Faculty of Science and Engineering
Department of Chemistry

Chemical Markers of Decomposition for Forensic Science

Lisa Swann

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:……..

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For Alex

and Charlotte

ABSTRACT

This thesis describes investigations that were carried out to determine the chemical compounds produced during the decomposition of an animal model in the absence of a soil matrix. In order to do this, several analytical methods were developed for various classes of compounds. Stillborn piglets and whole adult pig carcasses were used to model the decomposition process.

Samples for analysis were collected from field trials conducted at decomposition research facilities in Perth and Canada. Two separate locations were used to provide a 'compare and contrast' approach to the identities of compounds detected following the analysis of decomposition fluid.

Gas chromatography-mass spectrometry was used for preliminary studies into short chain fatty acids that have the potential to show reproducible patterns over certain postmortem intervals. Samples were analysed following a simple aqueous dilution and filtration. Additional compounds were detected, including several long chain fatty acids, which were also investigated for their potential as indicators of postmortem interval. Samples collected from the two separate locations, Western Australia (Perth) and Southern Canada (Oshawa) were analysed. This enabled a comparison of components to be carried out under significantly different climatic conditions. To verify the identity of the compounds, the predicted fragmentation patterns and possible mechanisms based on the library search results were also determined and compared with the obtained mass spectral traces from the fluid samples.

A simple capillary zone electrophoresis method with detection by ultraviolet absorbance spectrophotometry was developed for the determination of biogenic amines and amino acids. Resolution and total analysis time was improved after the method was subject to optimisation utilising a chemometric approach. A screening design followed by a central composite design was carried out, with peak resolution and total analysis time as response factors.

The optimised method was applied to porcine decomposition samples with target analytes identified by migration time and spiking. Samples were analysed following a 1:4 dilution with methanol, followed by filtration.

A method utilising liquid chromatography-electrospray-mass spectrometry was employed for the determination of 23 amino acids and amines in decomposition fluid. The effect of a complex sample matrix was investigated and found to have little to no effect on the analyte signal. Decomposition fluid samples required no sample preparation, other than filtration. To avoid overloading the column, optimum sample injection volume was 0.1 μL. Compounds were identified through precursor → product ion transition(s). The specificity of the LC-ESI-MS system enabled identification of all target compounds as being present in decomposition fluid. The identity of compounds that showed apparent trends in decomposition fluid was verified by predicting possible mechanisms for the precursor → product ion transition(s).

Analysis of data from each developed analytical method was conducted to establish any distinct relationship between the levels of particular compounds produced with respect to time and temperature. Preliminary results indicate that fatty acids show an 8-day cyclic trend, whilst total amino acid abundance shows a 14-day cyclic trend. Other compounds such as indole and putrescine showed general increasing trends over the course of the field trials.

Several analytical methods to analyse target compounds in decomposition fluid have been presented in this thesis, however, suggestions for future work are presented in the final chapter.

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ABBREVIATIONS

ADD	Accumulated degree-days
APCI	Atmospheric pressure chemical ionisation
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
CDH	Cumulative degree hours
CE	Capillary electrophoresis
CIA	Capillary ion analysis
CSF	Cerebrospinal fluid
CZE	Capillary zone electrophoresis
DRIFT	Diffuse reflectance infrared Fourier transform
ESI	Electrospray ionisation
FID	Flame ionisation detector
FTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HIBA	d,l-alpha-hydroxybutyric acid
HPLC	High performance liquid chromatography
Hx	Hypoxanthine
ISD	Internal standard
ISE	Ion selective electrodes
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LPIC	Low pressure ion chromatography
MRM	Multiple reaction monitoring
NRN	Ninhydrin reactive nitrogen
OPA	o-Phthalaldehyde
PBI	Postburial interval
PMI	Postmortem interval
PLOT	Porous layer open tubular
VFA(s)	Volatile fatty acid(s)
VOC(s)	Volatile organic compound(s)

SIA	Sequential injection analysis
SPE	Solid phase extraction
SPME	Solid phase micro extraction
TD/GC-MS	Thermal desorption/Gas chromatography - mass spectrometry
TIC	Total ion chromatogram
TMA	Trimethylacetic acid
UOIT	University of Ontario Institute of Technology
UV-Vis	Ultraviolet-Visible

CHAPTER 1 -INTRODUCTION

1.1 INTRODUCTION

The study of mammalian soft tissue decomposition is an emerging area in forensic science, with a major focus of the research being the use of various chemical and biological methods to study the fate of human remains in the environment. Such techniques have major roles to play in locating clandestine gravesites [1] and assisting in the estimation of postburial interval [2].

Decomposition of mammalian soft tissue is a postmortem process that, depending on environmental conditions and physiological factors, will proceed until complete disintegration of the tissue [3]. An understanding of this process is extremely important for investigations of suspicious deaths as it complicates determination of cause of death and makes the estimation of postmortem interval very difficult. The major stages of decomposition involve complex reactions which result in the chemical breakdown of the body's main constituents; lipids, proteins, nucleic acids and carbohydrates [4]. As decomposition proceeds, these macromolecules degrade to their structural components which include amino acids, fatty acids, and glucose [5]. The precise details of the biochemical pathways involved, along with a detailed knowledge of the temporal and environmental variation that may be observed have yet to be examined in detail. It has also been suggested that temporal variation of certain chemical species, in particular volatile fatty acids, could be used to age decomposing remains and potentially aid in the determination of postmortem interval [6].

The first step to understanding this chemistry is identifying the compounds present in decomposition fluids and determining when they are produced. However, decomposition fluid is a challenging sample to study, as it is a complex chemical mixture with associated insect life, microbial organisms and other debris. While methods using colourimetry or Fourier-transform infrared spectrophotometry can provide useful trend information, more selective analytical techniques are required to provide the level of chemical knowledge needed to fully understand the fundamental chemistry of decomposition.

Separation science techniques such as gas chromatography, liquid chromatography and capillary electrophoresis offer this level of specificity. This chapter provides an overview of decomposition chemistry and reviews recent advances in this area utilising analytical separation science.

1.2 MAMMALIAN DECOMPOSITION

After death, many physicochemical processes take place within the corpse, with the resulting changes from these occurring in a predictable order. The rate of change is strongly influenced by environmental factors, the most significant being temperature [7-10]. The physical principle known as Van't Hoff's rule, or the rule of ten, states that the velocity of chemical reactions increases two or more times with each 10 °C rise in temperature [10]. All decomposing bodies will go through the same decomposition process, but it is the variability in temperature that will determine the length of each stage and the overall velocity of the process [10]. A deviation in the body's ambient temperature to warmer or cooler surroundings will affect cellular metabolism by affecting the enzyme catalysts that regulate reactions within the body [10]. Within a few hours of death, investigators may use the rate of cooling of the body, postmortem lividity, potassium levels in vitreous fluid, and rigor to estimate postmortem interval. However these methods can only be used shortly after death, and have limited accuracy [7, 8]. Thus, once visible decomposition changes commence, the techniques available to the forensic scientist are greatly reduced.

Decomposition commences almost immediately after death [11], sometimes within the first 4 minutes [5, 12], depending on environmental conditions and surroundings. It can be characterised by two main stages, pre and post skeletonisation [6]. Pre-skeletonisation can be further broken down into four subsequent stages; fresh, bloated, decay and dry, as first described by Reed in 1958 [13]. A summary of the physical and chemical changes observed in each of these stages can be seen in Table 1.1.

TABLE 1.1. OVERVIEW OF MAMMALIAN DECOMPOSITION.

STAGE OF DECOMPOSITION	DESCRIPTION	REFERENCE
Fresh	Commences immediately after death	**[13]**
	Autolysis	**[4, 5]**
	Fluid filled blisters on the skin, skin slippage	**[4, 5, 12, 14]**
	Marbling of skin due to livor mortis	
Bloating	Putrefaction – destruction of soft tissues by microorganisms	**[5, 11]**
	Greenish discolouration of the skin	**[4, 5, 12]**
	Gas and fluid accumulation followed by purging	**[12]**
	Anaerobic fermentation	
Active Decay	Bloating has ceased	**[13]**
	Skin usually broken in one or more places	**[5, 13]**
	Rapid leaching from body	
	Large numbers of aerobic and anaerobic bacteria	**[5, 11, 14]**
	Extensive insect activity	**[14]**
	Possible carnivore activity	
	Collapse of abdominal cavity	**[12, 14]**
	Loss of internal organs through insect activity or autolysis	**[14]**
	Possible adipocere formation	
Dry	Diagenesis	**[12-14]**
	No carrion fauna remaining	**[13]**
	Small amount of decaying tissue	
	Mummification of remaining skin	**[14]**
	Bone exposure and skeletonisation	**[11, 14]**

The first identifiable process is autolysis, occurring during the fresh stage and literally meaning self-digestion of cells. Autolysis occurs first in the most metabolically active cells, i.e. cells where characteristically high rates of ATP production are more sensitive to anoxia [10], a total decrease in the level of oxygen. It is thought to be triggered by the decrease in intracellular pH occurring as a result of the decreased oxygen levels after death [4]. The onset of autolysis can be observed by the presence of fluid filled blisters on the skin, and skin slippage [5, 12] as cellular membranes dissolve, releasing nutrient rich cellular fluids into the body [12]. Tissues containing cells with the highest rate of ATP synthesis and membrane transport should typically decompose first [10].

Following autolysis, the second process in decomposition is the bloated stage, where the beginnings of putrefaction are seen. This is the destruction of the soft tissues of the body by the action of microorganisms (bacteria, fungi and protozoa) in a mostly anaerobic environment. Bloating may also be accompanied by moderate maggot activity and a strong odour [14]. Putrefaction is identifiable by a green/purple discolouration of the skin and occurs usually between 36 and 72 hours after death [5, 10, 11]. The discolouration seen on the body is the result of variously coloured pigments released into the tissues of the abdomen by lysing pancreatic cells [10]. The result of putrefaction is the catabolism of carbohydrates, proteins and lipids present in soft tissues into gases, liquids and small molecules [5, 10, 12]. The trapped volatile gases and fluid are responsible for the bloating of the carcass and a build up in pressure can result in purging from the body, often severe enough to cause additional postmortem injuries [5, 12].

Following the purging of gases, the active decay stage begins. Additional volatile fatty acids are produced from the continued breakdown of muscle, and the decomposition of protein and fat producing phenolic compounds and glycerols [5, 12]. Decomposition products such as putrescine and cadaverine are also detectable in this stage and insect activity is at a premium [6].

Putrefaction is still occurring during active decay, as chemical constituents are still being degraded and released.

The dry stage is the final stage of the decomposition process. In this stage, any remaining moist skin and tissue is converted to a 'leathery-like' sheet that adheres to bone [5, 12]. Skeletonisation is characterized by the appearance of exposed bone in over 50% of the body, but erosion of the skeletal elements has not yet begun [14]. This process will proceed until only the harder and more resistant bone, teeth and cartilage remain [11]. Chemical weathering of these remains continues but the time taken for this deterioration is considerably longer than previous stages of decomposition. Diagenesis, or the exchange of ionic species between bones and their surrounding environment, occurs, but at a rate dependent on the changing ratios of calcium and phosphate in a variety of soils [10].

Although decomposition is divided into these four stages, the distinction between the stages can be difficult to identify. Alternatively, stages may be absent altogether.

1.2.1 Factors affecting mammalian decomposition

The interrelated chemical and physical changes observed during mammalian decomposition are strongly influenced by environmental conditions with ambient temperature having the greatest impact [5, 9, 15]. Climatic conditions such as temperature, humidity, rainfall can affect the presence or absence of insects [5, 9, 16], a major contributor to the rate of decomposition. The accessibility of insects is also governed by clothing, burial depth and wounds on the carcass [9, 15, 17]. Other factors that can contribute to decomposition rates are carnivore activity, soil pH and size and weight of the original carcass [9, 17].

Variability in temperature is the most important and significant factor influencing the rate of decomposition of a corpse [6, 9, 12, 14]. High temperatures can increase the rate of decomposition, while temperatures that

are low or freezing can have an adverse affect by severely decreasing the decomposition rate. This finding is supported in a study by Archer in which higher temperatures were found to increase the mass loss rates and decomposition stage progression rates of exposed neonatal remains [16]. Temperature can also affect the presence or absence of insects and bacteria as well as the breakdown of proteins and carbohydrates present in the body [6]. Vass et al state that decomposition will occur down to 0°C due to salt concentrations in the body [6]. However, this statement is not supported with experimental evidence, so the temperature at which decomposition processes cease is not accurately known.

Meygesi *et al*. suggest that decomposition rates and a more accurate estimation of post-mortem interval (PMI) can be achieved when accumulated temperature is taken into account rather than individual daily temperatures [18]. Accumulated degree-days (ADD) are calculated by taking the sum of the average daily temperature for the length of time the corpse has been decomposing [18]. Vass suggests that the use of cumulative degree hours (CDH) is a more accurate technique [5]. CDH uses a twelve-hour temperature cycle in which the average temperature is calculated for every complete cycle rather than the average daily temperature. Both are linear models that can be used to estimate the PMI by succession and development of carrion insects if the temperature data is known for a specific area [19].

The second most important factor affecting decay rates and hence the resultant calculations to determine PMI is the accessibility and activity of insects [9, 14, 17, 20]. Flies and other insects are attracted to a corpse at various stages during decomposition, depending on the volatile compounds produced and associated odours that are emitted. The overwhelming majority of soft tissue destruction during decomposition is due to feeding by insects [9]. If insects are absent from the corpse, it can be assumed that decomposition will proceed at a significantly slower rate [9, 20, 21].

When a subject is buried, decomposition proceeds at a much slower rate. This is possibly due to the restricted access of insects to the corpse [14, 17, 20] and the decreased exposure and variation of environmental temperatures and rainfall [6]. The cumulative effect of these factors makes burial a significant consideration when examining rates and stages of decomposition [6, 9, 17]. Other factors that influence the rate of decomposition of a corpse include body size and weight, the presence or absence of carnivores, humidity, clothing, trauma to the body and soil pH [9]. The difficulty in estimating postmortem interval is that each factor does not stand-alone. Each factor is significantly inter-related with another.

1.2.2 Estimation of postmortem interval

Postmortem interval is a fundamental question in forensic investigations when reconstructing events surrounding suspicious deaths. This essential knowledge establishes a time frame that enables forensic personnel to focus investigative efforts around the time of the death, leads to greater efficiency of resource allocation and can save vital time. Scientific evidence of time of death is preferable to circumstantial information or witness testimony, which is vulnerable to mistakes or deception; however, it is notoriously difficult to estimate. Scientifically measured parameters that can be used to estimate time of death may be obtained from the body and its immediate surroundings [7, 8].

There are several methods currently recognized as useful in the estimation of postmortem interval including the extent of mortis. This covers algor mortis (the body acclimated to ambient temperature), livor mortis (discolouration of the skin) and rigor mortis (gelling cellular cytoplasm due to increased acidity leading to progressive stiffening and relaxing of the muscles in the body) [5]. However, these techniques are limited to the early periods after death (i.e. first 48 hours) [20, 22].

Other methods for the estimation of PMI include detection of levels of potassium and hypoxanthine in vitreous humor [5, 23-25], the extent of DNA

deterioration [26] and cooling of the corpse to ambient temperature [27]. These techniques are also limited and less reliable in the advanced stages of decomposition. The investigation into DNA degradation by Perry *et al.* proved promising for early to intermediate stages in the decomposition process, although sample preparation is complicated and not easily performed. This method of determining PMI is also yet to be field-tested.

Determinations that rely on the above methods show differences when compared with each other in estimating PMI [27]. They are also subjective, and rely on the experience of the examiner, leaving them open to a wider range of error [5, 20].

In the later stages of decomposition, forensic entomology has been found to be a useful tool in the estimation of postmortem interval [5]. Forensic entomology involves the recognition of arthropods (mostly insects), coupled with their sequential arrival times on a corpse and the developmental timetables of their offspring, to estimate the time and sometimes cause of death [28]. This technique has been studied and used with a certain degree of success [13, 17, 21, 28-32]. There are, however, situations in which the corpse has reached complete skeletonisation, or factors have affected the decay process (such as burial or low temperatures, insect access and presence of drugs)[33-36]. In such an event, the estimation of PMI via entomology becomes difficult if not impossible [37].

Vass and co-workers suggested that the use of various time dependant biomarkers, such as fatty acids, amino acids and neurotransmitters produced during soft tissue decomposition, might be a valuable alternative method in estimating time since death (TSD) [6]. Vass used soil solution analysis to look at five particular volatile fatty acids (VFAs), also known as short chain acids, and the distinct variation in the acids over time, with regards to accumulated temperature. The conclusion by Vass is that VFAs can be accurate and reliable biomarkers indicating various stages of decomposition. The estimation given by Vass using VFAs and neurotransmitters is ±2 days and ±2 weeks respectively.

However, the conclusion is not supported by relevant experimental evidence, and therefore, extrapolations to various environmental conditions cannot be made.

1.3 EARLY STUDIES INVOLVING BIOMARKERS (NON-CHROMATOGRAPHIC)

1.3.1 CHEMICAL TECHNIQUES FOR LOCATING CLANDESTINE HUMAN REMAINS

A major area of investigation within the field is the application of physical and chemical techniques for locating clandestine human remains. In many instances, bodies are concealed by burial, which has a confounding effect on the search and location of the remains. Numerous techniques have been applied to forensic search and recovery situations including the use of cadaver dogs [38], botanical evidence associated with grave sites [39, 40], probing [41], thermal imaging [39], and the use of geophysical equipment such as the ground penetrating radar [42-44]. Each of these techniques has associated limitations and is best applied in a sequential manner for locating buried human remains [39].

Recently, chemical analysis of soils related to decomposition sites has been proposed as an alternative method for detecting clandestine graves [45-47]. The process of soft tissue decomposition results in a significant pulse of nutrients into the soil surrounding a cadaver. A proportion of these nutrients result from the release of nitrogen-containing compounds within the body, which include proteins, peptides, amino acids, and amines [11]. These nitrogenous compounds have the ability to react with ninhydrin, a chemical that is commonly available in police and forensic laboratories due to its use as a chemical enhancement technique for fingerprint visualisation [48]. In 2008, Carter *et al.* hypothesised that the decomposition of a body would result in an increase in ninhydrin reactive nitrogen (NRN) and may provide a new technique for identifying gravesoil (i.e. soil associated with a decomposing cadaver) [45, 49].

Their study involved the burial of juvenile rats (*Rattus rattus*) in three contrasting soil sites followed by sequential harvesting at pre-assigned postburial intervals (PBI). Gravesoil collected from beneath the decomposing

cadavers was analysed using an NRN chemical protocol which involved the extraction of nitrogenous-compounds from the soil, reaction with ninhydrin reagent, and determination of the absorbance at 570 nm using spectrophotometry [50]. The concentration of NRN was determined by comparison with a leucine standard. The gravesoil samples demonstrated a 1.4-2.2-fold increase in NRN when compared to the control samples. The authors suggested that the significant increase of NRN in soil following decomposition of a cadaver had the potential to be used as an investigative technique for locating clandestine gravesites [45].

An additional study applied the NRN technique proposed by Carter *et al.* [45] to an investigation of swine (*Sus domesticus*) carcass decomposition in both burial and surface decomposition scenarios [47]. The study involved the use of larger swine carcasses as analogues for human decomposition. Six carcasses were placed in shallow gravesites and a sequential destructive sampling regime was used to collect gravesoil samples at specific postburial intervals. This study expanded on the study conducted by Carter *et al.* [45] by investigating the lateral diffusion of NRN from the decomposition site. The authors hypothesised that NRN concentrations would increase as decomposition proceeded, but would decrease as the distance from the cadaver increased [47]. To study this hypothesis, gravesoil samples were collected from the centre and edge of each gravesite once a month for a period of six months, as well as from the base and walls of the graves at the time of exhumation. Additionally, five swine carcasses were placed on a soil surface and allowed to decompose for 97 days during which time soil samples were collected from beneath the decomposing carcasses. The ninhydrin analysis was adapted from Carter *et al.* [45] and the absorbance was scanned from 500-640 nm using a UV-Visible Spectrometer. The results indicated that NRN was a useful technique for detecting gravesoil in the early postmortem period (~2 months postburial) and that the lateral diffusion of nitrogen influx was narrow in a gravesite. In contrast, the NRN concentration was significantly higher during both the early and later postmortem stages for the carcasses decomposing on the surface. In addition to its potential application as an investigative tool for locating clandestine buried

remains, the authors also proposed its use for detecting the original site of decomposition for cadavers that have been scavenged or subjected to postmortem relocation.

A case summary presented in 2009 details the application of the NRN technique to an outdoor death scene following disturbance of the remains, most likely through the activity of scavengers [46]. The colourimetric technique was used to assist with determining the location of death and the origin of decomposition. Soil samples were collected from several locations including the presumptive primary decomposition site (head and torso), the lower body, arm and hand, scalp, and control sites. The soil collected from the presumptive decomposition site demonstrated a significantly higher concentration of NRN than the other sites and it was concluded that decomposition had occurred in this location. The value of this method as a quantitative chemical alternative for locating decomposed human remains and decomposition sites was thus confirmed [46]. However, exactly what constitutes NRN in the context of these decomposition studies has yet to be established.

A novel NRN technique using headspace collection has recently been proposed [51] and is based on a purge and trap method using a motorized pipette and an activated, alumina-coated, porous layer open tubular (PLOT) column. The study utilized feeder rat carcasses placed on either the soil surface or buried within the soil, then sealed in pressed wooden boxes. Samples were collected by drawing the headspace through PLOT columns, which were pierced through septum ports placed on the compartment lids. The adsorbed compounds were eluted by forcing a 2M KCl solution through the PLOT column and into a vial containing ninhydrin reagent. Following dilution with an ethanol solution, the absorbance of the sample was measured at 570 nm using a diode-array UV-Vis spectrophotometer. The method was successful at detecting NRN with only a 5 min headspace collection period. The average recovered masses of NRN were higher for both exposed and buried carcasses when compared to the blank (soil) samples. NRN was not detected during the first 4 weeks of decomposition but was detected in the headspace of the compartments in weeks 5, 6, 10, and

20. In contrast to the study by Van Belle *et al.* [25], slightly higher masses of NRN were detected for the buried carcasses compared to the exposed carcasses. However, the experimental setup differed considerably between both studies (i.e. one was in a natural environment and the other was in a controlled environment) and this result may not be representative of all forensic scenarios. The study was useful in proposing an alternative use of the NRN technique for locating clandestine graves.

1.3.2 Chemical techniques for the identification of adipocere

Adipocere, a postmortem decomposition product that forms from the neutral fats present in soft tissue, may be found directly on decomposed remains or in the surrounding soil environment, especially in burials. Spectroscopic techniques have also been applied to forensic decomposition studies to identify fatty acid concentrations indicative of adipocere formation. The use of infrared spectroscopy as a qualitative tool for determining the lipid profile of soft tissue has been well documented [52-54]. In 2000, Stuart *et al.* [53] proposed a diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy technique to study soil samples believed to contain adipocere from human remains. The technique involved minimal sample preparation and was useful in demonstrating the degradation of triglycerides into fatty acids. Carbonyl bands in the 1740-1700 cm^{-1} region were identified as being characteristic of adipocere formation and assisted in determining the degree of formation that had occurred. DRIFT spectroscopy was shown to be a rapid and effective method for detecting and characterising adipocere formation in soils [53]. The same technique was also successfully applied to adipocere samples formed in aqueous environments [54]. The chemical composition of pig and human adipose tissue degradation was investigated over a period of 21 days. The lipid profile for both pig and human tissue demonstrated a decrease in carbonyl bands relating to triglycerides and a concomitant increase in bands relating to free fatty acids as immersion time increased. The formation of fatty acids in the human tissue samples appeared to occur more rapidly than in the pig tissue samples.

An alternative Fourier transform infrared (FTIR) spectroscopy method has been applied to the investigation of adipocere formed in soils and mock coffins [52]. Attenuated total reflectance (ATR) infrared spectroscopy is a surface analytical technique that requires little to no sample preparation [55]. Samples can be extracted using a solvent and cast as a film on a slide, then placed directly onto the ATR crystal. This method also proved to be effective in characterising the formation of adipocere in soils based on changes in the carbonyl region of the spectrum. Analysis by ATR spectroscopy was also valuable in demonstrating the affect of the burial environment on the rate of adipocere formation.

1.4 CHROMATOGRAPHIC STUDIES OF DECOMPOSITION PRODUCTS

While the non-chromatographic approaches described above have been valuable in providing a qualitative profile of the decomposition fluid as it is formed and released into the surrounding environment, further information is required regarding the biochemical pathways involved in the process. For this reason, separation methods are being investigated with the goal of providing quantitative data that will assist in strengthening the knowledge base relating to decomposition chemistry as it applies to forensic science.

Initial studies into the use of chromatographic methods for the analysis of decomposition products were very much based on the principle of identifying chemical markers that could be used to give an estimation of postmortem interval. The use of a chemical biomarker would aid in the elimination of examiner bias as well as providing an orthogonal method that would aid in offering a more reliable estimate of postmortem interval than existing techniques on their own. The earliest reports of this approach were made in the 1960's, focusing on the measurement of potassium ion (K^+) concentration in vitreous humor by flame photometry [56, 57], Whilst several studies have continued this work, there has been a shift away from the use of these early techniques towards separation science, which are less prone to matrix interferences [58]. Initial studies made use of gas chromatography, but more recently, liquid chromatography and capillary electrophoresis have been

applied in an attempt to utilise the temporal variation of the chemical markers of decomposition in order to estimate postmortem interval [5, 6, 59-62]. A summary of chromatographic techniques applied to various decomposition matrices is shown in Table 1.2, and discussed below.

TABLE 1.2. SUMMARY OF CHROMATOGRAPHIC METHODS USED IN THE ANALYSIS OF MAMMALIAN SOFT TISSUE DECOMPOSITION PRODUCTS AND TO AID IN THE ESTIMATION OF POSTMORTEM INTERVAL. (NB. ALL ACRONYMS AND ABBREVIATIONS DETAILED ON PAGE XXII OF THESIS)

AUTHORS	ANALYSIS METHOD	MATRIX	PMI OF SAMPLES	ANALYTES	RUN TIME	DETECTION LIMIT	APPLICATION
Patrick and Logan [63]	Technicon TSM amino acid analyser	Vitreous humor	Up to 96 hours	Free amino acids	Not given	Not given	Estimation of PMI in infants
Rognum *et al.* [64]	HPLC – UV (254 nm)	Vitreous humor	<120 hours	Hypoxanthine (Hx)	Not given	None given	Determination of PMI (Intercept: 7.6 μmol/L, Slope: 5 °C: 4.2, 10 °C: 5.1, 15 °C: 6.2, 23 °C: 8.8 μmol/L/hr)
Vass *et al.* [6]	GC-FID	Soil solution	Up to 70 days	VFAs	Not given	Not given	Estimation of PMI
Madea [65]	HPLC	Vitreous humor, Cerebrospinal Fluid (CSF)	Known but not given	Hx	Not given	Not given	Estimation of PMI in infants
James *et al*. [25]	HPLC-UV (250 nm)	Vitreous humor	Known but not given	Hx	Not given	Not given	Estimation of PMI, comparison of previously published equations.
Ferslew *et al.* [66]	CIA - indirect UV (214 nm)	Vitreous humor	Not given	K^+	7 mins	None given	Direct detection of cations for forensic analysis
Tagliaro *et al.* [67]	CZE – indirect UV (214 nm)	Vitreous humor	5-96 hours	K^+	3 mins	9.0 μM	Validation of a new method/ Determination of PMI
Tagliaro [58]	CE – indirect UV (214 nm)	Vitreous humor	7-144 hours	K^+	3 mins	Not given	Variability in potassium concentrations between two eyes of identical PMI

Authors	Analysis Method	Matrix	PMI of samples	Analytes	Run time	Detection Limit	Application
Bocaz-Beneventi *et al.* [68]	CZE –indirect UV (214 nm)	Vitreous humor	3-144 hours	K^+, NH_4^+, Na^+, Ba^{2+} (ISD)	3 mins	None given	Improving estimation of PMI with artificial neural networks
Munoz Barus [69]	HPLC	Vitreous humor	1-28 hours	Hx	Not given	Not given	Estimation of PMI
Vass *et al.* [5]	GC-MS	Soil solution	Up to 3 weeks	Amino acids, putrescine, cadaverine,	Not given	Not given	Estimation of PMI
Forbes *et al.* [70]	GC-MS	Adipocere	Not given	Long chain fatty acids, methyl ester derivatives	Not given	Not given	Detection of adipocere in grave soils
Vass *et al.* [71]	TD/GC-MS	Headspace analysis	Up to 1.5 years	VOCs	50 mins	Not given	Cadaver dog training, odour database
Statheropoulos *et al.* [72]	TD/GC-MS	Headspace analysis	3-4 weeks	VOCs	40 mins	Not given	Characterisation of VOCs from human decomposition
Munoz [73]	HPLC-UV (200-400 nm)	Vitreous humor	Not given	Hx	Not given	LOD:0.02 µM LOQ:0.08 µM	Estimation of PMI
Zhou *et al.* [74]	LPIC – conductivity det.	Vitreous humor	1-27 hours	K^+	6.7 mins (for K^+)	LOD: 1 mmol/L LOQ: 2 mM/L	Estimation of PMI
Statheropoulos *et al.* [75]	TD/GC-MS	Headspace analysis	Approx 4 days	VOCs	40 mins	Not given	Characterisation of VOCs from human decomposition

AUTHORS	ANALYSIS METHOD	MATRIX	PMI OF SAMPLES	ANALYTES	RUN TIME	DETECTION LIMIT	APPLICATION
Girela *et al.* [76]	HPLC – Fluorometric det.	Vitreous humor, CSF	5-60 hrs	Free amino acids, OPA derivatives	25 mins	Not given	Cause of death and estimation of PMI
Notter *et al.* [77]	SPE-GC-MS	Pig adipose tissue	Not given	Long chain fatty acids	26 mins	LOD: 5 mg/mL (S/N 3) LOQ: 10 mg/mL (S/N 3)	Quantitation of fatty acids in adipocere
Hoffman [38]	SPME-GC-MS	Headspace Analysis	Not given	VOCs	23.5 mins	Not given	Characterisation of VOCs from human remains for cadaver dog training
Notter *et al.* [54]	GC-MS	Pig adipose tissue, human skin and subcutaneous fat	Not given	Long chain fatty acids	Not given	Not given	Characterisation of adipocere
Dekeirsschieter *et al.* [78]	TD/GC-MS	Headspace analysis	Approx 2 months	Volatile organic compounds	36 mins	Not given	Comparison of VOCs from carcasses in 3 biotopes
Swann *et al.* [59]	GC-MS	Decomposition fluid	Up to 60 days	Volatile fatty acids Long chain fatty acids	25 mins	Not given	Characterisation of decomposition fluid

AUTHORS	ANALYSIS METHOD	MATRIX	PMI OF SAMPLES	ANALYTES	RUN TIME	DETECTION LIMIT	APPLICATION
Swann *et al.* [61]	CE-UV-Vis (200 nm)	Decomposition fluid	Up to 60 days	Biogenic amines Amino acids	12 mins	5.8-8.6 mg/L	Characterisation of decomposition fluid

1.4.1 VITREOUS HUMOR

In the early postmortem stages, one of the most widely used biochemical methods to estimate postmortem interval is the determination of potassium ion (K^+) and hypoxanthine (Hx) concentrations in vitreous humor [56, 57, 64, 79, 80]. The vitreous humor refers to the fluid that fills the vitreous body (or posterior chamber) of the eye [66]. Its potential usefulness for postmortem chemical analysis results from it being anatomically confined [67] and thus somewhat 'protected' and preserved for longer time periods from bacterial contamination, enzymatic activity and putrefactive changes [25, 57]. It has also been stated that vitreous humor has advantages for chemical analysis for biomarkers compared to the use of blood and cerebrospinal fluid (CSF) due to accessibility [57], ease of sampling [25], less susceptibility than blood to rapid chemical changes [80] and relative independence from environmental influences [81].

The following section discusses the most prominent analytes, potassium ions and hypoxanthine, which have been analysed in vitreous humor in an attempt to estimate postmortem interval. To date, no reliable method for postmortem interval estimation based on measurement of either Hx or potassium ions has been developed. There has been significant disagreement on how Hx and potassium ion concentrations vary in vitreous humor, and there is a wide variability in measurements taken between samples [25, 57, 64, 79, 80, 82, 83]. This unreliability limits the use of individual measurements of both potassium ions and Hx as indicators of postmortem interval. Various statistical approaches have been applied to attempt to improve the accuracy in estimation. Typically, the postmortem interval has been used as the independent variable and the potassium ion concentration as the dependent variable in linear regression analysis. Recently, it has been suggested that using potassium ion concentration as the independent variable will improve the postmortem estimation [69, 84]. This has also been the suggestion for the use of Hx in determining postmortem interval. However, a review in 2006 found that in a random sample of 492 cases, only 153 were within the predicted postmortem interval with the remaining

339 having a systematic overestimation [84]. Several authors have suggested that a combination of both Hx and potassium ion determination would be more effective in estimating postmortem interval than either individual measurement [25, 64]. However, problems arise for two reasons. From an analytical perspective, method development needs to be established specifically for the vitreous humor matrix and not based on other matrices such as serum or urine [24] and consistent analytical protocols need to be employed to ensure reliable, reproducible results between samples and between laboratories. From a forensic viewpoint, each determination of postmortem interval needs to take into account several factors including cause of death [69], environmental conditions [83, 85, 86], body temperature [85], age of deceased [85], duration of terminal episode [83, 85], chronic illness [57, 79, 83] and alcohol consumption [86]. The combination of all of these factors makes biochemical indicators in vitreous humor a potential complementary technique rather than a stand-alone method for postmortem interval estimation.

1.4.1.1 POTASSIUM IONS (K^+)

A correlation between concentration of potassium ions and postmortem interval was first reported by Jaffe in 1962 [56]. This study showed an increase in potassium concentration in 36 determinations up to 125 hours postmortem [56]. Several studies regarding the usefulness of this as a biochemical indicator of postmortem interval have since been reported [57, 79-82]. A summary of non-chromatographic methods for the determination of analytes in vitreous humor is shown in Table 1.3.

TABLE 1.3. SUMMARY OF NON-CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF POTASSIUM IONS IN VITREOUS HUMOR.

