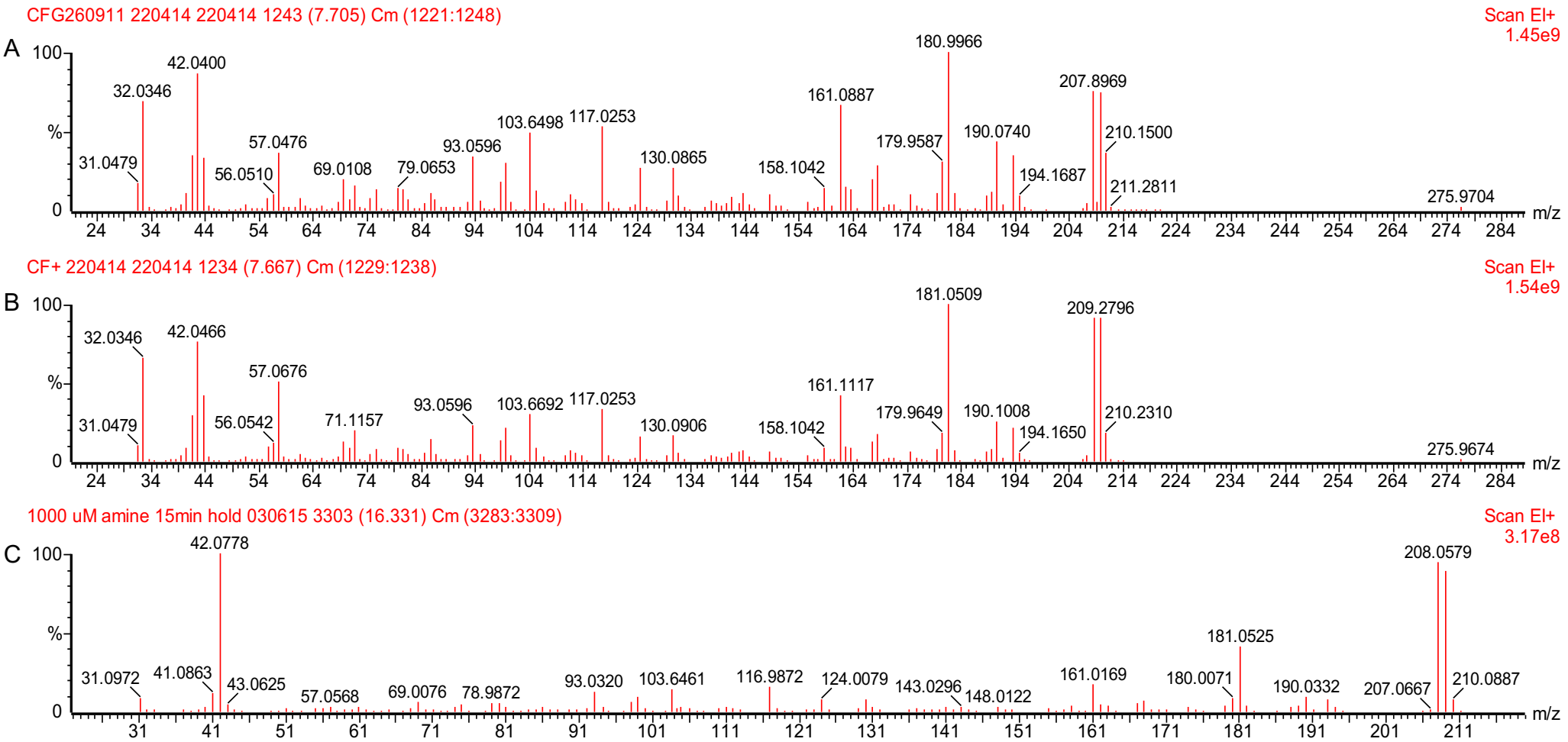

Figure 37 Mass spectra for methylamine from Cranfield leachate sample 26-09-11 (A), a positive control (B) and another positive control using GC temperature program b) (C)

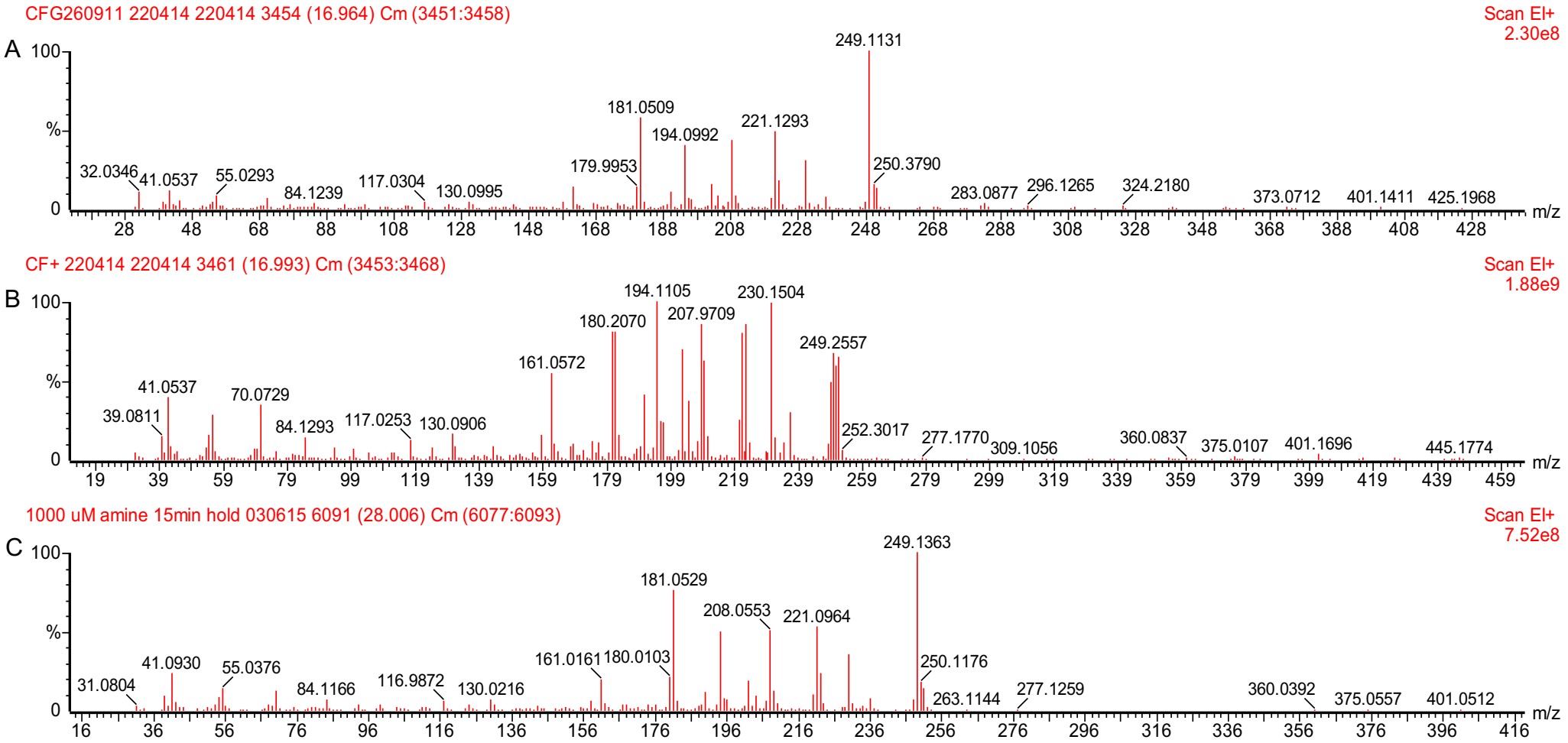

Figure 38 Mass spectra for putrescine from Cranfield leachate sample 26-09-11 (A), a positive control (B) and another positive control using GC temperature program b) (C)

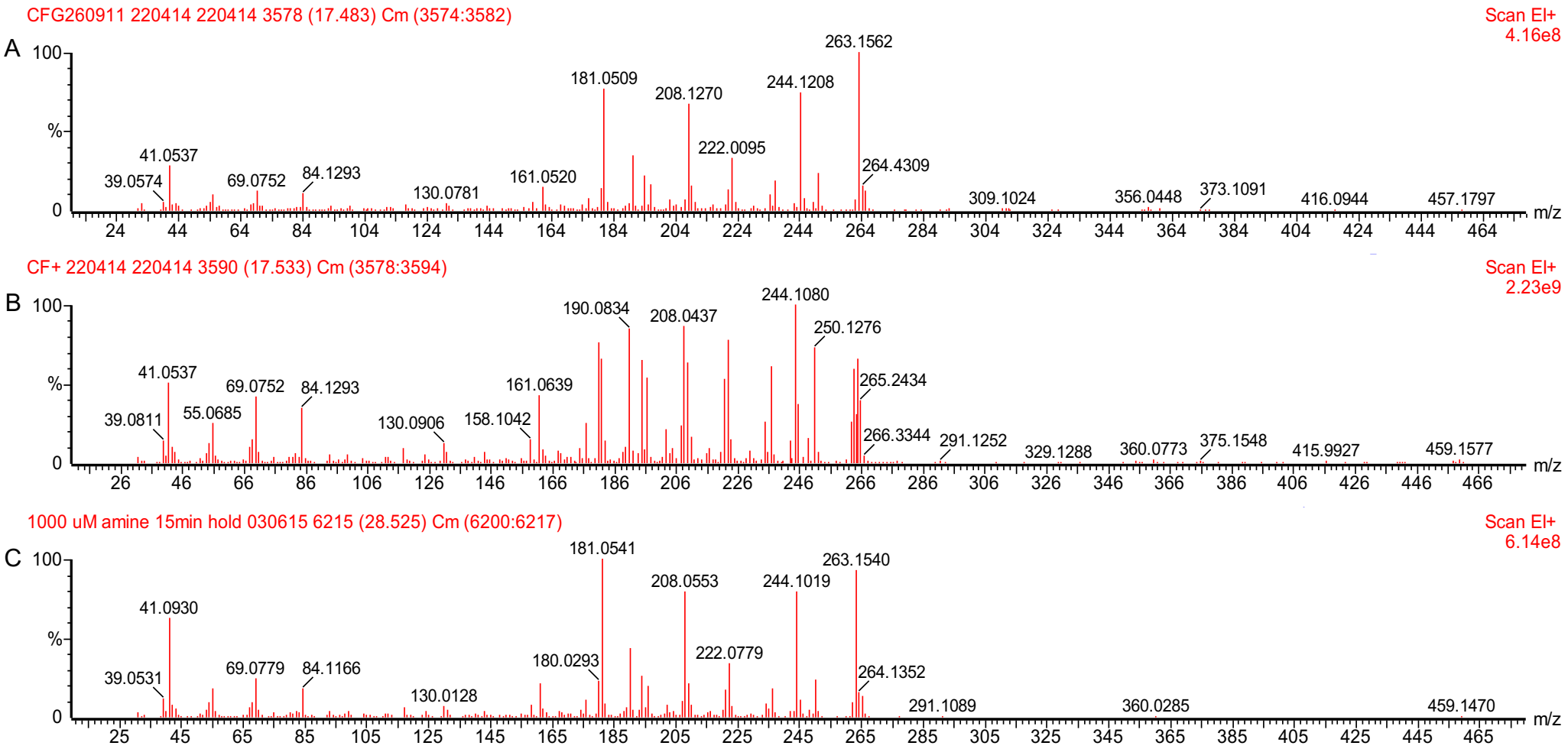

Figure 39 Mass spectra for cadaverine from Cranfield leachate sample 26-09-11 (A), a positive control (B) and another positive control using GC temperature program b) (C)

Calibration graphs (amine concentration vs signal abundance) for putrescine, cadaverine and methylamine within a concentration range of 0.1 µmol L^{-1} to 1000 µmol L^{-1} were plotted using Microsoft Excel, (see Figure 40, Figure 41 and Figure 42). The 95% confidence interval (the red line) of the calibration graph and the 95% prediction interval (the green line) of the data points were calculated and also plotted. The calibration line fits the model well as only one data point (x = 500 µmol L^{-1}) for each amine falls outside the 95% confidence range of the calibration line (also see section on outliers, page 122). However, the data point still fits inside the 95% prediction interval and therefore indicates that this data point could be observed during analysis. The coefficient of determination, R^2, of each calibration graph expresses a good fit between the calibration points and the linear trend line ($R^2 > 0.995$).