AUTHOR	NUMBER OF CASES	ANALYSIS	PMI OF SAMPLES (HOURS)	SLOPE (MMOL/L.HR)	INTERCEPT (MMOL/L)	ERROR IN PMI ESTIMATION (HOURS)
Jaffe [56]	36	Flame photometry	Up to 125	-	-	-
Adelson [79]	209	Flame photometry	Up to 25	0.17	5.36	+/- 10
Sturner [57]	54	Flame photometry	104	0.14	5.6	+/- 4.7
Hughes [87]	135	Flame photometry	100	0.12	4.98	+/- 20
Hansson [82]	108	Flame photometry	Up to 120	0.17	8	+/- 20
Coe [80]	160	Flame photometry	24	Up to 6 hours: 0.332 >6 hours: 0.1625	Up to 6 hours: 4.99 > 6 hours: 6.19	+/- 12
Blumenfeld [88]	127	Flame photometry				+/- 26
Madea [83]	170	Flame photometry	Up to 120	0.19	5.88	± 20 ± 10.5(first day)
James [25]	100	Flame photometry	Not given	0.23	4.2	± 18
Rognum *et al.* [64]	87	Flame photometry	Up to 120	0.17 (5 °C) 0.20 (10 °C) 0.25 (15 °C) 0.30 (23 °C)		5
Sparks *et al.* [89]	91	Ion selective electrodes				

Potassium was chosen as an analyte over other components present in vitreous humor for a variety of reasons. Instrumentation at the time of this work (1960's) was not sensitive enough to detect very low levels of some constituents, such as the levels of total proteins and albumins [80]. Other components such as alkaline phosphotase were uniformly negative with respect to concentration and associated error. Of the electrolytes analysed (sodium, chloride and potassium), sodium and chloride remained stable for long periods of time after death [57, 80], discounting them as indicators of postmortem interval. Vitreous potassium was the only analyte that showed an increase in concentration after death, which allowed it to be expressed by a linear mathematical function [80].

While early work focused on methods using flame photometry [56, 79, 80, 82] and ion selective electrodes (ISE) [86, 89], (Table 1.3) it is well documented that several discrepancies exist between studies in the estimation of postmortem interval using vitreous potassium ion concentrations [83, 85, 86]. Several papers report linear equations for the determination of postmortem interval [25, 57, 64, 79, 80, 82, 83], however, the intercept varies markedly, thereby, altering the estimation of postmortem interval. These studies also have a wide range of associated uncertainty, ranging from ± 4.7 hours [57] to ± 34 hours [83]. Suggested reasons for these discrepancies include ambient temperature [83, 85], elevated urea levels [86], alcohol consumption [86], sample acquisition and storage [67] and analytical instrumentation [67, 85]. Such variation limits the use of vitreous potassium ion concentration as a reliable indicator of postmortem interval.

The first reported shift away from flame photometry for the analysis of potassium ions in vitreous humor to determine postmortem interval was published by Ferslew *et al.* in 1998 [66]. Ferslew compared capillary electrophoresis (CE) [66], with UV detection at 214 nm, with an ion selective electrode (ISE) method for the determination of vitreous potassium in 25 human subjects. CE is useful at handling samples that are particularly complex or 'dirty' in nature [90-92] as can often be the case with work involving

decomposition samples. The use of CE allowed for rapid analysis time (<5 mins), improved separation, more symmetrical peak shapes, enhanced detection limits and direct analysis of the specimen [66], whilst only requiring very small sample volumes [58]. Although analysis time was relatively quick, the detection limits achieved were mmol/L, which is comparable with both flame photometry and ISE methods. The data analysis reports a significant linear correlation (r^2 = 0.9642) between potassium ion concentrations obtained by both CE and ISE methods.

The disadvantage of the CE method was the requirement of a complex buffer system for analysis, involving reagents capable of chemical complex formation, 4-methylbenzylamine, 18-crown-6-ether and d,l-alpha-hydroxybutyric acid (HIBA) [66]. Addition of the 18-crown-6-ether was used to prevent co-migration of the potassium and ammonia peaks [58, 66, 67]. Complete separation was essential to provide the possibility of accurate quantitation of potassium concentrations. HIBA was used in the run electrolyte as a chelating agent to control the flow of buffer through the capillary in order to improve peak resolution and shape [66]. The addition of HIBA was reported as affecting the complete resolution of all the cations studied [67]. UV-absorbing additives, such as 4-ethylbenzylamine and imidazole, were added as their electrophoretic mobilities match that of the analytes. They were therefore suitable for detection of ammonium, alkali earth and transition metal cations by CE [66, 67].

This complex buffer system has been used in several reported CE studies [58, 67, 68]. A comparison between CE-UV-Vis and flame photometry for samples with a known postmortem interval of 5-96 hours, resulted in a correlation coefficient of 0.9333 for the potassium ion concentrations in 12 real samples [67]. This work reports detection limits down to μmol L^{-1} (based on S/N of 3), 1000 times better than flame photometry [67]. A significant linear correlation (r^2 = 0.904) has also been reported between potassium ion concentration and postmortem interval using the CE-UV-Vis method for 20 subjects who were deemed to have died a violent death, however no information was given in regards to uncertainty in the estimation [67]. Using the same CE method, a

similar correlation was reported in later work by the same authors, comparing potassium ion concentrations from both eyes [58]. Postmortem interval for these samples ranged from 7-144 hours. Although the determination of potassium ion concentrations gave similar results, the reliability and improved accuracy of CE, as well as the need for small sample volumes, make it advantageous for the determination of vitreous potassium over non-separative methods [67].

An attempt to improve the statistical correlation between vitreous potassium and postmortem interval using CE with indirect UV detection was achieved by utilising a chemometric approach [68]. Using the complex buffer system described in previous reported studies and applying artificial neural networks, the authors reported a good linear correlation (r^2 = 0.98) in comparison to the traditional linear least squared (LLS) method, improving the prediction of postmortem interval by a factor of 5 [68]. The advantage of a chemometric approach is the decrease in the number of required experiments, a decrease in analysis time once the method had been optimised, plus an enhanced potential for statistical evaluation of any data [68].

In 2007, low pressure ion chromatography with conductivity detection was used for the determination of vitreous potassium concentration as an alternative to CE, with applications to postmortem interval [74]. The study involved 62 samples from autopsy cases with known postmortem interval ranging from 1-27 hours achieving a detection limit of 1 mmol L^{-1} and limit of quantitation of 2 mmol L^{-1}. The advantage of this method is the simplicity of the solvent system and sample preparation making it more cost effective than CE [74].

1.4.1.2 Hypoxanthine (Hx)

Hypoxanthine (Hx) is a degradation product of adenosine [65] and is elevated due to hypoxia prior to death [64]. Hypoxanthine has previously been analysed by High Performance Liquid Chromatography (HPLC) with applications

generally in the pharmaceutical and medical fields [93]. The potential for the use of vitreous Hx as a postmortem interval indicator was first described by Rognum *et al.* in 1991 [64]. HPLC with UV detection was successful in demonstrating a linear relationship between Hx and postmortem interval (r=0.93) in 87 subjects with a known postmortem interval of less than 120 hours [64]. Controlled conditions were employed to study the effect of four different storage temperatures on the estimation of postmortem time. Using HPLC methods that were previously described in the literature, an improvement in the detection limit was obtained. The greater sensitivity of HPLC demonstrates its ability to determine analytes over a greater time span than previously used flame photometry and ISE. Rognum *et al.* also concluded that the uncertainty associated with the analysis of Hx by HPLC showed a smaller range of scatter when compared with the determination of potassium by flame photometry for the same samples, particularly in the first 24 hours. The gradient for the determination of Hx becomes steeper with increasing ambient temperature [64]. This is in accordance with the reported literature, which states that temperature is the factor with the greatest impact on rate of decomposition and subsequently, the determination of postmortem interval [6, 9, 14].

In 1994, Madea *et al.* published results that contradicted previous literature reports of the linear relationship between Hx and postmortem interval [65]. Vitreous Hx was analysed by HPLC, using a method based on the work of Rognum *et al.*, but concluded that the smaller margin of error was associated with the determination of potassium ions by ion selective electrodes rather than the HPLC analysis of Hx. It should be noted that Rognum *et al.* determined potassium ion concentrations using flame photometry. Also, potassium ions and Hx were shown to rise linearly immediately postmortem and not after 24 hours as had been reported [64]. Since both studies used similar analytical methods, this discrepancy is accounted for by the authors as a difference in sample collection itself, and the duration of hypoxia prior to death [65]. In 1997 [25], an effort was made to compare the linear equations provided by the 3 separate studies of Madea [65], Bocaz-Beneventi [68] and Rognum *et al.* [64]. HPLC with

UV detection was used to determine Hx whilst flame photometry was used to measure vitreous potassium ion concentration [25]. It was concluded that there was a linear correlation between both Hx and potassium ion concentration with postmortem interval. However, the experimental method used, sampling techniques and the make-up of the sample group itself were the reasons behind the observed differences in the resulting linear equations used to estimate postmortem interval [25]. The result of this work combined results from both Hx and vitreous potassium measurements to arrive at a new equation that improved the accuracy of postmortem interval determination. This was achieved by taking the mean of the individual estimates of postmortem interval given by the two separate equations, reducing the standard deviation of the overall estimate [25].

In 2002, the estimation of postmortem interval was improved using the hypothesis that cause of death is a factor in the level of Hx in the vitreous humor [69]. The analysis determined Hx using HPLC and only investigated cases where the cause of death was the same (death by hanging). Using 206 samples from 176 subjects with a known postmortem interval ranging from 1.08 - 28.91 hours, detection limits in μmol L^{-1} range were obtained (exact numbers not given). The linear correlation between postmortem interval and both potassium ions (r^2=0.818) and Hx (r^2=0.757) was reported by using an inverse prediction statistical method. The inverse prediction method was again used for the determination of Hx in vitreous humor from 134 samples [73]. A revised HPLC method showed a decrease in analysis time and improved reliability [73]. Although no information is given in regards to the separation conditions in the 2002 paper, in 2006, Munoz *et al.* state that the sample preparation is significantly faster and detection limits are improved down to 0.02 μmol L^{-1} for Hx.

More recently Passos *et al.* moved away from a chromatographic approach for the determination of Hx and instead used a sequential injection analysis (SIA) system that enabled the simultaneous determination of both potassium and Hx in vitreous humor [94]. SIA is an automated flow based technique that allows

multianalyte determinations within a single instrument [95]. Detection limits of 1.02 μmol L^{-1} for Hx and 5 x 10^{-5} mmol L^{-1} for potassium ions· were achieved respectively. The Hx levels showed a greater linear correlation to postmortem interval (r^2 = 0.998) than potassium ions, which was in agreement with some of the earlier literature reports [25, 64]. While this approach shows great promise for multianalyte determinations of this kind, SIA instrumentation is not widely available and requires customising for specific applications [95].

1.4.1.3 Amino acids

Vitreous humor has also been analysed for free amino acids. Erdei and Vass used paper chromatography to identify 13 amino acids, but this early research did not attempt to correlate concentrations with postmortem interval [96]. Prior to this, most amino acid analysis had been performed using non-separative methods such as amino acid nitrogen analysis [97-99]. Very few publications deal with the amino acid content in vitreous humor, particularly in relation to postmortem interval. The work done in 1988 by Patrick and Logan [63] was the first report of a chromatographic separation showing a relationship between postmortem interval and free amino acid concentration in vitreous humor [63]. Analysis was by cation exchange chromatography using a Technicon TSM amino acid analyser. This work was completed on 120 cases of infants less than 1 year old, with samples having a postmortem interval up to 96 hours. Twenty-seven amino acids showed a logarithmic linear relationship with postmortem interval although at different rates. The conclusion of this work was that samples showed a reliable result for the estimation of postmortem interval but only if they were collected within the first 24 hours.

In 2008, HPLC was used to determine free amino acids in both vitreous humor and cerebrospinal fluid [76]. This work used an o-phthalaldehyde (OPA) derivatisation procedure prior to analysis for 58 samples with an average postmortem interval of 23.9 hours (range 5-60 hours). No information is given in regards to detection limit, analysis time or error in estimation of postmortem interval. While the authors claim a linear relationship between selected amino

acid concentrations in vitreous humor and postmortem interval, the correlation co-efficients presented ($0.3191 \leq r \leq 0.4508$), would not support this contention to any great extent.

1.4.2 Decomposition products

When considering the following body of work it should be noted that due to the ethical issues involved in the use of human cadavers, pig (*Sus dometica*) carcasses are often used to model the human decomposition process [13, 21, 37, 100]. They are considered to be an acceptable substitute due to their similarity to human torsos in weight, fat to muscle ratio, hair coverage, biochemistry and physiology [11, 39].

1.4.2.1 Decomposition fluid and soil solution analysis

A method with application to both early and late stages of decomposition would be invaluable to forensic investigators in cases of suspicious deaths. In the later stages of decomposition, forensic entomology (Section 1.2.2), has offered insight into the estimation of postmortem interval, however, there are situations where entomology becomes redundant, such as burial or low temperature and the presence of drugs in the body (Section 1.2.2).

Small organic molecules, such as short chain volatile fatty acids (C_2-C_5), were the primary focus of early studies into the chemistry of decomposition in the mid-late postmortem interval. Work by Vass *et al.* [5, 6] used soil solution analysis (the liquid phase between particles) to analyse five microbially produced volatile fatty acids (VFAs); propionic, butyric, iso-butyric, valeric and iso-valeric acid, and their variation over time, with regards to accumulated temperature (Figure 1.1). An advantage of using VFAs and other blood proteins is that they can remain biologically active and detectable for a considerable amount of time [62], thus extending their use potentially into the late postmortem interval. For this reason, Tuller used VFAs in the analysis work from known execution and gravesites in the former Yugoslavia [62].

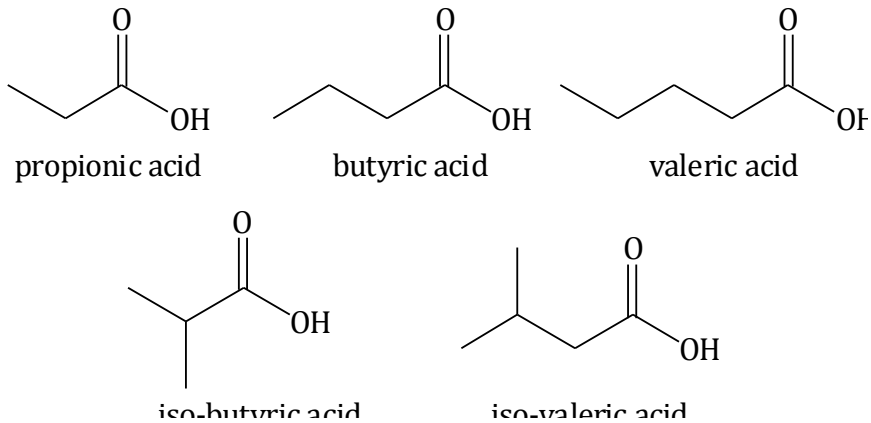


FIGURE 1.1. SHORT CHAIN VOLATILE FATTY ACIDS (VFAS).

The analysis of VFAs had typically been carried out using gas chromatography (GC). GC provides a rapid and reliable method [101-104] for the analytical separation and subsequent analysis of volatile and thermally stable mixtures, showing good sensitivity and reproducibility. Early studies attempted to deal with peak tailing and ghosting as well as finding the most effective column for separation [103, 105].

Vass identified and quantified 5 VFAs using a relatively simple aqueous dilution and filtration method to prepare samples for analysis [6]. Each sample was then acidified prior to being analysed using packed column GC with flame ionisation detection (FID). The addition of formic acid increased reproducibility by minimising peak tailing and ghosting and improved the analyte volatility [101, 105]. Vass also stated that VFAs became more volatile at pH<7.0 [6]. Vass used accumulated degree-days (ADD) to show specific ratios in concentrations of propionic, butyric and valeric acids during decomposition. The estimate given by this method in determining postmortem interval is currently within ± 2 days [5].

In more recent studies decomposition fluids in the absence of a soil matrix were investigated, using pig carcasses in place of human cadavers. Samples were analysed using capillary GC with mass spectrometric (MS) detection [59]. Capillary GC has several advantages over packed column GC including the ability to produce faster and more efficient separations with a higher degree of

resolution as well as greater sensitivity and greater chemical inertness to the analyte [106-108]. In contrast to the sample preparation published [6], formic acid was eliminated from the procedure when it was shown to have no significant effect on the volatility or reproducibility of standards run in triplicate [59]. This meant that sample preparation was now a simple aqueous dilution and filtration prior to analysis.

An increase in the column temperature and the use of GC-MS also allowed the identification of several previously unreported compounds present in decomposition fluid that were eluting late in the analysis time (Figure 1.2). The main compounds identified were long chain acids, as well as phenylacetic acid, phenylpropionic acid, 2-piperidone and iso-caproic acid, which were of potential forensic significance. In contrast to previous findings, the authors found no patterns in the VFAs with ADD, however, the presence of long chain fatty acids (unreported using Vass's GC-FID method) showed cyclic trends that were tentatively related to the fly life cycle [60].

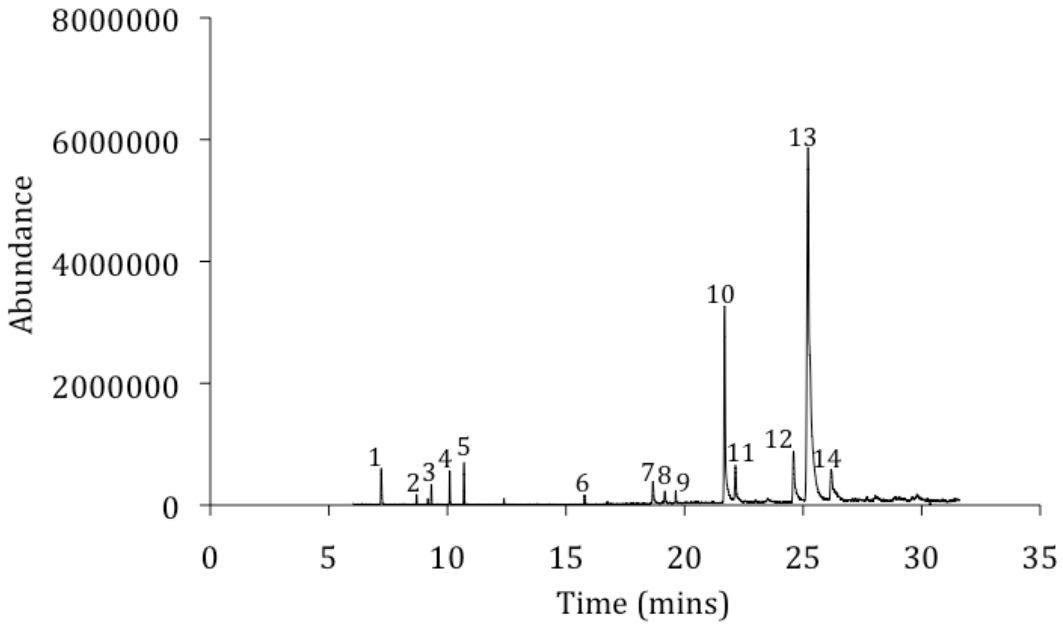


FIGURE 1.2. CHROMATOGRAM SHOWING COMPOUNDS PRODUCED DURING A PORK RASHER TRIAL WITH IDENTIFIED PEAKS: 1. ACETIC ACID, 2. PROPIONIC ACID, 3. TRIMETHYLACETIC ACID (TMA), 4. BUTYRIC ACID, 5. ISO-VALERIC ACID, 6. 2-PIPERIDONE, 7. PHENYLACETIC ACID, 8. PHENYLPROPIONIC ACID, 9. MYRISTIC ACID, 10. PALMITIC ACID, 11. PALMITOLEIC ACID, 12. STEARIC ACID, 13. OLEIC ACID, 14. LINOLEIC ACID [59, 109].

Vass and coworkers, in 2002, used GC-MS to analyse amino acids, neurotransmitters and decomposition by-products (cadaverine and putrescine) as a new method for determining postmortem interval [5], revealing patterns useful for postmortem interval estimation of up to 3 weeks. The sample preparation involved two separate derivatisation procedures, one for the amino acids and one for the cadaverine and putrescine, as well as two separate temperature programs on the instrument. The samples were derivatised in order to increase their volatility, thermal stability and mass-spectral detection [5], however, the need for two derivatisation procedures significantly increased the sample preparation time and the potential for loss of analyte. Subsequent analysis of the data showed that neither putrescine nor cadaverine were useful indicators of postmortem interval and were disregarded from further work. Of the compounds analysed, Vass found that ratios between oxalic acid, gamma amino butyric acid (GABA), proline and methionine, phenylalanine, tyrosine, iso-leucine and histidine showed linear relationships with postmortem interval [5], however, these results have not been replicated. Of these, oxalic acid was seen to be the most valuable indicator and its abundance was determined as a combination of both oxalic and glycolic acids, as these co-eluted during the analysis. The different acids were identified by their respective molecular ions; m/z = 261 (oxalic acid) and m/z = 247 (glycolic acid).

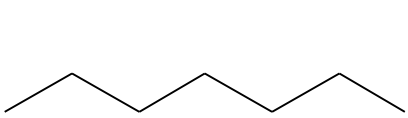
While the main focus of the above studies has been the estimation of postmortem interval, chemical analysis can also be used to establish the origin of identified compounds. This information can then be related back to the original composition of the carcass, as a large proportion of the decomposition products produced should reflect the fat and protein content of the remains. Methods of production for the compounds in decomposition fluid remain largely unproven, with most suggested pathways being based on previous microbial fermentation studies [110, 111].

The limitations associated with the use of GC resulted in the development of new analytical methods to deal with the variety of compounds present in decomposition fluid. The 'dirty' nature of the sample, lends itself to frequent

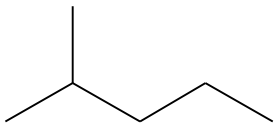
instrument maintenance, thus slowing down sample throughput. Also, GC is unable to detect both long chain and short chain acids (both shown to be present in a sample of decomposition fluid) in a quantitative way due to the polarity difference in the molecules. Detection of these compounds requires either derivatisation of samples, or the use of more complex methods, such as tandem GC to deal with this problem, thereby significantly increasing sample preparation times. In addition to this, compounds that are not volatile or thermally stable are unable to be analysed in free form by GC.

1.4.2.2 VOLATILE ORGANIC COMPOUNDS (VOCS)

Volatile organic compounds (VOCs) associated with decomposition have long been used as a means of training cadaver dogs to aid in the detection of clandestine graves [38, 71, 112, 113]. The most abundant compounds reported from several studies [38, 71, 72, 75, 78] can be seen in Figure 1.3. A decomposition odour analysis database was established in 2004 to identify VOCs produced during the decomposition process [71]. This work used thermal desorption-gas chromatography-mass spectrometry (TD/GC-MS) to identify 424 compounds (and several more unidentified) that evolved from the cadavers with either no external signs of decomposition or in the very early stages. These compounds were then grouped into classes for ease of data analysis. These classes include cyclic and non-cyclic hydrocarbons, nitrogen and oxygen containing compounds, acids/esters, halogens, sulfur compounds, and other/miscellaneous compounds [71].

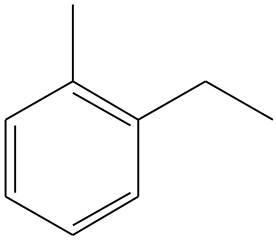


heptane

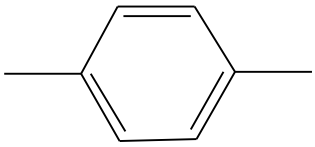


2-methylpentane

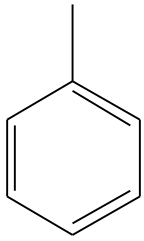
non-cyclic hydrocarbons



1-methyl-2-ethylbenzene

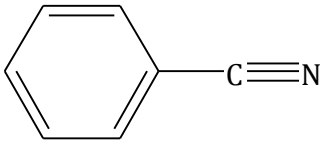


p-xylene



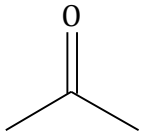
toluene

cyclic hydrocarbons

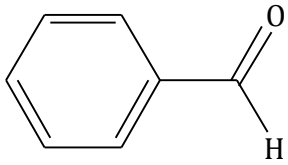


benzonitrile

nitrogen compounds

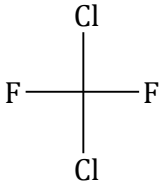


2-propanone

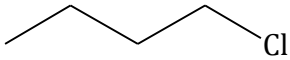


benzaldehyde

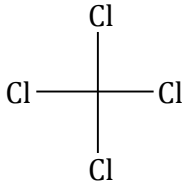
oxygen compounds



dichlorodifluoromethane

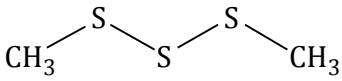


1-chlorobutane

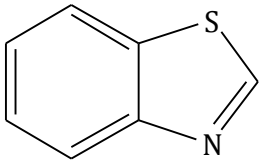


tetrachloromethane

halogen compounds



dimethyltrisulfide



benzothiazole

sulfur compounds

FIGURE 1.3. VOLATILE ORGANIC COMPOUNDS (VOCS) IDENTIFIED IN DECOMPOSING HUMAN REMAINS.

VOCs evolved from a decaying body were analysed by TD/GC-MS to provide a chemical profile of decomposition [72, 75]. Both of these studies yielded a chemical profile of decomposition but with significantly less compounds (30 [75] and 80 [72]) than previous work [71]. This is possibly due to sampling only taking place over a 24 hour period for cadavers with a known postmortem interval of 4 days [75] and 3-4 weeks [72] compared with 1.5 years [71] as well as preliminary data from a 12-year-old gravesite [71]. Recent work in this area showed 35 core compounds from 5 main classes evolved from the decaying body, with analysis again by TD/GC-MS [78]. The results of this work showed that the volatile pattern changes over time, which is in accordance with the reported literature [71].

1.4.2.3 ADIPOCERE

Whilst not directly related to the estimation of postmortem interval, one of the most widely studied decomposition products is adipocere (see section 3.2). While FTIR analysis has been useful in studying trends, analysis of adipocere using GC-MS has allowed a more detailed understanding of its composition [114, 115] through rapid and simple methods with good reproducibility [77]. The major components identified were long chain fatty acids particularly myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$), which are present in characteristic ratios [77, 114, 115].

There are reports in the literature of trace levels of 10-hydroxystearic acid [115] and other unsaturated fatty acids, including linoleic ($C_{18:2}$) [77], palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids. The disadvantage of the analysis of adipocere is the requirement for sample pre-treatment, which is necessary to achieve a reliable and accurate quantitative determination by GC-MS [54, 77]. Methods such as thin layer chromatography and column chromatography [116, 117] have been utilised to separate adipocere from interfering compounds, however, these methods are often affected by low recoveries of lipids, large solvent requirements and oxidation of unsaturated fatty acids due to long term exposure to the ambient environment [54, 77].

1.5 AIMS

The main focus of this research is to expand the current knowledge base relating to decomposition chemistry and its application to forensic investigations. It aims to provide an understanding of the variability in chemical composition of decomposition fluids produced under varying environmental conditions. The significance of different climatic conditions on the decomposition process and the identity of compounds produced is not established in the literature. To further understand the chemistry involved in the decomposition process, and in an effort to characterise the chemical compounds produced in decomposition fluid, analytical methods were developed using gas chromatography-mass spectrometry (Chapter 3), capillary electrophoresis with ultaviolet visible detection (Chapter 4) and liquid chromatography-mass spectrometry (Chapter 5).

Biosynthetic pathways are proposed for components identified in decomposition fluid. This approach provides a framework for targeted analysis of decomposition products, thus allowing a clearer understanding of the chemistry of decomposition.

The observation of trends in decomposition fluid was investigated to establish and identify key chemical markers of decomposition that will improve the ability to date decomposing tissue during forensic investigations.

CHAPTER 2 – DECOMPOSITION FIELD TRIALS

2.1 INTRODUCTION

The estimation of postmortem interval in relation to suspicious deaths is one of the most critical questions faced by forensic investigators. Field studies are often conducted to increase the knowledge base in this area and aim to simulate real-life conditions [6, 9]. However, there are a large number and variety of variables affecting the decomposition process and the high degree of inter-mingling between variables limits the ability to adequately control and isolate them. Isolation of even one variable to give controlled conditions, gives a biased view of a complicated puzzle [9]. In contributing to the knowledge of postmortem interval, it is essential that details such as nature of the terrain where the body is placed and season of placement be known [9] during field studies. Also vital, is information such as daily temperature, rainfall, humidity and insect activity, all of which are significant factors that are known to affect the decomposition process [9, 16, 17, 118]. Field trials of this nature pose significant challenges to researchers in this area, however, there is little to no available literature discussing these issues.

One of the major challenges in setting up a research facility for decomposition studies is finding a suitable location. Generally, a location close to the researchers is ideal but the requirement of seclusion and prevention of access from the general public is paramount. Depending on the specific area of research, field trial areas are often chosen based on types of vegetation and soil [100, 119], the level of drainage in the area [100], and comparable levels of sunlight and shade for individual carcasses within the trial [120]. For studies that require entomological data, the placement of carcasses can also be an issue within the area. Studies involving entomology associated with decomposition processes require that carcass placement be at least 20 metres apart to avoid overlapping of insect species between carcasses for valid results [120]. Work by Vass *et al.* requires that placement of a fresh carcass in a trial must not be within 10 metres of previous carcass placement [5] to avoid potential cross-contamination of compounds from previous field trials. These requirements give some idea as to the difficult criteria required for an adequate field trial area

as well as the size required, particularly for trials that require large sample sets and replicates.

Once a potential location has been identified, the significant cost involved with the establishment and continued maintenance of a decomposition research facility is a major consideration. The initial set-up of a facility requires clearing of land, as well as appropriate fencing to prevent unwanted access by the general public. Fencing is required to be dug into the ground to prevent potential scavengers digging underneath. The nature of this research involves the use of several subjects with the need for replicate experiments, with the cost often out of reach of many researchers [16]. The completion of a relevant comparison between trials requires experiments to be conducted at the same time period over several years. Other initial set-up costs can include weather stations, protective cages for carcasses and protective clothing for researchers. The use of land for a decomposition research facility often also precludes its use for other research purposes.

A variety of samples can be collected and studied during the decomposition process including entomology [119-121], adipocere [53, 77, 114, 122], tissue samples [123], decomposition fluid [59, 60, 109], volatile organic compounds [72, 75, 78] and soil solution [6]. The majority of these sample types require the movement and disturbance of carcasses for sample collection. Most researchers agree that minimal movement of the carcass to obtain samples is required to prevent disturbance of the feeding insects and minimise destruction of postmortem artifacts [6, 17, 119, 120, 124]. The effects of physical disturbance have not been reported in extensive detail in the literature apart from a 2007 study by Adlam and Simmons [124]. The effects of disturbance were observed for weight loss, carcass temperature, soil pH and decomposition using rabbit carcasses. This study concluded that both weight loss and carcass temperature were affected by repeated disturbance of the feeding insects, although the extent of the effect was not investigated.

Several studies report the problems faced by the effects of scavenging during observations of the decomposition process. As early as 1965 [118], the need for protective fencing around purpose built facilities as well as custom-made cages to protect carcasses was evident and the majority of studies utilise these [78, 100, 118, 120]. Although this is done to protect each carcass in order to generate decomposition products for analysis, this is an example of a controlled variable that would otherwise be present in a real-life situation. Carnivores and rodents can cause extensive destruction to the body and often eat the soft tissues present, resulting in an increased decomposition rate [9].

The choice of subject type is also important in decomposition studies in order to provide a realistic view of the decomposition process. Various animals have been studied including dogs [13], cats [125], rabbits [124], rats [126], chickens and birds [118], mice [127] and foxes [128]. These animals, however, were shown to be ineffective in decomposition studies due to the presence of feathers and fur, discrepancies in the uniformity of size, availability of intact specimens and dissimilarities to humans such as fat and protein content as a percentage of the mass of the body [11, 39, 120]. Pet mince has also been utilised in a laboratory-based environment to generate data from an entomological based perspective [36]. Extremely small carcasses, such as lizards and toads, have been reported to decompose and mummify very quickly showing no marked stages of decomposition [120], thus making them ineffective models of decomposition.

The subject size is also relevant in a decomposition field trial. Stillborn piglets and adult pigs have both been used as an acceptable substitute for human cadavers in research trials [77, 78, 119, 120, 122], as described in Chapter 1. Research conducted by Archer [16] used stillborn piglets to simulate neonatal remains in a field trial observing the effects of rainfall and temperature. Archer reports that there are significant differences in the decomposition patterns observed for piglets and those seen for adult pigs and human cadavers [16]. Initial work in this PhD thesis also used stillborn piglets to generate decomposition fluid for analysis, however, it was found that the piglets dried

out quickly and failed to produce a significant amount of fluid for analysis (see Chapter 3). In comparison, whole adult pig carcasses produced large amounts of fluid and the field trials lasted for a significantly longer period of time, allowing observations of other factors affecting the decomposition process. Payne [118] also used piglets in a decomposition study to observe the associated insect species and noted that the dry stage of the decomposition process was reached within 8 days of initial carcass placement. A 22 kg adult pig is the recommended subject choice as the size is approximately equivalent to an adult male torso, where the decomposition process is generally focused [120].

This chapter outlines the set up of two distinct field trial locations that were utilised to provide a 'compare and contrast' approach to compounds detected during the analysis of decomposition fluid. With respect to the literature discussed, the chosen field trial areas showed similarities in carcass location with respect to shade and sunlight, uniformity in ground level and good drainage. The flora and fauna surrounding each carcass placement was considered to be essentially the same. Both piglets and whole adult pigs, approximately 20-25 kg, were utilised to create a complete overview of the decomposition process.

2.2 DECOMPOSITION RESEARCH FACILITIES

Preliminary decomposition studies were carried out at two distinct locations; Perth, Western Australia and southern Ontario, Canada, to provide a comparative overview of compounds detected in decomposition fluid, as well as a qualitative overview of the processes occurring during decomposition under different climatic conditions.

As part of the author's Honours research project, initial studies in Western Australia were performed with pork rashers (belly pork, *Sus scrofa*) to aid in method development [59, 109]. Further trials were carried out with stillborn piglets (Perth) and adult pig carcasses (*Sus scrofa*, Canada and Australia) to

create a more realistic model of decomposition and to gain a more accurate understanding of the composition of decomposition fluid.

2.2.1 Perth, Western Australia

The field trial area for this project was located in, Bentley, Western Australia (32°01′S, 115°53′E), 6 kilometres southeast of Perth. It is a partially cleared scrub area, approximately 4 m x 6 m (Figure 2.1). Field trials were conducted in April 2007 (piglets), September 2007 (adult pigs) and February 2008 (adult pigs).

Figure 2.1. Photograph of field trial area located on campus at Curtin University of Technology.