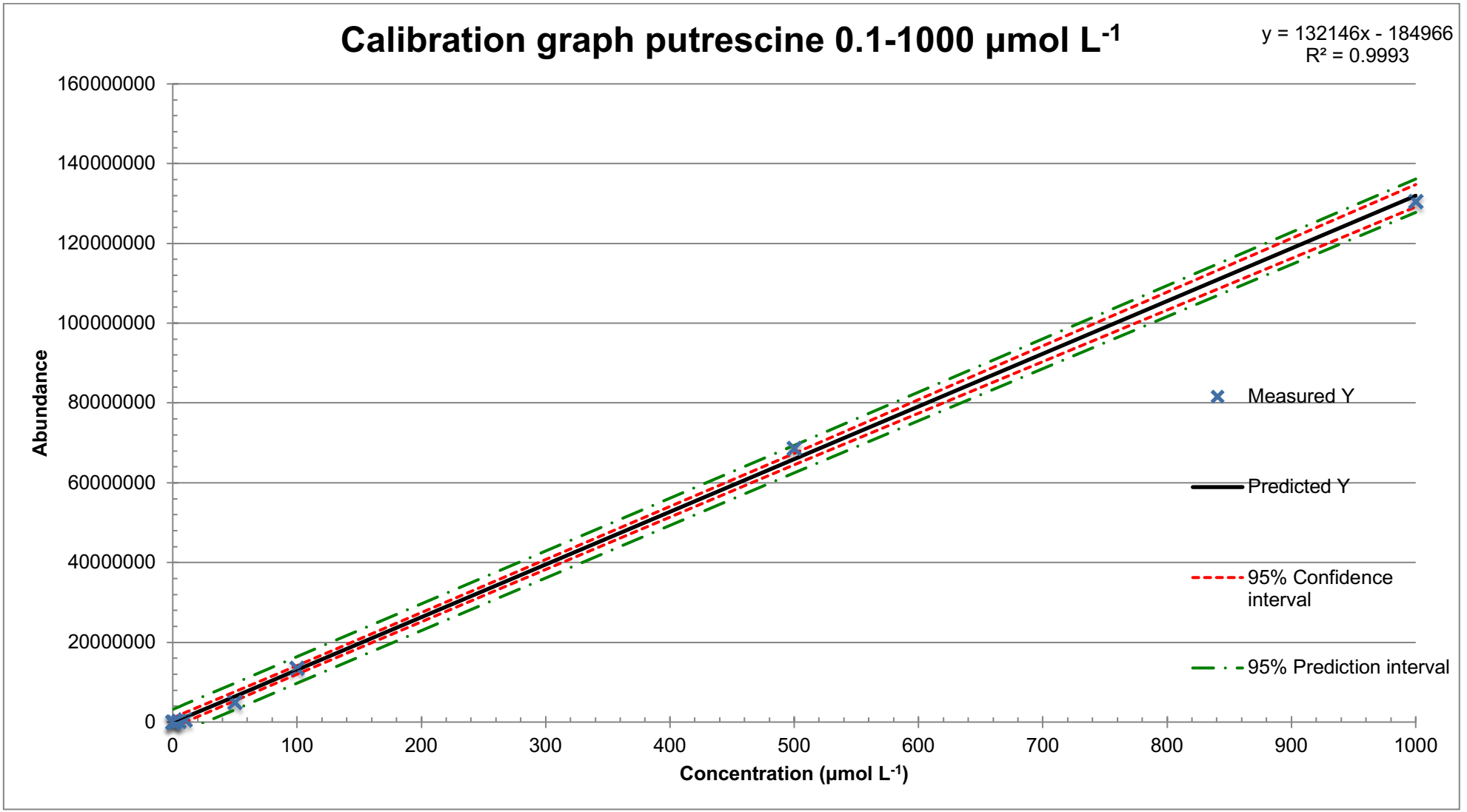

Figure 40 Calibration graph for putrescine using GC-MS analysis

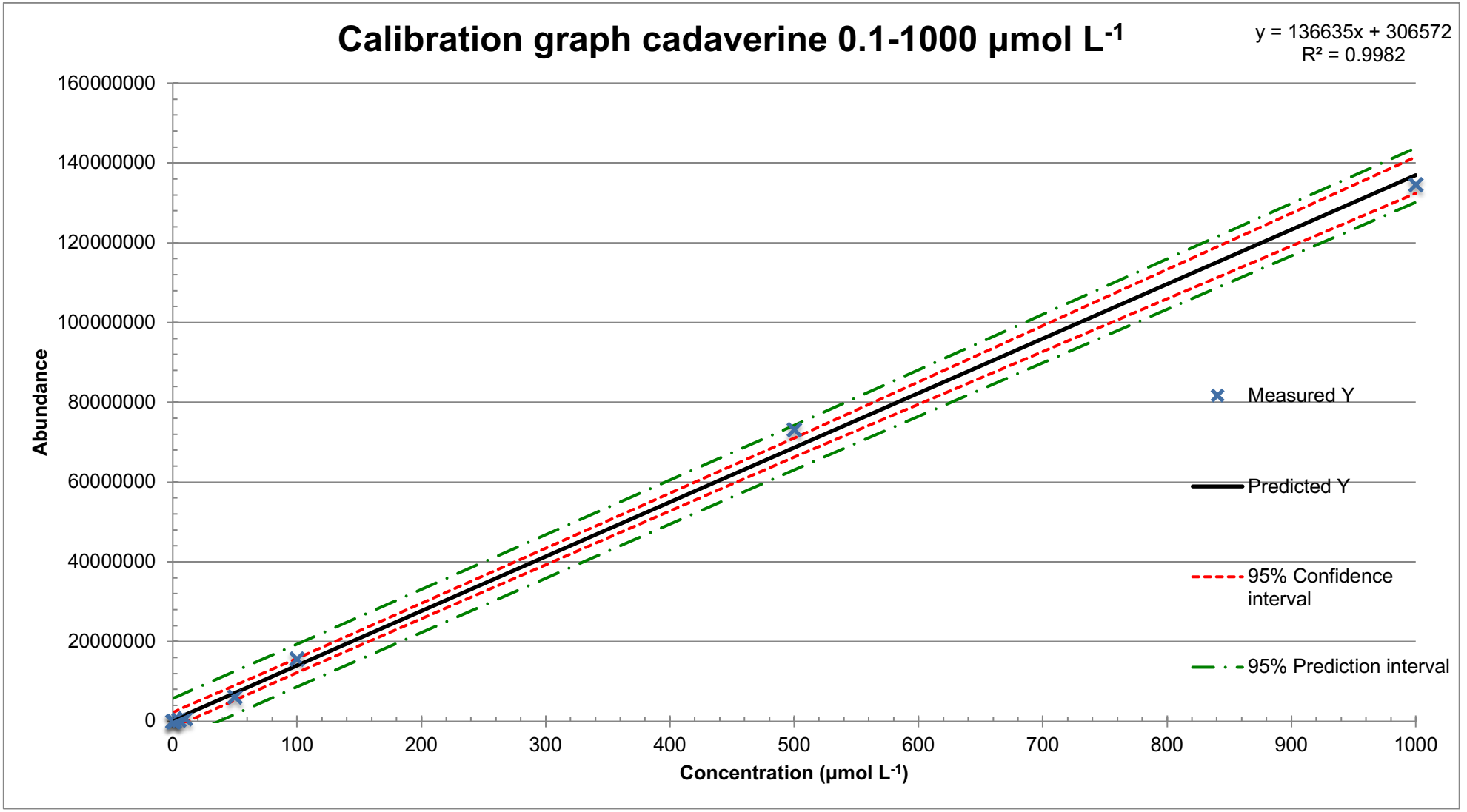

Figure 41 Calibration graph for cadaverine using GC-MS analysis

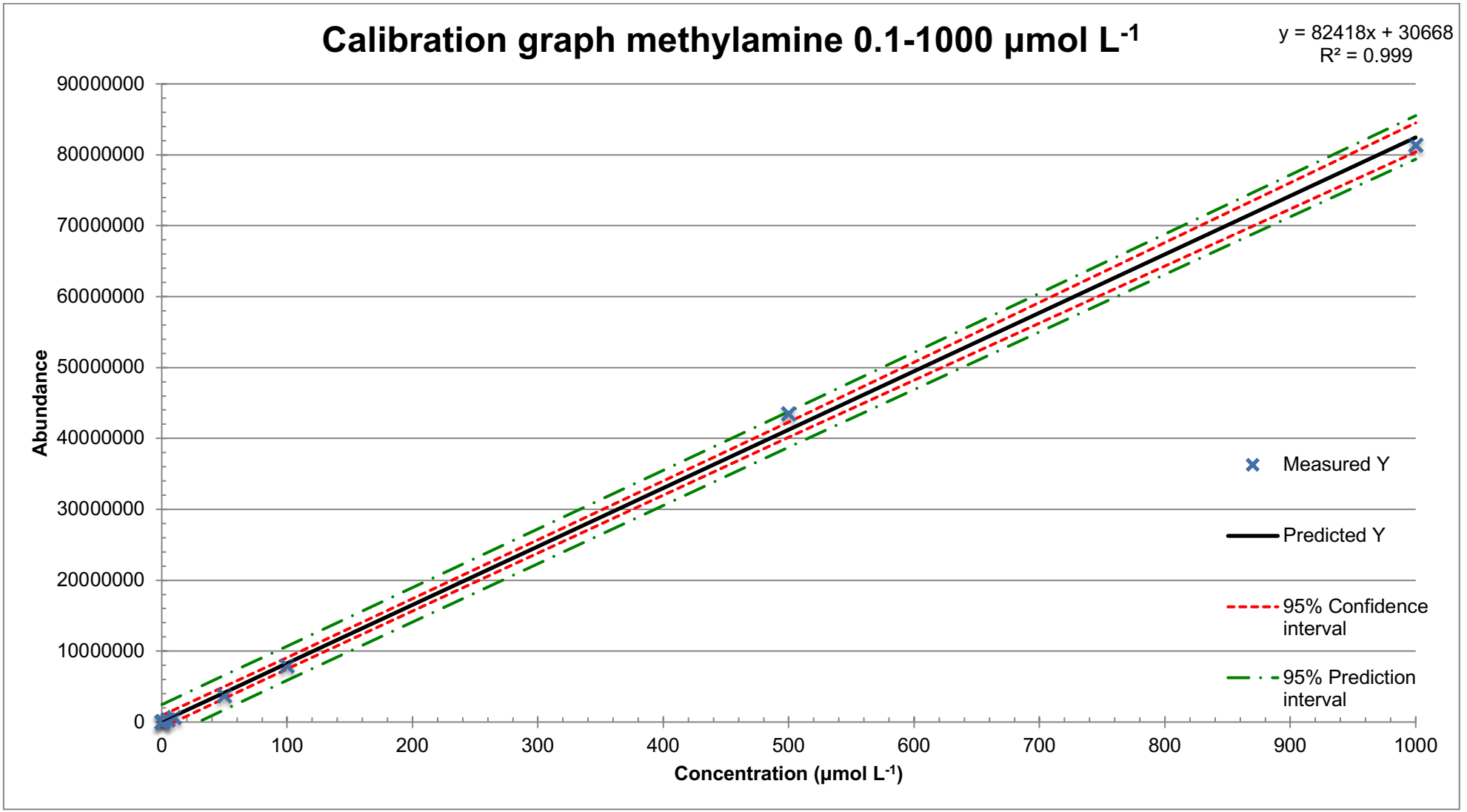

Figure 42 Calibration graph for methylamine using GC-MS analysis

The linearity for the detection of putrescine was determined through calculating the product-moment correlation coefficient, r, and the coefficient of determination, R^2, which should equal the squared r-value if a linear relationship is present, $r^2 = R^2$, (See Equation 1 and Table 41 in Appendix II). The correlation coefficient of derivatised putrescine within a concentration range of 0.1 µmol L^{-1} to 1000 µmol L^{-1} was 0.99964, this was very close to +1 indicating a positive linear correlation was present. As correlation coefficients could be easily misinterpreted (Miller & Miller 2010), the t-value (n-2) was calculated using Equation 2 (t = 259.36), which was compared to tabulated critical t-values to test the null hypothesis. The critical t-value 3.50 at the 99% confidence interval, obtained from Miller & Miller (2010), rejected the null hypothesis and also confirmed the presence of a linear correlation. The linearity values of cadaverine and methylamine were also calculated and are displayed in Table 28 along with the putrescine data. Table 28 highlights, along with the calibration graphs, that a positive linear correlation was present for putrescine, cadaverine and methylamine over 5 decades from 0.1 µmol L^{-1} to 1000 µmol L^{-1} after GC-MS analysis.

Table 28 Highlighting a linear correlation for putrescine, cadaverine and methylamine

Compound	Putrescine	Cadaverine	Methylamine
r-value	0.99964	0.99910	0.99950
$r^2 = R^2$	0.99927	0.99820	0.99899
t-value	259.36	164.93	220.70
t-critical 99% CI (p = 0.01)	3.50	3.50	3.50
Linear correlation present	Yes	Yes	Yes

As a linear relationship was observed for the derivatised amines, the best-fit regression line and the 95% confidence interval of the slope and intercept were calculated, Equation 3. The slope, intercept and their confidence limits for the derivatised amines are visible in Table 29. The least squares method, used to calculate the best-fit regression line, assumes that all errors are associated to the Y-axis and thus minimises the deviation in the Y-direction between the experimental data points and the calculated trend line (Miller & Miller 2010). Excel utilises the least square method to calculate the trend line, as the data in Table 29 is identical to the calibration graphs above, confirming this methodology is commonly used to create a best-fit straight line calibration graph.

Table 29 Slope and intercept information including their 95% confidence intervals and uncertainty for putrescine, cadaverine and methylamine

Analyte	Putrescine	Cadaverine	Methylamine
Slope least squares	132146	136635	82418
Intercept least squares	-184966	306572	30668
95% CI (p = 0.05) slope	± 3183	± 5178	± 2333
95% CI (p = 0.05) intercept	± 1192050	± 1939269	± 873792
Average uncertainty of unknown sample (%)	313	2059	3972

Despite the high R^2 values Table 29 indicates that the average uncertainty of determining the concentration of unknown samples over the entire calibration range was 313% for putrescine and much higher for cadaverine and methylamine. As the uncertainty was especially large at the lower range of the calibration series (Table 30), new calibration graphs were created for putrescine, cadaverine and methylamine between a concentration range of 0.1 µmol L^{-1} to 10 µmol L^{-1} using the same dataset. As visible in Table 30 the average uncertainty for putrescine decreased from 313% to 29% for the low-range calibration graph, the average uncertainty values of cadaverine and methylamine reduced to 28% and 22%. Even though the uncertainty was reduced using this approach a relatively high difference was observed between the lowest prepared concentration and its calculated concentration, 0.49 – 0.10 = 0.39 µmol L^{-1}, as the calculated concentration was almost five times larger than the prepared concentration. The large relative difference between the measured and calculated concentration for the lowest three samples (0.1 µmol L^{-1}, 0.5 µmol L^{-1} and 1.0 µmol L^{-1}) could be explained by the limit of quantification (see section below).

Table 30 Highlighting the average uncertainty for putresicne between the two calibration graphs using the different concentration ranges form the same data

0.1 – 1000 µmol L^{-1} calibration putrescine			**0.1 – 10 µmol L^{-1} calibration putrescine**		
Prepared concentration (µM)	Calculated concentration (µM)	Uncertainty (%)	Prepared concentration (µM)	Calculated concentration (µM)	Uncertainty (%)
0.1	1.4020	761.24	0.1	0.4918	51.25
0.5	1.4206	751.29	0.5	0.5363	46.93
1.0	1.4615	730.26	1.0	0.6343	39.56
5.0	3.2052	332.89	5.0	4.8133	5.07
10.0	5.4211	196.74	10.0	10.1242	2.96
50.0	39.4572	26.89			
100.0	103.5176	10.18			
500.0	520.9713	2.12			
1000.0	989.7434	1.35			
Mean		**312.55%**	**Mean**		**29.15%**

The decrease in uncertainty between the two calibration series in Table 30 could be explained through the residuals (measured y-value – predicted y-value), which are used to estimate unobservable model error (Yan & Su 2009). A residual is an observable estimate of an unobservable statistical (model) error, so larger residuals indicate a larger error. Thus the decrease in the uncertainty of putrescine could be explained due to the decrease in the sum of squared residuals as the squared residuals are lower in the first five samples than the other four (Table 41, Column 8).

A possible reason for the larger residuals in the remaining four calibration standards was due to an influential data point in the calibration series that had disproportionate effects on the position of the regression line as a result of bias or leverage (Barwick 2003). Bias occurs when an outlier is present in the middle of the calibration series, shifting the regression line. Leverage could happen when an outlier is present at the extremes of the calibration range, tilting the calibration line, but could also occur when a calibration point is a distance away from others along the x-axis, even if it is not an outlier (Barwick 2003). Through visual inspection no outliers (see section below) were observed indicating that the influence occurred through leverage due to unequal spacing of the calibration points along the x-axis (see section below). The leverage was calculated for each calibration standard of putrescine for the 9-point calibration series to the 5-point calibration series, see Table 31. The calibration standard with the highest leverage, in bold, continues to

be in the largest calibration standard indicating unequal spacing of the calibration points along the x-axis (due to the calibration graph ranging over five decades). Table 31 highlights that calibration standard 1000 µmol L^{-1} in the 9-point calibration series influences 70% (0.6959) of the trend line and the influence remains above 60% using the other series. According to Barwick (2003) a relatively small error in the measured response has a significant effect on the position of the regression line as leverage affects both the gradient and intercept.

Table 31 Calculated leverage of each calibration standard for putrescine

Measured concentration	Leverage (influence)				
0.1	3.59%	3.35%	6.49%	6.41%	14.57%
0.5	3.58%	3.31%	6.27%	5.95%	11.18%
1.0	3.56%	3.27%	6.00%	5.41%	7.57%
5.0	3.40%	2.96%	4.08%	1.97%	3.97%
10.0	3.22%	2.60%	2.20%	0.06%	**62.72%**
50.0	1.92%	0.54%	7.93%	**80.19%**	
100.0	0.76%	0.13%	**67.04%**		
500.0	10.39%	**83.84%**			
1000.0	**69.59%**				
Sum:	**100%**	**100%**	**100%**	**100%**	**100%**

One of the best known methods to detect outliers is through the use of Cook's squared distance, also called Cook's distance (Miller & Miller 2010). Table 32 shows the Cook's distance values of each calibration point from the 9-point calibration series to the 5-point calibration series. According to Miller & Miller (2010) values with a calculated Cook's distance above 1.0 justifies the omission of a suspected data point, which are highlighted in bold in Table 32. These highlighted values were also highlighted in Table 31 as experiencing the most leverage because the leverage is taken into account when calculating Cook's distance (Cohen et al. 2003; Mickey et al. 2004; Miles & Shevlin 2001). Thus the calibration points experiencing high leverage were considered outliers through the use of Cook's distance and a different approach was adapted to determine the presence of outliers through the use of standardised residual plots (Miller & Miller 2010). Standardised residuals are commonly used to detect outliers through determining the goodness of fit of each calibration point and should show random scatter around the zero line (Taylor 2015; Yan & Su 2009). Residual z-scores were calculated through dividing the y-residuals by $s_{y/x}$, which were plotted in standardised residual plots (Figure 43), residuals larger than three times that of the standard deviation ($s_{y/x}$) may be considered an outlier (Yan & Su 2009).