2.2.2 Ontario, Canada

The field trial was conducted in temperate woodland in southern Ontario, Canada. The study area was located approximately 51 km northeast of Toronto (43°56'N, 78°54'W) across a slight (5°) gradient, with some drainage/depression areas. Dominant vegetation at the site included eastern

white cedar (*Thuja occidentalis*), maples (*Acer spp.*) and trembling aspen (*Populus tremuloides*) (Figure 2.2).

FIGURE 2.2. PHOTOGRAPH OF FIELD TRIAL AREA LOCATED ON CAMPUS AT THE UNIVERSITY OF ONTARIO INSTITUTE OF TECHNOLOGY.

The whole adult pig trial utilising carcasses approximately 20 kg in weight was conducted over the Canadian summer (July-August 2007).

2.3 WEATHER DATA

The weather data for both locations was recorded on a daily basis using temperature loggers (Thermodata Pty Ltd) to record ambient temperature every 30 minutes in both locations.

For the trials conducted in Western Australia, data from the three closest official weather-recording stations (Perth metro, Perth airport and Jandakot airport) was obtained on a daily basis from the Bureau of Meteorology for comparative purposes.

The average daily temperature was recorded in order to derive the accumulated degree-days (ADD). ADD was calculated by taking the sum of the average daily temperature (average of the maximum and minimum, ° C) for the length of time the corpse had been decomposing. Meygesi *et al*. suggest that decomposition rates and a more accurate estimation of PMI can be achieved when accumulated temperature is taken into account rather than individual daily temperatures [18]. Results for all trials are presented using calculated ADD.

2.4 FIELD TRIAL SET-UP AND SAMPLE COLLECTION

2.4.1 PIGLET TRIAL

Stillborn piglets were used to model the decomposition of neonatal remains in the ambient environment. They have been used in previous studies [16, 118, 121], although their application to investigations of postmortem interval in the literature is scarce. They were primarily used for ease of sampling and availability at the time of the field trial at Curtin University. Piglets were considered to be a step-up from pork rashers in furthering the knowledge base in this area and the next logical progression in understanding the overall decomposition process.

Four stillborn piglets (supplied by Linley Valley Pork, Northam, Western Australia) were placed in a custom made rack which fitted to the inside of a 15 L plastic storage box. Two of the piglets were completely covered with fly-wire and the remaining two were left exposed (Figure 2.3). All four piglets were protected inside a custom-made wire cage (80 x 70 x 60 cm) to prevent access by feral cats and crows.

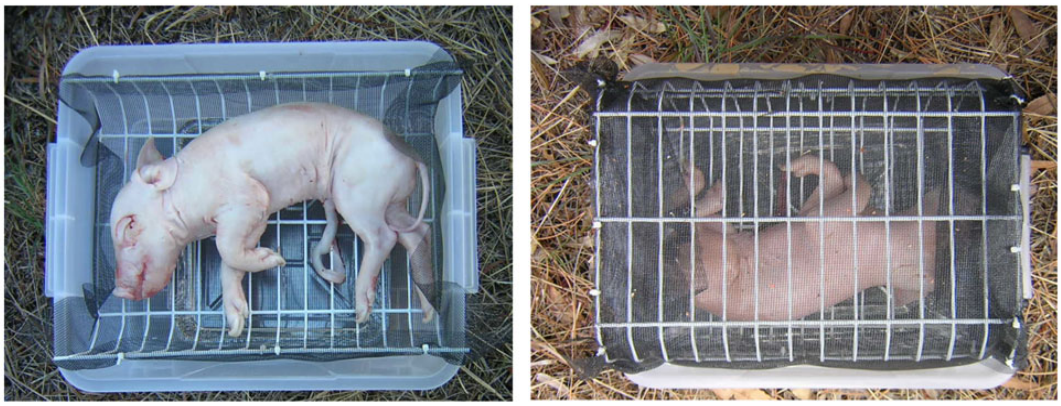


FIGURE 2.3. SET UP OF STILLBORN PIGLETS SHOWING ONE PIGLET EXPOSED TO INSECT ACTIVITY AND ONE COVERED WITH FLYWIRE.

The piglets were placed in the field trial area on the 13th April 2007 (autumn). Decomposition fluid first appeared on day 11 (ADD 195) and samples were collected from piglets labeled 1 and 3. Sampling continued for these piglets on a daily basis until day 17 (290 ADD). Fluid was collected using a 5 mL sterile syringe and placed directly into a pre-weighed centrifuge tube. Collected samples were placed directly into the freezer until time of analysis.

The difference in daily temperature compared with previously run field trials using pork rashers (belly pork) accounted for the delay in fluid production. It was evident that fly and maggot activity was much greater in the warmer months compared with the cooler months. This allows the inference that increased insect activity leads to a greater production of decomposition products at an increased rate. This is supported in the literature by Mann [9], who states that the majority of soft tissue destruction is by insect activity. The piglets that were protected from insect activity showed little to no production of decomposition fluid. This is also in accordance with the literature where Payne reports that carcasses protected from insect activity [118] produced little to no decomposition products and reached a mummification stage much more rapidly. This highlights the importance of the need for a much larger sample set due to the complex and variable nature of the decomposition process as well as the importance of the source of the decomposition fluid for field trials.

2.4.2 Whole adult pigs, Ontario, Canada

Two whole adult pig carcasses were placed in the field trial area, exposed to the ambient environment, on 17th July 2007 (summer), recorded as day zero, in southern Canada in a dedicated research facility (Figure 2.4). Each pig was placed on a wire rack fitted over a 'grave' in the sand, which was lined with a tarpaulin for fluid collection. Each pig was then covered with chicken wire to prevent scavenging from feral animals. Fly activity was immediately apparent and maggot activity was evident from day 2 (ADD 44). Decomposition proceeded at a fast rate with an average maximum daily temperature of 26.45 °C. The need for a larger sample set was yet again emphasised by the differing rate of decomposition for both adult pigs which were placed within metres of each other and exposed to comparable levels of sunlight and shade.

Figure 2.4. Photograph showing set-up and placement of adult pig carcasses during the Canadian field trial, July 2007.

Decomposition fluid first appeared on day 5 (ADD 126) and this was the first sampling day for both pigs. The main issue encountered during this trial was the large number of maggots present in the collection pit making sampling of fluid difficult. This occurred predominantly on days 7-8 (ADD 163-183) when insect activity was deemed to be at a maximum. Samples were collected daily until day 14 (ADD 313), when insect and maggot activity had ceased and only mummified skin and skeletal remains were evident. A detailed record of the appearance of each pig as well as sampling and entomological information can be seen in Appendix 1. A comparative summary of the timing and approximate length of each decomposition stage for all field trials can be seen in Table 2.1.

TABLE 2.1. COMPARATIVE SUMMARY OF TIMING OF DECOMPOSITION STAGES AFTER CARCASS PLACEMENT SHOWING APPROXIMATE ONSET AND END DAY. NUMBER IN BRACKETS INDICATES ASSOCIATED ADD.

DECOMPOSITION STAGE	DECOMPOSITION FIELD TRIAL			
	Piglets (Perth, April 2007)	Adult pigs (Canada, July 2007)	Adult pigs (Perth, Sept 2007)	Adult pigs (Perth, Feb 2008)
Fresh	0-2 (18-60)	0-2 (24-64)	0-2 (11-36)	0-1 (20-42)
Bloated	3-7 (77-140)	2-4 (64-100)	3-10 (55-166)	2-4 (62-109)
Active Decay	8-13 (157-243)	4-8 (100-183)	11-24 (180-356)	5-7 (134-191)
Dry	14-17 (258-289)	8-14 (183-313)	27 – end (400-765)	8-14 (222-372)

It should be noted that this summary is a generalisation of decomposition stages. For all adult pig trials, the data presented for the onset and end days were taken as averages of all pigs included in the trial. Decomposition stages for each pig were noted to overlap considerably with respect to timing, particularly for the trials conducted in the cooler months. The most common overlap was that of the bloat stage and active decay stage where significant discolouration

and bloating was still present in the upper torso, but the lower torso had significant maggot activity, consistent with active decay. Also, the active decay and dry stages often overlapped. The limbs and head regions of the carcass were often skeletonised while the main torso was still in active decay.

2.4.4 Whole adult pigs, Perth, Western Australia

Further field trials were conducted in Perth under differing environmental conditions to observe differences in the rate of decomposition for adult pigs. An overview of the set-up in the field is seen in Figure 2.5. Analysis of subsequent fluid collection would allow direct comparison of the compounds produced during the decomposition process between distinct locations.

Figure 2.5. Photograph showing set-up and placement of adult pig carcasses during the Perth field trial, September 2007.

2.4.3.1 AUGUST - OCTOBER 2007

Three adult pigs were placed in the ambient environment on 31st August 2007, recorded as day zero. Each pig was placed on a wire rack fitted over a 'grave' in the sand, which was lined with a tarpaulin for fluid collection. Each pig was then covered with a purpose built wire cage to prevent scavenging from feral animals. Fluid was collected from each pig, starting on day 5 (ADD 87). Fluid was collected on an almost daily basis, excluding weekends, from all pigs until 19th October 2007 (ADD 765). It should be noted that in the event of rain, pigs were left uncovered. The 'dilution' effect on the decomposition fluid was unknown and to the best of our ability, a representative sample was taken. A detailed record of the appearance of each pig as well as sampling and entomological information is described in Appendix 1.

2.4.3.2 FEBRUARY - MARCH 2008

Three adult pigs were placed in the ambient environment on 20th February 2008, recorded as day zero. The set up of the field trial was the same as described in Section 2.4.3.1. Insect activity was observed almost immediately on pig 2, with the first fluid collection on day 2 for pig 1 (ADD 42) and day 3 for pigs 2 and 3 (ADD 63). As expected from previous trials, the warmer ambient temperature facilitated a predictably faster rate of decomposition. Mummification of the pig carcasses was expected in this trial as the warmer weather dried the skin out. This was observed for all pigs as early as day 6 (ADD 162). The rapid onset of mummification prevented significant levels of insect activity, although activity was observed to be at a premium on days 3-5 (ADD 85-135). At this time all pigs were classified as being at the end of putrefaction and beginning the active decay stage of the decomposition process. Overall, the decomposition process was completed within a much shorter time span compared with previous trials conducted in cooler ambient temperatures. Fluid was collected from all pigs until day 14 (ADD 373) and this was deemed to be the end of the trial. The pigs were mostly mummified and skeletal at this stage with little to no insect activity. At this point, decomposition fluid was no longer

being produced. A detailed record of the appearance of each pig as well as sampling and entomological information is described in Appendix 1.

2.5 Observations in experimental field trials

2.5.1 Variability in rate of decomposition

One of the main issues in studying rates of decomposition in field trials is the variability in rate of postmortem change for each subject in the ambient environment [9, 18, 129] Although all of the pig carcasses studied were subject to the same environmental conditions, were of similar size and weight and had the same time of death recorded, pigs were observed to decompose at a different rate, both between trials and between pigs in the same trial. An example of this can be seen in Figure 2.6, which shows 2 adult pig carcasses photographed during the Canadian field trial on day 8 (ADD 167). Pig 1 shows complete deflation of the carcass and is almost a complete maggot mass. Pig 2 shows partial deflation and only has maggot masses on the head and hind leg/lower torso region.

FIGURE 2.6. VARIABILITY OBSERVED BETWEEN TWO PIG CARCASSES IN THE SAME FIELD TRIAL (CANADA). BOTH PIGS WERE PHOTOGRAPHED ON DAY 8 (ADD 167).

It was also evident that trials conducted in the cooler months were more likely to proceed through all the stages of the decomposition process as described in Chapter 1. Although there may have been overlap between individual stages, each stage was identifiable and lasted for a significantly longer period of time than stages conducted in the warmer-hotter months. Trials that took place over summer months reached the mummification and skeletonisation phase of

decomposition more rapidly than trials in the cooler months. Individual decomposition stages were often difficult to identify and may not have been present at all. Another significant observation was that not all stages of decomposition are applicable to all areas of the body. As described by Megyesi [18], limbs do not bloat and they do not purge decomposition fluid.

Differences were also observed in the rate of decomposition between the carcasses that were exposed to insect activity and those that were protected. Carcasses that were protected produced little to no decomposition fluid and tended to reach the mummification phase of the decomposition process more rapidly. This is supported in the literature, where Mann [9] reports that the overwhelming majority of soft-tissue decomposition is due to feeding by insect larvae. Thus, the prevention of insect activity significantly reduces the decay process [15]. This was also observed in earlier work [60, 109]. An example of this can be seen in Figure 2.7, where the stillborn piglets used in the trial, whether covered or exposed, decomposed at a different rate, even though they were in close proximity to each other and subjected to the same environmental conditions and surroundings. This is another indication that a larger subject group is vitally important. A clear distinction can be made, however, for the rate of decomposition of the piglets exposed to maggots and those that were not. The exposed piglets (1 and 3) reached a state of advanced decomposition considerably faster than the two in which maggot activity was excluded (2 and 4).

FIGURE 2.7. PHOTOGRAPHS SHOWING DIFFERENCES IN PIGLET DECOMPOSITION ON DAYS 1, 4, 9, 15 (APRIL 2007). (N.B. PIGLET 1 WAS UNCOVERED; PIGLET 2 WAS COVERED WITH FLYWIRE).

2.5.2 SAMPLING ISSUES

The sampling of the decomposition fluid from the collection pits meant that on each sampling session, the racks holding the pigs needed to be lifted to allow access to the fluid. The effect of disturbing the pigs and thereby the feeding

maggots and insects is unknown. However, for the purposes of these field trials, the collection design worked well.

The consistency of the fluid itself caused problems during the sampling process, particularly during the trials conducted in the warmer months. As decomposition progressed and the level of insect activity reached a premium, 'fluid' collection became increasingly difficult. As the temperature increased, fluid that had accumulated in the pit dried out much more rapidly. Pits were sampled from 3 different locations at each session, although the chances of a representative sample were now minimised further as mixing of the fluid in the pit could not be achieved.

As the contents of the pit were essentially decomposing remains, this was a protein source for the feeding insects and the pits often contained large maggot masses (Figure 2.8). This also made collection of the fluid difficult as quite often, large groups of maggots were collected in what essentially was a solid mass.

FIGURE 2.8. COLLECTION PIT SHOWING LARGE MAGGOT MASS AND DECOMPOSITION FLUID. PHOTOGRAPH TAKEN ON DAY 8 (ADD 183) IN CANADA.

The lack of fluid production from piglets made sample collection difficult as in the warmer months, samples of fluid were often dried out before collection. The small amount of fluid produced, however, was expected and can be related back to the initial composition of the carcass. Widdowson [130] found that the fat content of stillborn piglets is approximately 1 % of their total body weight. The majority of decomposition fluid should represent the level of protein and fat in the remains [6, 11], so it would be expected that the piglets would have minimal production.

2.6 Conclusions

This chapter has provided an overview of the field trials that have been reported in the literature to study the decomposition process. The reviewed literature highlights issues that are faced by investigators, including the control of environmental variables, choice of location and subject, and the financial cost in initial establishment of a field trial that requires replicate experiments over a significant time frame. The choice of decomposition product for further analysis and investigation is also important if relevant extrapolations to case studies are to be made.

The approach taken with regards to the design and set-up of field trials conducted during this research took into account the difficulties and challenges faced by previous researchers. Decomposition fluid samples that were collected from the trials conducted in both Canada and Western Australia were used for subsequent analysis as detailed in Chapters 3-5.

CHAPTER 3 – ANALYSIS OF VOLATILE FATTY ACIDS (VFAS) BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS)

3.1 INTRODUCTION

Volatile fatty acids (VFAs) comprise a variety of low molecular weight aliphatic carboxylic acids, primarily C_2 to C_7 [111]. VFAs are produced by anaerobic microbial fermentation processes of non-digestible carbohydrates [111, 131, 132] during the digestion process in humans and farm animals [131-133], particularly cattle and sheep [111]. The production of VFAs from the degradation of carbohydrates and lipids [131] represents the major source of absorbed energy for the host species [132]. Recent research suggests that VFAs also have several other advantages. VFAs have been shown to stimulate water absorption, mineral uptake [134] and induce an insulin response [135]. Butyric acid has also been reported to reduce inflammation in the large intestine [136].

Apart from being formed by degradation of carbohydrates, acetic (C_2), propionic (C_3) and butyric (C_4) acids [111, 133] are also produced by deamination of amino acids at pH 6-7 [111]. Deamination results in the production of VFAs, carbon dioxide, hydrogen and ammonia [111]. A less common pathway for the production of VFAs is decarboxylation of amino acids, induced at pH 5-6.

3.1.1 ANALYSIS OF VOLATILE FATTY ACIDS

Gas chromatography (GC) has typically been the preferred analytical method for the determination of VFAs and has been widely applied in areas such as environmental and waste-water monitoring [101, 104, 137, 138], food science [139] and clinical analysis [103]. GC provides a rapid and reliable method [101-104] for the analytical separation and subsequent analysis of mixtures, showing good sensitivity and reproducibility of results. Early studies attempted to deal with peak tailing and ghosting as well as finding the most effective column for separation [103, 105].

Ghost peaks occur when peaks from a previous run appear in the following chromatogram at a similar retention time [105]. In work by Ackman and Burgher, formic acid was added to samples and standards to eliminate the phenomenon of ghost peaks [105], although formic acid itself gave no response

in the flame ionisation detector. The requirement of formic acid was supported by Willig *et al.*, who suggest that the injection of aqueous formic acid was required after every sample run to prevent the ghosting effect [102]. Hordijk disagrees with the addition of formic acid in the experimental design, as he was unable to detect formic acid in the ion chromatogram and did not observe peak ghosting [101]. This was also confirmed by initial investigations into the optimisation of the GC method during the author's Honours research [109]. The use of formic acid was questioned when separations of a calibration series involving mixtures of volatile fatty acid standards performed in the absence of formic acid, showed no obvious, immediate difference in peak area or height. Peak tailing or ghosting was also not evident [59].

The second function of the formic acid is to further acidify the solutions containing the volatile fatty acids and thereby improve volatilisation in the GC [101]. Vass also found that VFAs became more volatile at pH < 7.0 [6].

In the analysis by Van Den Bogaard, the comparison of different pre-treatment methods and different stationary phases for the quantitative analysis of VFAs is achieved [103]. It was concluded that a carbowax column was the best for analysis, dramatically reducing the run time and giving a low and reproducible limit of detection for VFAs C_2-C_7. The carbowax column, a polar column, also showed complete separation of the peaks produced. This was confirmed by Hordijk *et al.*, who achieved good separation of peaks with a free fatty acid phase (FFAP) column [101].

Van Den Bogaard also concluded that an aqueous extraction of VFAs was the simplest and the fastest in terms of pre-treatment of samples [103]. Aqueous treatment of samples was also used by Vass, who used a simple extraction method for soil solution before analysis of volatile fatty acids by GC [6].

A widely used method for the analysis of VFAs is the esterification to methyl esters to increase volatility of the acids. The main problem with this method

was the non-quantitative recoveries for the ester derivatives of the short chain acids [140].

Other methods of extraction included liquid-liquid extraction using ether [104]. This method proved to be problematic in relation to calibration and reproducibility. Problems arose for the lower volatile fatty acids, namely C_2 and C_3, which showed non-quantitative recoveries (i.e. did not allow for the complete extraction of all VFAs) and required extra steps in preparation and subsequent data manipulation. Organic extraction methods are more complicated and thereby much slower in comparison to aqueous methods [103]. The main advantage of an organic extraction method, however, was the ability to quantify fatty acid concentrations from different biological systems [141].

3.1.2 Decomposition chemistry

Volatile fatty acids are produced by the aerobic degradation of soft tissues, are water-soluble, and leach from the corpse earlier in decomposition than long chain hydrophobic hydrocarbons [6]. Branched chain carboxylic acids (such as iso-valeric and iso-butyric) are the result of microbial fermentation of the branched chain amino acids valine and leucine [102].

An advantage of using volatile fatty acids and other blood proteins is that they can remain detectable for a considerable amount of time [62]. For this reason, Tuller used VFAs in the analysis work from known execution and gravesites in the former Yugoslavia [62]. However, the experimental determination of VFAs produced during decomposition is yet to be validated with a statistically sound and robust method. The effects of a controlled sample size and factors that affect decomposition and hence the appearance of VFAs is still to be investigated, particularly for forensic investigations.

GC was first used in relation to decomposition chemistry by Vass in 1992 [6] where data was collected on various short chain volatile fatty acids following

analysis using packed column GC coupled with flame ionization detection (FID). Vass analysed soil solution samples (the liquid phase between soil particles) taken from seven cadavers placed in the natural environment at various times of the year. A simple dilution and filtration method was used, followed by an acidification with formic acid to prepare each sample for analysis. Samples were analysed using a Chromosorb W column, typically used for the analysis of polar compounds. Using accumulated degree-days (ADD, Section 2.3), a technique first described by Edwards *et al.* in 1987 [142], Vass found that the VFA concentrations, regardless of subject or season, were the same [6]. With this information, Vass found it possible to estimate the postmortem interval for any individual. Currently, the best estimation reported by Vass for the estimation of postmortem interval is ± 2 days [6].

Work by Vass *et al.* [5, 6] attempted to use VFAs as time dependant biomarkers of decomposition to obtain a more accurate estimation of PMI. Vass identified 5 main VFAs (propionic, n-butyric, iso-butyric, n-valeric and iso-valeric (Figure 3.1)) that showed distinct patterns in concentration during decomposition, for any given accumulated degree-days (ADD). The estimate given by Vass in determining PMI is currently ± 2 days using VFAs although none of the published research papers are supported with experimental evidence [5].

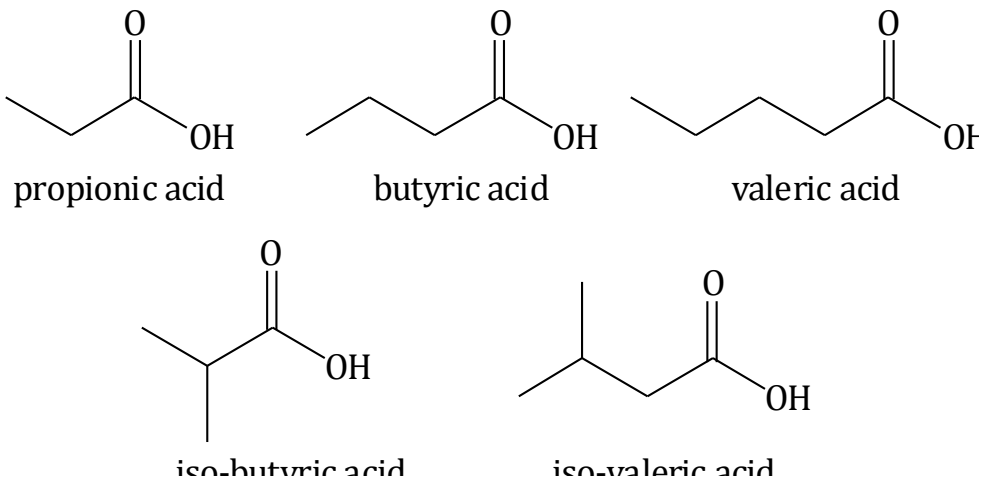
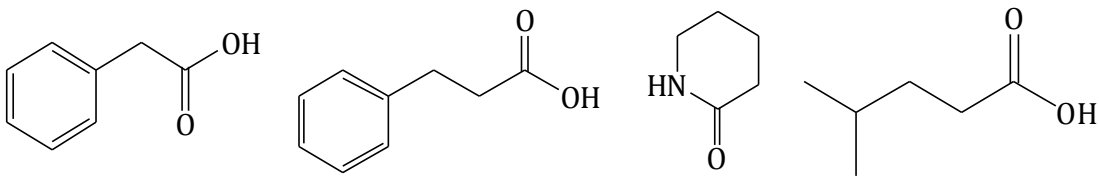


FIGURE 3.1. VOLATILE FATTY ACIDS DETECTED AND IDENTIFIED BY VASS ET AL. [6] DURING DECOMPOSITION OF HUMAN REMAINS.

In 2005, studies were initiated in Victoria (South Eastern Australia) to investigate the potential of soil solution analysis as a means of identifying patterns in decomposition fluid [19, 143], by replicating the original work by Vass [6]. The individual short chain acids were identified using capillary gas chromatography-mass spectrometry (GC-MS). The results from these preliminary experiments indicated that while interesting patterns in the production of the volatile fatty acids (VFAs) emerged, further studies are required. These include complete characterisation of decomposition fluids produced under different environmental conditions as well as investigations of factors that may affect the fluid production.

Research then commenced in 2006, in Perth, as part of the author's Honours research project using pork rashers (belly pork) and stillborn piglets to investigate the identity and temporal variation in the compounds produced during the decomposition process [109]. Pork rashers (belly pork) were used to generate decomposition fluid samples that could be used to aid in method development. The complex and highly variable nature of the decomposition sample itself was confirmed during this research.

Newly resolved compounds were identified during this research and the relative abundance and the possibility of trends in these components were considered to be important, as they had previously not been described in decomposition fluid. Their validity in the estimation of PMI was also unknown. These compounds were identified by mass spectral fragmentation patterns, spiking and retention time as being phenylacetic acid, phenylpropionic acid, 2-piperidone and iso-caproic acid (Figure 3.2). The mass spectral fragmentation patterns can be seen in Appendix 2.



phenylacetic acid phenylpropionic acid 2-piperidone iso-caproic acid

FIGURE 3.2. ADDITIONAL COMPOUNDS DETECTED IN DECOMPOSITION FLUID WITH INCREASED TEMPERATURE IN THE GC COLUMN.

3.1.3 AIMS

This chapter describes the application of an optimised gas chromatography-mass spectrometry method to the analysis of fluid samples produced during the decomposition process, specifically the detection and identification of fatty acids. Replicate experiments using stillborn piglets were conducted to confirm the validity of results from previous work. An extension of this was the use of whole adult pig carcasses to gain a more realistic view of the decomposition process.

3.2 EXPERIMENTAL

3.2.1 CHEMICALS AND REAGENTS

A stock solution (80 mM) of each of the five target short chain volatile fatty acids; propionic (Ajax Chemicals, Australia; Sigma-Aldrich, Canada), n-butyric (Chem Supply, Australia; Sigma-Aldrich, Canada), iso-butyric (Aldrich, Australia; Sigma-Aldrich, Canada), n-valeric (Sigma, Australia; Sigma-Aldrich, Canada) and iso-valeric acid (Aldrich, Australia; Sigma-Aldrich, Canada), was prepared in deionised water. A set of standard calibration solutions in the concentration range 0.2 – 10 mM was prepared by serial dilution of the stock solution. All acids used were of analytical grade (>99 %). The internal standard, trimethylacetic acid (TMA, 2.5 mM) (Sigma, Australia; Sigma-Aldrich, Canada), was added to each of the calibration standards in a 1:1 ratio immediately prior to analysis. Calibration and stock solutions were stored at 4 ^{0}C before and after analysis. All calibration standards were analysed in triplicate by GC-MS.

A 20mM formic acid (Sigma, Aus) solution was prepared and added (1:1) to trimethylacetic acid (2.5 mM). This mixture was then added to a set of calibration solutions (1:1) and analysed in triplicate by GC-MS.

A mixture containing trimethylacetic acid (2.5 mM) and deionised water (1:1) was prepared and added to a set of calibration solutions (1:1). The solutions were analysed in triplicate by GC-MS.

3.2.2 Sample preparation

The collection of decomposition fluid samples is described in Chapter 2. The recovered fluid samples were allowed to equilibrate at room temperature. A 1:2 dilution was prepared of the fluid with deionised water. The mixture was filtered (0.8/0.2 µm Acrodisc® filter, PALL Life Sciences) and 0.5 mL filtrate and 0.5 mL trimethylacetic acid (2.5 mM) solution was prepared immediately prior to analysis by GC-MS.

3.2.2.1 Chemical analysis: stillborn piglets and adult pig carcasses (Perth, Western Australia)

Volatile fatty acid calibration standards and samples were analysed by Gas Chromatography (Hewlett Packard 5890 Series II) interfaced with a Mass Selective Detector (MSD) (Hewlett Packard HP5971). A 1 µl aliquot was introduced into the split/splitless injector by means of a Hewlett Packard 7673A autosampler. The gas chromatograph was fitted with a 50 m x 0.22 mm ID x 0.25 µm (df) BP20 SGE column. GC conditions are described in section 3.2.3. Data acquisition and analysis was performed using Chemstation software.

3.2.2.2 Chemical analysis: adult pig carcasses (Ontario, Canada)

Chromatographic analysis was performed on a Thermo-Finnigan Trace GC Ultra coupled with a Thermo-Finnigan Polaris Q mass spectrometer. A 1 µL aliquot was introduced onto a HP-INNOWAX (J&W Scientific 19091N-133) capillary column (30 m x 0.25 mm ID x 0.25 um df) by means of a Thermo Triplus autosampler. GC conditions are described in section 3.2.3.

3.2.3 Analytical conditions

The GC oven was programmed from 100 °C to 121 °C at 3 °C min^{-1}, then 15 °C min^{-1} to 265 °C and held for 7 min. The injector was operated at 240 °C and a split ratio of 10:1. Helium was used as the carrier gas at a constant pressure of 20 psi (1.1 mL min^{-1} for FID and 1.3 mL min^{-1} for MS). Typical MSD conditions were: ionisation energy 70 eV, source temperature 160 °C and electron multiplier voltage 2000 V. Full instrumental conditions are listed in Table 3.1.

TABLE 3.1. INSTRUMENTAL PARAMETERS FOR GC-FID AND GC-MS.

PARAMETER	CONDITIONS	
Split injection ratio	10:1	
Head pressure	20 psi	
Injector temperature	240 °C	
Oven temperature	100 °C	
Injection volume	1 µL	
Data collection	Chemstation	
	FID	**MS**
Carrier gas (Helium)	1.1 mL/min	1.3 mL/min
Air flow	350 mL/min	
Nitrogen flow	25 mL/min	
Detector temperature	280 °C	160 °C
Transfer line temperature		265°C
m/z (scan mode)		20-420 amu
Temperature programs	$A: 100^\circ \xrightarrow{3^\circ\ min^{-1}} 121^\circ \xrightarrow{15^\circ\ min^{-1}} 200^\circ$	$A: 100^\circ \xrightarrow{3^\circ\ min^{-1}} 121^\circ \xrightarrow{15^\circ\ min^{-1}} 200^\circ$ $B: 100^\circ \xrightarrow{3^\circ\ min^{-1}} 121^\circ \xrightarrow{15^\circ\ min^{-1}} 265^\circ$
Analysis run time	15.93 mins	A: 19.23 mins B: 31.6 mins

3.3 RESULTS AND DISCUSSION

The optimised method for the analysis of VFAs was developed during the author's Honours research [109]. When compared to the GC methods used by Vass [6] and in preliminary work conducted at Deakin University [19, 143], a higher final column temperature of 265 °C was used. An increased column temperature improved the resolution of compounds produced in the decomposition process as well as the detection of previously unidentified components.

3.3.1 ANALYTICAL FIGURES OF MERIT

Calibration was performed with mixed acid standards (0.2 mM-10 mM) with the initial aim of identifying and quantifying analytes (Table 3.2). Calibration standards were analysed in triplicate.

TABLE 3.2. SLOPE AND INTERCEPT INFORMATION WITH STANDARD ERROR (SE) VALUES FOR CALIBRATION WITH TARGET MIXED VOLATILE FATTY ACID STANDARDS (0.2-10 MM).

VFA	SLOPE	SE	INTERCEPT	SE	r^2 VALUE
Propionic acid	0.552	0.005	-0.0612	0.027	0.997
Iso-butyric acid	0.75	0.004	-0.0577	0.021	0.998
Butyric acid	0.78	0.004	-0.0772	0.024	0.997
Iso-valeric acid	0.959	0.004	-0.0698	0.022	0.998
Valeric acid	0.96	0.006	-0.0853	0.033	0.998

The standard deviation in the slope and intercept were determined using the LINEST function in Microsoft Excel. The standard deviation in the slope and the intercept ranged from 0.4-0.6 % and 2.0-3.3 % respectively. These values are deemed reliable enough to be used in the determination of unknowns.

Instrumental precision was determined using low (0.2 mM), mid (2.0 mM) and high (8.0 mM) range standards (n=5). The relative standard deviation (% RSD) for peak area and retention time was then calculated (Table 3.3).

TABLE 3.3. PRECISION ANALYSIS OF VOLATILE FATTY ACIDS AT LOW (0.2 MM), MID (2.0 MM) AND HIGH (8.0 MM) RANGE CALIBRATION STANDARDS (N=5).

VFA	% RSD PEAK AREA			% RSD RETENTION TIME		
	0.2 mM	**2 mM**	**8 mM**	**0.2 mM**	**2 mM**	**8 mM**
Propionic acid	3.44	3.79	2.81	0.10	0.05	0.04
Iso-butyric acid	2.67	3.15	2.96	0.12	0.07	0.04
TMA	3.76	0.66	2.47	0.14	0.07	0.02
Butyric acid	2.50	3.37	4.08	0.14	0.06	0.03
Iso-valeric acid	3.99	2.04	3.80	0.11	0.04	0.03
Valeric acid	3.05	3.19	2.50	0.09	0.04	0.03

The limit of detection was calculated at three times the signal to noise (S:N) ratio. Three detection limits were determined for the five target VFAs (Table 3.4):

TABLE 3.4. DETECTION LIMITS FOR TARGET VOLATILE FATTY ACIDS.

VFA	DETECTION LIMIT (mM)
Propionic acid	0.09
Iso-butyric acid	0.06
Butyric acid	0.06
Iso-valeric acid	0.04
Valeric acid	0.04

3.3.2 TEMPORAL VARIATION IN CHEMICAL CONTENT

Fatty acids have been reported as having the potential to show reproducible patterns over certain postmortem intervals during decomposition in the absence of a soil matrix [6]. Data analysis was conducted to establish any distinct relationship between the levels of particular compounds produced with respect to time and temperature, in the trials run in both Perth and Canada.

3.3.2.1 PIGLET TRIAL

The piglet trial, conducted in April 2007, was seen as the next logical progression from pork rashers in understanding the overall decomposition process. This was conducted as a replicate experiment to confirm results of previously reported studies [59]. A sample chromatogram of decomposition fluid from piglet 2 can be seen in Figure 3.3.