Table 32 Cook's distcance value of each calibration standard for putrescine

Measured concentration	Cook's distance (outlier test)				
0.1	.00172	.06446	.05205	.11916	.54896
0.5	.00086	.05147	.03862	.06643	.00385
1.0	.00022	.03778	.02529	.02470	.31098
5.0	.00322	.00099	.00022	.03670	.06396
10.0	.02060	.02680	.03987	.30353	**1.89030**
50.0	.09633	.36599	.56596	**61.27320**	
100.0	.00952	.00722	**10.10971**		
500.0	.77224	**8.54536**			
1000.0	**11.47699**				

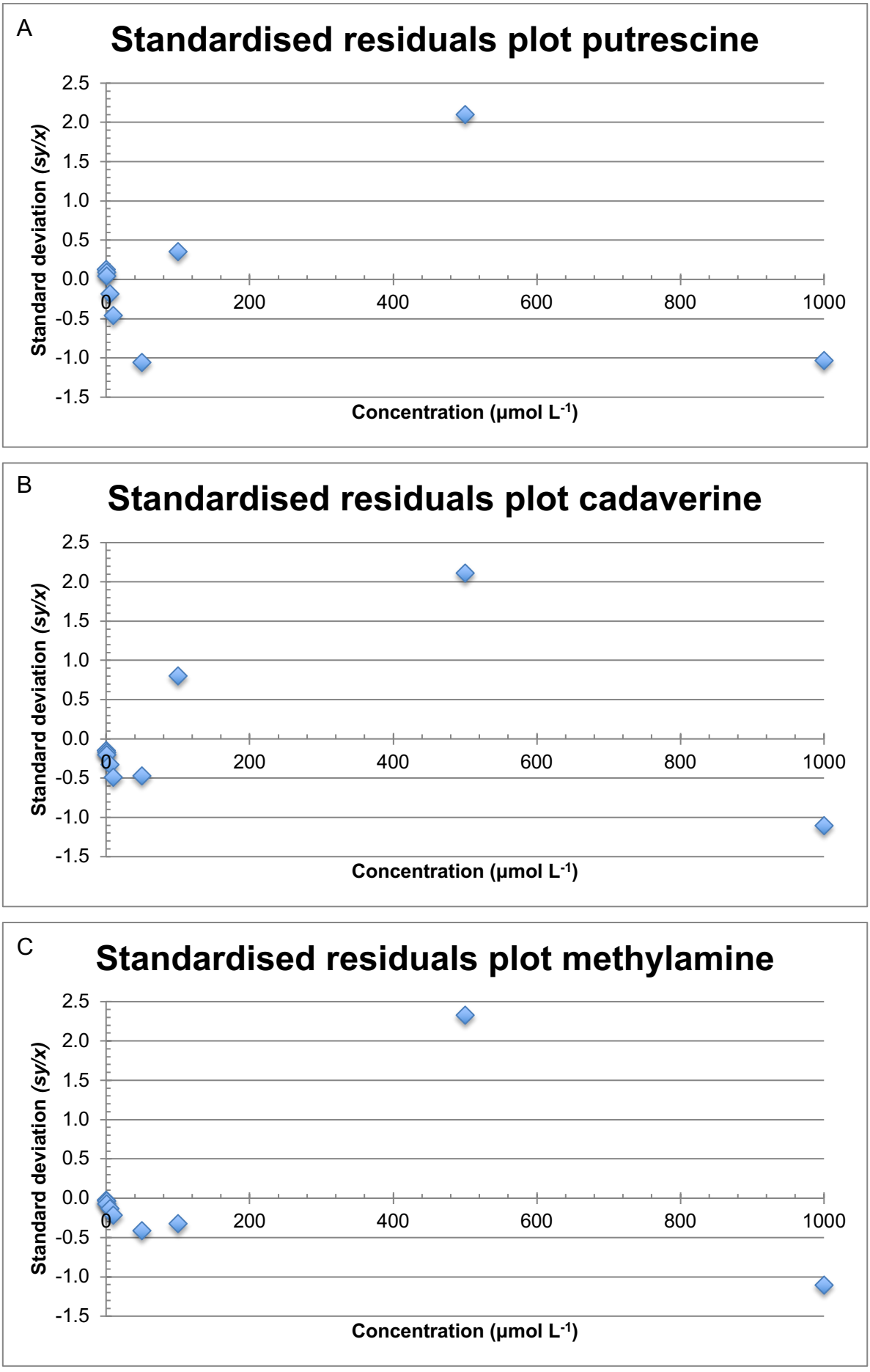

Figure 43 Standardised residual plots for putrescine (A), cadaverine (B) and methylamine (C)

The standardised residual plot of putrescine, Figure 43A, was satisfactory as it showed random scatter around zero and no obvious pattern was observed. The residual at 500 µmol L^{-1} could be an outlier although it was still below three standard deviations. The residuals for cadaverine (Figure 43B) were also satisfactory, it again highlights random scatter around zero, no pattern was observed and the residual 500 µmol L^{-1} could also be a potential outlier but was still below three standard deviations. Methylamine's residuals (Figure 43C) on the other hand were not satisfactory as all of the residuals except the residual at 500 µmol L^{-1} were negative, indicating that the regression line was biased. Taylor (2015) stated that a residual plot containing more than three points in the positive or negative direction is worth investigating for the possibility of bias at those concentrations, especially when they occur at the extremes of the calibration range.

The omission of calibration standard 1000 µmol L^{-1} was investigated, as a negative residual was presented, indicating that this calibration standard was below the fitted trend line. In addition, very high leverage was observed for this particular data point, which as discussed earlier could have a significant effect on the regression line (Barwick 2003). When calibration standard 1000 µmol L^{-1} was removed, the calibration graph changed as expected, the slope increased, the intercept decreased and the regression (coefficient of determination) improved (Figure 44). The new calibration graph contained a predicted trend line up to 1000 µmol L^{-1}. As visible in Figure 44 the instrumental observed value of 1000 µmol L^{-1} fell far outside the predicted trend line suggesting that this value introduced bias when used in the calibration series and was thus excluded from further calculations (this also applied to putrescine and cadaverine). A possible explanation for the decreased response of calibration standard 1000 µmol L^{-1} is that at this particular concentration the mass spectrometer was getting overloaded and resulted in slight plateauing of the calibration curve. Due to the potential overloading and decrease of linearity the upper limit of quantification was set at 500 µmol L^{-1}, the next largest calibration standard included. No further analysis was performed to confirm the possibility of detector overloading and decrease in linearity as most of the samples analysed exhibited amine concentrations below 500 µmol L^{-1}.

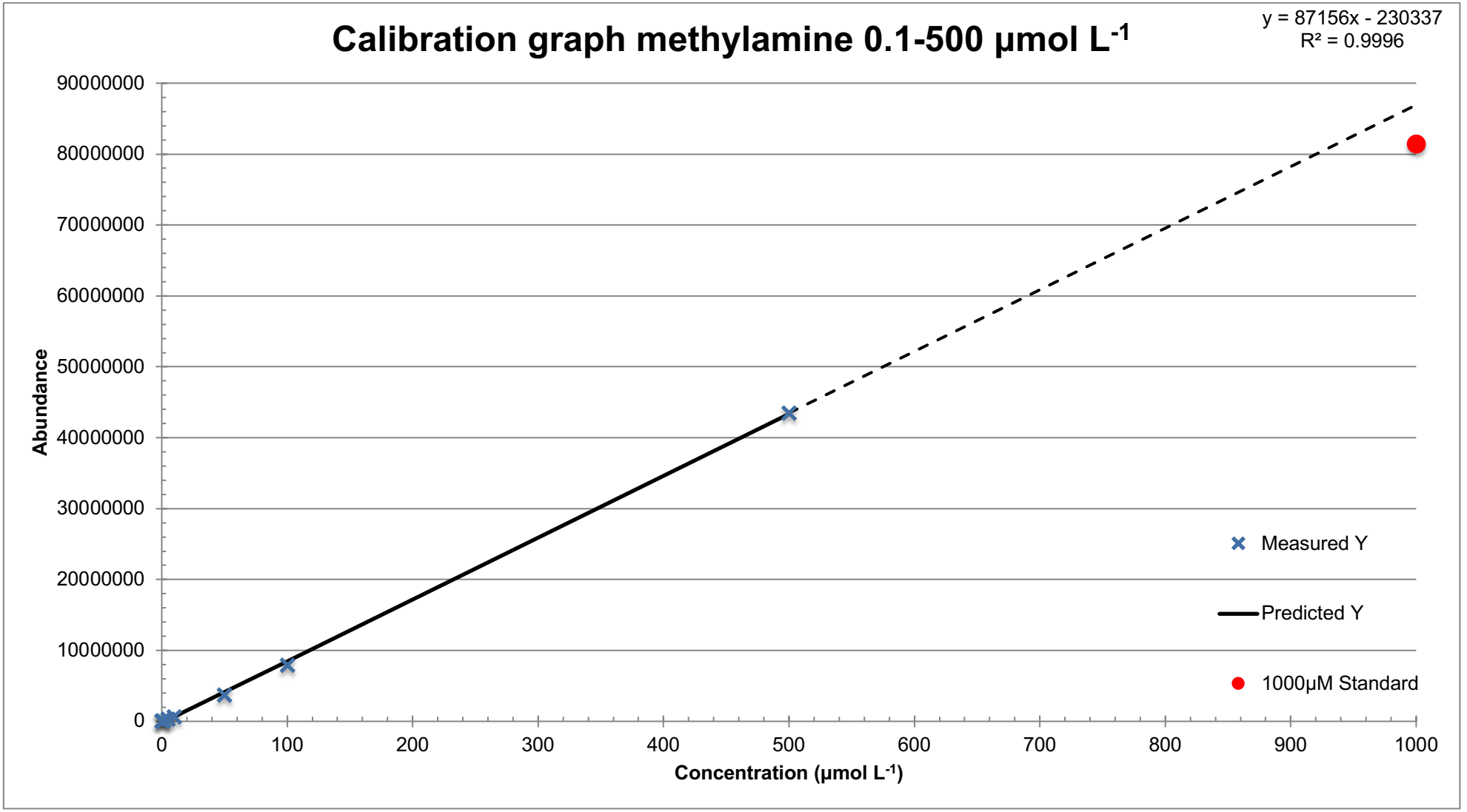

Figure 44 Calibration graph of methylamine without datapoint 1000 µmol L^{-1}

Table 33 displays the limit of detection and limit of quantification for putrescine calculated using two separate methods. The first method (left side of Table) is commonly used in analytical chemistry and is calculated by dividing the random error in the y-direction ($s_{y/x}$) by the slope of the trend line (Miller & Miller 2010), whilst Method 2 (adopted from Rosier et al. (2014) utilised the standard deviation of the intercept instead of the $s_{y/x}$, resulting in lower values. The limit of quantification of Method 2 is around 1.0 µmol L^{-1}, this value is assumed to be less accurate due to the significant uncertainty associated with the 1.0 µmol L^{-1} sample. The limit of quantification of Method 1 is just above 2.0 µmol L^{-1} and is thus assumed to more accurately reflect the limit of quantification for putrescine. However when the signal-to-noise ratio for putrescine was determined at concentrations of 0.1, 0.5 and 1.0 µmol L^{-1} using Turbomass chromatography software (see Table 34), the limit of quantification (ten times signal to noise ratio) for putrescine indicated to be between 0.5 µmol L^{-1} and 1.0 µmol L^{-1}. It was thus decided that the detection and quantification limits for putrescine cadaverine and methylamine should be calculated using Method 2. The detection limit of putrescine, cadaverine and methylamine were calculated to be 0.29 µmol L^{-1}, 0.27 µmol L^{-1} and 0.18 µmol L^{-1}, whilst the quantification limits were determined to be 0.98 µmol L^{-1}, 0.90 µmol L^{-1} and 0.61 µmol L^{-1}, see Table 35.

Table 33 Detection and quantification limits calculated using standard extrapolation method and method adopted from Rosier et al. (2014)

Determination method	$\frac{s_{y/x}}{b}$ (Method 1)		$\frac{s_a}{b}$ (Method 2)	
	Minimum	Maximum	Minimum	Maximum
Detection limit (µmol L^{-1})	0.643	0.677	0.285	0.301
Quantification limit (µmol L^{-1})	2.143	2.257	0.951	1.002

Table 34 Signal-to-noise ratio calculated from chromatogram using Turbomass software

Concentration	Putrescine	Cadaverine	Methylamine
1.0 µmol L^{-1}	22.79	17.16	18.64
0.5 µmol L^{-1}	7.60	3.97	12.24
0.1 µmol L^{-1}	ND[1]	ND[1]	10.34

[1] Not detected during analysis

The detection and quantification limits discussed in the previous paragraph were determined using extrapolation, which are not as reliable as analysis made near the expected detection limit. It has been recommended by the "National Association of Testing Authorities, Australia" that samples with a concentration near the estimated limit of detection should be analysed to confirm they could be detected appropriately. As the calculated detection limits of the amines were between the two lowest calibration standards, they were compared to their peaks signal-to-noise ratios. The signal-to-noise ratios for putrescine and cadaverine (Table 34) indicated that their detection limit is indeed above 0.1 µmol L^{-1} and their quantification limit above 0.5 µmol L^{-1}, however the detection and quantification limits of methylamine should be lower than calculated. It has been decided to set the lower limit of quantification for all amines at 1.0 µmol L^{-1} of which the signal-to-noise ratio for each amine is above 10:1, see Table 34.

Table 35 Detection and quantification limites for putrescine, cadaverine and methylamine

Analyte	Putrescine	Cadaverine	Methylamine
Detection limit (µmol L^{-1})	0.29	0.27	0.18
Detection limit (ppb)	25.56	27.59	5.59
Quantification limit (µmol L^{-1})	0.98	0.90	0.61
Quantification limit (ppb)	86.39	91.96	18.95

After establishing the quantification limits for putrescine, cadaverine and methylamine new calibration calculations were performed on the remaining calibration standards and are displayed in Table 36. It is observed that the R^2 values of the calibration series are all above or at 0.995 thus providing accurate results.

Table 36 Slope and intercept information including their 95% confidence intervals for putrescine, cadaverine and methylamine

Analyte (concentration range)	Slope	95% CI[1]	Intercept	95% CI[1]	R^2 value
Putrescine (1-10 µmol L^{-1})	55138	± 3696	-26812	± 12712	0.995
Putrescine (10-500 µmol L^{-1})	138704	± 2875	-735932	± 491138	0.999
Cadaverine (1-10 µmol L^{-1})	65627	± 40623	-30614	± 13974	0.996
Cadaverine (10-500 µmol L^{-1})	147407	± 3443	-378355	± 588117	0.999
Methylamine (1-10 µmol L^{-1})	64746	± 2695	-4089	± 9268	0.998
Methylamine (10-500 µmol L^{-1})	87425	± 1353	-336525	± 231114	1.000

[1] Confidence Interval.

Good accuracy was observed as the coefficient of determination of the amines were above 0.995 indicating that the accuracy of the current method was above 99.5%. Table 37 illustrates that the calculated concentration ± the uncertainty does correspond to the actual measured concentration. The average error of the calculated concentrations was around 10% for putrescine and cadaverine and lower for methylamine at 6.89% further suggesting good accuracy of the current methodology. The methodology was also determined to be reproducible as duplicate derivatised positive control samples containing 1000 µmol L^{-1} concentrations of putrescine, cadaverine and methylamine had relative standard deviation values of 3.38%, 3.05% and 3.62% respectively. Although the method will be less reproducible towards the lower limit of quantification (LLOQ) a relative standard deviation < 20% is allowed at the LLOQ (Anderson et al. 2015). The relative standard deviation for methylamine using GC-FID (0.20%) and GC-MS (3.38%) could be due to differences in instrumentation as discussed by Cicchetti et al. (2008).

Table 37 Highlighting the average error in quantification for putrescine, cadaverine and methylamine

Analyte	Putrescine		Cadaverine		Methylamine	
Prepared concentration (μmol L^{-1})	Calculated concentration (μmol L^{-1})	Error (%)	Calculated concentration (μmol L^{-1})	Error (%)	Calculated concentration (μmol L^{-1})	Error (%)
1.0	0.63 ± 0.65	36.57	0.63 ± 0.60	36.78	0.73 ± 0.40	27.25
5.0	4.81 ± 0.63	3.73	4.91 ± 0.58	1.87	4.92 ± 0.39	1.67
10	10.12 ± 0.77	1.24	10.08 ± 0.71	0.79	10.06 ± 0.48	0.62
50	41.56 ± 9.97	16.87	44.13 ± 11.22	11.74	46.78 ± 7.43	6.44
100	102.60 ± 9.86	2.60	108.99 ± 11.10	8.99	94.89 ± 7.36	5.11
500	500.31 ± 12.93	0.06	498.85 ± 14.55	0.23	501.29 ± 9.67	0.26
Mean		10.18**%**		10.07**%**		6.89**%**

5.4 Analysis of Cranfield Leachate

The data for the samples received in April 2012 and analysed in March 2013 highlighted that methylamine was present in a significant abundance in comparison to putrescine and cadaverine as seen in Figure 45. Putrescine, cadaverine and methylamine were detected from one-month post burial to at least six months' post burial. These amines could be detected over a longer period of time (at least 669 days post burial) as visible in Figure 47. In addition, putrescine and cadaverine were detected up to at least 902 days' post burial using GC-FID in the Keele leachate samples (Blom 2012). Based on the high relative abundance of methylamine in comparison to putrescine and cadaverine, in Figure 45, methylamine has the potential to be an important biomarker for the detection of clandestine graves using chemical based techniques and has not previously been reported as a decomposition product within the field of Taphonomy.

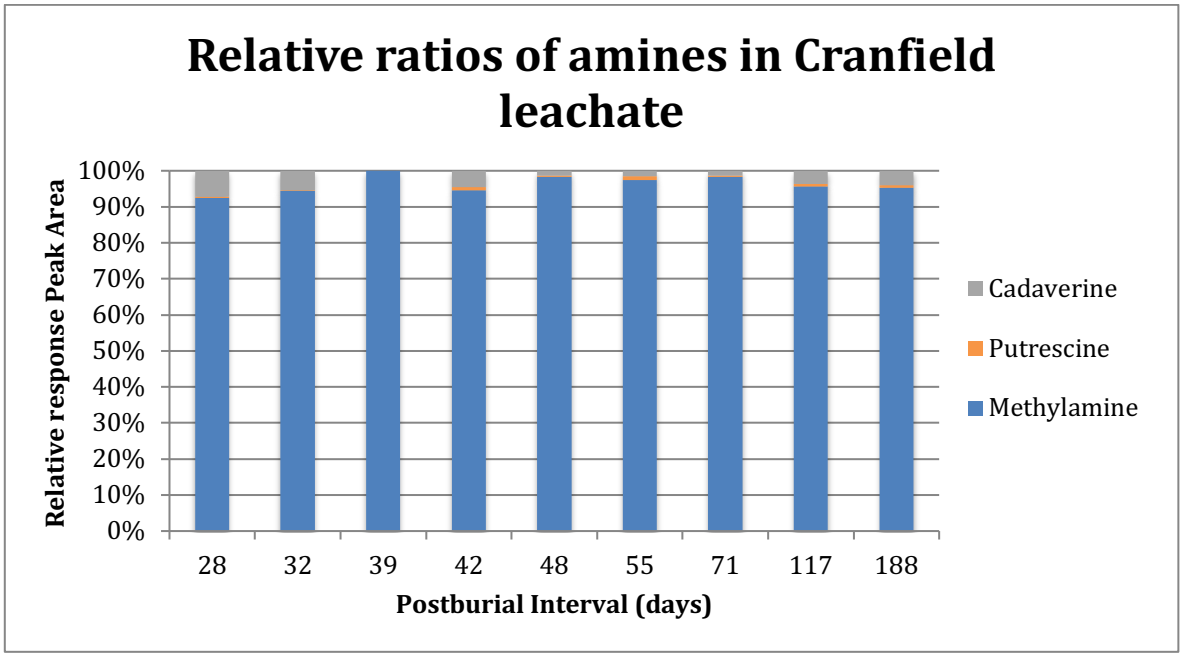

Figure 45 Bar chart for the relative ratios of amines in Cranfield leachate samples over time since burial

Duplicate leachate samples (according to the labels) were received in March 2014 and analysed in April 2014, which produced different results as methylamine was less abundant in the duplicate samples (Figure 46). A potential reason for these differences could have been the conditions under which the samples were stored. According to S˙liwka-Kaszyńska et al. (2003) storage conditions could affect the chemical composition of the leachate samples. In addition, Forbes et al. (2014) demonstrated that freezing blood samples negatively affected its chemical composition, which could explain the significant variation in the presence of putrescine, cadaverine and methylamine between the duplicate leachate samples as earlier data demonstrated good reproducibility for the derivatisation and GC-MS analysis (see Section 5.7 Storage Experiments).

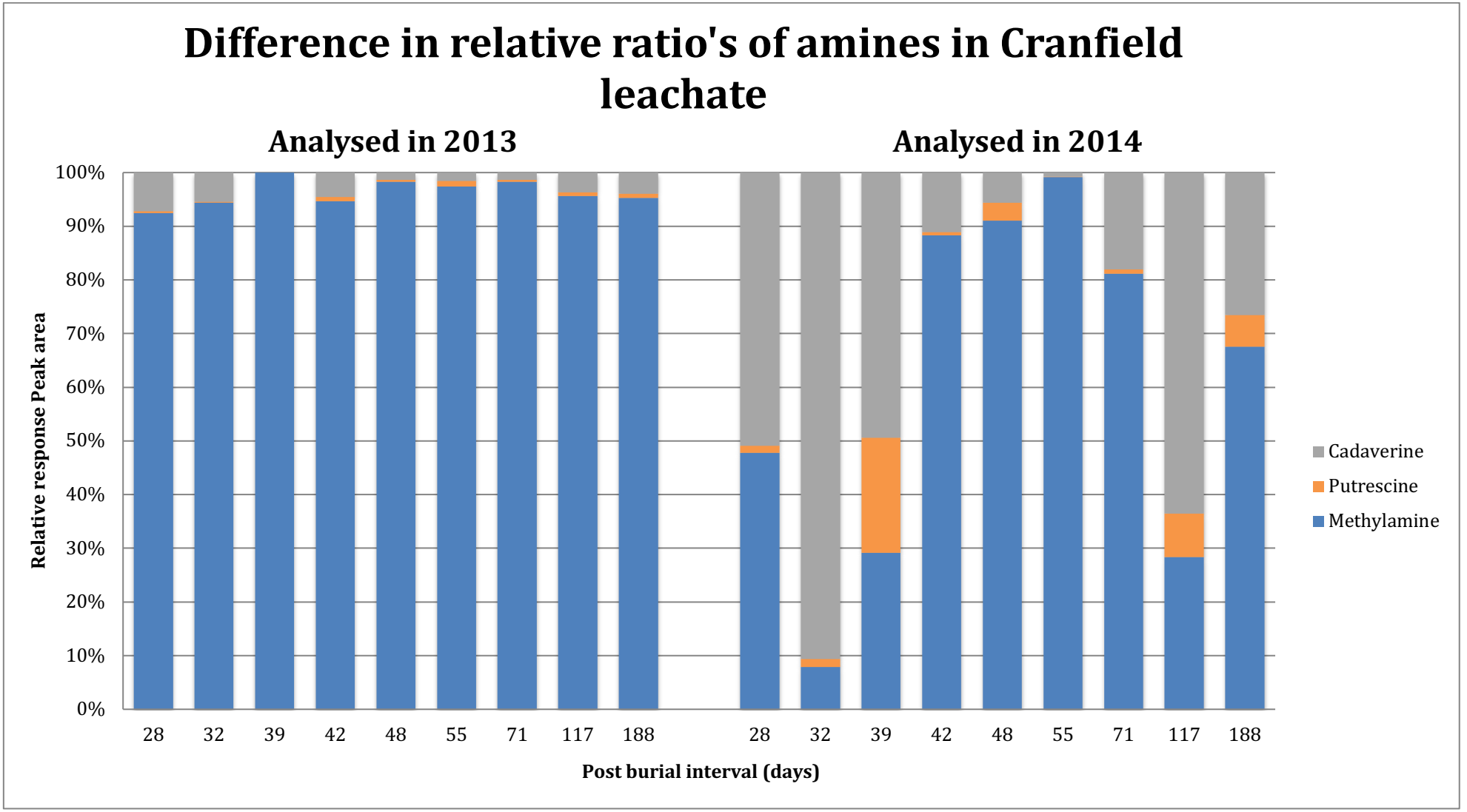

Figure 46 Bar chart for the difference in relative ratios of amines in Cranfield leachate samples between duplicate samples

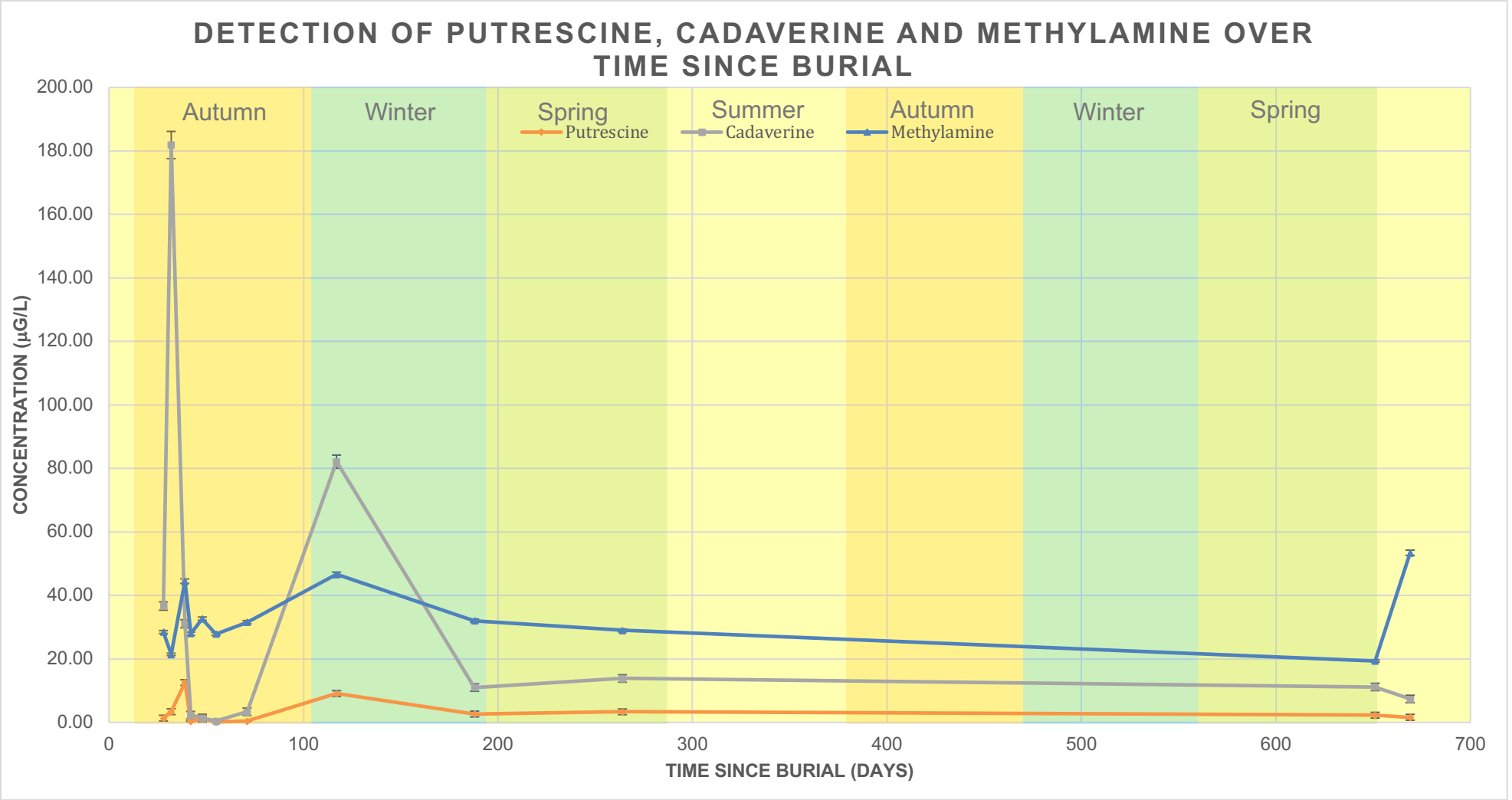

Figure 47 Chart displaying the concentrations of putrescine, cadaverine and methylamine detected in the leachate samples over time since burial using Cranfield leachate samples obtained in March 2014.

Figure 49 displays the concentration of putrescine cadaverine and methylamine detected in the Cranfield leachate samples obtained in March 2014 over time since burial. The period for which the amines were most concentrated was shortly after burial up to 117 days post burial and was in the autumn and winter. No obvious patterns emerged to interpret the data except that methylamine was present in larger concentrations than putrescine and cadaverine in all samples except for samples 28 and 32 days post burial. The data did not show any temporal changes that were linear or allowed regression, and thus did not allow any determination towards post mortem interval calculation as Vass et al. (2002) had previously indicated. Samples were not provided for the period between 264 and 651 days post burial, although putrescine, cadaverine and methylamine were still detected up to 669 days post burial.

The longevity for the detection of putrescine and cadaverine in this study was far greater than the research performed by Bonte & Bleifuss (1977), Vass et al. (2002) and Swann et al. (2012). This study detected putrescine, and cadaverine in the samples collected for nearly two years post burial, whilst Bonte & Bleifuss (1977), Vass et al. (2002) and Swann et al. (2012) detected putrescine and or cadaverine in mammalian decomposition up to 55 days, approximately 21 days and 44 days post mortem respectively. However, their decomposition process was aboveground and this project studied belowground decomposition, which resulted in different decomposition rates (Gunn 2006; Statheropoulos et al. 2011; Vass 2011). Furthermore, Bonte & Bleifuss (1977) determined that anaerobic environments accelerate amine production and could also explain the differences in the detection of these amines between this study and others. Fiedler et al. (2004) detected cadaverine in soil samples fifteen years post burial, although no confirmation had been given regarding the exact post burial interval. In addition, the bodies were buried in a coffin and adipocere was detected, which both slow down the decomposition rate (Dent et al. 2004). Samples were taken at one particular time point since burial and thus only provided limited information regarding the longevity for the detection of cadaverine.

The data from this study indicated that in addition to the detection of putrescine, cadaverine and methylamine, other primary amines were detected in the leachate samples, see Figure 48. A reconstructed ion chromatogram has been created using m/z values 181 and 208, which are characteristic fragments for the derivatised

amines as m/z 208 corresponds to the α-cleavage product ion $[C_6F_5\text{-}CH\text{=}N\text{-}CH_2]^+$ and m/z 181 corresponds to a stable fragment of the fluorinated benzene ring $[C_6F_5\text{-}CH_2]^+$ (Deng et al. 2006; Llop et al. 2010a). This resulted in the detection of seven additional primary amines in the leachate samples of which one compound (peak four) was positively identified as n-butylamine through comparison of the fragmentation patterns to the literature and a positive control sample, see Table 38. Four compounds (peaks one, two, three and five) were identified with a relatively high certainty to be ethylamine, n-propylamine, isobutylamine and n-pentylamine respectively, through comparison of the fragmentation patterns to the literature. In addition, isobutylamine, having a similar fragmentation pattern to N-butylamine, was the only structural isomer that had its base peak ion at m/z 208 and lacked the fragment ion at m/z 222, and was thus confirmed to be isobutylamine through interpretation of the molecular structure and its fragmentation. The boiling point of isobutylamine was 67°C, which is lower than n-butylamine (76°C) furthermore confirming its elution before n-butylamine. The mass spectrum of peak six was very similar to that of n-pentylamine but lacked the m/z 236 fragment ion, indicating the compound could be a structural isomer of n-pentylamine such as isopentylamine. Although, the intermolecular forces of a n-alkane are larger than that of is structural isomers, thus experiencing higher boiling points so making this unlikely (Brown et al. 2007). Peak seven was not identified, it did have a base peak ion at m/z 208 but the next fragment was at m/z 255 indicating that this compound was not a straight chain alkane. It should also be noted that both signals presumptively identified as ethylamine and n-pentylamine were detected in both grave and control samples but significant differences we observed between their concentrations.