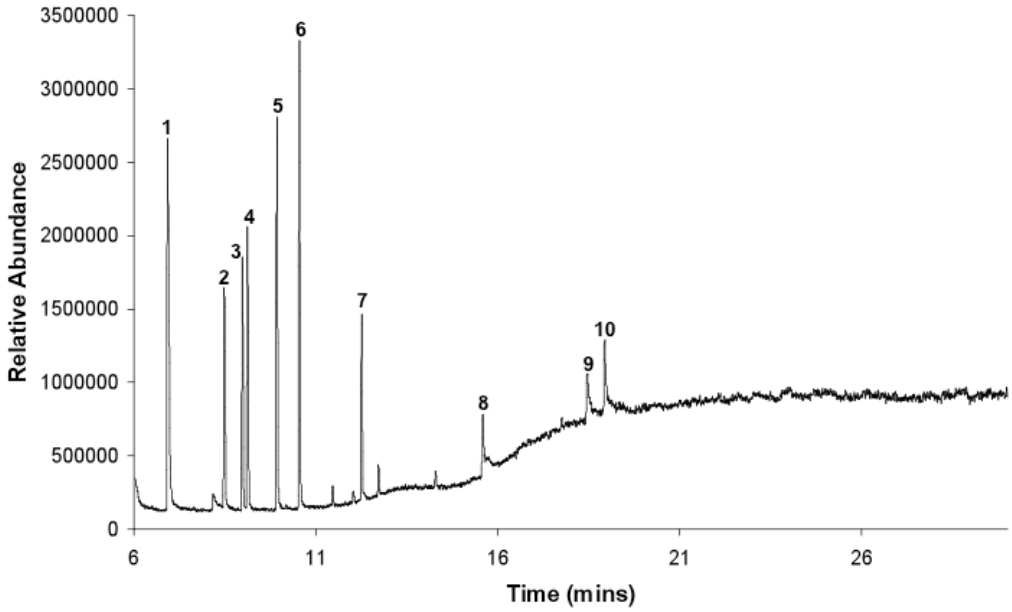


FIGURE 3.3. SAMPLE CHROMATOGRAM FROM PIGLET 2 ON DAY 14 (ADD 243) CONDUCTED IN PERTH, WESTERN AUSTRALIA, APRIL 2007, SHOWING IDENTIFIED PEAKS 1. ACETIC ACID, 2. PROPIONIC ACID, 3. ISO-BUTYRIC ACID, 4. TMA, 5. BUTYRIC ACID, 6. ISO-VALERIC ACID, 7. ISO-CAPROIC ACID, 8. 2-PIPERIDONE, 9. PHENYLACETIC ACID, 10. PHENYLPROPIONIC ACID.

It was demonstrated clearly during the piglet trial that short chain acids produced during decomposition show significant variation in concentration (0.19-7 mM). On the limited collection days, all of the target short chain acids were detected and identified, with the exception of valeric acid. Other compounds identified during this trial include 2-piperidone, phenylacetic acid, phenylpropionic acid, 4-methylvaleric acid, palmitic acid and oleic acid [59], although these had not been reported in previous decomposition studies, with the exception of palmitic and oleic acids [53, 70, 77]. Discrepancies also existed in the rate of decomposition between piglets exposed to maggot activity and piglets protected from maggots. This is in accordance with the author's Honours research [109] and is also supported in the literature [15, 118].

The limited amount of collection days for the piglet trial prevented any identification of trends, both for individual acids and ratios between acids.

The range of compounds that has been reported in previous decomposition studies [59] was not seen in this subsequent stillborn piglet trial. The sporadic appearance of long chain fatty acids detected during the piglet trial eliminates their use in establishing trends in production. Widdowson [130] found that the fat content of stillborn piglets is approximately 1% of their total body weight. The lack of long chain acids in the piglet trials can be related to the absence of adipose tissue since long chain acids are produced by the cleavage of triglycerides during aerobic degradation of fats and soft tissues [11].The timing of this trial also has some significance. Archer reports that the mass loss rate and the decomposition stage progression rate of stillborn piglets increases with increasing rainfall [16]. This trial was conducted in mid-Autumn 2007, when the average rainfall in Perth increases to 144 mm from the average 65 mm in summer [144]. The increasing rainfall in conjunction with the lack of adipose tissue in the piglets, accounts for the limited number of sampling days provided by this trial.

3.3.2.2 ADULT PIG TRIAL, CANADA

All of the initial C_2-C_5 target acids were detected in each fluid sample during this trial. Figure 3.4 shows the variation in compounds produced and their relative abundance for pig 1.

With the exception of isobutyric acid, target short chain acids passed through a maximum on day 6 (ADD 144), the point at which maggot activity was observed to be the highest. This is in contrast to the previous pork rasher trial [60], where the short chain acids passed through a maximum on day 15 (ADD 359), possibly due to the higher level of humidity, and therefore increased insect activity, experienced in Ontario compared with Western Australia. The acids appear to follow a cyclic trend, tending towards a second minimum on day 10 (ADD 223) and then increasing again until the end of the trial on day 14 (ADD 313) (Figure 3.5). No fluid was collected on day 16 which would allow completion of a second cycle to confirm this theory.

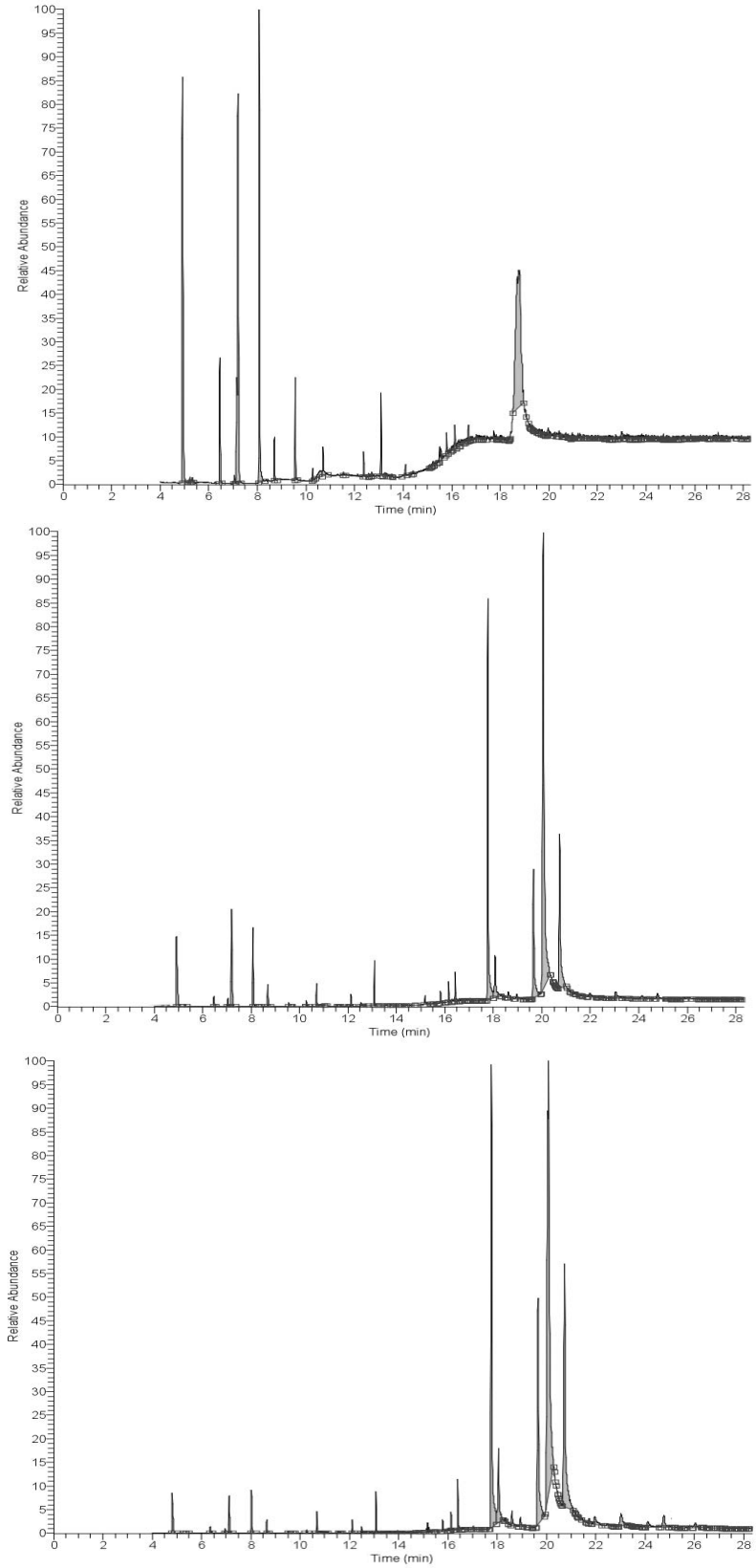


FIGURE 3.4. CHROMATOGRAMS REPRESENTING CHANGES IN COMPOUNDS DETECTED IN DECOMPOSITION FLUID FOR PIG 1 ON DAYS 5 (ADD 110), 10 (ADD 207), 14 (ADD 297).

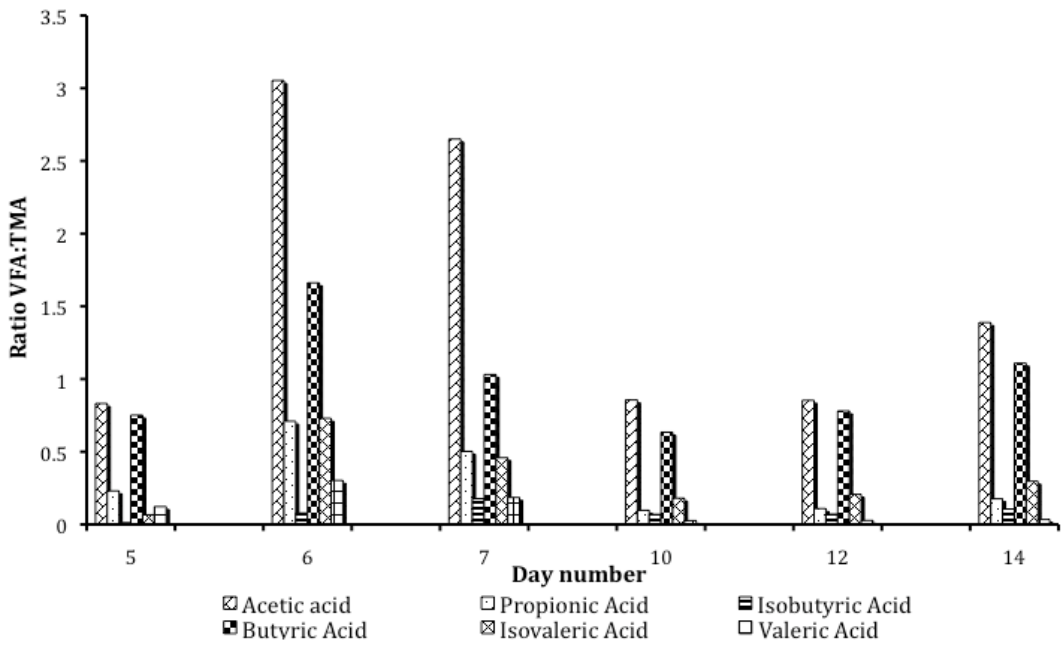


FIGURE 3.5. CYCLIC TRENDS OBSERVED IN SHORT CHAIN FATTY ACIDS DURING THE ADULT PIG TRIAL CONDUCTED IN CANADA.

The initial increasing trend shown by all C_2-C_5 acids suggested the possibility that all acids were produced by a common source. The patterns seen in the short chain acids can be related to initial work by Vass who suggested their abundance is at a maximum between 350 and 450 ADD [6]. Short chain acids in this adult pig trial reach a maximum on day 6 (ADD 144). Short chain VFAs were first detected on day 5 (ADD 126), although Vass suggests their appearance does not occur until at least 150 ADD. Unlike the work completed by Vass, valeric acid was not detected in the pig trial, although acetic acid was detected in abundance but is not mentioned in Vass's previously published work.

The detection of long chain acids was expected due to the fat content in an adult pig. These acids (oleic, stearic, palmitic, linoleic) showed an increasing trend over the 14-day time period for which the trial was run (Figure 3.6). All long chain acids reached a maximum on day 14 (ADD 313) and due to time constraints and the difficulty in sampling no further fluid samples were collected to demonstrate the continuation of this trend.

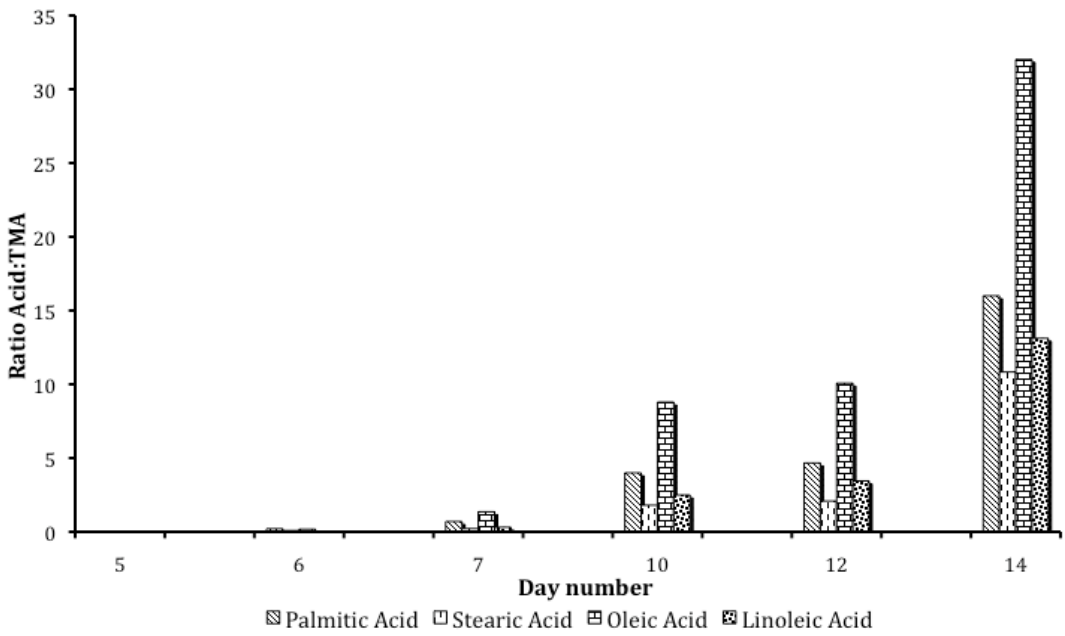


FIGURE 3.6. TRENDS OBSERVED IN LONG CHAIN ACIDS PRODUCED DURING THE ADULT PIG TRIAL IN SOUTHERN CANADA OVER COLLECTION DAYS 5-14.

In this trial, long chain acids were first detected on day 6 (maximum maggot activity) with a calculated ADD of 144. When compared to previously run trials, long chain acids can be first detected at ADD 192. When comparing this field study and the pork rasher trial, both show a maximum for the long chain acids peak around day 14-15. These have comparable ADD values of 313 and 359 respectively. This apparent 8-day increase (day 6-day 14) in the level of long chain acids can be tentatively related to the level of fly activity, the feeding cycle of the maggots and the level of adipose tissue in the carcass (the proposed origin of the fatty acids) [145]. Once the eggs are laid, it can take up to 24 hours for them to hatch. During this time, production of long chain fatty acids would be expected to be relatively low, as seen on days 5-6. The following 5-6 days are spent feeding and growing until full size is reached [145] and the production of long chain fatty acids steadily increases. Once the first maggots have grown to full size (7-8 days total), they migrate away from the food source to an environment suitable for pupation [145] and production will decrease. The pupation period lasts an additional 8-10 days [145]. After pupation, the adult fly can now emerge and leave in search of a food source.

The life cycle of the flies and maggots was not investigated in this trial, and therefore, further trials are needed to relate the 8-day feeding cycle to production of long chain fatty acids. If this trial had continued for an extended period of time, the level of long chain fatty acids would be expected to decrease on the next sampling session and then show another cyclic trend. This apparent trend was first reported in the literature in 2010 [60].

The ratio between individual long chain acids was analysed for patterns that would indicate their use as an effective indicator of PMI. The ratios between oleic:palmitic, oleic:stearic, oleic:linoleic, palmitic:stearic, palmitic:linoleic and stearic:linoleic acids showed no obvious trends. Vass concluded that long chain acids were indicative of decomposition in a cold environment and could be used to verify possible movement from a cold to warm area [6]. The presence of large, water insoluble, long chain fatty acids in a warm weather environment is contrary to these findings by Vass.

3.3.3 Mass spectrometry

The compounds produced in the pork rasher and piglet trials were identified using the WILEY 275 mass spectral library database (Table 3.5). However, to verify the identity of the compounds, the predicted fragmentation patterns and possible mechanisms based on the library search results were also determined and compared with the obtained mass spectral traces from the fluid samples.

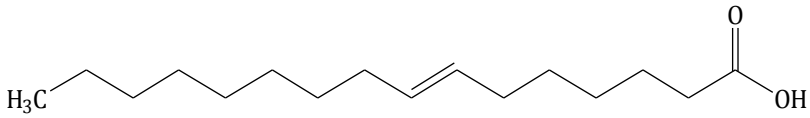
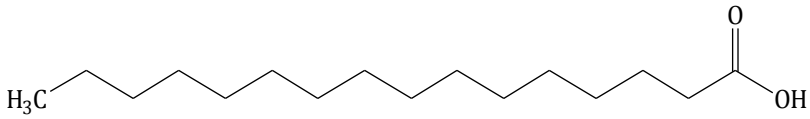
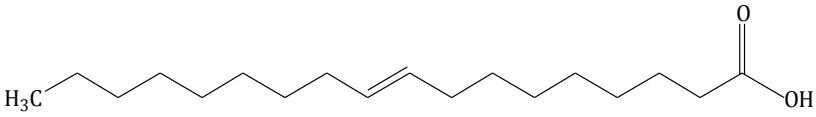
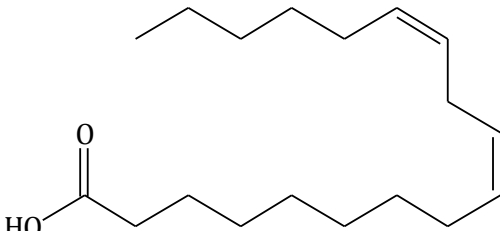
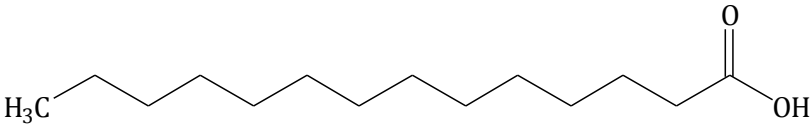
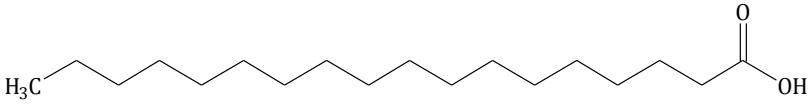
TABLE 3.5. COMPOUNDS PRODUCED DURING DECOMPOSITION OF PORCINE FLESH.

COMPOUND	MEAN RETENTION TIME (MINS)	SD	% RSD	TRIAL (PIGLET/PORK RASHER)
Acetic acid	7.3	0.03	0.4	Both
Formic acid	8.53	0.007	0.813	Piglet
Propionic acid	8.78	0.02	0.263	Both
Iso-butyric acid	9.25	0.02	0.232	Both
Butyric acid	10.1	0.022	0.217	Both
Iso-valeric acid	10.7	0.02	0.182	Both
Valeric acid	11.7	0.003	0.029	Piglet
Iso-caproic acid	12.4	0.007	0.053	Both
2-piperidone	15.8	0.015	0.097	Both
Phenylacetic acid	18.7	0.013	0.069	Both
Phenylpropionic acid	19.2	0.015	0.077	Both
Myristic acid	19.6	0.005	0.024	Rashers
Palmitic acid	21.7	0.015	0.069	Rashers
9-hexadecenoic acid	22.2	0.011	0.049	Rashers
Stearic acid	24.6	0.013	0.055	Rashers
Oleic acid	25.2	0.023	0.09	Rashers
Linoleic acid	26.2	0.014	0.054	Rashers

3.3.3.1. LONG CHAIN FATTY ACIDS

In long chain acids, the spectrum consists of two series of peaks. The retention of charge is either on the oxygen containing fragment (m/z 45, 59, 73, 87…) or the alkyl fragment (m/z 29, 43, 57, 71, 85…). Predicted fragmentation patterns for the long chain acids can be seen in table 3.6. The hydrocarbon pattern also shows peaks at m/z 27, 41, 55, 56, 69, 70 [146]. This is consistent with the spectra assigned to palmitic acid that can be seen in Appendix 2.

TABLE 3.6. STRUCTURES AND EXPECTED FRAGMENTATION PATTERNS FOR LONG CHAIN FATTY ACIDS.

COMPOUND	EXPECTED FRAGMENTATION PATTERN (m/z)
9-Hexadecenoic acid (palmitoleic acid) 	29, 41, 55, 60, 69, 83, 88, 97, 110, 124, 137, 152, 171, 192, 227, 236, 254
Palmitic Acid 	29, 43, 55, 60, 71, 85, 97, 115, 129, 157, 171, 185, 199, 213, 227, 256
Oleic acid 	29, 41, 55, 69, 83, 97, 111, 125, 137, 151, 157, 166, 180, 193, 222, 264, 282
Linoleic acid 	29, 41, 55, 60, 67, 81, 95, 109, 123, 137, 150, 161, 221, 265, 280
Myristic acid 	29, 43, 60, 73, 85, 97, 129, 171, 185, 228, 239
Stearic acid 	29, 43, 55, 60, 73, 85, 97, 111, 129, 171, 185, 199, 227, 241, 255, 284

The molecular ion peak for straight chain acids is also expected to be weak [146, 147] and this is again in agreement with the assigned spectra. The palmitic acid molecular ion (m/z 256) can be clearly seen on the mass spectrum but is low in abundance.

The most characteristic peak for long chain acids is m/z 60. This can be attributed to a McLafferty rearrangement (Figure 3.7) with the general mechanism as follows:

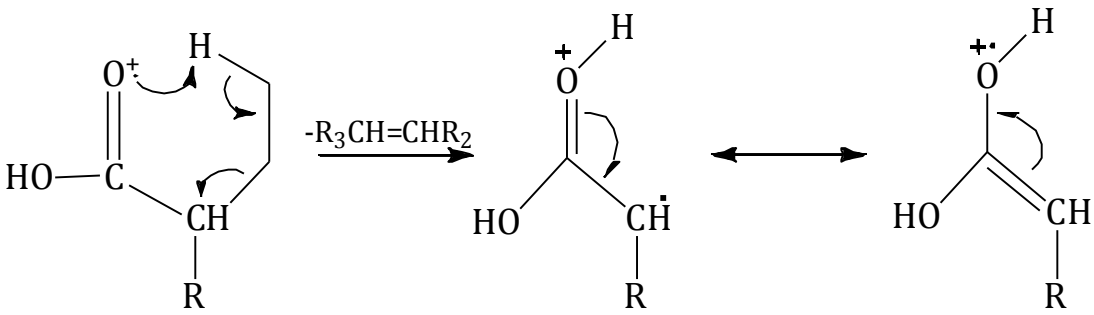


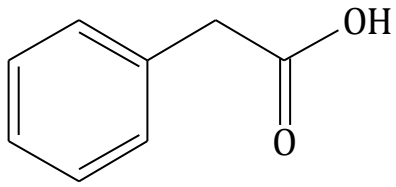
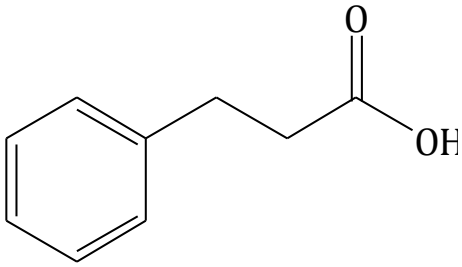
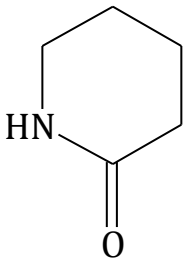
FIGURE 3.7. MCLAFFERTY REARRANGEMENT MECHANISM FOR LONG CHAIN CARBOXYLIC ACIDS [146].

In short chain acids, such as iso-caproic acid (Figure 3.8), peaks at M-OH and M-CO_2H are prominent. These peaks in the mass spectrum represent cleavage of bonds next to C=O [146].

3.3.3.2. AROMATIC ACIDS AND CYCLIC AMIDES (LACTAMS)

Table 3.7 shows the predicted mass spectral fragmentation patterns for the aromatic acids and cyclic amides that were observed in the decomposition trial of the whole adult pig carcasses.

TABLE 3.7. STRUCTURES AND EXPECTED FRAGMENTATION PATTERNS OF IDENTIFIED AROMATIC ACIDS AND CYCLIC AMIDES.

COMPOUND	EXPECTED FRAGMENTATION PATTERN (m/z)
Phenylacetic acid 	28, 39, 51, 65, 76, 91, 112, 136
Phenylpropionic acid 	28, 51, 65, 77, 91, 104, 150
2-Piperidone 	27, 30, 42, 43, 55, 70, 99

In aromatic acids, the molecular ion peak is prominent [146]. This was observed in the mass spectrum for both phenylacetic and phenylpropionic acid which can be seen in Appendix 2.

A base peak of m/z 91 indicates the presence of the resonance stabilised tropylium ion ($C_7H_7^+$) or the formation of a benzylic carbocation [148], a primary carbocation. The unstable benzylic carbocation can rearrange to form the tropylium ion and as this is more stable, it is the most likely outcome. It is formed by cleavage at the carbon α to the ring (Figure 3.8) [148].

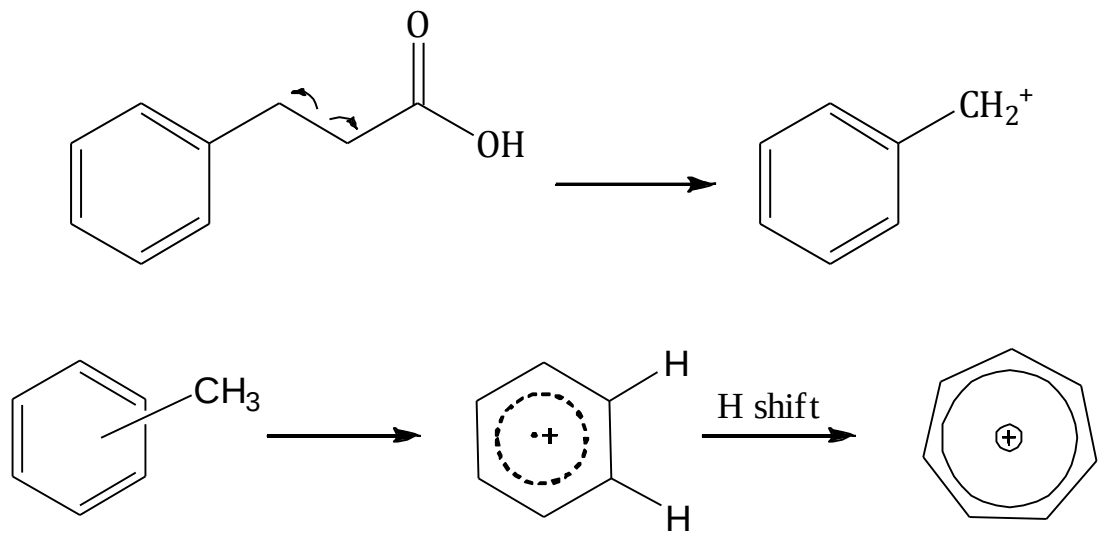


FIGURE 3.8. FORMATION OF TROPYLIUM ION AND BENZYLIC CARBOCATION IN AROMATIC ACIDS [146].

Formation of a peak at m/z 77 is not a prominent peak and is probably due to the formation of a $C_6H_6^+$ ion. The relatively low abundance of this peak is expected due to unlikely cleavage at the benzylic position [146]. This is observed in the proposed spectra for phenylpropionic acid.

The peak present at m/z 65 results from the elimination of a neutral acetylene molecule from the tropylium ion (Figure 3.9) [146]:

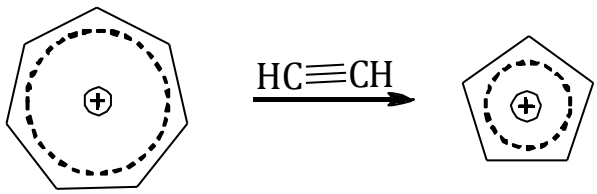


FIGURE 3.9. LOSS OF ACETYLENE MOLECULE FROM TROPYLIUM ION IN AROMATIC ACIDS.

The next abundant peak, m/z 51, represents a loss of $-CH_2$ from the cyclopentadienyl ion to give a $C_4H_3^+$ ion or a loss of –HCCH- from $C_6H_5^+$ [147].

Cyclic amides, have a prominent peak at m/z 70, which is consistent with the loss of two heavy atoms from a six-membered ring [A/Prof K. Lim, 2006, pers. comm.], in this case, the loss of CH_2NH from piperidone (Figure 3.10). The assigned mass spectral trace for piperidone can be seen in Appendix 2.

A radical cation is then formed:

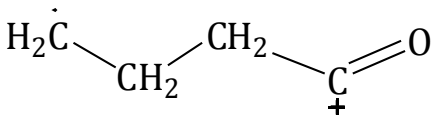


FIGURE 3.10. RADICAL CATION FORMED BY LOSS OF CH_2NH FROM PIPERIDONE.

The peak at m/z 55 is formed by further fragmentation of the radical cation at m/z 70 (Figure 3.11):

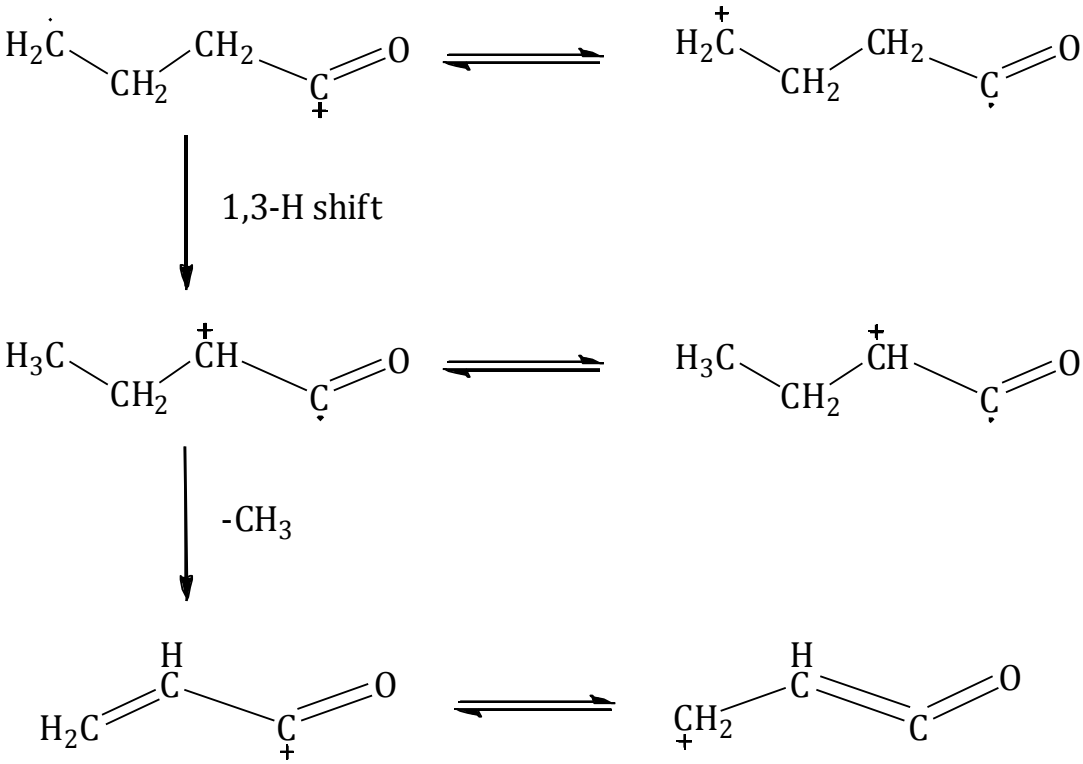


FIGURE 3.11. FORMATION OF M/Z 55 PEAK FOR PIPERIDONE.

The formation of m/z 43 and 42 are possibly due to loss of O=C=N(+)-H and loss of $CH_2C=O$ from the original piperidone molecule.

The most abundant peak at m/z 30 is formed by loss of two heavy atoms from an open-chain form of the parent molecule. The most likely fragmentations are loss of either a $CH_3N(+)H$ cation or $C(+)H_2NH_2$ cation. Fragmentation to produce m/z 27 is formed by loss of neutral C=O to give $CH_2=C(+)H$. This fragment is formed by subsequent fragmentation of the m/z 55 fragment [K. Lim, 2006. personal communication].

The predicted fragmentation pattern for 2-piperidone can be verified by the obtained mass spectral trace; however, the WILEY library search does not contain either of the isomers of 2-piperidone. Although the library search assigned the spectra to 2-piperidone, it is difficult to say whether or not the isomers, 3-piperidone and 4-piperidone, are present in the fluid samples.

3.3.4 Proposed biosynthetic pathways

Decomposition processes are the result of bacterial and enzymatic action under aerobic and anaerobic conditions [11]. A large proportion of the decomposition products produced should reflect the amount of fat and protein content of the remains. The major contributors to the products found in decomposition fluid will be protein, carbohydrate and fats [11], with the majority coming from adipose tissue and amino acid degradation.

3.3.4.1 Adipose tissue

Adipose tissue is specialised connective tissue that functions as the major storage site for fat in the form of triglycerides [11]. The body's adipose tissue comprises, on average, 5-30% water, 2-3% proteins and 60-85% lipids (fats), of which 90-99% are triglycerides [11]. Triglycerides are composed of one glycerol molecule attached to three fatty acid molecules (Figure 3.12). During decomposition, triglycerides are cleaved by enzymatic action to produce a glycerol molecule and three separate fatty acid chains.

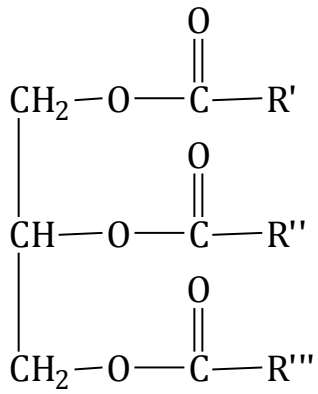


FIGURE 3.12. STRUCTURE OF A TRIGLYCERIDE MOLECULE.

The degradation of adipose tissue to free fatty acids is an essential component of the formation of adipocere. The work by Forbes *et al*. [70, 114] has shown that the long chain fatty acids palmitic, stearic and myristic are present in characteristic amounts when adipocere is formed

In research by Dent *et al.* into adipose tissue, monounsaturated oleic acid was by far the most prominent, followed by linoleic, palmitoleic and palmitic acids [11]. This held true for the oleic acid in the pig trial but not for the other long chain acids. Although oleic acid is the most abundant in the triglyceride molecules, it is also formed by the hydrogenation of linoleic acid. In turn, stearic acid is formed via the hydrogenation of oleic acid.

The second most abundant acid found in the trial was palmitic acid. Palmitic acid can be formed by hydrogenation of palmitoleic acid and beta-oxidation of oleic acid [149].

All six of the long chain acids found in the adult pig trial are considered to be possible components of adipose tissue [11, 70].

3.3.4.2 AMINO ACIDS

Amino acids are carboxylic acids that contain an amino group at the α-carbon atom. Hydrolysis of a protein cleaves the peptide linkage and individual amino acids are released. Amino acids can then either be deaminated or decarboxylated to produce straight and branched chain volatile fatty acids [133].

The short chain volatile fatty acids have a wide variety of amino acid sources; acetic acid (alanine, glycine, serine), propanoic acid (lysine, threonine), butyric acid (alanine, glutamic acid, aspartic acid), isovaleric acid (leucine, isoleucine), valeric acid (proline) and isobutyric acid (valine) [133].

Amino acid degradation by anaerobic bacteria always involves oxidation-reduction reactions. The ultimate reduction products include a variety of short chain fatty acids, δ-amino valeric acid and molecular hydrogen [110].

At a neutral pH (pH 6-7), deamination is the major pathway for metabolic degradation of amino acids (Figure 3.13) [111]. Deamination results in the production of VFAs, CO_2, H_2 and NH_3:

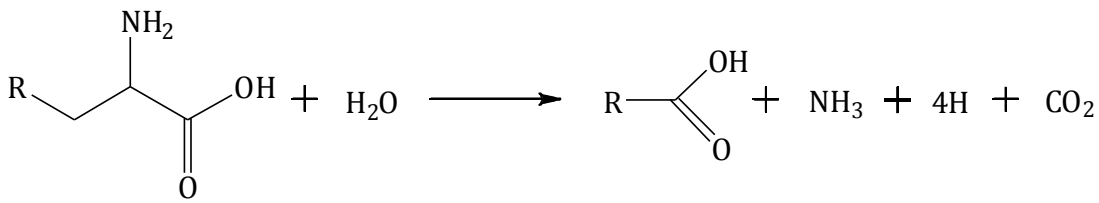


FIGURE 3.13. DEAMINATION PATHWAY FOR AMINO ACIDS.

Amino acid decarboxylase is induced at pH 5-6 but is a much less common degradation pathway (Figure 3.14) [111]. The general reaction is:

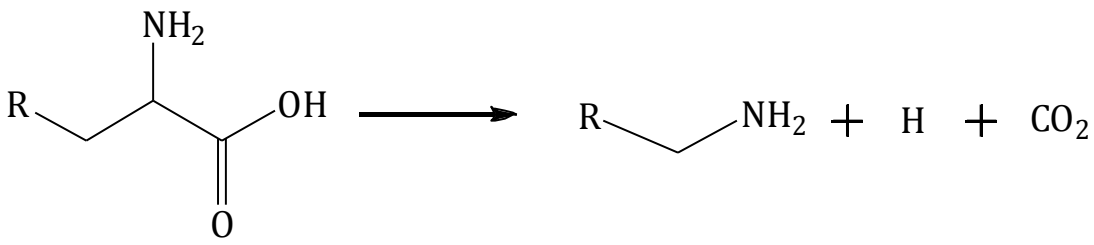


FIGURE 3.14. DECARBOXYLATION PATHWAY FOR AMINO ACIDS.

Proposed origins from amino acids for the compounds found in the field trials are as follows:

The amino acid phenylalanine, on putrefaction, yields 3 different aromatic acids; phenylacetic acid (produced by oxidation), phenylpropionic acid (reduction) and benzoic acid [150] (Figure 3.15). Benzoic acid was not detected in any of the fluid samples.

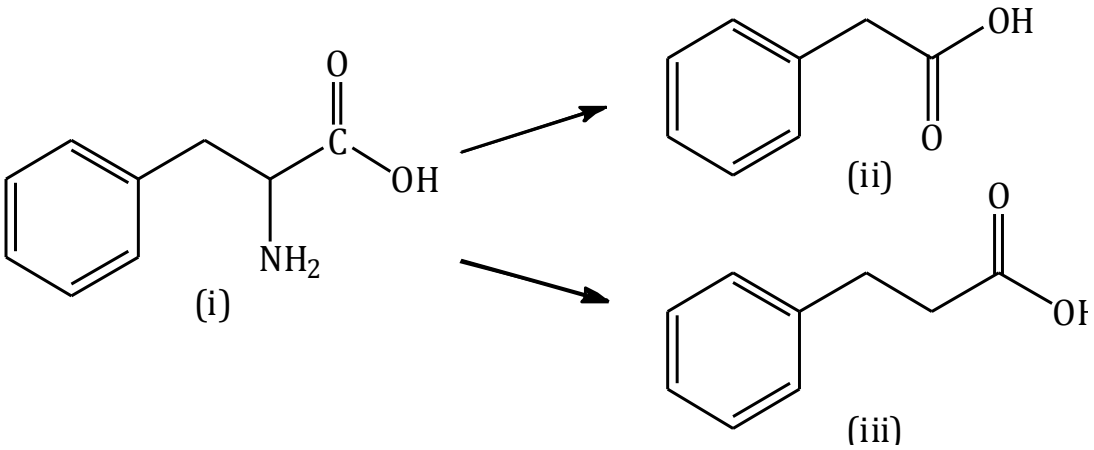


FIGURE 3.15. DEGRADATION PATHWAY OF PHENYLALANINE (I) TO PRODUCE PHENYLACETIC (II) AND PHENYLPROPIONIC (III) ACIDS.

Phenylacetic and phenylpropionic acids are also produced by the deamination of tyrosine (Figure 3.16) by anaerobic bacteria [133].

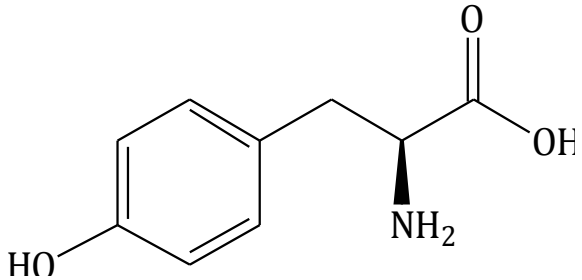


FIGURE 3.16. STRUCTURE OF TYROSINE.

2-piperidone is a lactam (cyclic amide) that was found to be a metabolite of cadaverine, along with 1-piperidine and 5-aminopentanoic acid [151]. Cadaverine is a volatile amine produced by the decarboxylation of the amino acid lysine (Figure 3.17) [111, 133]:

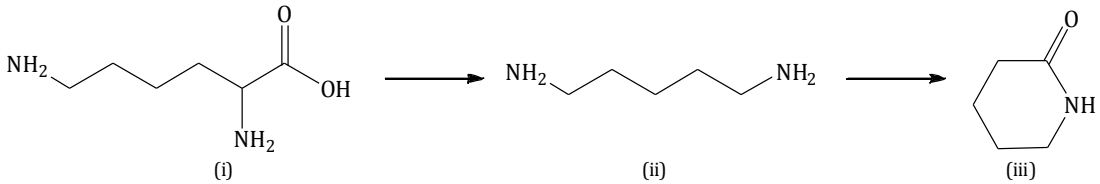


FIGURE 3.17. DEGRADATION PATHWAY OF LYSINE (I) VIA CADAVERINE (II) TO PRODUCE 2-PIPERIDONE (III).

Isocaproic acid (4-methylvaleric acid) is formed via the reductive deamination of the amino acid leucine (Figure 3.18) [133, 152]:

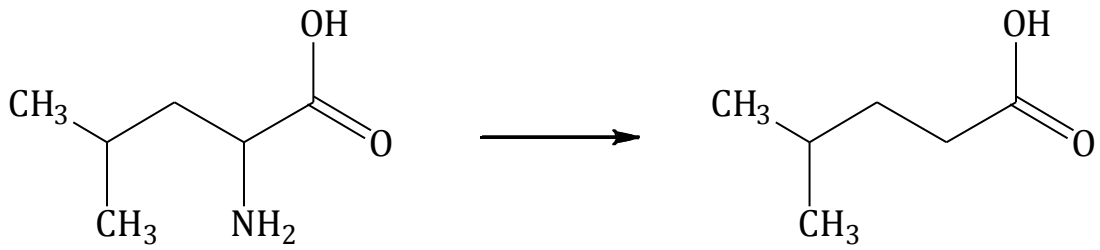


FIGURE 3.18. REDUCTIVE DEAMINATION OF LEUCINE TO PRODUCE ISO-CAPROIC ACID.

Another suggested pathway for the origin of iso-caproic acid via leucine is the enzymatic cleavage of the cholesterol side chain [153, 154].

It should be noted that the proposed degradation pathways were taken from literature predominantly concerned with fermentation processes, particularly for the breakdown of amino acids [110, 111]. Further analytical study is required to confirm these biodegradation pathways. The majority of studies cited accept the cleavage of triglyceride molecules as the origin of long chain fatty acids [11].

3.4 CONCLUSIONS

A simple aqueous dilution and filtration followed by analysis with gas chromatography-mass spectrometry was successful in confirming the presence of target compounds in decomposition fluid. The presence and identity of compounds that had been previously unreported in decomposition fluid was also established.

The importance of the choice of subject as a source of fluid was recognised, as stillborn piglets did not produce enough decomposition fluid over a large enough time frame to establish trends in the production of compounds. However, for whole adult pig carcasses, a possible 8-day cyclic trend was observed for the production of short chain acids. An 8-day increase was also observed for long chain fatty acid production.

Two decomposition research facilities with different climatic conditions showed similarities in classes of compounds produced over the course of the field trials.

Chapter 4 – Analysis of free biogenic amines and amino acids by capillary electrophoresis (CE)

4.1 INTRODUCTION

Biogenic amines are defined as basic nitrogenous compounds that are mainly formed by decarboxylation of amino acids [155, 156]. These organic bases can be produced by the enzymes of raw products in food and beverages [155], and can be related to both food spoilage and shelf life quality. Alternatively, biogenic amines can be manufactured by the microbial, vegetable and animal metabolism [155] of living organisms. Food products likely to contain these compounds include fish and fish products [90, 91, 156], meat products [157, 158], eggs [159] and cheeses [160], beer and wine [161, 162]. Their presence, particularly in meat products, is a concern due to their toxicological implications [155, 157].

Tyramine is one of the main biogenic amines involved in food poisoning and is formed by decarboxylation of the amino acid tyrosine. It has been reported to have both vasoactive and psychoactive effects [155, 156]. Tyrosine has also previously been reported as present in the soil solution analysis of decomposition products [6]. Tryptophan, a precursor of serotonin and indole also biodegrades to form tryptamine, another compound involved in food poisoning. The presence of indole in decomposition fluid was confirmed during previous GC-MS studies as detailed in Chapter 3. The detection of indole led to the conclusion that the pre-cursor compound must also be present in decomposition fluid. The detection of this and other amines and amino acids would require appropriate analytical methods to be developed and validated.

Compounds such as putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) are essential components in the regulation of nucleic acid function and protein synthesis in living cells [155, 163]. Putrescine is derived from the decarboxylation of the amino acid arginine, while cadaverine results from lysine [111, 133]. Both cadaverine and putrescine have been shown to have significant pharmacological effects [156] and both have been previously reported as present in decomposition fluid [6].

4.1.1 Capillary electrophoresis

Capillary electrophoresis (CE) is defined as the differential movement of charged species (ions) by attraction or repulsion in an electric field, carried out in narrow-bore capillaries [164, 165]. This method of separation was first demonstrated in 1937 by Tiselius [166] where analytes in a protein mixture were separated at a rate based on individual charge and mobility with respect to a buffer solution and strength of an applied electric field. This work received little attention until the 1960's, when a paper was published by Hjerten [167] in 1967, detailing one of the first CE instruments. Hjerten was able to separate inorganic ions, nucleic acids, proteins and viruses using capillary zone electrophoresis (CZE). Jorgenson and Lukacs [168] then demonstrated the simplicity of the instrumental set-up combined with the capabilities of high resolving power in the 1980's and it was at this time that significant interest in CE progressed.

Like Hjerten's work, much of the early research involving CE had applications which focused particularly on biomolecules, such as large proteins and peptides, including DNA [169], traditionally separated by gel electrophoresis. The application of CE to DNA analysis was seen as an extension of this methodology [169]. CE is also able to separate small biologically active molecules [170], such as pharmaceuticals, where HPLC has been the established technique for analysis [169]. The introduction of chromatographic mechanisms [171] has allowed electrophoresis to be applied to water-insoluble neutral species [169, 171]. This was first demonstrated by Terabe [172] with the introduction of micelles into the buffer solution, known as micellar electrokinetic chromatography (MEKC). CE now has a wide variety of operational modes and detection capabilities allowing various analytes to be separated and detected with high levels of sensitivity and selectivity, making it ideal for incorporation into forensic laboratories.

4.1.2 Analysis of biogenic amines and amino acids by capillary electrophoresis

CE has been applied to separations of biogenic amines in environmental samples, foods and beverages, with derivatisation of the amines often being necessary to enable detection [90-92, 162] In cases where derivatisation is not required, the use of micelles is often employed [173, 174] or complex buffer systems are needed for detection [162, 173]. In these methods, substantial analysis times often result [90, 92, 161]. Zhang *et al.* determined seven biogenic amines in soy sauce, fish and wine samples by micellar electrokinetic chromatography (MEKC) with laser induced fluorescence detection after derivatisation of the amines with the fluorescent label 3-(4-fluorobenzoyl)-2-quinolinecarboxaldehyde [91]. Krizek and Pelikanova separated biogenic amines as N-substituted benzamide derivatives in fish meat by MEKC with UV-visible detection [90]. Liu and Cheng analysed lake water using MEKC with post-column chemiluminescence detection of N-(4-aminobutyl)-N-ethylisoluminol labeled amines. Direct analysis of amines in selected food samples without derivatisation has been achieved using separation on a fluorinated ethylene-propylene co-polymer analytical capillary with conductivity detection [173]. Biogenic amines have also been separated by CE without derivatisation using an ammonium acetate buffer containing acetonitrile or methanol as an organic modifier at pH 7.5. Separation was found to be best achieved with acetonitrile as the modifier, however there was no application to any real samples [175]. Cortacero-Ramirez *et al.* analysed bottled beer for 18 amines and amino acids without the need for derivatisation or micelles, however, there was not complete baseline resolution of all the compounds of interest. This method also has a substantial analysis time (>30 min) [161]. Whilst the vast majority of papers presented achieve separation and detection of similar amines, to the best of our knowledge, no analytical methodology using CE as a separation technique for the determination of the chemical species present in decomposition fluid has been reported.

4.1.3 Application to forensic analysis

The use of CE for forensic analysis has been demonstrated since the 1980's with a wide range of applications [176-180], particularly in cases where the sample matrix can be extremely complex [169]. Samples can contain an array of endogenous compounds that require separation from the analyte of interest prior to analysis [169]. A range of forensically important compounds are amenable to separation by CE and have successfully been analysed; these include drug molecules, gunshot and explosive residue, DNA, body fluids and pen inks [176, 179, 181, 182].

The first application of CE to decomposition products was reported in 1998 by Ferslew [66], who used CE as a new methodology for direct detection of the potassium ion concentration in vitreous humor of 25 human subjects. The aim of this work was to estimate postmortem interval by showing a linear correlation between potassium ion concentration and time since death, as described in Chapter 1. This was followed by the work of Tagliaro *et al.* [58, 67] and Bocaz-Beneventi [68] who also used CE with UV-Vis detection to develop and validate methods for the analysis of potassium ions in vitreous humor with potential application to postmortem interval. Artificial neural networks (ANN) were also applied with the aim of improving the linear correlation between the two [68]. Early work in this area focused on the analysis of potassium by means of flame photometry and ion selective electrodes [56, 79, 80, 82, 86, 89]. However, the use of CE allowed for more rapid analysis times, improved separation of small inorganic ions, more symmetrical peak shapes, enhanced detection limits, and direct analysis of the sample [66] whilst only requiring very small sample volumes [58].

4.1.4 Experimental design

An experimental design approach was used to improve resolution and thereby quantification in peaks that were poorly separated in the electropherogram. This consisted of a screening design to establish the appropriate experimental domain and evaluate the influence of four factors; buffer pH, background

electrolyte concentration, percentage of organic modifier and voltage. This was followed by a central composite design (CCD). This method was chosen because of its ability to allow the entire response surface to be viewed in relation to the value of the various factors.

4.1.5 Aims

When compared with similar existing methods developed using CE, this chapter reports a comparatively faster, simple (derivatisation and presence of micelles not required) and reproducible capillary electrophoresis method for selected biogenic amines and amino acids in decomposition fluid.

CE was chosen for analysis of decomposition samples as it shows a high level of resilience to 'dirty' or complex sample matrices, such as alcoholic beverages and food, particularly cheese and fish [90-92, 162]. It offers the potential for highly efficient and rapid separations, and thus is extremely useful when analyzing complex mixtures. CE can cope with analytes that are not suitable for gas chromatography (GC) due to thermal instability, whilst also providing more rapid separations than are achievable with liquid chromatography (LC), whilst still maintaining high resolution [164].

4.2 Experimental

4.2.1 Chemicals and reagents

Chemicals were obtained from the following suppliers: Tyramine, tryptophan and indole (Sigma-Aldrich, New South Wales, Australia), tyrosine, tryptamine and phenylalanine (Fluka, New South Wales, Australia), aniline, boric acid and sodium hydroxide (BDH, Victoria, Australia), benzylamine (ACROS, New Jersey, USA), methanol (LC Grade, Mallinckrodt, Missouri, USA). Standards (400 mg/L) were prepared in methanol:water (1:1). Ultrapure water was prepared in-house using a Purelab water system. Peak identification was carried out by the injection of known individual standards and by spiking samples with known standards.

The background electrolyte was prepared by mixing varying concentrations of boric acid (33 mM, 50 mM, 75 mM, 100 mM, 117 mM) with 0.1 M sodium hydroxide (~80:20 v/v); the pH was adjusted to the desired level with 0.1 M sodium hydroxide. Methanol (13.2 %, 20 %, 30 %, 40 %, 46.8 %) (v/v) was then added to the buffer solution.

All buffer solutions and standards were degassed by filtration through a 0.45 μm PTFE Gelman Acrodisc® syringe filter prior to injection and analysis.

4.2.2 Sample collection and preparation

The collection of decomposition fluid samples is described in Chapter 2. Initial studies were performed with pork rashers (belly pork, *Sus scrofa*) to aid in method development. Further trials were carried out with stillborn piglets and adult pig carcasses (*Sus scrofa*) to create a more realistic model of decomposition and to gain a more accurate understanding of the composition of decomposition fluid.

Samples were stored in the freezer prior to analysis. Samples were prepared by a 1:4 dilution in methanol, vortexed for 1 minute, followed by filtration through a 0.45 μm PTFE Gelman Acrodisc® syringe filter. Samples that were analysed by CE-UV-Vis had previously been analysed by GC-MS as described in Chapter 3.

4.2.3 Analytical instrumentation

Experiments were performed using an Agilent 3DCE Capillary Electrophoresis system equipped with a diode array detector. An uncoated 75 μm fused silica capillary of total length 65 cm and effective length 56 cm (Agilent) was used for analysis. Instrument control and data acquisition were performed using ChemStation software.

Sample introduction was achieved by pressure injection at 50 mbar for 2.5 seconds. During sample analysis, a constant voltage of 30 kV was applied,

resulting in an electrophoretic current of 26 μA. The temperature around the capillary was maintained at 30 °C. For all experiments, the wavelength of detection was 200 nm. The final optimized running buffer consisted of 70 mM boric acid adjusted to pH 9.5 with 0.1 M sodium hydroxide and 32 % methanol (v/v).

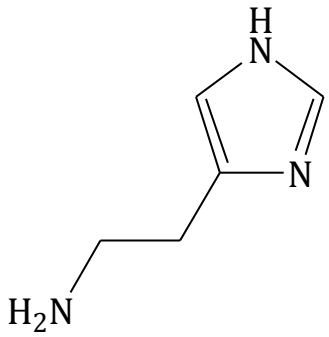
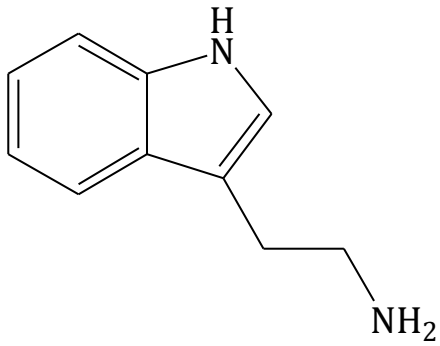
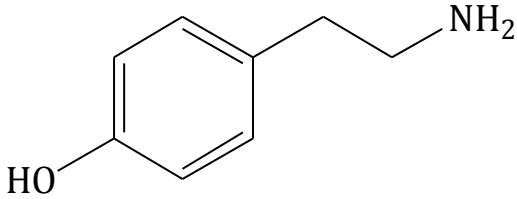
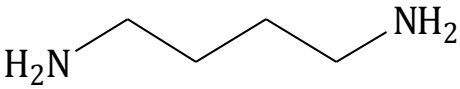
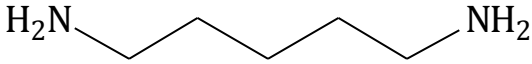
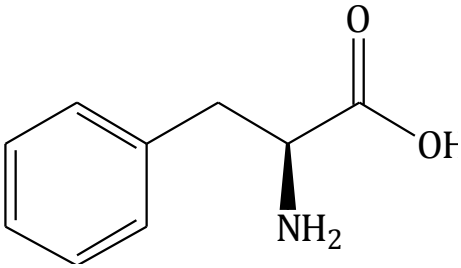
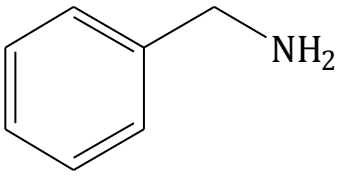
When a new capillary was used, it was conditioned for 30 minutes each with 1.0 M sodium hydroxide, ultrapure water and running buffer. At the beginning of each day, the capillary was flushed for 5 minutes with 1.0 M sodium hydroxide, ultrapure water and running buffer successively. For the screening design, the capillary was flushed for 10 minutes with the new running buffer (as described by conditions detailed in Table 4.2) between each run. Preconditioning before each run was achieved by flushing with running buffer for 3 minutes. Between runs, post-conditioning for 3 minutes with ultrapure water, 3 minutes with 0.1 M sodium hydroxide and 3 minutes with ultrapure water was performed. For the optimized system, when reproducible analysis was achieved, post-conditioning was reduced to 3 minutes with ultrapure water only.

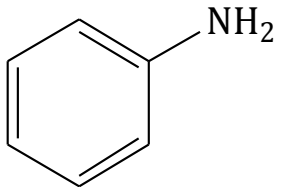
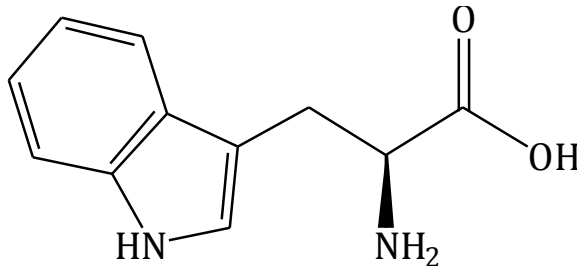
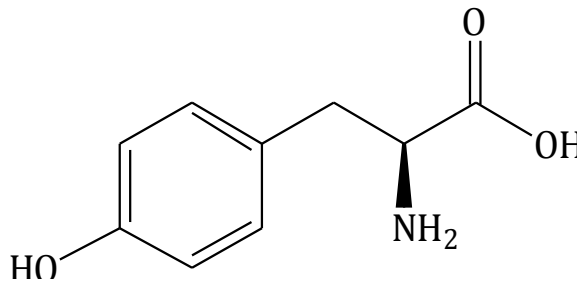
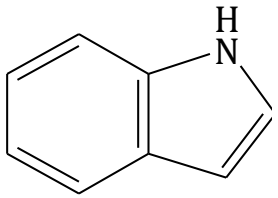
The screening design and central composite design were generated and graphed using SAS/JMP software version 7.

4.3 RESULTS AND DISCUSSION

The biogenic amines selected for this study are presented in Table 4.1. All of the compounds listed were chosen on the basis of their availability in the laboratory. Excluding benzylamine and aniline, all compounds were chosen due to previous reports in the literature of their presence in decomposition products [6] and the results from analysis by GC-MS, as described in Chapter 3. Compounds such as tyramine and tryptophan also have implications for food safety and shelf-life quality as well as significant toxicological and health implications [155, 156].

TABLE 4.1. SELECTED BIOGENIC AMINES AND AMINO ACIDS. * INDICATES SUCCESSFUL SEPARATION BY METHOD OUTLINED IN THIS WORK.

NAME	STRUCTURE	pK_a [REF]
Histamine		9.75 [183]
Tryptamine*		10.2 [183]
Tyramine*		9.74 [183]
Putrescine		9.35 [183]
Cadaverine		10.05 [183]
Phenylalanine*		9.31 [183]
Benzylamine*		9.33 [184]

NAME	STRUCTURE	pK_a [REF]
Aniline*		4.63 [184]
Tryptophan*		9.41 [183]
Tyrosine*		9.21 [183]
Indole		16.7 [184]

4.3.1 DERIVATISATION

Free amines and amino acids in their free form are notoriously difficult to analyse. Separation of biogenic amines by CE can be problematic especially because the amines have low hydrophilic properties, similar dissociation constant (pK_a) values, and the tendency to be adsorbed on the capillary wall [185]. A derivatisation procedure with the use of micelles is often employed to overcome these problems Derivatisation procedures enhance detection sensitivity of analytes [186] while the addition of micelles enables detection of both charged and neutral species [165].

On reviewing the literature [90, 91, 173], the derivatisation procedure chosen for further study was based on the method of Krizek and Pelikanova [90] due to

simplicity, availability of reagents and applicability to the analytes listed in Table 4.1. The derivatisation involved conversion to a *N*-benzamide derivative via a 12-step procedure using hazardous chemicals such as benzoyl chloride and dichloromethane. The running buffer required to achieve separation consisted of 50 mM sodium tetraborate, 40 mM sodium dodecyl sulfate, 25 % methanol (v/v) at pH 9.4. Investigations into the presence of micelles found that they were required for peak detection. In a similar fashion to the original authors it was found that the performance of the derivatisation was highly dependent on vigorous mixing of the reagent and analyte. A sample electropherogram from the derivatisation containing putrescine, cadaverine, tryptamine and tyramine can be seen in Figure 4.1. Mixed acid standards showed several unidentified peaks indicating the possibility of residual free amines present post-derivatisation. Peaks may also have been observed for benzoic acid that is readily formed during the derivatisation procedure from benzoyl chloride [90].

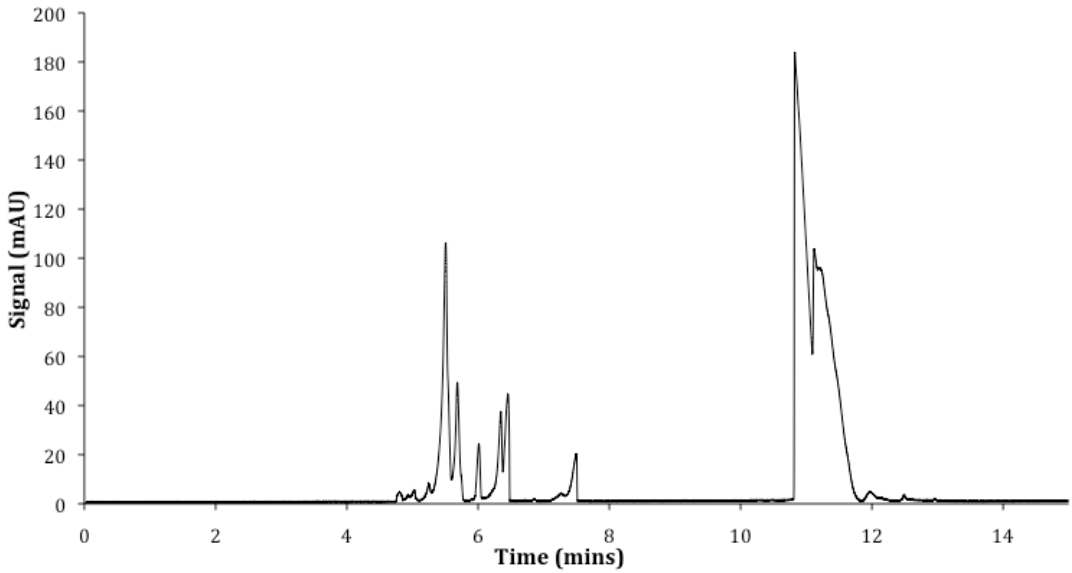


FIGURE 4.1. ELECTROPHEROGRAM SHOWING DERIVATISATION OF A MIXED ACID STANDARD CONTAINING 4 COMPOUNDS.

While the separation and detection was achieved, the results for the derivatisation were erratic and not reproducible. The variability in results achieved for the derivatisation procedure led to further investigations of the separation conditions. Successful separation and detection of a number of the amines and amino acids (Table 4.1) could be achieved without prior derivatisation or the presence of micelles. In comparison to the derivatisation procedure outlined by Krizek and Pelikanova, no sample preparation was required other than a simple dilution and filtration. Samples could be prepared and ready for analysis in a matter of minutes. Initial investigation determined that two amines (tryptamine, tyramine) and three amino acids (tryptophan, phenylalanine, tyrosine) could be separated within 8 minutes using a fused silica capillary with a simple background electrolyte consisting of boric acid (50 mM) with 25 % methanol (v/v) at a pH of 9.4. Benzylamine and aniline co-migrated and also partially overlapped with tyramine. All of the other compounds were baseline resolved (Figure 4.2). Indole, which was initially included in this study due to previous identification in decomposition fluid, had to be excluded at this stage as it was not able to be separated. This can be explained by the differences in pK_a values between indole (pK_a 16.2) and those compounds that were separated (pK_a range 9.21-10.2). The optimised running buffer was at pH 9.5. As expected without derivatisation, those compounds successfully separated and detected all have primary amine functionalities and a chromophoric benzene ring. For this reason, further studies excluded putrescine and cadaverine, which are both aliphatic compounds. A trial run with a decomposition fluid sample indicated the presence of the analytes in Table 4.1 except for benzylamine and aniline. Considering the rapid nature and simplicity of this approach we proceeded to optimize the separation conditions in order to achieve baseline resolution.

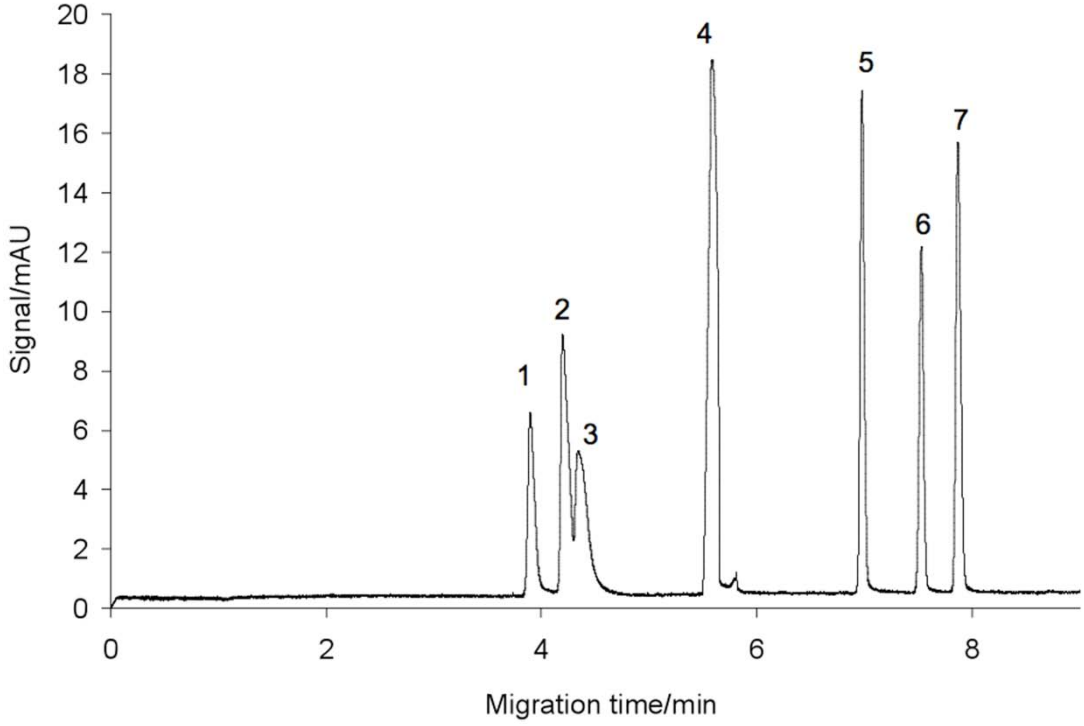


FIGURE 4.2. UNOPTIMISED SEPARATION. PEAK IDENTIFICATION: 1. TRYPTAMINE, 2. TYRAMINE, 3. BENZYLAMINE/ANILINE, 4. NEUTRAL, 5. TRYPTOPHAN, 6. TYROSINE, 7. PHENYLALANINE.

4.3.2 SCREENING DESIGN

When considering optimisation of this CE method, it was recognized that the potential existed for interactions between factors that will effect the separation. These include pH, background electrolyte [BGE] concentration, applied voltage and percentage of organic modifier. The pH of the buffer determines the degree of ionization for moderate and weakly basic or acidic analytes [187]. Manipulation of the buffer pH therefore affects the electrophoretic mobilities of the analytes in question [164, 187]. The concentration of the background electrolyte is important as the positive ions from the buffer are attracted to the negatively charged capillary wall, creating an electrical double layer and a potential difference, allowing the ions the freedom to migrate with the EOF [165]. Organic solvents can be used as an additive for hydrophobic species, however, it will increase the buffer viscosity [164]. Electrophoretic mobility of an analyte will differ when the viscosity of the buffer changes and also with the applied voltage [164, 165].

The relationship between the variables affecting a CE separation meant that a univariate (one-at-a-time) approach was likely to be ineffective for optimisation and as such a multivariate chemometric approach was used. This consisted of a screening design to determine the significant individual factors and interactions, followed by a central composite design to optimize the method with respect to resolution and time.

To establish the appropriate experimental domain and evaluate the influence of buffer pH, concentration of background electrolyte (boric acid), % of organic modifier (methanol) and applied voltage, a 2-level full factorial design (2^4) was used resulting in a total of 16 unique experiments. The screening design also established the significance of possible interactions between variables. All possible interactions were considered including secondary (2-way), tertiary (3-way) and quaternary (4-way) interactions.