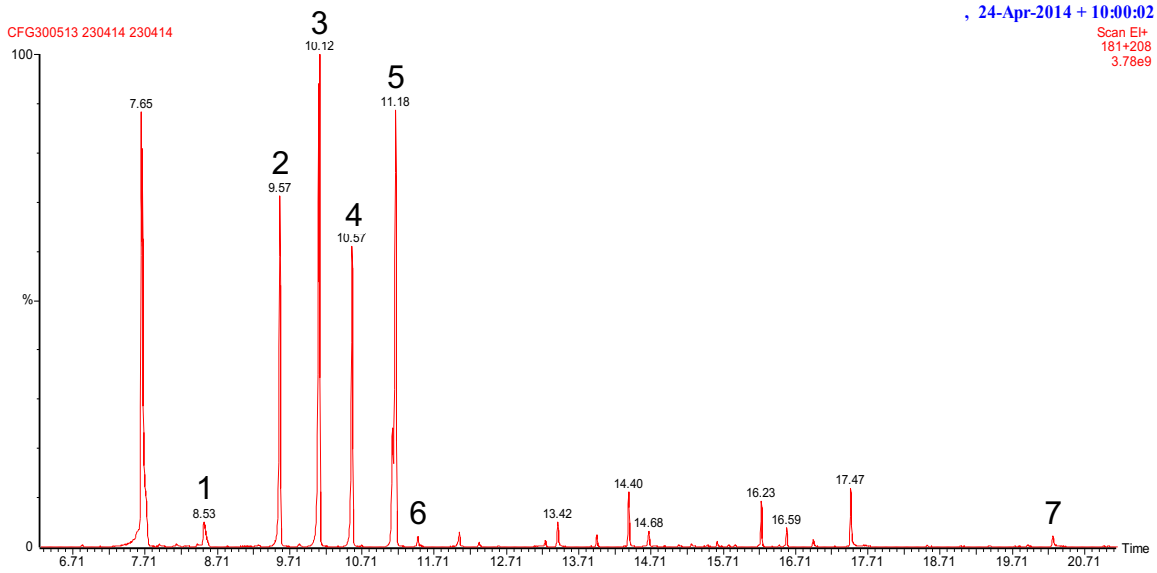

Figure 48 Reconstructed ion chromatogram of Cranfield grave sample 30-05-13 displaying additional signals related to the derivatisation (derivatised primary amines)

Table 38 Potential compound identification of other primary amines present in the leachate samples

Peak of interest no.	Possible compound	Notes
1	Ethylamine	• Mass spectra compared to (Ngim et al. 2000; Avery & Junk 1985): 208 (100), 181 (36), 194 (31) and 233 (23)
2	N-Propylamine	• Mass spectra compared to (Ngim et al. 2000; Avery & Junk 1985): 208 (100), 181 (42), 209 (35) and 161 (10) • Molecular ion present at 236 (7)
3	Isobutylamine	• Mass spectra compared to (Ngim et al. 2000; Avery & Junk 1985): 208 (100), 209 (90), 181 (86), 161 (20) and 250 (9)
4	N-butylamine	• Mass spectra compared to (Ngim et al. 2000; Avery & Junk 1985): 208 (100), 181 (65), 209 (52) and 190 (48) • Molecular ion present at 251 (15) • Confirmed using positive control
5	N-Pentylamine	• Mass spectra compared to (Ngim et al. 2000; Avery & Junk 1985): 208 (100), 190 (82), 181 (65) and 250 (61) • Peaks 209 (88) is also present in large quantity • Peak 264 (8) indicating to be the molecular ion peak
6	Unknown	• Similar to N-pentylamine although m/z 236 is low so could indicate iso-pentylamine
7	Unknown	• 208 basepeak 283 also significant • Most likely not straight chain alkane due to lack of fragments between 208 and 283

Green colour indicates positive identification
Yellow colour indicates identification with slight uncertainty due to lack of standard
Orange colour indicates some identification but still a lot of uncertainty
Red colour indicates no identification

Putrescine, cadaverine and methylamine are in the literature considered to be volatile and thus thought to be detected using headspace analysis such as solid phase micro-extraction (SPME) and sorbent traps combined with GC analysis (Dekeirsschieter et al. 2009; Hoffman et al. 2009; Statheropoulos et al. 2005, 2007, 2011; Tipple et al. 2014; Vass et al. 2004). In addition, Vass et al. (2004) hypothesised several reasons for the non-detection of these amines such as: a) putrescine and cadaverine are not volatile in a burial environment; b) putrescine and cadaverine are rapidly metabolised; c) putrescine and cadaverine are thermally labile; and d) putrescine and cadaverine are not produced in a burial environment. This study in combination with previous study by Blom (2012) indicates that statement a) is partly correct as these amines were not easily extracted from water due to their high polarity and alkalinity (Blom 2012; Hoshika 1977; Pan et al. 1997) and thus do not volatilise from moist environments including soil (see section below). Statement b) has not been seen in these samples, c) has not been observed using GC analysis and d) was disproved for our burial environments.

Researchers in the field of Taphonomy have reported that putrescine and cadaverine were not detected using headspace techniques due to a lack in volatility as discussed in the previous paragraph. Volatility is characterised by a chemicals' vapour pressure, allowing evaporation from various surfaces. However the term 'volatile' is not well defined and the vapour pressures of chemicals considered to be volatile can vary over several orders of magnitude (Herrmann 2010). Table 39 displays the vapour pressure of seven decomposition related VOC's published by Dr. Arpad Vass, including putrescine, cadaverine and methylamine (Vass et al. 2004). As visible in Table 39, methylamine is classed as the most volatile compound in this Table, putrescine and cadaverine are displayed in the middle of Table 39 indicating that these compounds are considered to be more volatile than nonanal and decanol. This highlights that the lack of detection of these amines using headspace techniques it is not due to volatility. A study performed by Blom (2012) indicated that when putrescine was dissolved in water the detection limit using SPME rose from approximately 7 µg L^{-1} to approximately 16 g L^{-1} for neat putrescine. This could be explained by a compounds partition coefficient (log $P_{o/w}$), which indicates hydrophobicity (polarity), which is below 0 for the amines. Table 39 lists whether evaporation from water-based media is possible through the use of Henry's law constant, evaporation for all compounds except the amines is expected

as highlighted in green. The amines do not volatilise from water-based media due to both their polarity, reducing their Henry's law constant, and their alkalinity as seen from their dissociation coefficient (Log D). Due to their alkalinity putrescine, cadaverine and methylamine will almost entirely exist in the cation form at environmental pH values (between 5 and 9) and thus will not volatilise form water-based media.

Table 39 Physico-chemical properties of VOC's reported by Vass (2004) including the bio-amines. Data taken from Hazardous Substances Data Bank

Compound	Vapour pressure (mm Hg 25°C)	Partition coefficient (Log P)	Dissociation coefficient (Log D)	Henry's law constant (atm-cu m/mol 25°C)
Methylamine	$2.65E^{3}$	-0.57	10.62	$1.11E^{-5}$
Carbon disulfide	359	1.94	No	$1.44E^{-2}$
Dimethyl disulphide	28.7	1.77	No	$1.21E^{-3}$
Toluene	28.4	2.73	No	$6.64E^{-3}$
Putrescine	2.33	-3.42	9.63 & 10.8	$3.54E^{-10}$
Cadaverine	1.01	-0.16[1]	9.13 & 10.25	$2.42E^{-9}$
Nonanal	0.37	3.27[1]	No	$7.3E^{-4}$
Naphtalene	$8.50E^{-2}$	3.3	No	$4.4E^{-4}$
Decanol	$8.51E^{-3}$	4.57	No	$3.2E^{-5}$
Hexadecanoic acid, methyl ester	$6.04E^{-5}$	7.38	No	$9.0E^{-3}$

Green colour indicates that evaporation from aqueous environments is expected
Red colour indicates that evaporation from aqueous environments is not expected
[1] Value is provided as an estimated value

5.5 Analysis of Water Casework Samples

Following GC analysis for the detection of the biogenic amines putrescine, cadaverine and methylamine it was observed that neither putrescine nor cadaverine were detected in any of the samples provided, see Figure 49. Methylamine was detected in the suspected grave samples but was also detected in the control samples. The data obtained from the Cranfield leachate samples highlighted a correlation between the detection of putrescine, cadaverine and methylamine during mammalian decomposition. However, this data indicates that methylamine is potentially not a suitable marker for mammalian decomposition as it has been detected in all the samples analysed and was not able to differentiate between the samples. This could potentially have been due to the significant time since burial (over 25 years) or that the body may have never been deposited at the specified location.

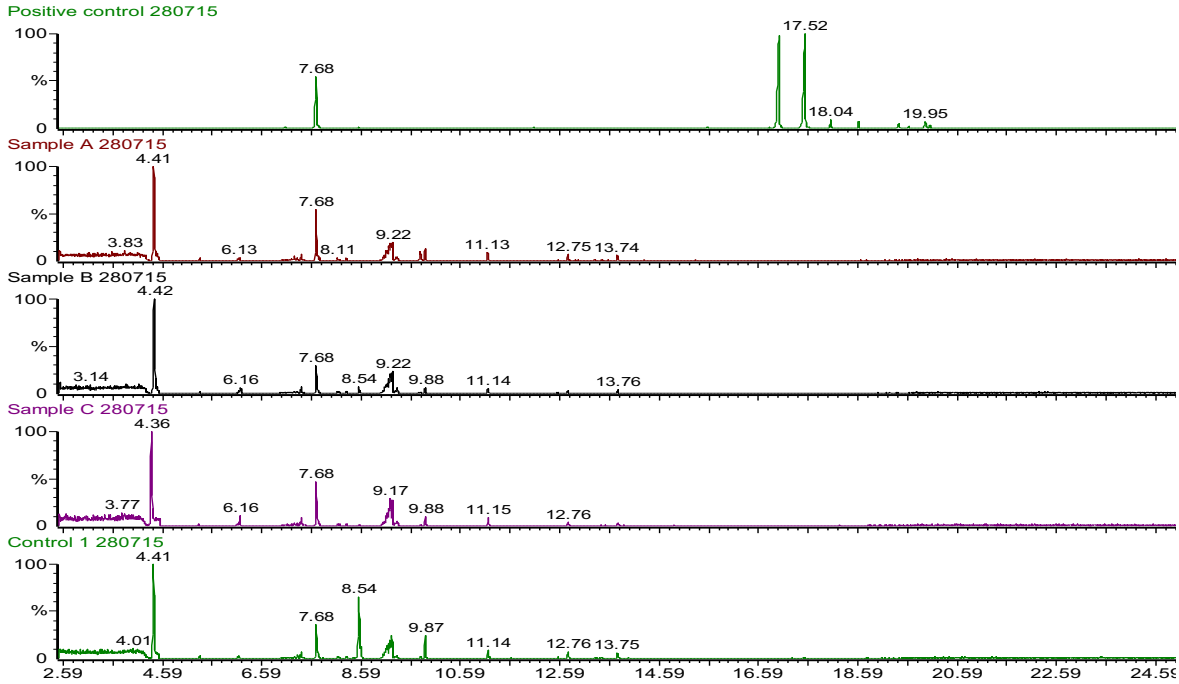

Figure 49 Chromatograms displaying the GC data for the detection of; methylamine (Rt 7.68), putrescine (Rt 17.01) and cadaverine (17.52) in the water samples (courtesy of Vincent Voorwerk)

5.6 Analysis of Soil Casework Samples

For the GC analysis, the recovery of the extraction was also calculated through the use of spiked soil samples and the data is visible in Table 40. As observed in Table 39, the recovery of the amines using the current extraction methodology is very poor, around 10% for methylamine and even lower for putrescine and cadaverine as the methodology used was not optimised for the extraction of amines. Fiedler et al. (2004) extracted putrescine and cadaverine through the use of a soxhlet extractor, however their recoveries are unknown and therefore these extraction methodologies can't be compared. Despite the low recovery, putrescine has been detected in sample 1 (grave location) at a concentration of nearly 150 ppb. Putrescine has also been detected in samples 6 (100m downhill) and 8 (near VRD indication) but were below the limit of quantification. Putrescine was not detected in the remaining samples (immediately downslope, or the control sample) and cadaverine was not detected in any of the samples, see Figure 50. On the other hand, methylamine has been detected in all samples including the negative control soil and is present at approximately the same quantities in all samples and is therefore unable to provide any additional information. In addition to the detection of putrescine, approximately eleven compounds were detected in sample 1 (see Figure 51) that were not detected or detected at significantly lower quantities in the other samples, however further research is required to determine if these compounds are derivatised amines or co-extractants during the derivatisation.

Table 40 Recovery of putrescine, cadaverine and methylamine from soil

Chemical	Spiked (µg·L^{-1})	Found (µg·L^{-1})	Recovery (%)
Methylamine	15.5	1.7	10.72
Putrescine	44.1	4.0	9.17
Cadaverine	51.1	3.7	7.32

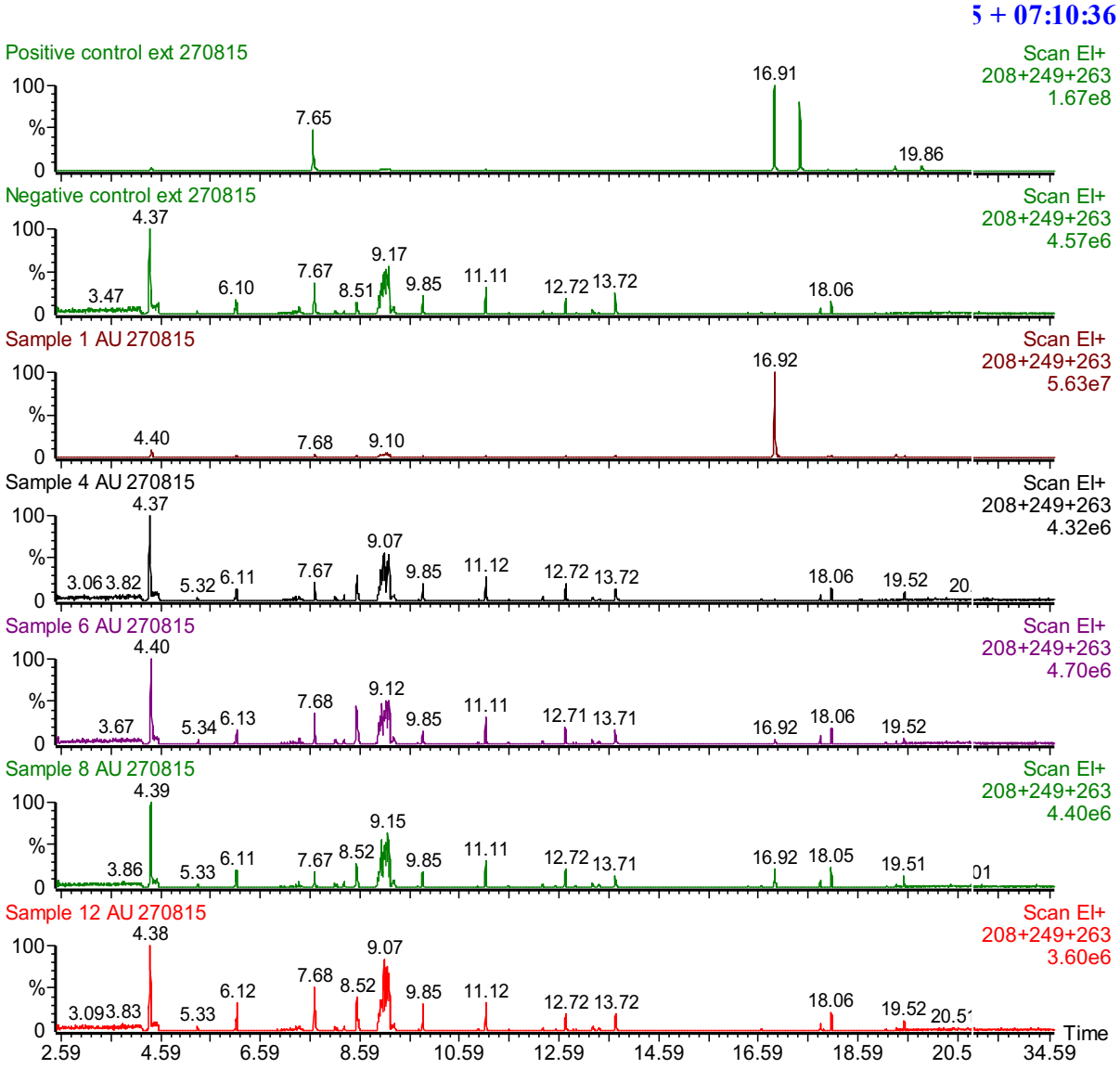

Figure 50 Chromatogram displaying the detection of putrescine (rt 16.91), cadaverine (rt 17.52) and methylamine (rt 7.65) in the different samples analysed

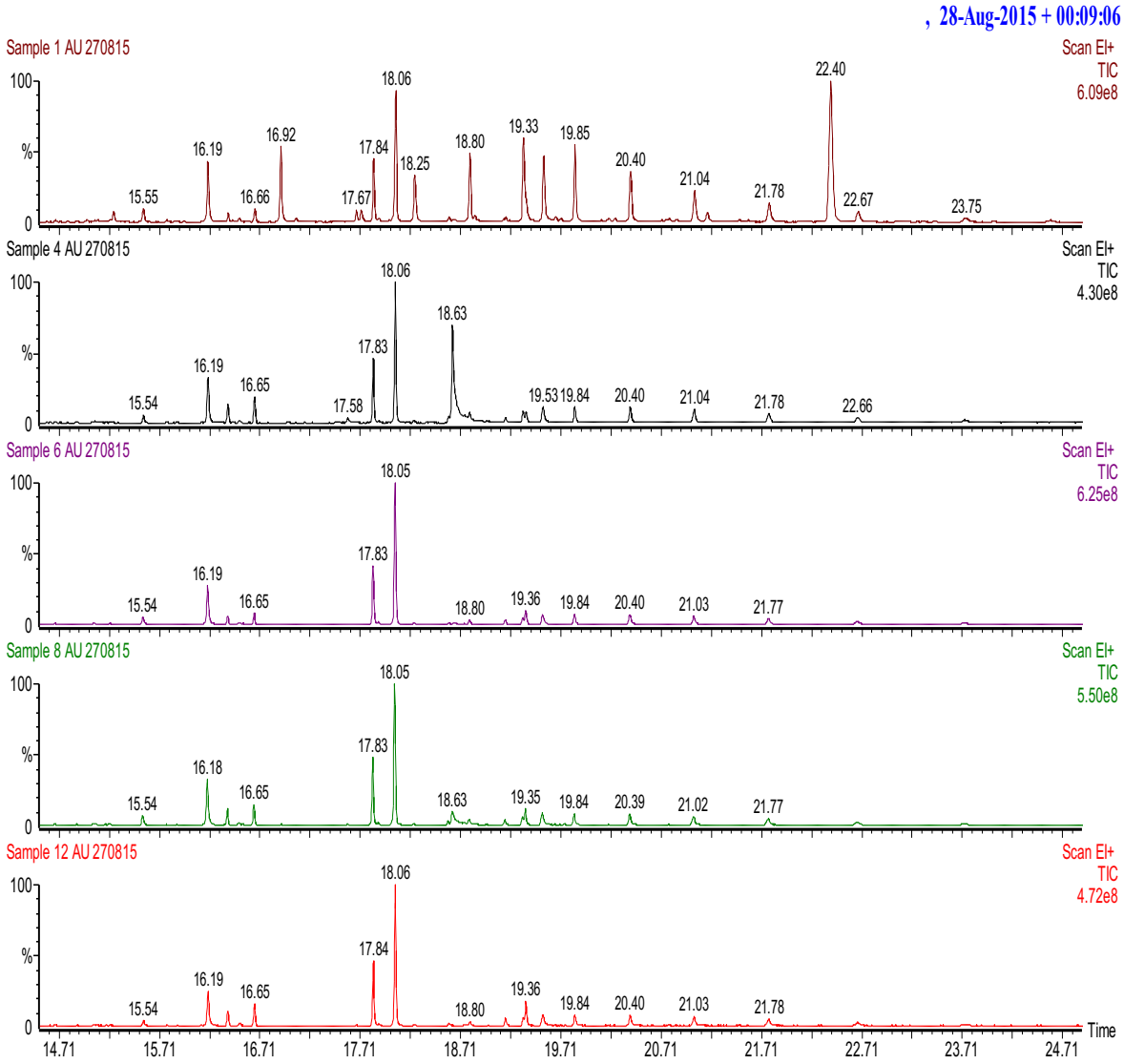

Figure 51 Chromatogram displaying the detection of several other components of interest in the grave samples in comparison to the other samples

The detection of putrescine in sample 6 and 8 could indicate migration of the chemical constituents and could explain why the PCA-plot displayed the most variation in these samples in relation to the other samples following the IC results (see Section 4.4 Analysis of Soil Casework Samples). Figure 52 displays a hypothesised pattern of chemical leaching downhill published by Dr. Laurance Donnelly in 2010 and could explain the detection of low quantities of putrescine downhill. The detection of trace levels of putrescine in sample 8 (slightly uphill) might have been through capillary action, where putrescine, alongside other water soluble chemicals migrated slightly uphill. The VRD also detected at this location and thus corroborates the hypothesis of chemical migration through capillary action. Phosphate was also found to be higher at sample 8 but otherwise the IC data was less conclusive than the GC data, although the relative ratio of nitrate between the upper and lower sample was found to be larger in the gravesite sample.

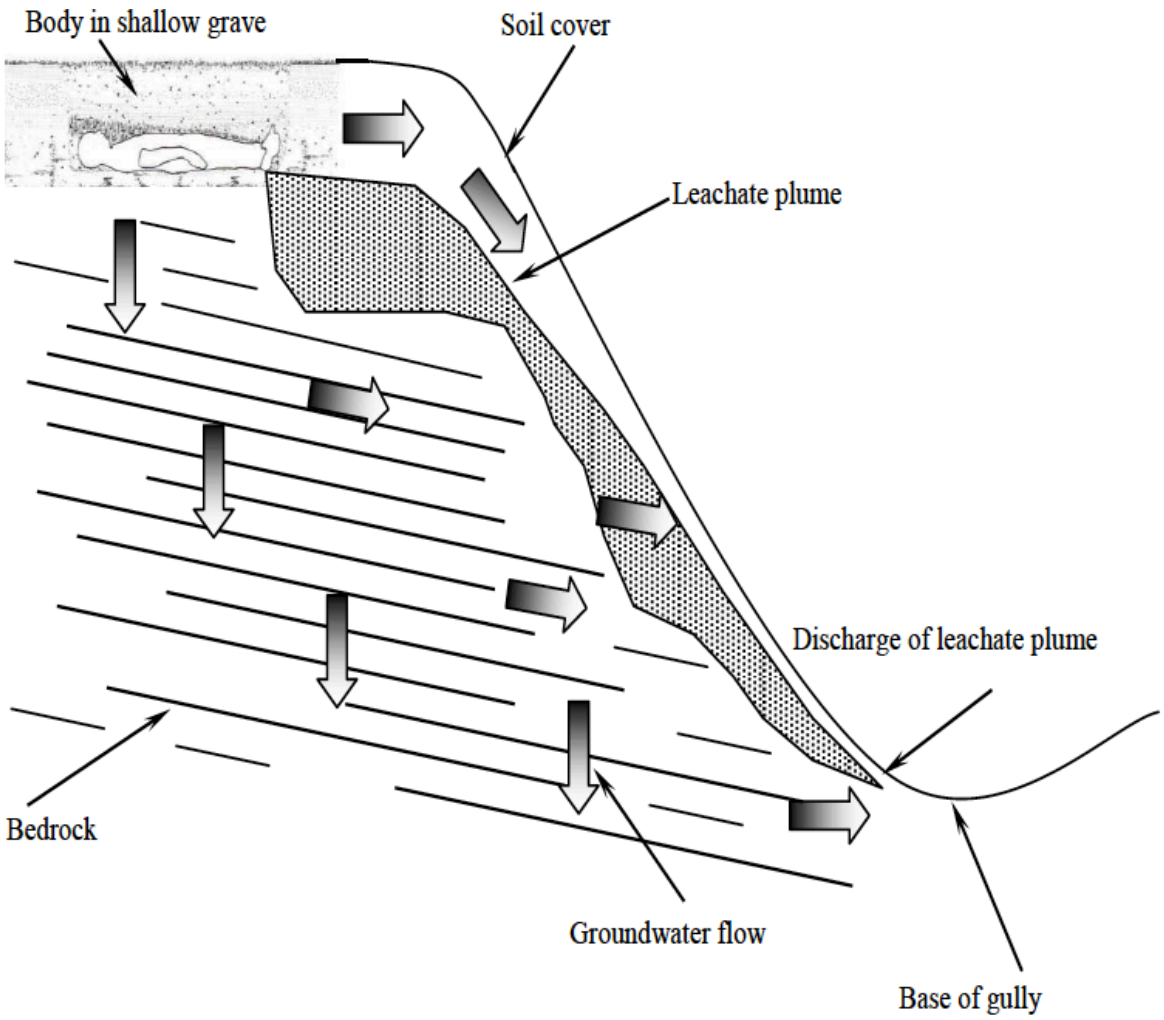

Figure 52 Schematic drawing highlighting the underground migration of decomposition products. Taken from Donnelly (2010)

5.7 Storage Experiments

5.7.1 Storage of Standard Solutions

On visual interpretation of the data, no gross differences were observed between the levels of putrescine, cadaverine and methylamine between the fresh samples and those from the different storage conditions. Thus a one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate the effect of different storage conditions. The dependent variables were the raw data for methylamine, putrescine and cadaverine, and the independent variable was the storage conditions containing five levels labelled; initial samples, room temperature, fridge, freezer and freezing/defrosting. No statistically significant difference ($p > 0.05$) was observed between the storage conditions on the combined dependent variables using Pillai's trace, V = 0.73, $F(12, 30) = 0.81$, $p = 0.642$, and resulted in no further testing and interpretation of the MANOVA data.

Analysis of Variance (ANOVA) was chosen to analyse the data from the storage experiment as it is the most powerful test of comparing samples. Using multivariate data, *e.g.* multiple dependent variables, there is an option of performing one ANOVA per dependent variable and combining the results or to perform a MANOVA (Boyd et al. 2006). A MANOVA is preferred as Type I errors (false positive) could be introduced by combining the results of multiple ANOVA's, and if separate ANOVA's are conducted, any relationship between the dependent variables is ignored losing important information (Boyd et al. 2006; Field 2013; Pallant 2010). The outcomes of the MANOVA were compared to separate ANOVA's and gave identical results.

A MANOVA has several multivariate tests to choose from; Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root. Out of these tests Wilks' Lambda is most commonly reported, however Pillai's trace is more robust when sample sizes are equal and thus less prone to violation of assumptions. The decision was made to use Pillai's trace instead of Wilks' Lambda as a small sample size was used for the significance testing (Field 2013). The significance of a MANOVA is indicated by the multivariate test used, Pillai's trace calculates the amount of variance in the dependent variables that is accounted for by the greatest separation of the independent variables thus V = 0.73 implies that 73% of the variance is explained by the different storage conditions (Norman & Streiner 2008).

The F value is the ratio between the mean square values and is expected to have a value close to 1.0 when the null hypothesis is true. A large F value indicates that the variation among group means is more than expected to see by chance resulting in the null hypothesis to be incorrect (Zar 2010). The F value of the MANOVA was 0.806 thus confirmed the null hypothesis was true.

Figure 53 displays a bar chart showing the effect of the storage conditions on the mean detection of putrescine, cadaverine and methylamine, 95% confidence interval error bars have been included to visualise the MANOVA results. As visible in Figure 53 no differences in the detection of putrescine, cadaverine and methylamine were observed between the different storage conditions within their 95% confidence interval.

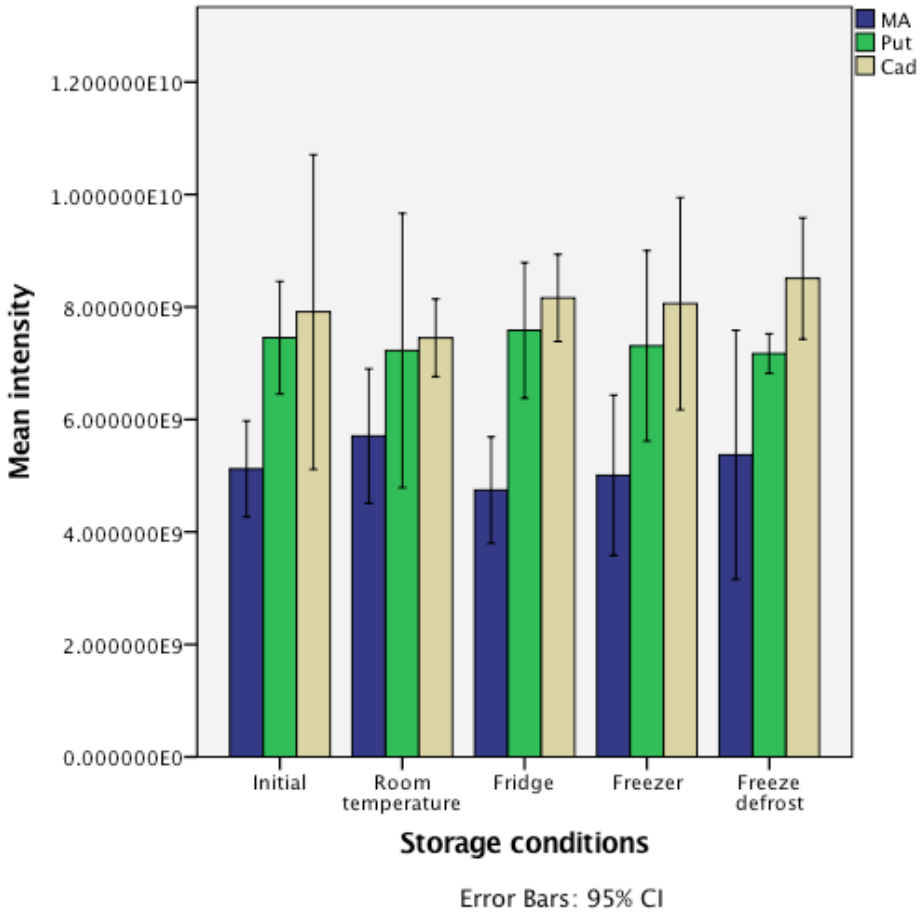

Figure 53 Bar chart displaying the average intensity and 95% confidence interval for 1.0 mmol L^{-1} putrescine, cadaverine and methylamine solutions stored for three months

5.7.2 Storage Casework Samples

On visual interpretation of the matrix effects data, no gross differences were observed between the levels of putrescine cadaverine and methylamine between the different samples. Thus a one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate the effects of the matrix on the derivatisation of the amines in the water samples. The dependent variables were the raw data for methylamine, putrescine and cadaverine, and the independent variable was the different spiked samples containing five levels labelled; positive control, sample A, sample B, sample C and sample control. A statistically significant difference ($p < 0.05$) was observed between the different samples and the combined dependent variables using Pillai's trace, V = 2.25, $F(12,15) = 3.75$, $p = 0.009$. When the results for the dependent variables were separately considered no significant difference was observed using a Bonferroni adjusted alpha level of 0.017. Thus it has been concluded that no significant difference was observed between the derivatisation of each individual amine between the spiked samples and positive control.

The effect of storage of the casework samples was investigated to establish if putrescine, cadaverine and methylamine could have been present in the samples but have been decomposed before analysis commenced. On visual interpretation of the data, no gross differences were observed between the levels of putrescine cadaverine and methylamine before and after storage. Thus a one-way analysis of variance (ANOVA) was performed on each amine to investigate the effect of storage on the amines. The dependent variables were the raw data for methylamine, putrescine and cadaverine, and the independent variable was the storage conditions; before and after storage. No statistically significant difference ($p > 0.05$) was observed between the storage and the detection of methylamine $F(1,15) = 0.43$, $p = 0.524$, and resulted in no further testing and interpretation of the ANOVA data. The same was concluded for putrescine $F(1,15) = 0.002$, $p = 0.962$, and cadaverine $F(1,15) = 0.057$, $p = 0.814$.

It was hypothesised that implementation of the developed IC and derivatisation GC methodologies on the water samples would be straightforward from an analysis perspective as the methodologies were both developed for the analysis of liquid

samples. The results discussed in the previous paragraphs highlighted that the water matrix did not interfere with the derivatisation of putrescine, cadaverine and methylamine individually. In addition, it also indicated that if putrescine and or cadaverine were present in detectable levels it would not have been decomposed during storage. It therefore seems that either the applied methodologies are not sensitive enough and/or these methodologies are not suitable to differentiate between decomposition and control samples in an aquatic environment. This is however unlikely as both the IC and GC methodologies have been able to detect the required analytes at low ppm and low ppb detection limits in their respective methods (see Chapter 4 Analysis of Inorganic Anionic Compounds Using Ion Chromatography and Chapter 5 Detection of Putrescine, Cadaverine and Methylamine in Mammalian Decomposition Fluids). Other possibilities for the inability to differentiate could have been limitations from water sampling or as discussed previously, the non-detection is related to the time since burial of the victim or the victim was never deposited at the specified location. It has been noted that natural water appears to be homogeneous but is in fact spatially and temporally heterogeneous, and stratification is common in lakes with variations in flow, chemical composition and temperature (Dean 1998). This indicates that sediment samples or water samples taken close to the sediment are likely to provide different results in comparison to surface water and could therefore be a reason why the IC and derivatisation GC methodologies were not able to differentiate between the water samples. In addition, no data is available for the detection of putrescine, cadaverine and methylamine in an aquatic environment up to 25 years post burial.