Resolution between peaks for each electropherogram was evaluated using Equation 1:

EQUATION 1: $$R = \frac{2(t_2 - t_1)}{w_2 + w_1}$$

where t_2 and t_1 are the migration times and w_2 and w_1 are the corresponding peak widths at the bases of adjacent peak pairs [188]. For overlapping and co-migrating peaks, the resolution was estimated using data from Snyder [189]. A single response objective function (Chromatographic Exponential Function; CEF, Equation 2) was used to evaluate the quality of each electropherogram with regard to resolution and time [190].

EQUATION 2: $$CEF = \left(\sum_{i=1}^{n-1} (1 - e^{a(R_{opt} - R_i)})^2 + 1\right)\left(1 + \frac{t_f}{t_{\max}}\right)$$

R_{opt} and R_i are selected optimum resolution and the resolution of the *ith* peak pair respectively, t_{max} and t_f are the maximum acceptable time (15 minutes) and the migration time of the final peak respectively, a is the slope adjustment factor (default = 1.5) and n is the number of expected peaks. The design matrix for the factors and their high (+1) and low values (-1), along with the calculated response factors, are summarized in Table 4.2. Experiments were run in a randomized order (using random number tables generated in MS Excel) but have been shown sequentially for ease of viewing.

TABLE 4.2. LEVELS OF THE FOUR DESIGN VARIABLES AS USED IN THE SCREENING DESIGN. (-1) INDICATES THE LOW LEVEL, (+1) INDICATES THE HIGH LEVEL. TABLE 1 ALSO DETAILS THE COMPONENTS OF THE RUNNING BUFFERS.

EXPERIMENT	pH	[BGE]	% METHANOL (V/V)	VOLTAGE	CEF
1	9 (-1)	50 (-1)	20 (-1)	20 (-1)	591.3
2	9	50	20	30 (+1)	512.9
3	9	50	40 (+1)	20	13.9
4	9	50	40	30	11.5
5	9	100 (+1)	20	20	1141.3
6	9	100	20	30	983.1
7	9	100	40	20	15.6
8	9	100	40	30	12.5
9	10 (+1)	50	20	20	2544.2
10	10	50	20	30	687.9
11	10	50	40	20	8721.3
12	10	50	40	30	2716.1
13	10	100	20	20	16152.8
14	10	100	20	30	11238
15	10	100	40	20	12490.4
16	10	100	40	30	15916.4

The calculated CEF value was input into the screening design analysis tool using JMP version 7 software. A high CEF value indicates a poorly resolved electropherogram, therefore, CEF was optimized to a minimum. The evaluated data showed that pH (p-value=0.0013) was the most significant individual factor with the greatest effect on the quality of the electropherogram. The significant effect of pH is expected in CE as pH determines the degree of ionization for moderate and weakly basic or acidic analytes [187]. Manipulation of the buffer pH therefore affects the electrophoretic mobilities of the analytes in question [164, 187]. The concentration of the background electrolyte (p-value=0.0106) was also a major factor. Voltage and the % organic modifier had the least impact (p-values=0.4382 and 0.6378 respectively), with the only apparent effect being a decrease in the analysis time at the higher voltage, although often at the expense of resolution.

The secondary interaction between pH*[BGE] was the most significant (p-value=0.0119) as seen in Table 4.3, with pH having a high, positive effect at both high and low concentrations of background electrolyte. Although the percentage of organic modifier did not have a significant individual effect, its secondary interaction with pH (p-value=0.0285) is of value. The pH had a high effect at both low and high levels of organic modifier. For this reason, the percentage of methanol added to the buffer system cannot be excluded from further optimisation studies. All of the secondary interactions involving voltage were insignificant. Voltage had little to no effect at both high and low levels of the other three factors. Voltage was deemed to have no effect on the quality of the electropherogram and was therefore excluded from the central composite design to optimise the remaining parameters. The associated p-values for all interactions are listed in Table 4.3.

TABLE 4.3. P-VALUES FOR INDIVIDUAL FACTORS AND ALL INTERACTIONS SHOWING STATISTICAL SIGNIFICANCE.

INDIVIDUAL FACTORS AND INTERACTIONS	p-VALUE (* INDICATES A SIGNIFICANT EFFECT)
pH	0.0013*
[BGE]	0.0106*
% Organic Modifier	0.6378
Voltage	0.4382
pH*[BGE]	0.0119*
pH*% Organic Modifier	0.0285*
pH*Voltage	0.4191
[BGE]*% Organic Modifier	0.5102
[BGE]*Voltage	0.6223
% Organic Modifier*Voltage	0.7443
pH*[BGE]*% Organic Modifier	0.5761
pH*[BGE]*Voltage	0.5596
pH*% Organic Modifier*Voltage	0.7161
[BGE]*% Organic Modifier*Voltage	0.2535
pH*[BGE]*% Org Modifier*Voltage	0.2875

As expected, none of the tertiary interactions were significant statistically (p-values >0.05). The four-way interaction between all factors was also not statistically significant in terms of effect on resolution and time.

4.3.3 CENTRAL COMPOSITE DESIGN

A central composite design (CCD) was chosen to optimise the separation of target compounds because of its usefulness in viewing the entire response surface and how a response relates to the value of various factors [187]. A CCD uses coded variables to model the response surface and give a direct measurement of the importance of each effect and interaction [87].

A 2-level, 3-factor central composite design is detailed in Table 4.4. The design consists of 8 (2^3) factorial points at all possible combinations of ± 1, with 7 star points, including a central point of the form (0, 0, 0, 0) and six points of the form (± 1.68, 0, 0, 0), with a recommended 5 replicates in the centre of the form (0, 0, 0, 0) giving a total of 20 experiments (Figure 4.3) [191].

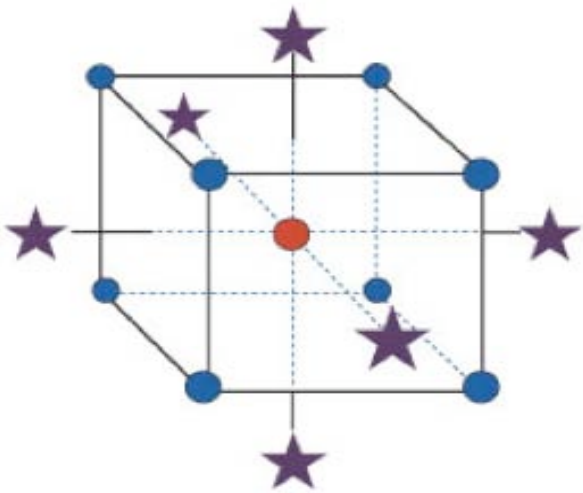


FIGURE 4.3. SCHEMATIC DIAGRAM OF A CENTRAL COMPOSITE DESIGN SHOWING STAR POINTS EQUIDISTANT FROM THE CENTRE POINT AND HIGH AND LOW VALUES OF VARIABLES.

The replicates give an estimation of the error associated with the design. All star points are equidistant from the origin and are placed at $\pm\sqrt[4]{2^f}$ where f is the number of factors, equaling 1.68 for three factors [191]. The star points correspond to, at coded positions ± 1.68 from the centre, pH values of 8.66 and 10.34, [BGE] values of 33 mM and 117 mM and organic modifier at 13.2 % and 46.8 %. The purpose of the star points is to test the robustness of the system. The unique coded experiments can be seen in Table 4.4. Experiments were randomised (using random number tables generated in MS Excel) but have been shown sequentially for ease of viewing.

TABLE 4.4. CODED THREE-FACTOR CENTRAL COMPOSITE DESIGN CONSISTING OF THREE LEVELS, DETAILING STAR POINTS AND REPLICATION.

	pH	[BGE]	% ORGANIC MODIFIER
FULL FACTORIAL	-1	-1	-1
	-1	-1	+1
	-1	+1	-1
	-1	+1	+1
	+1	-1	-1
	+1	-1	+1
	+1	+1	-1
	+1	+1	+1
STAR POINTS	0	0	0
	0	0	-1.68
	0	0	+1.68
	0	-1.68	0
	0	+1.68	0
	-1.68	0	0
	+1.68	0	0
REPLICATION	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0

Using the JMP software, a response surface was generated (Figure 4.4). As CEF was optimized to a minimum, the minimum point on the curve was used to detail the optimum parameters for the separation conditions.

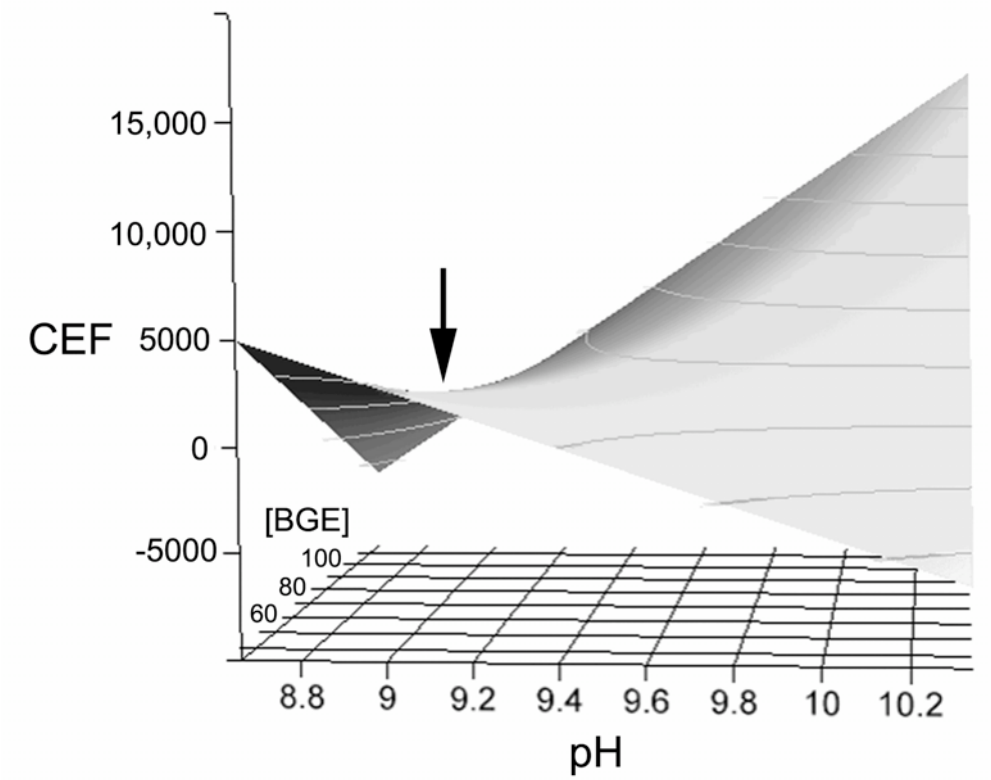


FIGURE 4.4. RESPONSE SURFACE GENERATED USING JMP FOR THE CENTRAL COMPOSITE DESIGN, OPTIMIZING CEF TO A MINIMUM. ARROW INDICATES OPTIMUM POINT ON RESPONSE SURFACE.

From the curve, the range of values obtained for each parameter was; pH 9 - 9.2, [BGE] 65-75 mM, % organic modifier 30-35 %. As the values obtained were ranges, the middle point of each was chosen as the specific optimum conditions for each factor (i.e. pH 9.1, [BGE] 70 mM, % MeOH 32.5 %). However, although not the lowest point on the response surface, a pH of 9.5 was chosen for the optimum running conditions as the pK_a values for the target analytes fell within ± 1-2 units of this. A comparison of a standard mixture containing eight known compounds at the pre- and post-optimum conditions is shown in Figure 4.5, with migration time data presented in Table 4.5.

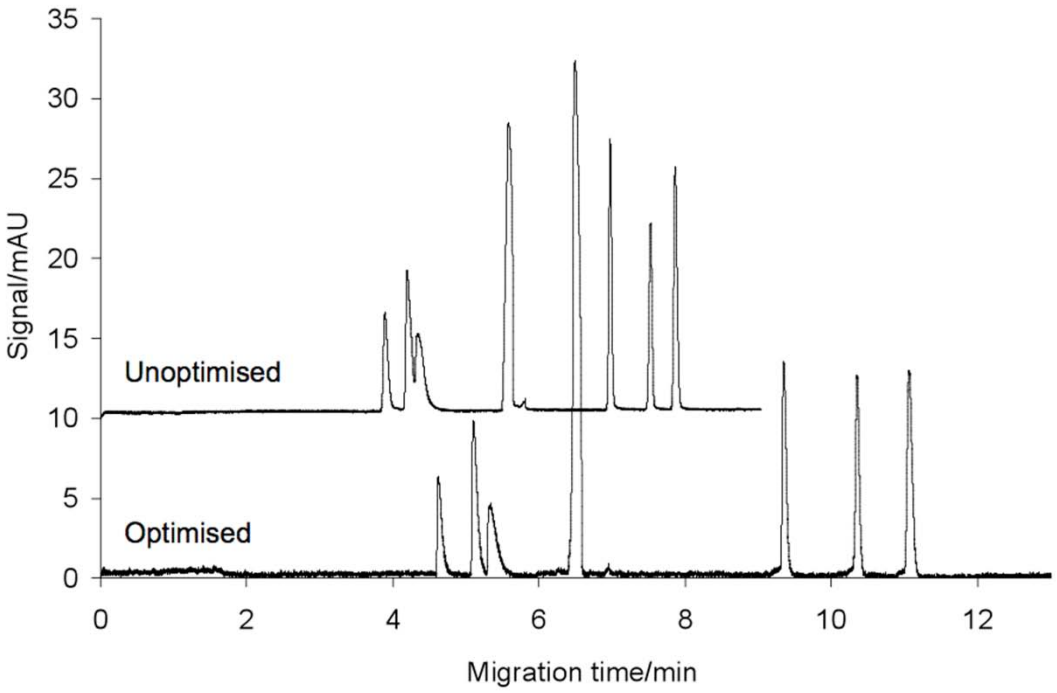


FIGURE 4.5. COMPARISON OF THE STANDARD MIXTURE AT PRE- AND POST-OPTIMUM CONDITIONS.

TABLE 4.5. PEAK IDENTIFICATION AND MIGRATION TIMES (MINS) FOR STANDARD MIXTURE CONTAINING EIGHT BIOGENIC AMINES AND AMINO ACIDS AT PRE- AND POST-OPTIMISATION CONDITIONS BY CAPILLARY ELECTROPHORESIS.

PEAK NUMBER	PEAK IDENTIFICATION	MIGRATION TIME (MINS) PRE-OPTIMISATION	MIGRATION TIME (MINS) POST-OPTIMISATION	MIGRATION TIME RSD (%) (N=10)
1	Tryptamine	3.895	4.760	2.0
2	Tyramine	4.197	5.279	2.6
3	Benzylamine/Aniline	4.341	5.498	-
4	Neutral Marker	5.584	6.607	-
5	Tryptophan	6.974	9.861	3.9
6	Tyrosine	7.525	10.971	4.3
7	Phenylalanine	7.865	11.773	4.3

The resolution between peaks 2 and 3 was used as the indicator for the quality of the separation. In the pre-optimised conditions, distinct separate peaks are detectable but not baseline resolved. The analysis time for this sample is less than 8 mins. In the post-optimised standard, peaks 2 and 3 are completely baseline resolved. Although the analysis time for this separation is slightly longer (12 mins), the considerable improvement in resolution is an acceptable compromise.

The optimum pH of the background electrolyte was established to be 9.5, and this is to be expected considering the pK_a values for the successfully separated analytes. It would also be expected that indole would not be separated under these conditions. However, there is at this time no explanation for the co-migration of benzylamine and aniline, as the reference value for the pK_a for aniline is different from the other analytes and one would not have expected any separation of this compound.

4.3.4 Analytical figures of merit

While the intention of this study is primarily qualitative, preliminary studies into whether this method could also be applied quantitatively were carried out. Mixed standards containing the selected analytes over the concentration range of 4–80 mg/L were prepared and analysed. The resulting calibration functions are presented in Table 4.6. Detection limits, ranged from 5.8 mg/L for tyramine to 8.6 mg/L for phenylalanine. These are comparable to those reported by Cortacero-Ramirez [161] and Lange *et al.* [192] who obtained detection limits in the 5.6 and 6.0 mg/L, respectively using UV–Vis absorbance detection. In order to apply this method quantitatively it will require the use of an internal standard. Either benzylamine or aniline would appear to be good candidates as they are absent in decomposition fluid samples, but this requires further study.

TABLE 4.6. CALIBRATION DATA FOR SELECTED BIOGENIC AMINES AND AMINO ACIDS.

ANALYTE	CALIBRATION EQUATION	R^2	LOD (mg/L)
Tryptamine	y=0.1100x + 0.1701	0.9965	5.9
Tyramine	y=0.1393x + 0.3306	0.9967	5.8
Tryptophan	y=0.278x + 0.8537	0.9959	6.5
Tyrosine	y=0.255x + 0.764	0.9935	8.2
Phenylalanine	y=0.227x + 0.7494	0.9929	8.6

4.3.5 APPLICATION TO DECOMPOSITION SAMPLES

The optimized running conditions were applied to decomposition fluid samples collected as described in Chapter 2. The complex sample matrix meant that samples were expected to have a higher signal:noise (S:N) and potentially have more compounds present than in the standard mixture, thus requiring a longer analysis time. An example electropherogram produced by a decomposition fluid sample analysed using the optimised CE conditions can be seen in Figure 4.6.

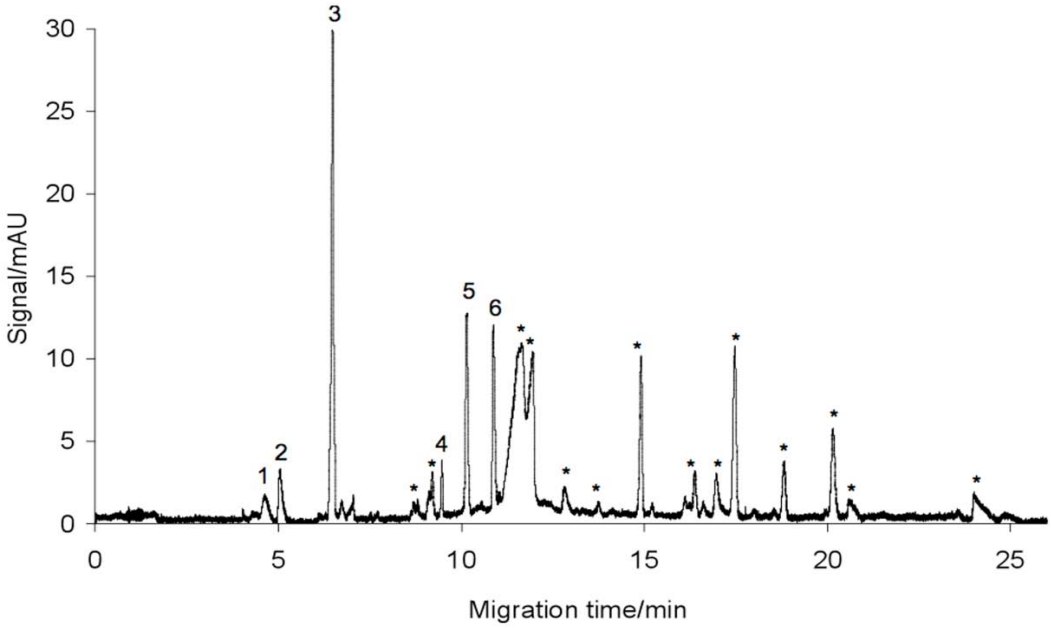


FIGURE 4.6. ELECTROPHEROGRAM AT OPTIMIZED RUNNING CONDITIONS FOR THE DECOMPOSITION FLUID SAMPLE. PEAKS IDENTITIES ARE: 1. TRYPTAMINE, 2. TYRAMINE, 3. NEUTRAL, 4. TRYPTOPHAN, 5. TYROSINE, 6. PHENYLALANINE. * UNIDENTIFIED COMPONENTS.

All the compounds present in the standard mixture were detected and identified in the decomposition sample by spiking and migration time, with the exception of benzylamine/aniline. These two compounds are not expected in decomposition fluid, as there is no relevant biodegradation pathway that would suggest their presence after degradation of fats, lipids or carbohydrates [11, 111]. A number of unidentified peaks were also observed (see Figure 4.7), and these will be the subjects of further study in order to investigate how they vary during the course of decomposition. Capillary electrophoresis-mass spectrometry and liquid chromatography-mass spectrometry will be investigated for their potential to elucidate the identity of these unknowns.

4.3.6 Observations of trends in target compounds in decomposition fluid

The samples analysed by capillary electrophoresis were taken from the adult pig trial conducted in Perth, Western Australia in September 2007. All of the target amines and amino acids were detected in the fluid samples during this trial. Figure 4.7 shows a sample electropherogram from pig 1 during this trial.

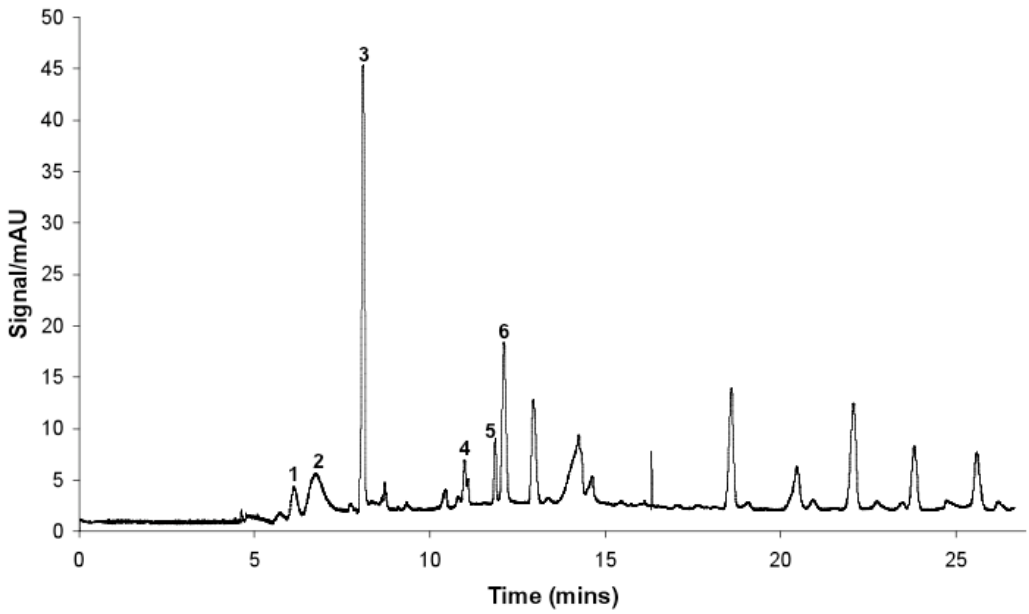


FIGURE 4.7. ELECTROPHEROGRAM OF DECOMPOSITION FLUID PRODUCED BY PIG 1. PEAK IDENTITIES ARE 1. TYRAMINE, 2. INTERNAL STANDARD, 3. NEUTRAL, 4. TRYPTOPHAN, 5. TYROSINE, 6. PHENYLALANINE.

Tyramine is derived from the amino acid tyrosine and follows a general increasing trend over the course of the trial (Figure 4.8), with the exception of the sample collected on day 11. This increase can be explained by the degradation of the precursor tyrosine. As tyrosine biodegrades, the level of by-product should increase. Tyramine was first detected on day 5 (ADD 87), the first sampling day for this particular trial.

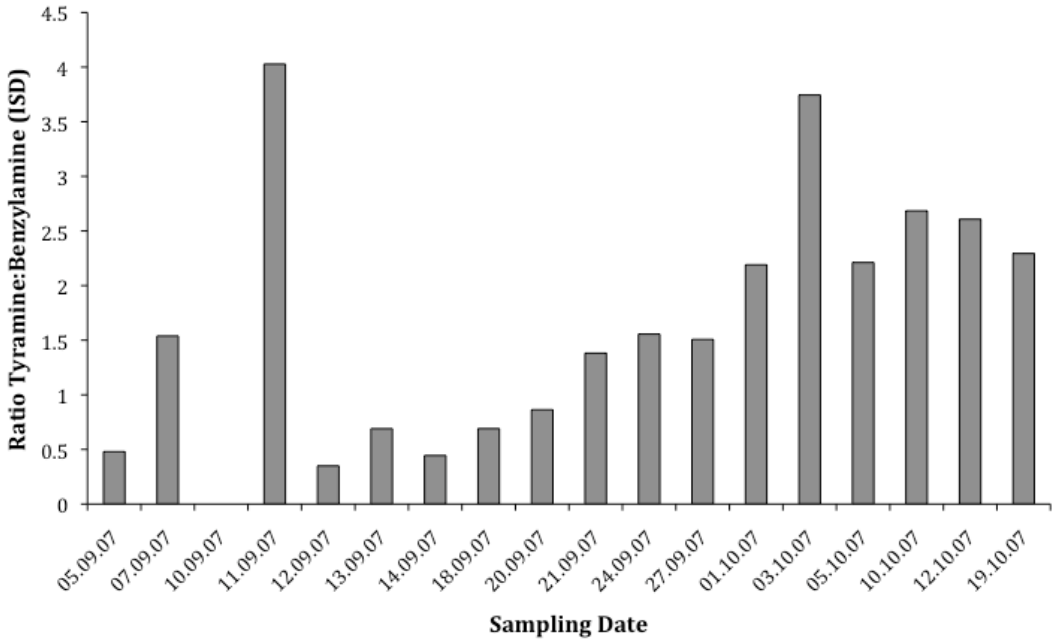


FIGURE 4.8. GENERAL INCREASING TREND OBSERVED IN TYRAMINE.

Tryptamine was only occasionally detected throughout this trial, so it was unable to be used in the establishment of trends. The occasional appearance of tryptamine could either be due to sensitivity issues associated with the use of a UV-Vis detector, or the absence from the decomposition fluid entirely. The total biogenic amine content was also analysed for trends, however, the sporadic detection of tryptamine meant that the trend for total biogenic amine content was almost identical to the appearance of tyramine. This also meant that the ratio between biogenic amines (tyramine:tryptamine) could not be analysed for trends.

The amino acid tyrosine appears to follow a cyclic trend, although slightly dissimilar to that observed in the fatty acids as described in Chapter 3. The apparent cycle observed in tyrosine appears to be an 8-day cycle followed by a 14-day cycle. Tyrosine and the other amino acids targeted in this trial were not detected until day 10 (ADD 166). This can tentatively be related to the early stages of insect activity, which during the early postmortem interval, are yet to fully break down the fat, protein and carbohydrates present in the carcass tissue to component amino acids. Of the three amino acids targeted in this trial,

tyrosine was the only one that made a consistent enough appearance to allow the observation of potential trends in formation. Tryptophan and phenylalanine were only detected sporadically throughout this trial. This lack of amino acids needs to be further investigated to establish whether sensitivity issues with the detector are the cause or whether compounds are absent from the fluid altogether. However, the trend for the total of all amino acids was investigated for potential trends. A cyclic trend was apparent and appeared to occur over a 14-day period, with amino acids passing through a maximum on day 12 (ADD 194), followed by a second maximum on day 33 (ADD 494). This trend is shown in Figure 4.9.

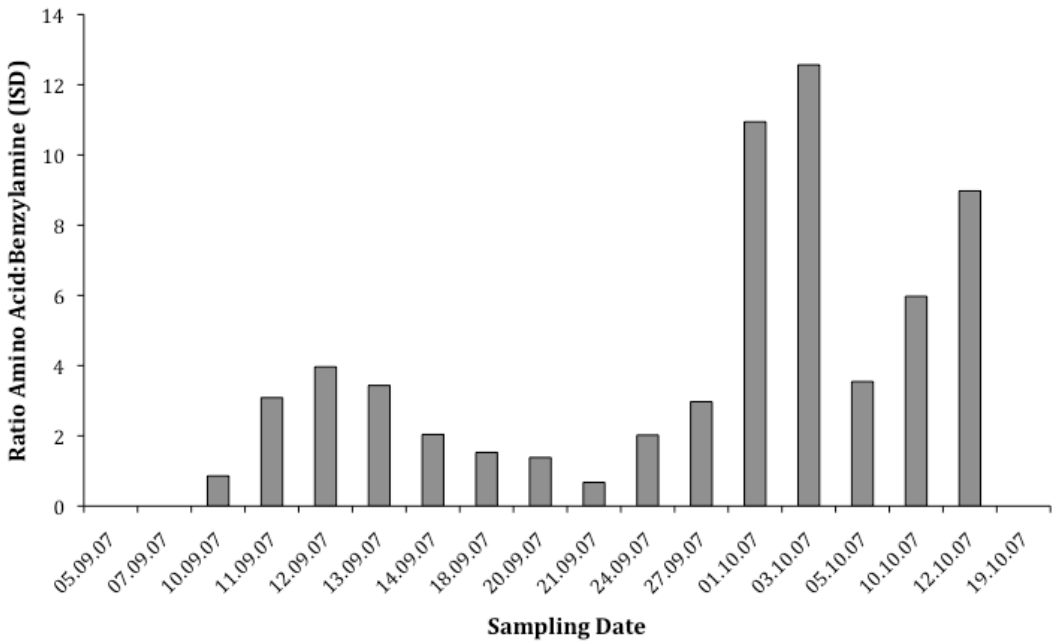


FIGURE 4.9. CYCLIC TRENDS OBSERVED FOR THE SUM OF AMINO ACIDS.

The trends observed in the amino acids and biogenic amines do not follow any of the observations seen for the long chain and short chain fatty acids as described in Chapter 3. When viewing trends in analytes according to accumulated degree-days, all four classes of compounds peak at different times, show differences with respect to abundance and appearance as well as differences in the length of cycle.

4.4 CONCLUSIONS

A simple, rapid and robust CE method for the determination of biogenic amines and amino acids was developed. A chemometric approach was found to be useful for rational and rapid optimization of the separation parameters. The method was successfully applied to porcine decomposition fluid samples, wherein the presence of tyramine, tryptamine, phenylalanine, tryptophan and tyrosine were identified by migration time and spiking. The temporal variation of these compounds in mammalian decomposition fluids was investigated. More sensitive analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS) are required to confirm the presence or absence of target compounds in decomposition fluid.

The potential also exists for application of the method to the rapid determination of tryptamine and tyramine in particular for pharmaceutical analysis and studies into food spoilage [90, 92, 162].

Chapter 5: Analysis of amino acids and amines by liquid chromatography – mass spectrometry (LC-MS)

5.1 INTRODUCTION

5.1.1 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Liquid chromatography coupled with mass spectrometry (LC-MS) is a relatively new analytical technique, first made possible in 1976 with the use of a moving belt interface [193]. LC-MS combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry [194].

The most common 'hyphenated' technique is that of GC-MS, with interfaces to link together the gas chromatograph and mass analyser readily available for both packed and capillary columns [189, 195]. This allows compounds to be easily and efficiently transferred to the detector once they have passed through the chromatographic column [195]. The advantage of GC-MS is that any compound that is capable of passing through a GC column (i.e. volatile and thermally stable) can be ionised, and the full analytical capacity of the mass spectrometer employed [195]. However, the linking of LC and MS is not as simple. Two major obstacles exist for linking LC with MS. Firstly, the significant proportion of water contained in the chromatographic mobile phase and secondly, analytes likely to be separated by LC are relatively non-volatile and not amenable to ionisation [195]. To overcome these incompatibilities, an interface is required, with the primary purpose of removing the volatile LC mobile phase [164, 189] before the molecules enter the mass detector [164]. The high water content of the mobile phase is unable to be pumped through to the detector [189] due to the high flow rate produced by the LC. The flow of eluent through the system to the detector, typically 1 mL/min [195] in conventional LC systems, would overload the high vacuum system of the mass spectrometer, operating at around 10^{-6} torr [195], swamping the output signal of the detector [196].

As likely analytes for separation by LC are generally non-volatile and not amenable to ionisation [189], alternative methods for ionisation are required. A variety of interfaces to deal with this are available for LC-MS [197] including

electron ionisation, chemical ionisation, thermospray, matrix assisted laser desorption ionisation (MALDI), electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Figure 5.1 shows a schematic diagram of the mechanism of electrospray ionisation.

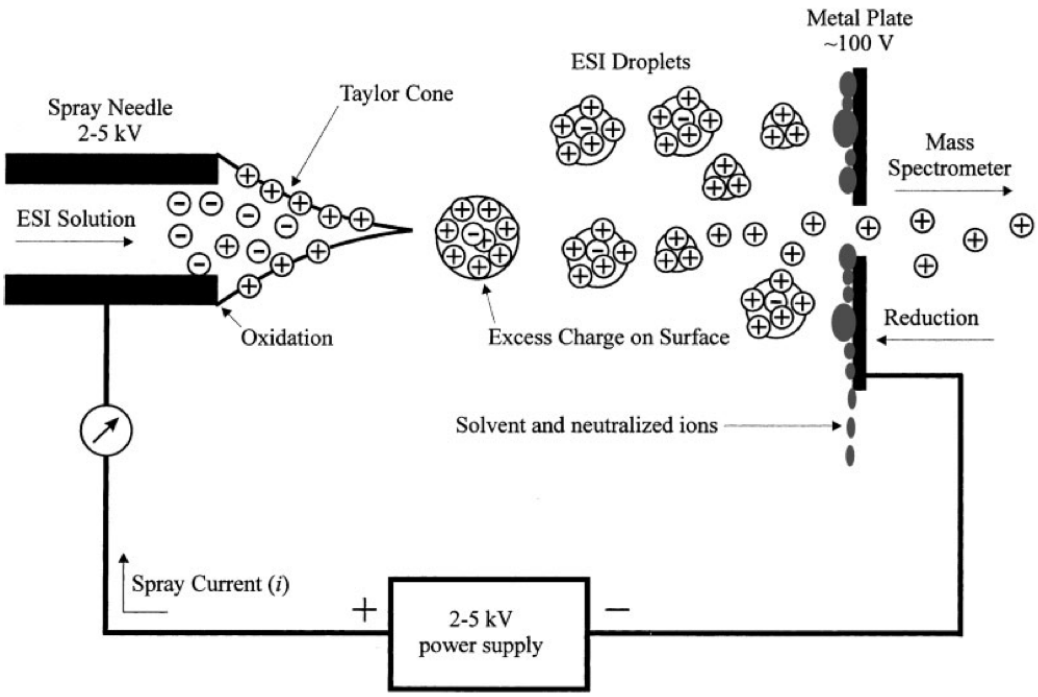


FIGURE 5.1. SCHEMATIC DIAGRAM OF THE ELECTROSPRAY IONIZATION PROCESS [198].