Chapter 6 Conclusion

This study sought to detect the non-volatile and semi-volatile decomposition products present in soil and water samples that could aid in the detection of clandestine gravesites and lead to the development of field based chemical tests to speed up the search process. The overall aim of this study was to determine if the detection of non-volatile and semi-volatile decomposition products, is a viable alternative to the current search methodologies available. The objectives to achieve this aim were:

- To develop a highly specific and sensitive methodology for the detection of putrescine and cadaverine in aqueous samples and determine if biogenic amines such as putrescine and cadaverine could be detected in mammalian decomposition.
- To explore why most of the researchers in the field of taphonomy were unable to detect the decomposition markers putrescine and cadaverine in their studies.
- To determine the usefulness of ion chromatography as a tool to analyse mammalian decomposition products.
- To determine the effectiveness of high performance liquid chromatography and liquid chromatography mass spectrometry as a tool to analyse products of mammalian decomposition.
- To determine if the developed analytical methodologies (gas chromatography and ion chromatography) can aid in the intelligence gathering process for locating clandestine gravesites.
- To develop an extraction methodology in order to allow for the analysis of soil samples as well as water samples.

This study has highlighted that the development of chemical tests is a viable alternative approach towards the already established search methodologies for the detection of clandestine gravesites. Three analytical methodologies have been developed for the detection of non-volatile and semi-volatile decomposition products within a soil or aqueous environment. These methodologies are capable to detect mammalian decomposition products and are able to differentiate between gravesite and control samples (see sections below). Even though some preliminary chemical marker identification has been performed further research is required in order to

identify the key decomposition products produced during the human decomposition process. When these compounds are identified field-based test would allow for easier and more rapid search procedures, to aid in the detection of clandestine graves. Such tests could be used in combination with victim recovery dogs to confirm or eliminate their alerts, or it can be used in a systematic approach to locate decomposition hotspots within a particular area and eliminate some of the disadvantages of the current search methods.

6.1 High Performance Liquid Chromatography

Liquid chromatography was utilised to explore the variety of decomposition products produced during mammalian decomposition through the use of HPLC-DAD and LC-MS. This work has sought to establish if alternative analytical techniques to the commonly used GC-MS methodologies could identify decomposition products that were not detected in the decomposition fluids by other researchers and aimed to determine the effectiveness of HPLC and LC-MS as a tool to analyse products of mammalian decomposition.

After exploring many different HPLC operating conditions, it was concluded that reversed phase chromatography using an octadecylsilyl (C18) column was more suitable to separate the decomposition products over normal phase chromatography (hilic). Due to the polarity of the decomposition compounds a buffered mobile phase was required, preferably at low pH to suppress ionisation of acidic compounds and stabilise the pH. The use of a formate buffer was preferred over the phosphate buffer due to its adaptability with LC-MS and similar HPLC conditions have been used by Swann et al. (2012) for the determination of amino acids and amines in decomposition fluids. HPLC-DAD is a useful tool for the detection of decomposition products and may have further use for quantification of key decomposition compounds but LC-MS is more suitable for the purpose of this study.

Sample clean-up was required for the analysis of the leachate samples using HPLC through the use of Whatman 0.45 µm PVDF syringe filters and was identical to the sample preparation performed by Swann et al. (2012). The use of SPE as a sample preparation/clean-up technique was disregarded as SPE was unable to concentrate the leachate samples due to insufficient sample quantities being available. A major

objective in this study was to identify the chemicals present in the leachate, which meant that all the compounds in the leachate were potentially of interest.

The data showed the potential for LC-MS to analyse and qualify the compounds present in the leachate samples, although further work is required before it will compete with the use of GC for the analysis of decomposition products. LC-MS is more suitable to detect the variety of compounds present in the leachate in comparison to HPLC-UV as it is a more versatile detector and allows analysis of a wide range of compounds without derivatisation. The data indicated that over 100 decomposition specific chemicals were present in the leachate samples and that some of these chemicals were detected over a relatively long period of time (over 30 months post burial).

The process of compound identification was difficult as no internal library was present and thus no compounds have been conclusively identified. A few amino acids and amines such as lysine, tyramine, phenylalanine and tryptamine were identified with reasonable certainty without the use of reference standards. Whilst identification of VFA was more difficult without the use of reference standards.

In summary, a suitable methodology has been developed for the analysis of non-volatile and semi-volatile decomposition products within the leachate samples using HPLC. Analysis was achieved through reversed phase chromatography using an octadecylsilyl (C18) stationary phase and a methanol:formate buffer pH 3.2 gradient mobile phase. To date no HPLC analysis nor any longitudinal studies have been performed on the leachate samples using HPLC, making this application novel. Minimal sample preparation was required as the samples only required filtering through the use of Whatman 0.45 µm PVDF syringe filters, other sample preparation techniques such as solid phase extraction could be beneficial, providing enough sample is available to perform the extraction and not dilute the analytes. LC-MS has for the first time been used for the analysis of soil leachate and has shown its potential to analyse and qualify decomposition products present in soil-water samples. The data highlighted that over 100 decomposition specific chemicals were present in the leachate samples in comparison to the detection of nineteen decomposition products detected by Swann et al. (2012) and some of these chemicals were detected over a relatively long period of time (over 30 months post

burial). However, further work is required to identify the wide range of chemicals present in the leachate samples as no compounds were conclusively identified in this study due to the lack of reference standards.

6.2 Ion Chromatography

This research set out to explore the use of ion chromatography for the analysis of decomposition products and explore its validity in a real world situation through analysis of samples collected from active murder enquiries. The aim of this study was to determine the usefulness of ion chromatography as a tool to analyse products of mammalian decomposition and determine if this methodology can aid in the intelligence gathering process to locate clandestine gravesites.

After the analysis of a range of leachate samples, the anionic compounds present in these samples were able to be identified. With the exception of a few signals the anionic compounds present in the leachate samples were identified as acetate, chloride, nitrite, nitrate, carbonate, phosphate and sulphate. Detection and quantification limits were determined to be in the low ppm to sub ppm range for the ions quantified.

As discussed in the previous paragraph, ion chromatography has the ability to detected anionic compounds produced during mammalian decomposition at low ppm to sub ppm concentrations. Therefore, it has the capability to distinguish between decomposition (gravesite) and control samples. This was expected as IC had been utilised for the quantification of decomposition products by Vass et al. (1992). Significant concentrations of acetate, up to 20 mg mL^{-1}, and carbonate, up to 15 mg mL^{-1}, have been detected during this decomposition study and could become markers for mammalian decomposition. Vass et al. (1992) highlighted that acetate is abundant in nature and too variable to be used for time since death estimation, but this study indicates it could be useful to locate clandestine gravesites, provided additional research will be conducted to determine the reproducibility of these results between cadavers, to determine their migration in soil and their natural abundance in different soil environments.

Following analysis of the casework samples, it was concluded that the IC methodology is capable to be applied to the analysis of forensic samples. It has

been able to differentiate between different soil samples and highlighted focus points through the use of statistical analysis. The samples that showed most variation through principal component analysis were the samples (slightly uphill near VRD indication and downhill of the grave) in which putrescine also was detected. The gravesite sample did indicate an interesting ratio for nitrate between the upper and lower portion of the soil which was only observed in the gravesite sample. However in general a lower quantity of extractable inorganic anions was detected in the gravesite sample, which was potentially due to improved drainage of the soil following excavation (Donnelly et al. 2018). It was however not able to differentiate between the different water samples but this could have been due to limitations regarding the sampling procedure used or time since burial. It was unlikely that the analysis methodology was not suitable as the detection limits for the anions are in the low ppm range. In addition, it has been applied within the field of taphonomy for the detection of decomposition products (Aitkenhead-Peterson et al. 2012; Vass et al. 1992), forensics to differentiate between forensic soil samples (Bommarito et al. 2007) and in the environmental science to determine the impact of cemeteries on the surrounding environment (Zychowski 2012).

In summary, ion chromatography has the ability to detect anionic compounds produced during mammalian decomposition up to low ppm detection limits and is the first study in its kind to identify and quantify the inorganic anions in the leachate over time since burial. It was therefore concluded that the methodology is capable distinguishing between decomposition (gravesite) and control samples, which was expected as Vass et al. (1992), Bommarito et al. (2007) and Aitkenhead-Peterson et al. (2012) all utilised IC to either detect decomposition related compounds or to distinguish between different soil samples. Following analysis of the case samples, it was concluded that the methodology developed is capable to be applied to the analysis of forensic samples. It has been able to differentiate between the different soil samples from this study to identify samples of interest through the use of statistical analysis. However, the developed methodology was unable to differentiate between the different water samples provided, which was most likely due to limitations in the sampling procedure or time since burial.

6.3 Gas Chromatography

This work set out to explore the detection of decomposition markers putrescine and cadaverine in mammalian decomposition following derivatisation GC analysis and explore the validity of the methodology in a real world situation through analysis of samples collected from active murder enquiries. This study has also sought to identify if, in addition to putrescine and cadaverine, other decomposition products could be identified, the time period these compounds can be detected post burial and why many researchers within the field of Taphonomy did not detect these markers in their studies. The detection of decomposition markers such as putrescine and cadaverine in the soil matrix could aid the Police in locating missing persons and murder victims through providing an additional complementary search technique.

A highly specific methodology has been developed for the detection and quantification of putrescine, cadaverine and methylamine in aqueous samples. This was achieved through derivatisation with pentafluorobenzaldehyde which is specific to primary amines and yields detection limits below 30 μg L^{-1} (30 ppb). Although this derivatisation mechanism produced geometrical isomers that could affect quantification, nevertheless good correlation (R^2 > 0.995) has been observed suggesting that the isomer ratios were consistent between 1.0 μmol L^{-1} and 500 μmol L^{-1}.

Derivatisation has been scarcely used for the analysis of decomposition products within the field of forensic taphonomy and the utilisation of pentafluorobenzaldehyde for the detection of these amines is therefore a novel application. Derivatisation has been mainly used for the determination of adipocere in soil and decomposing tissue samples (Forbes et al. 2003; Notter et al. 2008; Stuart et al. 2016) and had only been utilised for the determination of putrescine and cadaverine by Fiedler et al. (2004) for their detection in cemetery soil samples and by Vass et al. (2002) in decomposing tissue samples. The derivatisation reagent used by Fiedler et al. (2004) was using 9-fluoromethylchloroformate (carbamate formation) and utilised analysis by HPLC. Vass et al. (2002) utilised dimethylformamide dimethylacetal (schiff base formation) to derivatise putrescine and cadaverine followed by analysis using GC. Both of these derivatisation mechanisms would have been able to be utilised on the leachate samples as both

of these derivatisations work in aqueous environments. However both of these reagents are not amine specific and therefore would have created a less specific methodology which could have interfered with the analysis of putrescine and cadaverine. The use of pentafluorobenzaldehyde in particular was more suitable than for example benzaldehyde as pentafluorobenzaldehyde lowered the boiling point of the analytes thus decreasing retention times and improving sample throughput.

The developed methodology enabled the detection of putrescine, cadaverine and methylamine in the Cranfield leachate samples from 28 days post-burial up to at least 669 days post-burial. The detectability of putrescine and cadaverine over time since burial in this study far exceeded those published by other researchers. The studies published by Bonte & Bleifuss (1977), Vass et al. (2002) and Swann et al. (2012) only detected one or both of these amines over time and detected them up to 55 days, approximately 21 days and 44 days post mortem respectively. Making this the longest longitudinal study for the detection of putrescine and cadaverine, it should however be noted that the decomposition process of the other researchers was aboveground and this research studied belowground decomposition. Using the sample set provided for this study, no obvious patterns were able to be elucidated to predict and trend putrescine, cadaverine and methylamine levels. Following analysis of the casework samples, it was noted that putrescine was able to be detected in a soil environment approximately fifteen years after burial. It is interesting that putrescine was detected in this study and cadaverine was not as Fiedler et al. (2004) detected cadaverine in soil from a fifteen year old grave. This could possibly be inherent to the poor recovery of the soil extraction methodology used (see Section 5.6 Analysis of Soil Casework Samples). In addition, in the study published by Fiedler et al. (2004), the body was buried in a coffin and adipocere was detected. These parameters both affect the decomposition process (Dent et al. 2004) and could explain together with soil type the differences in detection of these amines.

In addition to the detection of putrescine and cadaverine, methylamine has been detected in the Cranfield leachate, which had not previously been associated with the mammalian decomposition process. However, following analysis of the case samples, methylamine transpired to be not as significant as was initially

hypothesised as it has been detected in both the soil and water casework samples, including the control samples but it should not be completely excluded either. These results has initiated further research with the aim to establish the natural abundance of methylamine and other primary amines within the environment. Other primary amines such as ethylamine, n-propylamine, isobutylamine, n-butylamine, n-pentylamine and amylamine were potentially also produced during the mammalian decomposition process, although further work is required to identify these compounds unambiguously.

Following analysis of the case samples using GC-MS, it was concluded that the derivatisation GC methodology developed is suitable for application to the analysis of forensic samples. Putrescine has been detected in the gravesite soil sample and was also detected in samples collected slightly uphill from the gravesite (near a VRD indication) and downhill from the gravesite. This indicated that migration of water soluble chemicals could migrate through the soil matrix both uphill and downhill. The uphill motion could be through capillary action and has been supported by the VRD indication and the IC data (due to differences in relative quantities of extractable inorganic anions). The downhill motion has been hypothesised by Donnelly (2010) through the flow of groundwater. Fiedler et al. (2004) also hypothesised this principle following the detection of cadaverine in control samples and the research published by Aitkenhead-Peterson et al. (2012) also suggested the migration of decomposition products downhill following their analysis. The GC methodology was however not able to detect putrescine or cadaverine in the water samples but this could have been due to limitations regarding the sampling procedure used, time since burial (over 25 years) or the absence of a body.

Research for the analysis of putrescine and or cadaverine in the field of forensic taphonomy has not readily been conducted, more often have these amines been reported as not detected within VOC characterisation studies (see Vass et al. 2004; Statheropoulos et al. 2005; 2007; 2011; Dekeirsschieter et al. 2009; 2012; Hoffman et al. 2009). Evidence in this study suggests that most researchers within the field of Taphonomy did not detect putrescine and cadaverine because these chemicals were according to Gill-King (1997) foul smelling and detectable by VRD, and therefore were believed to be detected using headspace techniques (sorbent traps and SPME). Putrescine, cadaverine and even methylamine are more volatile

than the majority of VOC's detected by other researchers, however due to their alkalinity become rapidly ionised in environmental pH conditions and therefore do not volatilise from moist environments such as soil or decomposing tissue, which explains why putrescine and cadaverine were not detected in other VOC characterisation studies.

Storage of the samples was never associated as one of the objectives in this study as the leachate samples had been stored for a significant period of time before the samples were sent to Staffordshire University for analysis. However, following the differences observed after analysing the duplicate leachate samples and the paper published by Forbes et al. (2014) highlighting that sample freezing could negatively impact sample integrity, several small scale storage experiments were conducted in this study. The data indicated that the different storage conditions may affect the chemical composition of the leachate samples, which could affect longevity studies, changes over time and operational work. However, no significant differences were observed in the storage experiment using a 1.0 μmol L^{-1} mixed amine solution in distilled water. Another storage experiment conducted on the casework water samples highlighted that neither the water matrix nor sample storage affected the detection of putrescine, cadaverine or methylamine in a significant manner. For the analysis of the soil samples it is unclear if the soil matrix inhibited the derivatisation or if the amines were extracted poorly. The latter is more likely as the recovery of the anions for the IC analysis was also poor and the applied methodologies were developed using soil-water samples. It has therefore been concluded that the different matrixes water or soil did not interfere with the analysis of the casework samples.

In summary, derivatisation GC analysis has proven itself to be a useful tool for the detection and identification of (semi-volatile) decomposition products. Sample preparation (*i.e.* derivatisation) was vital as it enabled the development of a highly specific methodology to quantify putrescine and cadaverine in aqueous samples at low ppb detection limits using pentafluorobenzaldehyde. Derivatisation has been scarcely used for the analysis of decomposition products within the field of forensic taphonomy and had only been utilised for the determination of putrescine and cadaverine by Fiedler et al. (2004) and Vass et al. (2002). These derivatisation mechanisms would have been able to derivatise the amines in the leachate samples

but are not amine specific and therefore would have created a less specific methodology and affected sample throughput. The longevity for the detection of putrescine and cadaverine over time since burial in this study far exceeded those published by other prominent researchers such as the studies published by Bonte & Bleifuss (1977), Vass et al. (2002) and Swann et al. (2012). In addition to the detection of putrescine and cadaverine, methylamine has been detected in this study which had not previously been associated with the mammalian decomposition process and other primary amines were potentially also produced during the mammalian decomposition process, although further work is required to identify these compounds unambiguously. Following analysis of the case samples, methylamine transpired to be not as significant as was initially hypothesised and led to the initiation of further research with the aim to establish the natural abundance of methylamine and other primary amines within the environment.

6.4 Soil Extraction

During this study a methodology was developed for the extraction of inorganic anions and amines from the soil matrix that has been applied to the analysis of the case samples. Based on the data obtained, it was concluded that a suitable extraction methodology was developed to aid in the analysis of soil samples. Unfortunately, due to time constraints associated with the analysis of the case samples a non-optimised methodology has been used with poor recoveries of around 20% and further optimisation of the extraction methodology is recommended for future analysis. No further optimisation was explored in this study as the stability of the compounds requiring analysis was unknown and extensive development would have been required in order to determine the effect of the different solvent conditions, which was not feasible within the turnaround time and equipment available. Nevertheless, a similar methodology has been used by Bommarito et al. (2007) for the extraction of anions to profile forensic soil samples and therefore was deemed suitable for the work discussed in this study.

In conclusion, this study highlighted the development and application of three novel alternative analytical methodologies to aid in locating clandestine gravesites through detection of the non-volatile and semi-volatile decomposition products within a soil or aqueous environment. The first methodology, has a novel approach to the detection of primary amines such as putrescine and cadaverine through

derivatisation with a highly specific derivatisation reagent in an aqueous environment. The application of the methodology, the data provided from the longitudinal study and the detection of methylamine (not associated with mammalian decomposition before) make this application and the data novel. The second methodology, has allowed for analysis of inorganic anions at low ppm concentrations and been able to differentiate between gravesite and control samples. This is the first study to identify and quantify inorganic anions over time since burial and therefore making it novel. The final methodology has shown capabilities to differentiate between grave and control samples and the potential to identify additional compounds produced during mammalian decomposition. Over 100 decomposition specific compounds have been detected and longitudinal data has been obtained using HPLC and LC-MS, making the methodology once again novel. Utilisation of these methodologies will lead to further identification of the key decomposition products produced during the human decomposition process and will allow for the development of field-based chemical test to aid in the detection of clandestine gravesites.

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Appendix I. Equations Used for Method Validation

- Product-moment correlation

$$r = \frac{\sum_i [(x_i - \bar{x})(y_i - \bar{y})]}{\{[\sum_i (x_i - \bar{x})^2][\sum_i (y_i - \bar{y})^2]\}^{1/2}}$$

- test for significant correlation, adapted t-test

$$t = \frac{|r|\sqrt{n-2}}{\sqrt{1-r^2}}$$

n= degrees of freedom, if t-table value is lower than calculated t-value a significant correlation is present.

- Coefficient of determination (linearity)

$$R^2 = \frac{\sum_i (y_i - \bar{y})^2 - \sum_i (y_i - \hat{y}_i)^2}{\sum_i (y_i - \bar{y})^2}$$

- Line of best fit, regression of y on x (method of least squares

$$b = \frac{\sum_i [(x_i - \bar{x})(y_i - \bar{y})]}{\sum_i [(x_i - \bar{x})]^2}$$

$$a = \bar{y} - b\bar{x}$$

- Error in the slope and intercept of the regression line

$$s_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}}$$

- standard deviation of slope

$$s_b = \frac{s_{y/x}}{\sqrt{\sum_i [(x_i - \bar{x})]^2}}$$

- standard deviation of intercept

$$s_a = s_{y/x} \sqrt{\frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x})^2}}$$

- Confidence limits slope and intercept

$$b \pm t_{(n-2)} s_b \qquad\qquad a \pm t_{(n-2)} s_a$$

t-value taken as n-2 degrees of freedom from table under confidence interval (usually 95%).

- Calculation of a concentration and its random error (uncertainty)

$$s_{x_0} = \frac{s_{y/x}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}}$$

$$x_0 \pm t_{(n-2)} s_{x_0}$$

Appendix II. Additional Method Validation Data

Table 41 Data of calibration standards

	x_i	x_i^2	y_i	$x_i - \bar{x}$	$(x_i - \bar{x})^2$	$y_i - \hat{y}$	$(y_i - \hat{y})^2$	$y_i - \bar{y}$	$(y_i - \bar{y})^2$	$(x_i - \bar{x})$ $(y_i - \bar{y})$
1	0.1	$1.00E^{-2}$	$3.06E^{2}$	$-1.85E^{2}$	$3.43E^{4}$	$1.72E^{5}$	$2.96E^{10}$	$-2.43E^{7}$	$5.90E^{14}$	$4.49E^{9}$
2	0.5	$2.50E^{-1}$	$2.76E^{3}$	$-1.85E^{2}$	$3.41E^{4}$	$1.22E^{5}$	$1.48E^{10}$	$-2.43E^{7}$	$5.90E^{14}$	$4.48E^{9}$
3	1.0	$1.00E^{0}$	$8.16E^{3}$	$-1.84E^{2}$	$3.39E^{4}$	$6.10E^{4}$	$3.72E^{9}$	$-2.43E^{7}$	$5.89E^{14}$	$4.47E^{9}$
4	5.0	$2.50E^{1}$	$2.39E^{5}$	$-1.80E^{2}$	$3.25E^{4}$	$-2.37E^{5}$	$5.63E^{10}$	$-2.40E^{7}$	$5.78E^{14}$	$4.33E^{9}$
5	10.0	$1.00E^{2}$	$5.31E^{5}$	$-1.75E^{2}$	$3.07E^{4}$	$-6.05E^{5}$	$3.66E^{11}$	$-2.38E^{7}$	$5.64E^{14}$	$4.16E^{9}$
6	50.0	$2.50E^{3}$	$5.03E^{6}$	$-1.35E^{2}$	$1.83E^{4}$	$-1.39E^{6}$	$1.94E^{12}$	$-1.93E^{7}$	$3.71E^{14}$	$2.60E^{9}$
7	100.0	$1.00E^{4}$	$1.35E^{7}$	$-8.52E^{1}$	$7.26E^{3}$	$4.65E^{5}$	$2.16E^{11}$	$-1.08E^{7}$	$1.16E^{14}$	$9.19E^{8}$
8	500.0	$2.50E^{5}$	$6.87E^{7}$	$3.15E^{2}$	$9.91E^{4}$	$2.77E^{6}$	$7.68E^{12}$	$4.44E^{7}$	$1.97E^{15}$	$1.40E^{10}$
9	1000.0	$1.00E^{6}$	$1.31E^{8}$	$8.15E^{2}$	$6.64E^{5}$	$-1.36E^{6}$	$1.84E^{12}$	$1.06E^{8}$	$1.13E^{16}$	$8.66E^{10}$
Means:	185.2	$1.40E^{5}$	$2.43E^{7}$	0	$1.06E^{5}$	0	$2.44E^{15}$	0	$1.85E^{15}$	$1.40E^{10}$
Sums:	**1666.6**	**$1.26E^{6}$**	**$2.19E^{8}$**	**0**	**$9.54E^{5}$**	**0**	**$1.21E^{13}$**	**0**	**$1.67E^{16}$**	**$1.26E^{11}$**

Equation 1 Calculcation of correlation coeficient and coeficient of determination

$$r = \frac{1.26\times10^{11}}{\sqrt{(9.54\times10^{5})(1.67\times10^{16})}} = 0.99964 \quad \rightarrow \quad r^2 = 0.996^2 = 0.99927$$

$$R^2 = \frac{1.67\times10^{16} - 1.21\times10^{13}}{1.67\times10^{16}} = 0.99927 \quad \rightarrow \quad r^2 = R^2$$

Equation 2 Calulation of t-value for the determination of a significant correlation

$$t = \frac{7}{\sqrt{1-0.99927}} = 259.36$$

Equation 3 Calibration and error calulations for putrescine

$$b = \frac{1.26\times10^{11}}{9.54\times10^{5}} = 132146 \qquad a = 2.43\times10^{7} - 1.32\times10^{5}\cdot 185.2 = -184966$$

$$s_{y/x} = \sqrt{\frac{1.21\times10^{13}}{7}} = 1.32\times10^{6}$$

$$s_b = \frac{1.32\times10^{6}}{\sqrt{9.54\times10^{5}}} = 1.35\times10^{3} \qquad s_a = 1.32\times10^{6}\sqrt{\frac{1.26\times10^{6}}{9\times9.54\times10^{5}}} = 5.05\times10^{5}$$

$$95\%\ CI\ slope = 2.36\cdot 1.35\times10^{3} = 3183$$

$$95\%\ CI\ intercept = 2.36\cdot 5.05\times10^{5} = 1192050$$

$$s_{x_0} = \frac{1.32 \times 10^6}{132146}\sqrt{1 + \frac{1}{9} + \frac{(1.31 \times 10^8 - 2.43 \times 10^7)^2}{132146^2 \cdot 9.54 \times 10^5}} = 13.3$$

$$95\%\ CI = 2.36 \cdot 13.3 = 30.1$$

Appendix III. Mobile phase combinations

Method	Instrument/ Detector	Stationary phase	Flow (ml/min)	Detection settings	Eluent		Curve	Run time (min)
1	HPLC-DAD	C18	1.0	260/ 280	I	20min 40% MeCN 60% H_2O	0	20
2	HPLC-DAD	C18	1.0	260/ 280	I	20min 30% MeOH 70% H_2O	0	20
3	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% H_2O 10min 50% MeOH 50% H_2O	0 1	20
4	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% H_2O 5min 50% MeOH 50% H_2O 3min 10% MeOH 90% H_2O	0 1 1	18
5	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% H_2O 3min 50% MeOH 50% H_2O 3min 10% MeOH 90% H_2O 4min 10% MeOH 90% H_2O	0 1 1 0	20
6	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% PO_4 Buffer (pH7.0) 5min 50% MeOH 50% PO_4 Buffer (pH7.0) 3min 10% MeOH 90% PO_4 Buffer (pH7.0)	0 1 1	18
7	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% HCOO Buffer (pH3.2) 5min 50% MeOH 50% HCOO Buffer (pH3.2 3min 10% MeOH 90% HCOO Buffer (pH3.2	0 1 1	18
8	HPLC-DAD	C18	1.0	260/ 280	G	10min 10% MeOH 90% PO_4 Buffer (pH3.0) 5min 50% MeOH 50% PO_4 Buffer (pH3.0) 3min 10% MeOH 90% PO_4 Buffer (pH3.0)	0 1 1	18
9	HPLC-DAD	C18	1.0	260/ 280	G	10min 0% MeCN 10% MeOH 90% HCOO Buffer 3min 30% MeCN 10% MeOH 60% HCOO Buffer 3min 30% MeCN 10% MeOH 60% HCOO Buffer 1min 0% MeCN 10% MeOH 90% HCOO Buffer 2min 0% MeCN 10% MeOH 90% HCOO Buffer	0 1 0 1 0	19
10*	HPLC-DAD	C18	1.0	260/ 280	G	5min 0% MeCN 10% MeOH 90% HCOO Buffer 3min 30% MeCN 10% MeOH 60% HCOO Buffer 3min 50% MeCN 10% MeOH 40% HCOO Buffer 3min 50% MeCN 10% MeOH 40% HCOO Buffer 2min 0% MeCN 10% MeOH 90% HCOO Buffer 4min 0% MeCN 10% MeOH 90% HCOO Buffer	0 1 1 0 1 0	20

Method	Instrument/ Detector	Stationary phase	Flow (ml/min)	Detection settings	Eluent		Curve	Run time (min)
11	HPLC-DAD	Silica	1.0	260/ 280	I	20min 90% MeCN 10% H_2O	0	20
12*	HPLC-DAD	Silica	1.0	260/ 280	G	5min 90% MeCN 10% H_2O 7min 70% MeCN 30% H_2O 3min 90% MeCN 10% H_2O 5min 90% MeCN 10% H_2O	0 1 1 0	20
13	HPLC-DAD	Silica	1.0	260/ 280	I	20min 95% MeCN 5% H_2O	0	20
14	HPLC-DAD	Silica	1.0	260/ 280	I	20min 98% MeCN 2% H_2O	0	20
15*	HPLC-DAD	Silica	1.0	260/ 280	G	5min 98% MeCN 2% H_2O 7min 70% MeCN 30% H_2O 3min 98% MeCN 2% H_2O 5min 98% MeCN 2% H_2O	0 1 1 0	20
16	HPLC-DAD	Silica	1.0	200/ 220	I	20min 98% MeCN 2% H_2O	0	20
17	HPLC-DAD	Silica	1.0	260/ 280	I	20min 98% MeCN 2% Acetate Buffer (pH 5.8)	0	20
18	HPLC-DAD	Silica	1.0	260/ 280	G	5min 98% MeCN 2% Acetate Buffer (pH 5.8) 7min 60% MeCN 40% Acetate Buffer (pH 5.8) 3min 98% MeCN 2% Acetate Buffer (pH 5.8) 5min 98% MeCN 2% Acetate Buffer (pH 5.8)	0 1 1 0	20
19	HPLC-DAD	Silica	2.0	260/ 280	I	20min 98% MeCN 2% Acetate Buffer (pH 5.8)	0	20
20	HPLC-DAD	Silica	1.0	260/ 280	I	20min 98% MeCN 2% Formate Buffer (pH 3.2)	0	20
21	HPLC-DAD	Silica	1.0	260/ 280	G	5min 98% MeCN 2% Formate Buffer (pH 3.2) 7min 60% MeCN 40% Formate Buffer (pH 3.2) 3min 98% MeCN 2% Formate Buffer (pH 3.2) 5min 98% MeCN 2% Formate Buffer (pH 3.2)	0 1 1 0	20
22	HPLC-DAD	Silica	0.5	260/ 280	I	20min 98% MeCN 2% Formate Buffer (pH 3.2)	0	20
23	HPLC-DAD	Silica	1.0	260/ 280	I	20min 10% MeCN 90% Formate Buffer pH (3.2)	0	20
24	HPLC-UV/FL	C18	1.0	210	G	10min 10% MeOH 90% H_2O 5min 50% MeOH 50% H_2O 3min 10% MeOH 90% H_2O	0 1 1	18

Method	Instrument/ Detector	Stationary phase	Flow (ml/min)	Detection settings	Eluent		Curve	Run time (min)
25	HPLC-MS	Cation + Anion exchange	0.35	DAD/ +/ -	G	5min 5% MeOH 90% H_2O 5% 10mM Ammonium Formate (pH 3.2) 20min 85% MeOH 5% H_2O 5% 10mM Ammonium Formate (pH 3.2)	0 1	25
26	HPLC-MS	Cation + Anion exchange	0.35	DAD/ +/ -	G	5min 5% MeOH 85% H_2O 10% 10mM Ammonium Formate (pH 3.2) 20min 90% MeOH 10% 10mM Ammonium Formate (pH 3.2)	0 1	25
27	HPLC-MS	PFP	0.35	DAD/ +/ -	G	5min 5% MeOH 90% H_2O 5% 10mM Ammonium Formate (pH 3.2) 20min 85% MeOH 5% H_2O 5% 10mM Ammonium Formate (pH 3.2)	0 1	25

* = Cleanup method

Appendix IV. Molecular Formulae Calculations

Peak detected is M+H = 88.4. M =87.4

The abundance for A+1 and A+2 need to be normalised, see table below.

A	A+1	A+2
4766155	187367	0
100%	3.93%	0%

3.93% is A+1, +/-10% = 4.32% and 3.54%.
So most likely 4 or 5 carbon atoms present in molecule.

87.4 has an odd mass number so according to the nitrogen rule it will have an odd number of nitrogen atoms. This will be 1 or 3 nitrogen atoms in the molecule.

4 carbon atoms, 4 * 12.01 = 48.04. 87.4 - 48.04 = 38.56 left
5 carbon atoms, 5 * 12.01 = 60.05. 87.4 - 60.05 = 27.35 left
1 nitrogen, 1 * 14.01 = 14.01
3 nitrogen, 3 * 14.01 = 42.03
So only 1 Nitrogen atom possible

When including oxygen to the formulae the following possibilities appear.

4 carbon Atoms	5 carbon Atoms
1. $C_4H_{25}N$	3. $C_5H_{13}N$
2. C_4H_9NO	

After calculation the rings and unsaturation's using the following formulae:
X - 0.5Y + 0.5Z + 1 = number of rings and unsaturation's.

X = the total amount of carbon and silicon atoms.
Y = the total amount of hydrogen, chlorine, fluorine and iodine atoms.
Z = the total number of nitrogen and phosphorous atoms.

4 – (25 / 2) + 0.5 + 1 = -7
4 – (9 / 2) + 0.5 + 1 = 1
5 – (13 / 2) +0.5 + 1 = 0

Molecule 1 has a negative number of rings and unsaturation's and is thus not possible, leaving molecules 2 C_4H_9NO and 3 $C_5H_{13}N$.