In ESI and APCI, the sample is ionised at atmospheric pressure and ions are then transferred into the high vacuum of the mass analyser [197]. Of the ionisation methods listed, ESI has become the preferred interface for analysis of biomolecules, because of its many advantages over other interfaces. It is capable of analysing a range of compounds from small, very polar compounds to [196, 197], large proteins and bioproteins of high molecular weight [197] as well as thermally labile compounds [199]. ESI also allows large, non-volatile analytes to be analysed directly from the liquid phase [198]. ESI can produce multiply charged ions from large molecules [197, 199] improving the sensitivity of the detector [197]. It is also sensitive to concentration rather than to the total amount of sample [197].

ESI is considered a 'soft' ionisation technique that results in little fragmentation of the molecule [196]. The ESI mass spectra of biological macromolecules correspond to molecular ions obtained through protonation $[M+H]^+$ or deprotonation $[M-H]^-$ with little to no contribution from fragment ions [197], thereby allowing observation of intact molecules. ESI is also compatible with tandem MS (MS/MS) allowing necessary structural information to be obtained for unknown analytes [200].

The powerful combination of LC-MS provides advantages such as selectivity of co-eluting peaks that can be isolated by mass selectivity, peak assignment in the presence of complex sample matrices and sample matrix adaptability [195, 201]. This allows for more definitive identification and quantitative determination of compounds that are not fully resolved chromatographically [189]. Liquid chromatography is also amenable to a wider range of compounds than can be analysed by GC, particularly those with high molecular weights. Analysis via GC also requires compounds to be both volatile and thermally stable.

5.1.2 Application of LC-MS to forensic science

LC coupled with tandem MS has become the technique of choice for the analysis of pharmaceutical samples and biological specimens, particularly with reference to small molecule drugs and metabolites in biological matrices such as blood, plasma, serum and other biological matrices [202]. The advantage of LC over widely established techniques such as GC is the ability for separation of compounds with high molecular weights, as well as the ability to deal with co-eluting compounds in a difficult sample matrix.

With respect to the literature searched, the vast majority of forensic papers detailing LC-MS separations involved illicit drugs, therapeutic drugs, metabolites and poisons [203-205]. Several reviews have also been written on the subject [206-212]. However, there are reports of LC-MS being used to separate amino acids [76] and hypoxanthine [69, 73] in vitreous humor and

cerebrospinal fluid with the potential to aid in postmortem interval estimation. LC-MS/MS has also been used for the analysis of gunshot residues [213], explosives [214-217] and chemical and biological warfare agents [218].

5.1.3 Analysis of amino acids and biogenic amines by LC-MS

Amino acids and biogenic amines that have been separated by liquid chromatography often require a derivatisation procedure to allow detection [219], as amino acids and amines generally lack a suitable chromophore or fluorophore [220]. However, the introduction of additional sample preparation and clean up procedures can lead to issues such as variability in results with different derivatisation procedures [220]. This variability can result from issues such as derivative instability and the occurrence of side reactions in the sample preparation [220]. Whilst the most widely used detector for LC analysis is UV absorption [195], it often lacks the selectivity and specificity required for analysis. However, use of a mass spectrometer increases the specificity by monitoring the mass to charge ratio of compounds. This reduces the need for complete separation of analytes, thus also reducing the analysis run time. Compared to detectors such as UV-Vis, MS also has the advantage of a significantly improved limit of detection [164, 189].

Traditionally, underivatised amino acids are separated by ion-exchange chromatography [221-224] or ion-pair reverse phase liquid chromatography [225-230], however these use non-volatile buffers [220]. The first successful separation of underivatised amino acids was achieved in 1993 by Van Leuken *et al*. by liquid chromatography-thermospray-mass spectrometry (LC-TSP-MS) [231]. However, thermospray has since been surpassed in use by softer ionisation techniques such as electrospray [220].

The first report of separation of free amino acids by LC-ESI-MS was by Chaimbault et al. [232] who successfully analysed and detected 20 free amino acids. This was followed by work from the same research group with the introduction of tandem mass spectrometry for the analysis of free amino acids

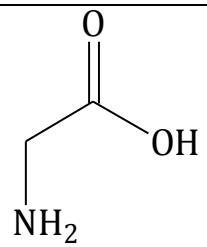
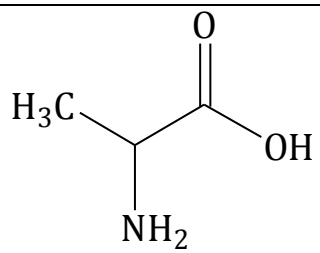
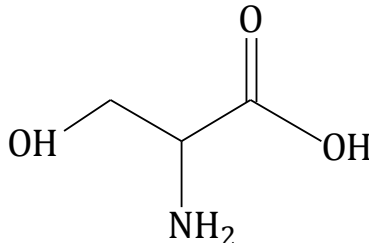
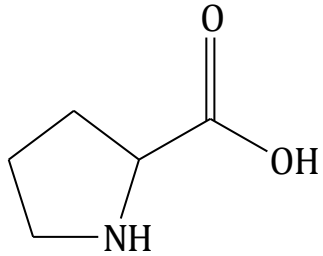
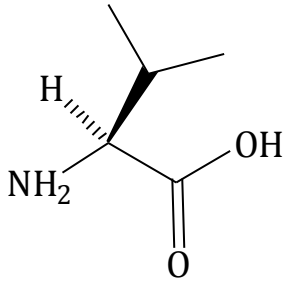
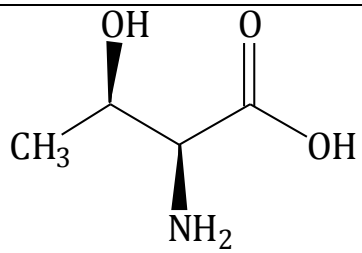
[233]. Direct detection of amino acids is preferential over derivatisation techniques as it avoids the problems of potential side reactions in the derivatisation process, derivative instability and reagent interferences [220].

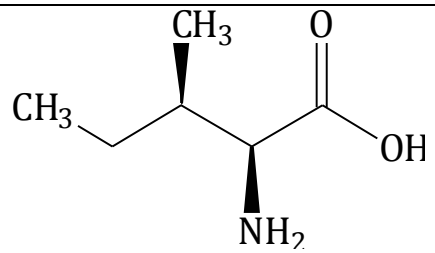
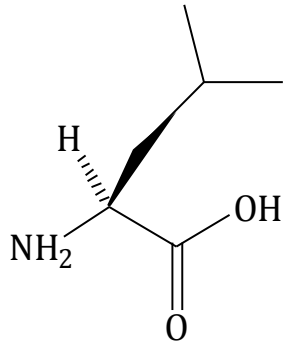
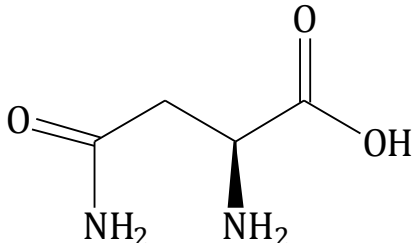
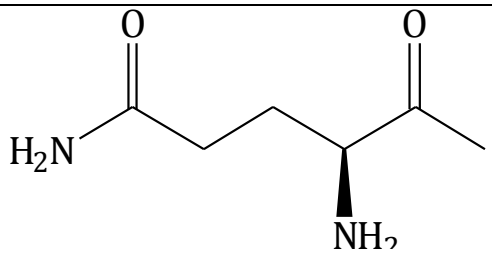
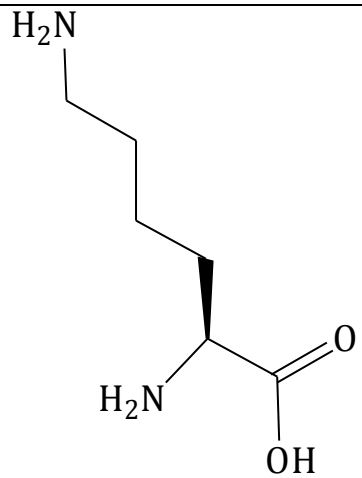
Amino acids and amines have been separated by LC-MS in a variety of matrices including surface and wastewaters [234], sewage treatment plant samples [235], foods including cheeses, fish and wine [160, 236-238] and biological samples [239, 240].

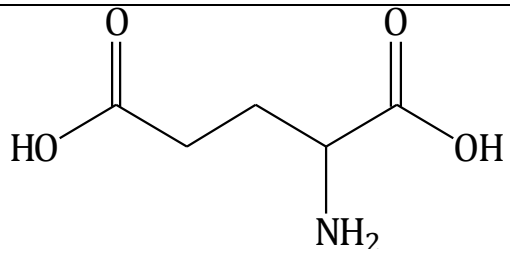
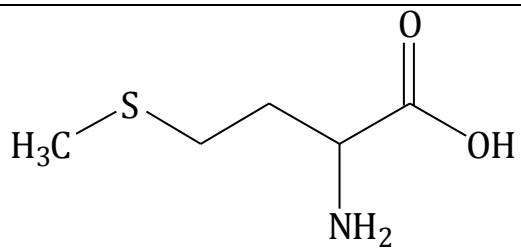
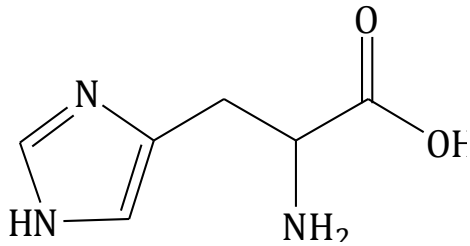
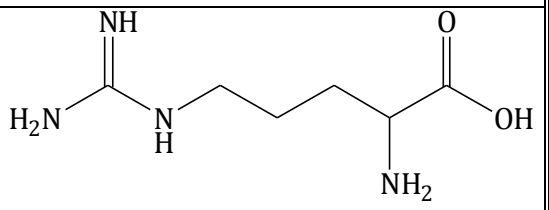
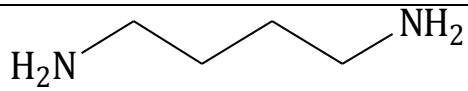
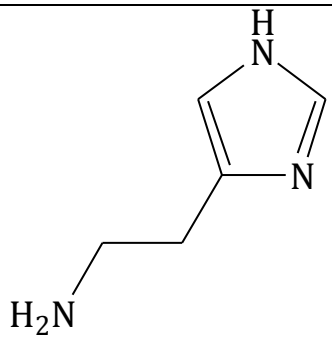
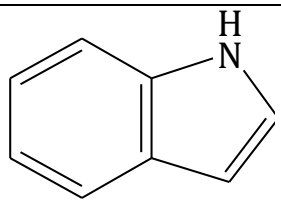
5.1.4 Aims

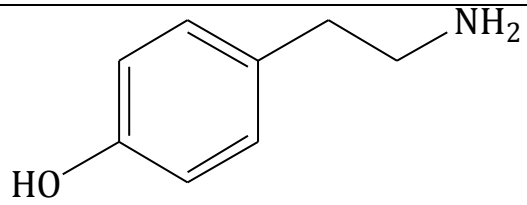
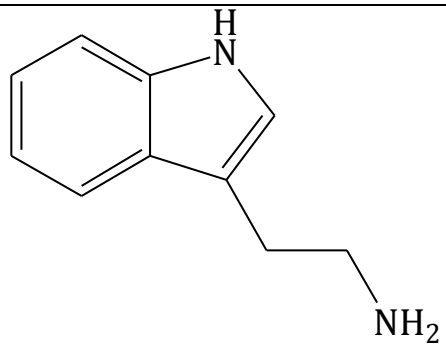
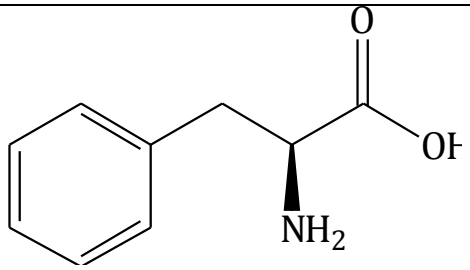
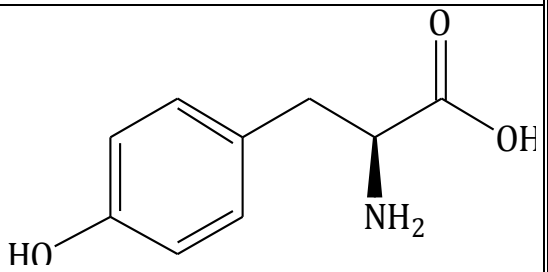
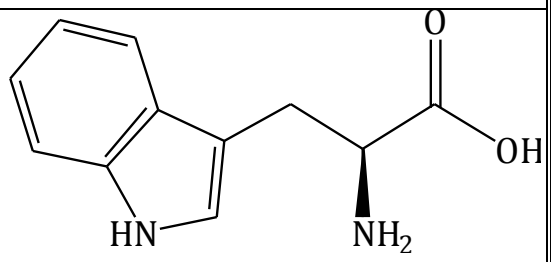
This chapter outlines the use of LC-ESI-MS for the analysis of biogenic amines and amino acids produced in mammalian decomposition fluid (Table 5.1). Compounds that had been the target of analysis in CE (Chapter 4) and identified in decomposition fluid by GC-MS (Chapter 3) were the starting point for compounds of interest. Pre-cursor amino acids were particularly targeted for potential trends in decomposition fluid with respect to time, particularly proline and methionine, which has been previously reported as potential indicators of PMI [5].

TABLE 5.1. STRUCTURES OF AMINO ACIDS AND BIOGENIC AMINES TARGETED FOR ANALYSIS BY LC-ESI-MS.

AMINO ACID	FORMULA	MW	STRUCTURE
Glycine	$C_2H_5NO_2$	75.1	
L-alanine	$C_3H_7NO_2$	89.1	
L-serine	$C_3H_7NO_2$	105.1	
L-proline	$C_5H_9NO_2$	115.1	
L-valine	$C_5H_{11}NO_2$	117.1	
L-threonine	$C_4H_9NO_3$	119.1	

AMINO ACID	FORMULA	MW	STRUCTURE
L-isoleucine	$C_6H_{13}NO_2$	131.2	
L-leucine	$C_6H_{13}NO_2$	131.2	
L-asparagine	$C_4H_8N_2O_2$	132.1	
L-glutamine	$C_5H_{10}N_2O_3$	146.2	
L-lysine	$C_6H_{14}N_2O_2$	146.2	

AMINO ACID	FORMULA	MW	STRUCTURE
L-glutamic acid	$C_5H_9NO_4$	147.1	
L-methionine	$C_5H_{11}NO_2S$	149.2	
L-histidine	$C_6H_9N_3O_2$	155.2	
L-arginine	$C_6H_{14}N_4O_2$	174.2	
Putrescine	$C_4H_{12}N_2$	88.152	
Histamine	$C_5H_9N_3$	111.15	
Indole	C_8H_7N	117.1	

AMINO ACID	FORMULA	MW	STRUCTURE
Tyramine	$C_8H_{11}NO$	137.18	
Tryptamine	$C_{10}H_{12}N_2$	160.22	
Phenylalanine	$C_9H_{11}NO_2$	165.2	
Tyrosine	$C_9H_{11}NO_3$	181.19	
Tryptophan	$C_{11}H_{12}N_2O_2$	204.23	

5.2 EXPERIMENTAL

5.2.1 CHEMICALS AND REAGENTS

Stock solutions of individual amino acid and biogenic amine was prepared as follows; L-alanine, L-histidine (BDH Chemicals), L-arginine, L-glutamine, L-leucine, L-proline, L-lysine, L-threonine, L-serine, L-tryptophan, L-isoleucine, L-methionine, L-valine (Sigma-Aldrich), L-asparagine, L-phenylalanine (Fluka), were all prepared at 5 mg/mL in a 30:70 (v:v) methanol:ultrapure water mixture. Tyramine, indole, L-glutamic acid, putrescine, histamine (Sigma-Aldrich), tryptamine, tyrosine (Fluka), glycine (BDH Chemicals) were all prepared at 1 mg/mL in 30:70 (v:v) methanol:ultrapure water mixture due to solubility issues at higher concentrations. The methanol (Mallinckrodt, Missouri, USA) used was analytical grade (>99 %). Formic acid was supplied by Ajax FineChem (Sydney, Australia). Ultrapure water was prepared in-house using a Purelab water system.

Mixed calibration standards containing all 23 analytical standards and covering the concentration range 0.01 ng/μL – 1000 ng/μL were prepared by serial dilution of a stock mixture with 30:70 (v:v) methanol:ultrapure water. All calibration standards were run in duplicate. All solutions were kept refrigerated at 4 °C to prevent degradation.

5.2.2 SAMPLE PREPARATION

The collection of decomposition fluid samples is described in Chapter 2. The recovered fluid samples from the field trial conducted in Perth, 2007, were allowed to equilibrate from frozen to room temperature and vortexed for 30 seconds. Preparation of decomposition fluid samples consisted of a simple filtration through a 0.45 μm PTFE Gelman Acrodisc® syringe filter. Fluid samples that were analysed by LC-MS had previously been analysed by GC-MS and CE-UV-Vis as described in Chapters 3 and 4, respectively.

To assess how matrix effects affected quantitative assessment of amino acids and biogenic amines, samples of decomposition fluid were diluted in 30:70 (v:v) methanol:ultrapure water in the ratios: no dilution, 1:10, 1:25, 1:50, 1:100 (v:v) and results were compared to injection of pure samples.

5.2.3 Analytical instrumentation

Calibration standards and decomposition fluid samples were analysed using an Agilent 1100 HPLC system (Palo Alto, USA) equipped with a solvent degasser unit, a quaternary pump and a 100 well-plate autosampler. LC separation was achieved with a Phenomenex (Torrance, USA) Gemini C18 column (250 mm x 3 mm I.D., 3μm particle size) at a flow rate of 150 μL/min. Other LC parameters are detailed in Table 5.2.

Table 5.2. Liquid chromatographic parameters for the separation of biogenic amines and amino acids in mixed standards and decomposition fluid samples.

Time (mins)	Eluent A (%)	Eluent B (%)	LC Conditions
0.00	10	90	**Eluent A:** MeOH with 0.1 % formic acid **Eluent B:** MilliQ water with 0.5 % formic acid **Flow rate:** 0.15 mL/min **Column:** Gemini C18 (250 mm x 3 mm I.D., 3 μm particle size) **Injection volume:** 0.1 μL
10.00	10	90	
20.00	100	0	
30.00	100	0	
31.00	10	90	
44.00	10	90	
44.10	10	90	
45.00	10	90	

The LC was coupled to a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Manchester, UK) fitted with an electrospray interface (ESI) operated in positive ion mode. For optimum signal, capillary and cone voltages were 3250 V and 25 V respectively. Other ESI and MS tuning parameters are listed in Table 5.3. An eluent containing a weak acid, such as formic acid, was chosen as the low pH facilitates the protonation of the analyte in +ESI mode

[198]. This is ideal for analysis of mixtures that contain compounds with weak basic functional groups [198].

Nitrogen and argon were both supplied by BOC Gases, Australia. Nitrogen gas (cryogenic liquid) was used as both desolvation and nebuliser gas, while high purity argon (99.997 %) was used as the collision gas for multiple reaction monitoring (MRM) experiments. Precursor → product ion transitions used for MRM experiments were selected based on their intensities in the MS/MS spectra (Table 5.4). For compounds that produced 2 stable transitions, the MRM ratio observed in the standard solution and in the samples was used to confirm the presence of the analyte in the sample

TABLE 5.3. GENERAL ESI (+) AND MS TUNING PARAMETERS FOR THE SEPARATION OF BIOGENIC AMINES AND AMINO ACIDS IN MIXED STANDARDS AND DECOMPOSITION FLUID.

ESI-MS TUNING PARAMETERS	SETTING
Capillary voltage (V)	3250
Cone voltage (V)	25
Hex.1, aperture, hex.2 (V)	0.0, 0.2, 0.3
Source temperature (°C)	145
Desolvation temperature (°C)	345
N_2 cone gas flow (L/hr)	47
N_2 desolvation gas flow (L/hr)	703
Quad. 1 and quad. 3 resolution	1
Ion energy quad. 1 Ion energy quad. 3	1.5 1.5
Multiplier (V)	750

TABLE 5.4. PRECURSOR AND PRODUCT IONS (+ESI) WITH OPTIMISED COLLISION ENERGY FOR THE ANALYSIS OF AMINES AND AMINO ACIDS IN DECOMPOSITION FLUID.

AMINO ACID	T_R (MINS)	PRECURSOR ION (m/z)	PRODUCT IONS (m/z)	COLLISION ENERGY (eV)
Histamine	6.36	112.15	95.3 83.1	12 12
Indole	33.67	118.1	91.3	20
L-alanine	6.44	90.1	72.1	10
L-arginine	7.07	175.2	158.1 70.3	12 15
L-asparagine	7.65	133.1	116.3 74.2	10 15
L-glutamic acid	8.04	148.1	130.2 102.2	10 11
L-glutamine	7.81	147.2	130.2 84.3	10 15
L-histidine	6.60	156.2	110.3 93.3	12 15
L-isoleucine	16.51	132.2	86.3 69.4	10 17
L-leucine	18.26	132.2	86.3 69.4	10 17
L-lysine	6.48	147.2	130.2 84.3	10 15

AMINO ACID	T_R (MINS)	PRECURSOR ION (m/z)	PRODUCT IONS (m/z)	COLLISION ENERGY (eV)
L-methionine	12.26	148.2	133.3 104.3	10 10
L-proline	8.78	116.1	70.2	12
L-serine	11.00	106.1	60.4	7
L-threonine	7.77	120.1	102.2 74.2	7 10
L-valine	9.76	118.1	72.3 55.4	12 20
Phenylalanine	29.77	166.2	120.2 131.2	15 12
Putrescine	6.13	89.15	72.4	10
Tryptamine	21.45	161.22	144.2	10
Tryptophan	30.93	205.23	146 188	12 10
Tyramine	19.75	138.18	121.5	15
Tyrosine	28.37	182.19	136.3	10

5.3 RESULTS AND DISCUSSION

5.3.1 METHOD DEVELOPMENT

The amino acids and amines selected for this study (Table 5.1) were chosen on the basis of availability in the laboratory. Several of these have been previously reported in decomposition fluid. As a result, these were the initial targets for identification and the observation of apparent trends.

5.3.1.1 OPTIMISATION OF MS/MS PARAMETERS AND MRM TRANSITIONS

Multiple reaction monitoring (MRM) was used over single ion monitoring (SIM) because of several advantages including high sensitivity, high selectivity and low risk of false positives [241]. Operating the mass spectrometer in the MRM mode allows for shorter analysis times since baseline separation and resolution of all analytes present is not required [235]. However, chromatographic separation may be still required for: (1) compounds measured at the same MRM transition (e.g. L-leucine and iso-leucine, which have the same precursor/product ion transition); (2) compounds that produce overlapping product ions upon fragmentation (e.g. L-glutamine and L-lysine); (3) compounds with the same product ion but with different precursor ions (e.g. L-proline and L-arginine).

Individual standards were directly infused to determine whether the biogenic amines and amino acids produced a better signal to noise (S:N) in ESI (+) or ESI (-). Infusion experiments also allowed for refinement of other MS tuning parameters, such as collision energy. Collision energy is responsible for the degree of fragmentation of the selected ions [200]. All compounds successfully produced $[M+H]^+$ adducts, therefore, ESI (+) was chosen for further analysis. Collision energy required specific optimisation for each analyte (Table 5.4).

Polar analytes can be charged in ESI through formation of adducts [198]. The inclusion of formic acid in the mobile phase ensured the formation of the proton adduct, $[M+H]^+$, as the most intense precursor ion [241]. The low pH of acidic

solutions facilitates analyte protonation as charge is carried by solvent clusters that are protonated, which then interact with the analyte [198]. This makes acidic solvents ideal for analysis of proteins and other organic molecules that possess basic functional groups [198].

The optimised MRM transitions for the individual amino acids and biogenic amines were generally in agreement with those reported in previously published literature [200].

5.3.1.2 MATRIX EFFECT

A matrix effect in LC-MS/MS is defined as the suppression or enhancement of the analyte signal induced by the co-eluting matrix [201]. Matrix effects result from competition between the analyte and the undetected matrix components reacting with primary ions formed in the LC-MS/MS interface [201, 241, 242] and can result in loss of sensitivity, precision and accuracy [201, 241, 242]. This effect was first described by Tang and Kerbarle [243], who showed that the response of organic bases using electrospray ionisation decreased, as the concentration of the analyte increased [243]. Determination of the effect of the matrix on the analyte in question allows assessment of the reliability and selectivity of the LC-MS/MS method [200] but is not often studied in detail [200].

Several methods have been proposed to deal with potential matrix effects including calibration methods, specific sample preparations, improvement of the chromatographic separation [201, 242, 244] and dilution of sample extracts [234, 245]. Sample preparation methods, such as solid-phase extraction (SPE), are reported as the most effective way of overcoming matrix effects [244] as it can be specific to the sample matrix and selectively purify the sample from the interferences [244]. Development of a method, such as SPE, is time consuming, expensive and often difficult as likely interferences are often similar to analytes with respect to polarity and retention in a reverse-phase column [241]. These

methods can also result in analyte losses or significant increases in analysis times [234].

Calibration techniques consist of the use of matrix matched standards, the use of internal standards or the standard addition method [234]. The standard addition method is often time consuming, as spiked samples must be analysed for each individual sample. This is not a practical task for large-scale decomposition field trials containing high numbers of samples. This method also requires a suitable matrix-matched blank to be used that may not always be commercially available or readily accessible. The use of a matrix-matched blank in this research would require a decomposition fluid sample without component biogenic amines and amino acids. This, of course, does not exist.

The use of internal standards can compensate for signal variations in ESI, however, a suitable standard was unable to be found for the decomposition samples in the limited time available. The cost of deuterated internal standards was also not an option given the large numbers of analytes present in the decomposition fluid.

An alternative approach for dealing with matrix effects is sample dilution or a reduction in injection volume [234, 244, 245]. This method has been described as ineffective for trace analysis [244], however, it worked well for our purposes as the analysis provided a qualitative overview of compounds rather than a quantitative approach.

The sample matrix effects in decomposition fluid were investigated by preparing samples in the ratios: no dilution, 1:10, 1:25, 1:50, 1:100 with 30:70 (v:v) methanol:ultrapure water with varying injection volumes, ensuring the same level of analyte was introduced into the column (Table 5.5).

TABLE 5.5. DILUTION FACTORS AND CORRESPONDING INJECTION VOLUMES FOR INVESTIGATIONS INTO THE MATRIX EFFECTS OF DECOMPOSITION FLUID.

DILUTION	INJECTION VOLUME (µL)		
No dilution	0.1	0.5	1.0
1:10	1.0	5	10
1:25	2.5	12.5	25
1:50	5	25	50
1:100	10	50	100

The matrix effect was measured as the change in peak area for each MRM transition. A successful result was deemed to be peak areas of similar value, thus signifying little to no matrix effects from the sample. The majority of the compounds identified in the sample showed little to no matrix effects (i.e. no significant difference in peak area between samples that had no dilution, 1:10 and 1:25) (Figure 5.2). From this it was concluded that smaller injection volumes with no dilution showed the best results. This would ultimately lead to minimal sample preparation time for future analysis.

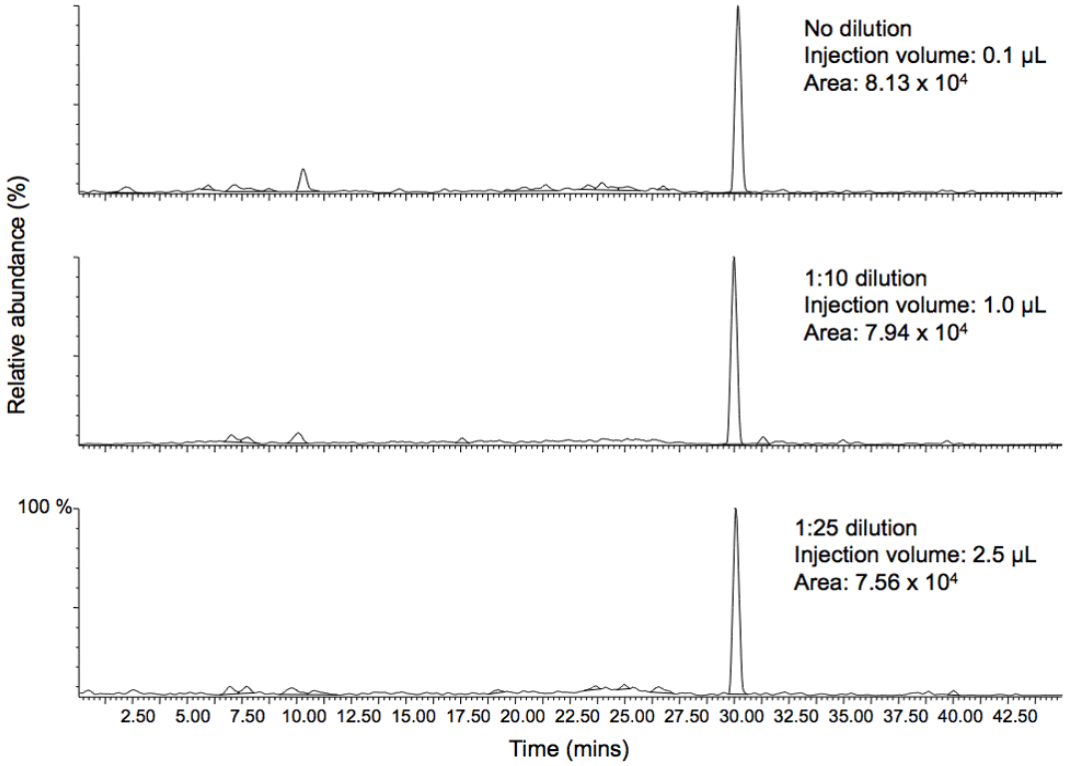


FIGURE 5.2. MATRIX EFFECT FOR PIG 1. MRM TRANSITION 205.1→188 (TRYPTOPHAN) AT RATIOS OF; NO DILUTION, 1:10 AND 1:25 DILUTION OF DECOMPOSITION FLUID.

At higher injection volumes, the reverse effect was seen. There was a significant difference in the peak area for undiluted sample at 1 µL and the corresponding 1:10 dilution (10 µL injection) and 1:25 dilution (25 µL injection). In this case the peak area for the undiluted sample was comparatively larger than for the diluted samples (Figure 5.3).

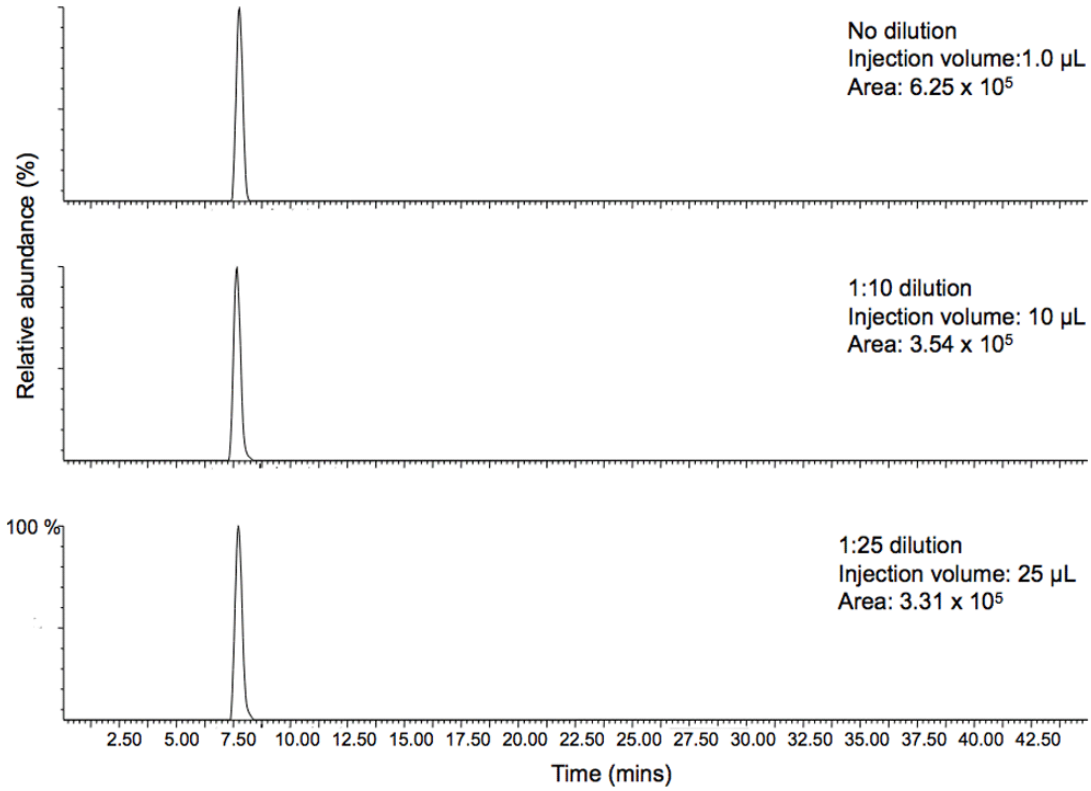


FIGURE 5.3. MATRIX EFFECT FOR PIG 1. MRM TRANSITION 148→102 (L-GLUTAMIC ACID) AT RATIOS OF; NO DILUTION, 1:10 AND 1:25 DILUTION OF DECOMPOSITION FLUID.

This effect can be explained as instead of the charge now being predominantly applied to the analyte in question, the charge is now shared between the matrix and the analyte, indicating suppression of the signal. Therefore, smaller injection volumes are better. From these results, it was concluded that the best sample introduction onto the column was direct analysis with no dilution factor at 0.1 µL injection volume. The low injection volume also prevented overloading the column with analyte.

5.3.2 ANALYTICAL FIGURES OF MERIT

Two sets of 20 calibration standards containing all 23 compounds was prepared by dilution of the stock solution over the concentration range 0.01 ng/µL – 1000 ng/µL. Calibration was performed by the standard addition method. Investigations into the correct dilution were investigated for the calibration. A 1:100 and 1:1000 dilution of the 23 compound standard mixture was prepared

in 30:70 (v:v) methanol:ultrapure water. Undiluted decomposition samples were spiked with an appropriate volume of either the 1:100 or 1:1000 dilution of the 23 compound mixture to make a total of 1 mL of calibration standard. All calibration standards were run in duplicate. Results indicated that a 1:100 dilution was successful in producing a linear calibration, without overloading the column and the MS detector. Resulting calibration data can be seen in Table 5.6. Limits of detection were calculated as three times the signal to noise ratio (S:N) from raw data for each pig.

TABLE 5.6. CALIBRATION DATA FOR AMINO ACIDS AND BIOGENIC AMINES ANALYSED BY LC.

ANALYTE	CALIBRATION EQUATION	r^2	LOD (ng/µL)		
			PIG 1	PIG 2	PIG 3
Histamine	y=79.430x + 20.76	0.9881	2.154	1.057	1.346
Indole	y=0.641x + 10.976	0.7055	2.780	1.244	2.300
L-arginine	y=495.93x + 80.853	0.9909	0.123	0.363	0.204
Asparagine	y=141.76x + 60.287	0.9739	0.0145	0.721	0.666
L-glutamic acid	y=54.004x + 69.511	0.9856	2.554	0.075	2.364
L-histidine	y=1282.6 + 133.73	0.9879	0.118	0.0125	0.0235
L-iso-leucine	y=1297.2x + 179.945	0.9929	2.091	4.781	0.0177
L-lysine	y=887.58x + 107.81	0.9862	0.389	0.174	0.129
L-methionine	y=505.35x + 115.38	0.9949	0.277	1.339	0.725
L-proline	y=1016.65x + 372.33	0.9945	0.201	0.564	0.565
L-serine	y=136.16x + 108.97	0.9659	1.591	2.027	1.759
L-threonine	y=479.84x + 122.59	0.974	1.260	1.444	1.268
L-valine	y=960.36x + 249.39	0.9856	0.786	0.636	0.297
L-phenylalanine	y=2902.16x + 8635.83	0.9502	0.980	0.642	0.869
Putrescine	y=6.872x + 29.544	0.9551	0.919	0.058	0.215
L-tryptophan	y=3190.53x + 5183.14	0.9909	0.246	0.169	0.269
Tyramine	y=0.1649x + 13.568	0.9272	0.788	0.428	0.072

Calibrations were generally linear over the range 0.05 – 25 ng/μL, for the majority of analytes, with the exceptions being tyrosine and L-leucine. This was most likely due to issues in sample preparation as both have successfully been separated and identified with the use of LC-MS on several occasions in various matrices [226, 239, 240, 246]. Both of these analytes were excluded from further analysis due to these poor results.

5.3.3 Application to decomposition fluid samples

The optimized LC method was applied to decomposition fluid samples collected as described in Chapter 2. An example total ion chromatogram (TIC) of a decomposition fluid sample analysed using the optimised LC method can be seen in Figure 5.4.

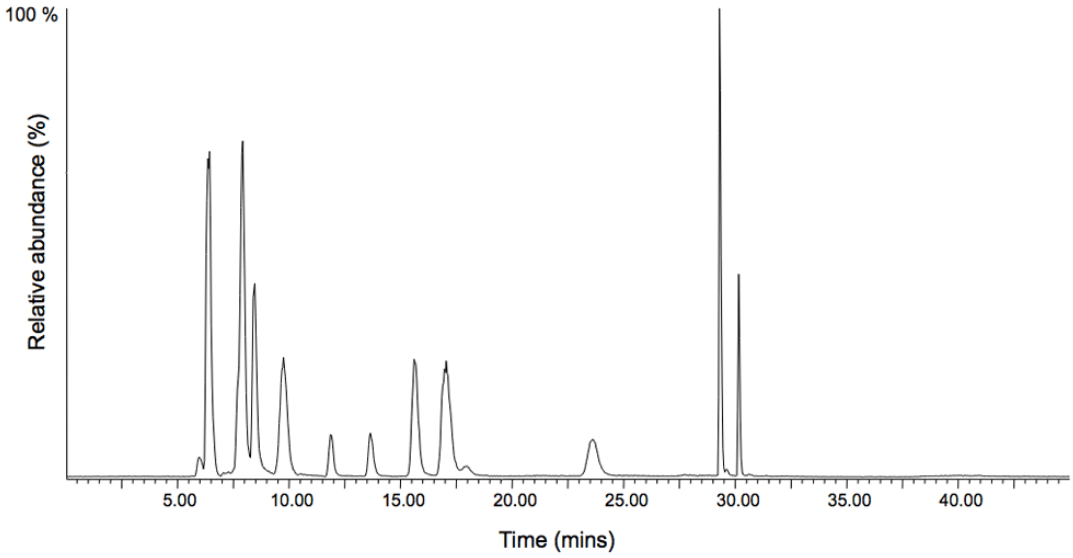


Figure 5.4. Total ion chromatogram (TIC) of decomposition fluid produced by pig 1 on day 13 (ADD 207) from the Curtin University trial, September 2007.

The first observation of the total ion chromatogram is the number of peaks present. Given the complex nature of the sample, it is expected that a complex chromatogram would result, particularly given the minimum sample preparation required before analysis by LC-MS. However, when viewing the MRM transitions for individual analytes, the majority of target compounds were

detected and identified. Figure 5.5 shows a typical extracted ion chromatogram for a decomposition fluid sample.

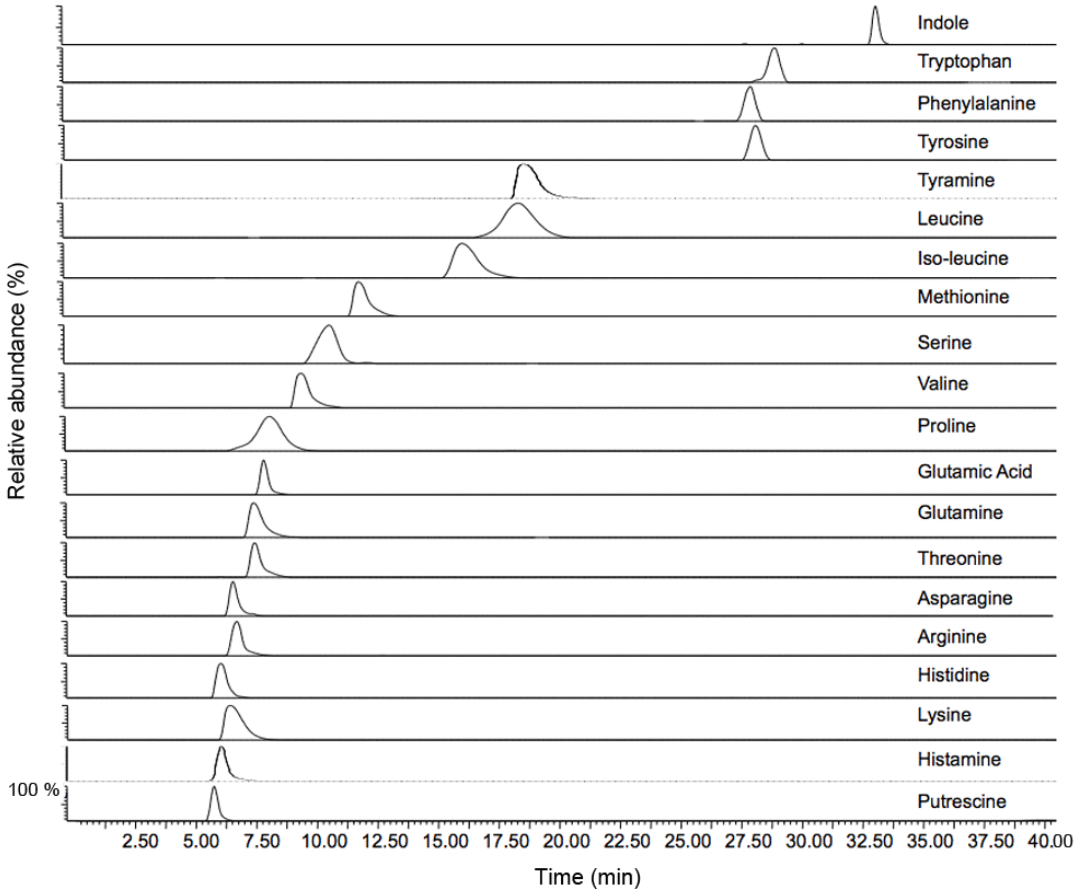


FIGURE 5.5. A TYPICAL CHROMATOGRAM FOR SEPARATION OF A DECOMPOSITION FLUID SAMPLE SHOWING IDENTIFIED COMPOUNDS.

One of the main advantages of LC-MS operated in MRM mode is the ability to distinguish compounds that are co-eluting. For example, L-threonine and L-asparagine are co-eluting at 7.77 and 7.65 minutes respectively. However, both compounds have significantly different precursor → product ion transitions (i.e. L-threonine 120.1 → 102.2 m/z and 74.2 m/z; L-asparagine 133.1 → 116.3 m/z and 74.2 m/z), which makes them distinguishable.

Similarly, compounds expected in decomposition fluid that have the same precursor ion are often also distinguishable. For example, indole and L-valine

have the same precursor ion, m/z 118, but the product ions produced in the mass analyser are m/z 91.3 for indole and m/z 72.3 and m/z 55.4 for L-valine. Moreover, L-valine and indole also have vastly different retention times of 9.76 minutes and 33.67 minutes respectively.

5.3.3.1 TRENDS OBSERVED IN DECOMPOSITION FLUID

Although the total ion chromatograms resulting from LC-MS analysis had peaks that were co-eluting, it was still apparent that these were showing variation between collection dates for samples from the same pig and on the same sampling date for different pigs (Figure 5.6).

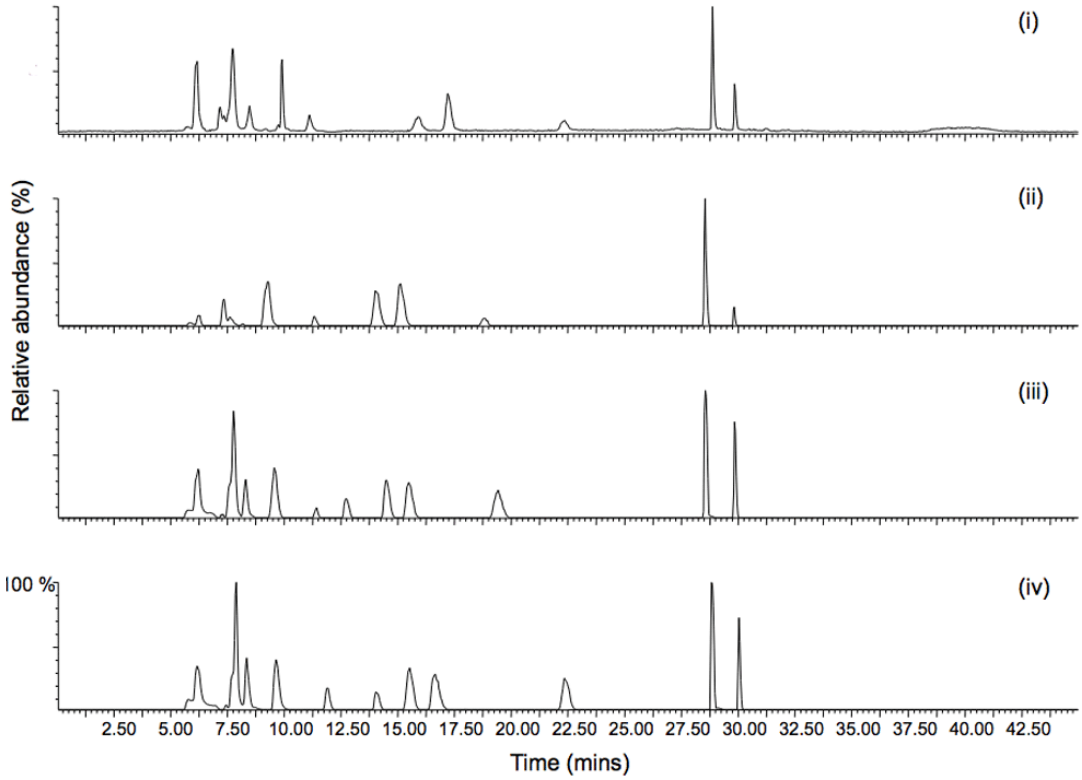


FIGURE 5.6. CHROMATOGRAMS FROM PIG 2 SHOWING VARIATION IN THE TOTAL ION CHROMATOGRAM ON DAYS 5 (ADD 86) (i), 13 (ADD 207) (ii), 31 (ADD 462) (iii), 42 (ADD 637)(iv).

Several amino acids, including phenylalanine, tyrosine, methionine and serine, have been reported as having the potential to be useful indicators of postmortem interval in human cadavers [5]. Data analysis was conducted to establish any distinct relationship between the levels of particular compounds produced with respect to time and temperature, in the adult pig trial run in Perth, 2007.

Tyramine and phenylalanine had also both been previously analysed by capillary electrophoresis. The results from CE analysis for tyramine showed a general increasing trend over the course of the field trial. This trend was confirmed by LC analysis, where tyramine showed a general increasing trend for all 3 pigs in the trial. Figure 5.7 shows the trend for pig 1.

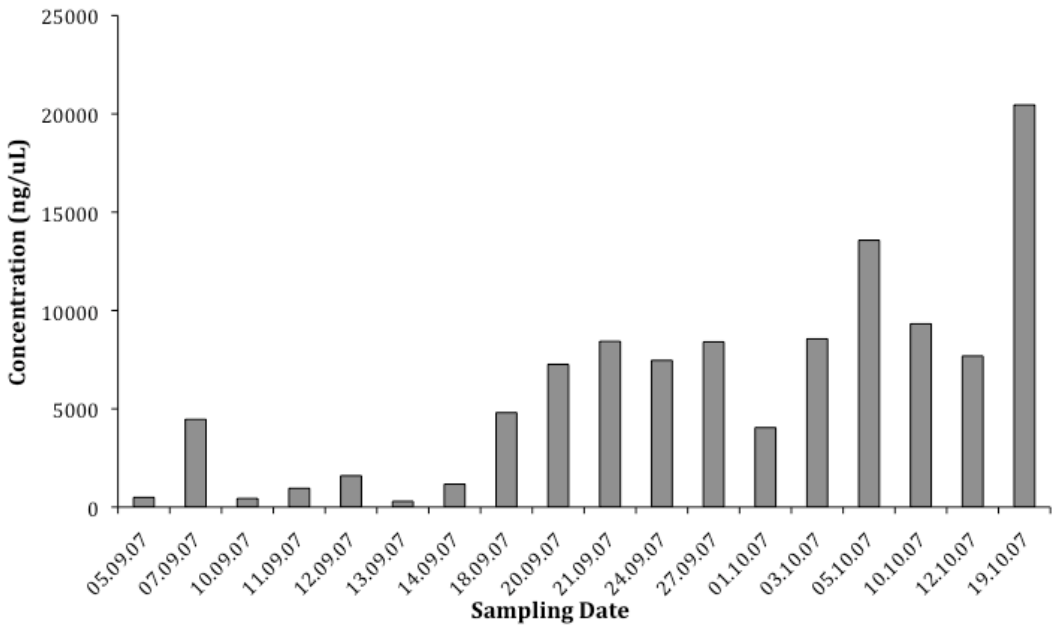


FIGURE 5.7. GENERAL INCREASING TREND OBSERVED FOR TYRAMINE IN DECOMPOSITION FLUID PRODUCED BY PIG 1, FOLLOWING ANALYSIS BY LC-ESI-MS.

As discussed in Chapter 4, the increasing trend observed for tyramine is expected over the course of the trial. As tyramine is derived from the pre-cursor amino acid tyrosine, it would be expected that degradation and a decrease in the precursor, would produce a corresponding general increase in the by-

product., however, this requires further investigation into the breakdown products of amino acids and biodegradation pathways.

One of the advantages of using LC-MS, was the improved sensitivity when compared with CE-UV-Vis. This was immediately apparent for the amino acids phenylalanine and tryptophan. Both of these compounds were targeted for analysis with CE-UV-Vis, however, the sporadic detection excluded them from further investigation as potential indicators of PMI. In LC-MS, however, both phenylalanine and tryptophan were detected on every sampling day for all three pigs. While CE-UV-Vis had limits of detection of 6.5 mg/L and 8.6 mg/L for tryptophan and phenylalanine respectively, and thus only detected them sporadically, LC-MS analysis detected both of these down to ng/μL. The range of concentrations observed was 5.62 – 223 ng/μL for tryptophan and 18.66 – 486.9 ng/μL for phenylalanine.

Tyrosine was also targeted as an analyte in CE-UV-Vis but proved problematic for LC-MS, most likely due to an error in sample preparation. The result of this was a calibration that was not linear (r^2=0.001109). The observation of the cyclic trend observed in tyrosine following CE-UV-Vis analysis could not be confirmed following analysis by LC-MS. However, in the results from the LC-MS tyrosine was first detected on day 5, but it was not detected by CE-UV-Vis until day 10 (ADD 166).

Potential trends were investigated for both tryptophan and phenylalanine following LC-MS analysis. These two amino acids were chosen for individual analysis as they were also target analytes in CE-UV-Vis. Individually, these amino acids showed no particular trend that might indicate their usefulness as a marker of PMI. However, when viewed together, they appeared to show a cyclic trend. This trend was discussed first in Chapter 4, and is confirmed by the data analysis from LC-MS. Figure 5.8 shows the apparent 14-day cyclic trend for the sum of phenylalanine and tryptophan. No other individual amino acids showed apparent trends in concentration that might indicate their reliability as useful markers of PMI. This was in contrast to previously published literature that

reported proline and methionine showed trends with reproducible results for specific ADD values [5].

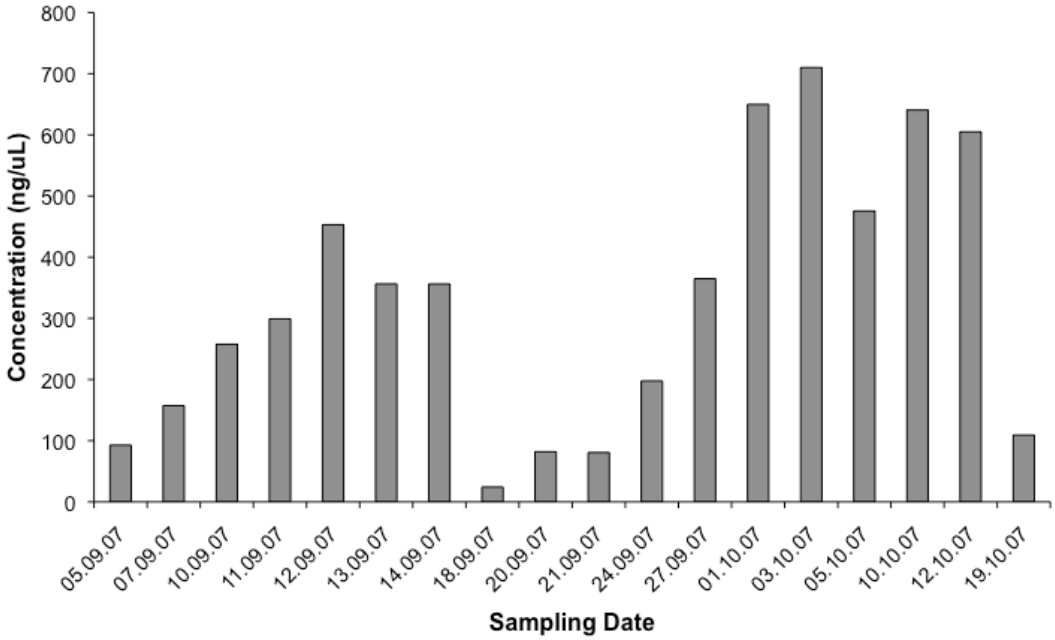


FIGURE 5.8. CYCLIC TREND OBSERVED FOR THE SUM OF PHENYLALANINE AND TRYPTOPHAN.

The next step was to investigate the trend in total amino acid concentration. With the exception of two sampling days, the sum of amino acid concentrations appeared to follow the same cyclic trend seen in tryptophan and phenylalanine. This cyclic trend appeared to occur over a 14-day period, with amino acids passing through a maximum on day 13 (ADD 207), followed by a second maximum on day 33 (ADD 462). This trend is shown in Figure 5.9.

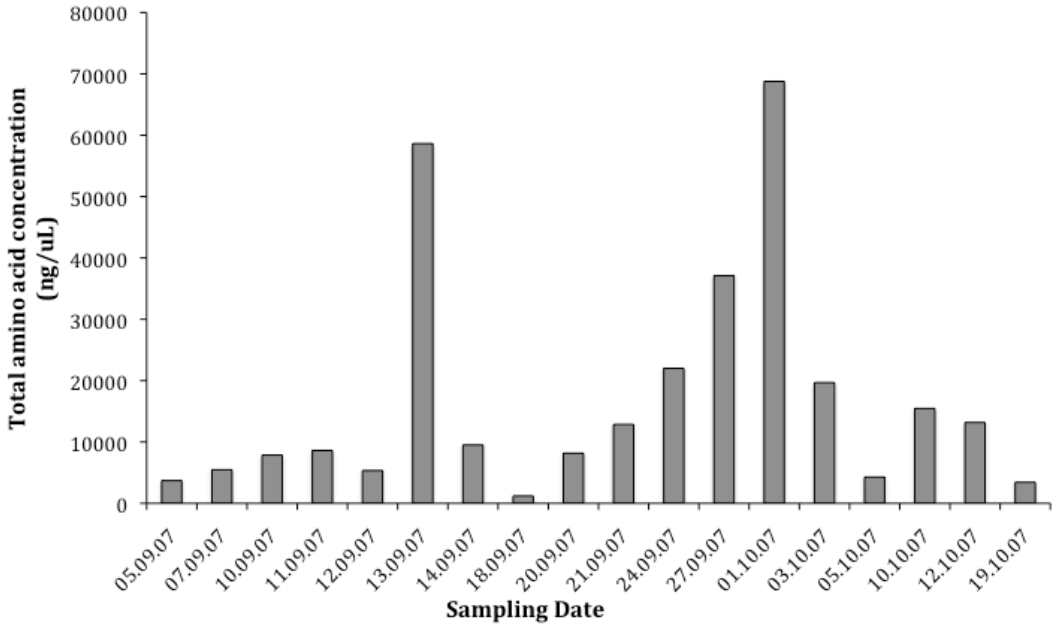


FIGURE 5.9. CYCLIC TRENDS OBSERVED FOR THE SUM OF AMINO ACID CONCENTRATIONS.

Two compounds that did show surprising results were indole and putrescine. Neither of these compounds was amenable to separation by CE-UV-Vis using the developed method; indole had a significantly different pK_a value compared to the pH of the background electrolyte and putrescine is an aliphatic amine lacking a chromophore, thus requiring a derivatisation step to enable detection by CE. Both indole and putrescine have previously been reported in decomposition fluid [5]. Indole was also detected during studies using GC-MS (Chapter 3), therefore, they were both of significant interest in LC-MS work.

When analysed by LC-MS, putrescine showed a general increasing trend for all three pigs (Figure 5.10) and indole showed the same increasing trend for pigs 1 and 3 but not for pig 2 (Figure 5.11).

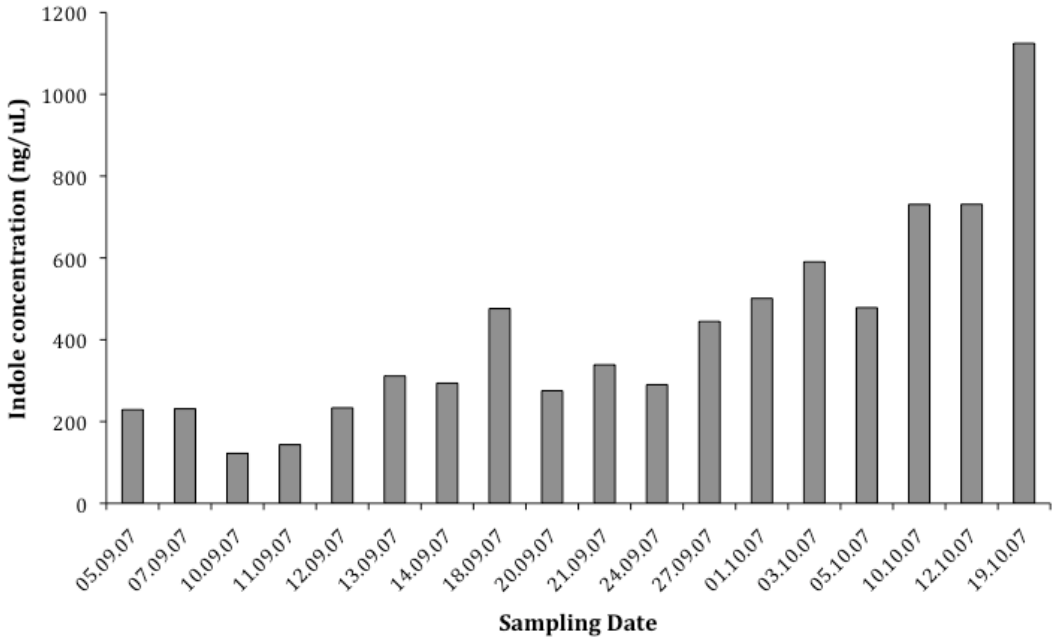


FIGURE 5.10. GENERAL INCREASING TREND FOR INDOLE AS SEEN IN PIG 1.

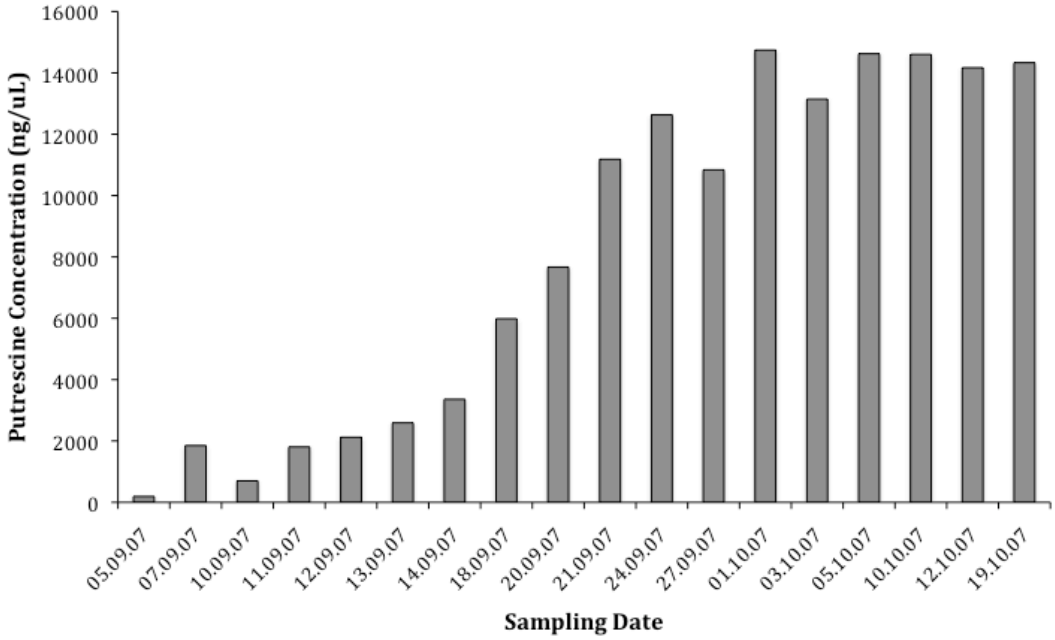


FIGURE 5.11. GENERAL INCREASING TREND FOR PUTRESCINE AS SEEN IN PIG 1.

5.4 (+) ESI MASS SPECTROMETRY

The formation of the product ion was investigated for selected compounds, particularly those that showed interesting trends over the course of the decomposition field trials. These included indole, tryptamine, phenylalanine, tryptophan and putrescine.

5.4.1 SELECTED AMINO ACIDS

TABLE 5.7. PRECURSOR AND PRODUCT IONS OF SELECTED AMINO ACIDS IN DECOMPOSITION FLUID.

COMPOUND	PRECURSOR ION (M/Z)	PRODUCT ION(S) (M/Z)
Phenylalanine	166.2	131.2, 120.2
Tryptophan	205.2	188, 146

The fragmentation pattern induced in LC-ESI-MS for tryptophan is dominated by the peak at m/z 188.2 (Figure 5.12). This peak represents a loss of 17 from tryptophan and is formed by α-cleavage of the –OH group next to the C=O, forming a carbocation on the remaining portion of the molecule [147].

The second most predominant peak in the tryptophan spectra can be seen at m/z 146. This is again represented by α-cleavage next to the C=O bond.

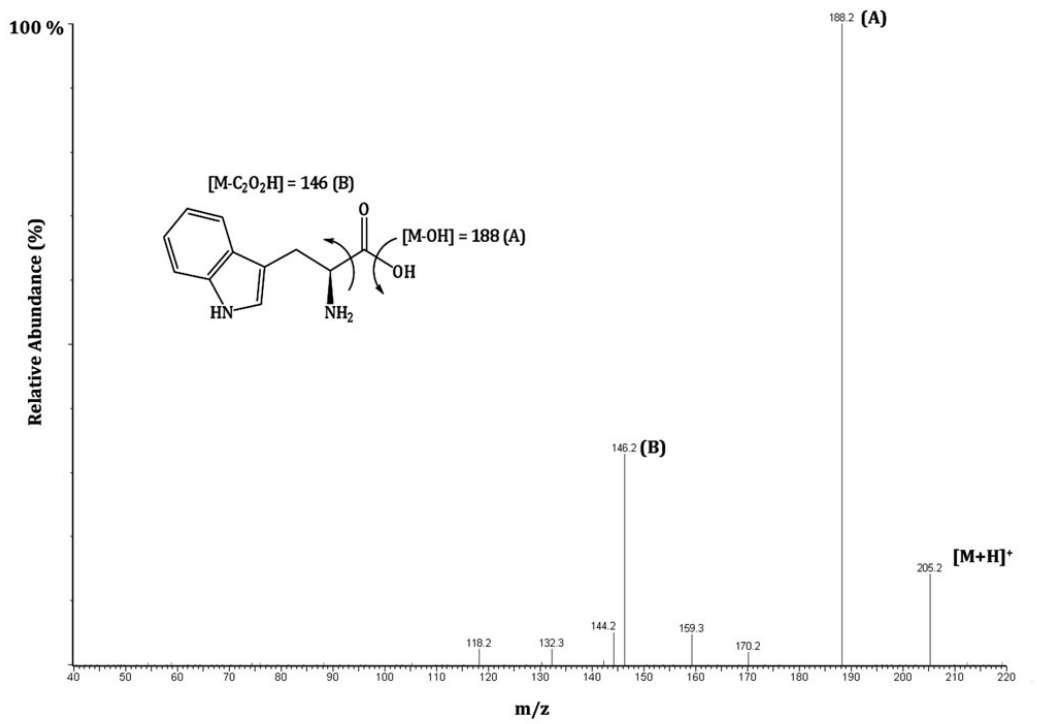


FIGURE 5.12. LC-ESI-MS OF TRYPTOPHAN SHOWING PRODUCTION OF DAUGHTER IONS.

The spectra produced by phenylalanine differs from that of tryptophan in the most abundant peak is not produced by the loss of the OH group. The loss of the OH group is represented by a relatively weak intensity peak at m/z 149.2 (Figure 5.13) and as such, was not used as a daughter ion in the identification of phenylalanine during analysis. Phenylalanine also shows a more intense $[M+H]^+$ peak, which is formed due to the addition of formic acid in the mobile phase. The intensity of these $[M+H]^+$ adducts varies between analytes.

The most abundant peak in the LC-ESI-MS spectra of phenylalanine is represented by the peak at m/z 120.2. This is typical of a compound containing an acid group which undergoes α-cleavage next to the C=O bond. The loss of the –COOH from phenylalanine which gives rise to the peak at m/z 120.2 can be seen in Figure 5.13.

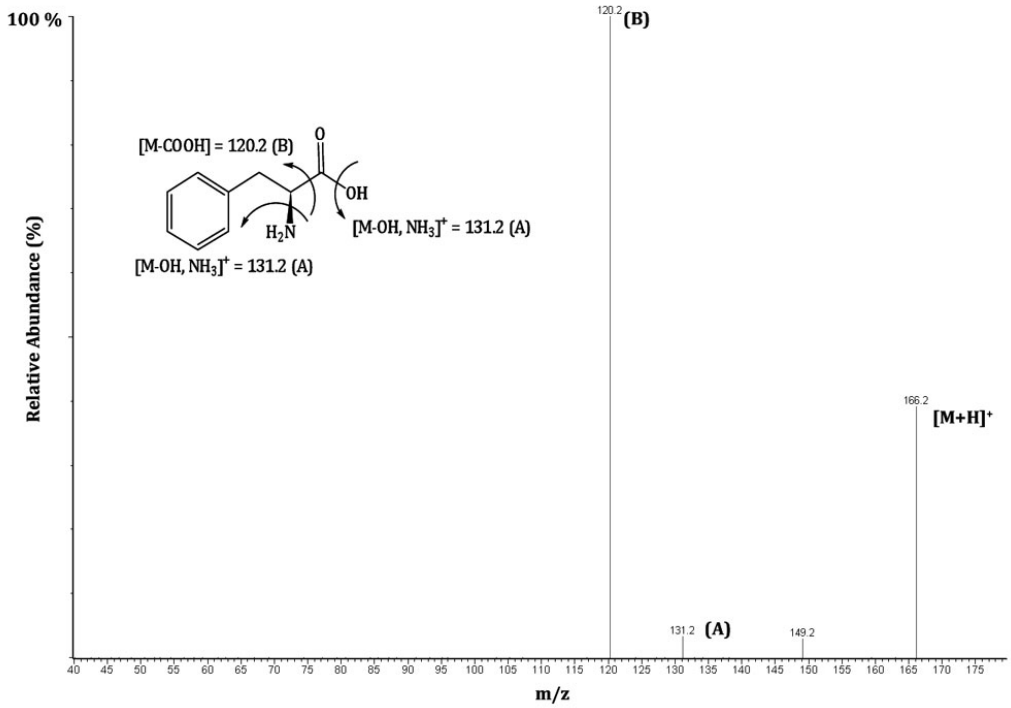


FIGURE 5.13. LC-ESI-MS OF PHENYLALANINE SHOWING PRODUCTION OF DAUGHTER IONS.

5.4.2 SELECTED BIOGENIC AMINES

Table 5.8 shows the relevant product ions produced by (+) ESI fragmentation for selected biogenic amines. As discussed earlier, ESI is a soft ionisation technique, producing little to no fragmentation in the mass analyser. Therefore, a prediction of a full mass spectral fragmentation pattern is not relevant.

TABLE 5.8. PRECURSOR AND PRODUCT IONS OF SELECTED AMINES IN DECOMPOSITION FLUID.

COMPOUND	PRECURSOR ION (M/Z)	PRODUCT ION(S) (M/Z)
Indole	118.1	90, 63
Tryptamine	161.1	144.2
Putrescine	89	72

Tryptamine is an aromatic biogenic amine with a ring structure that is similar to indole. It is also derived from the amino acid, tyrosine, by deamination [110, 111] To identify tryptamine by LC-ESI-MS in this work, the production of only one daughter ion was required. The peak shown at m/z 144.2 (Figure 5.14) represents a loss of the primary amine group [M-17] to form the most abundant peak.

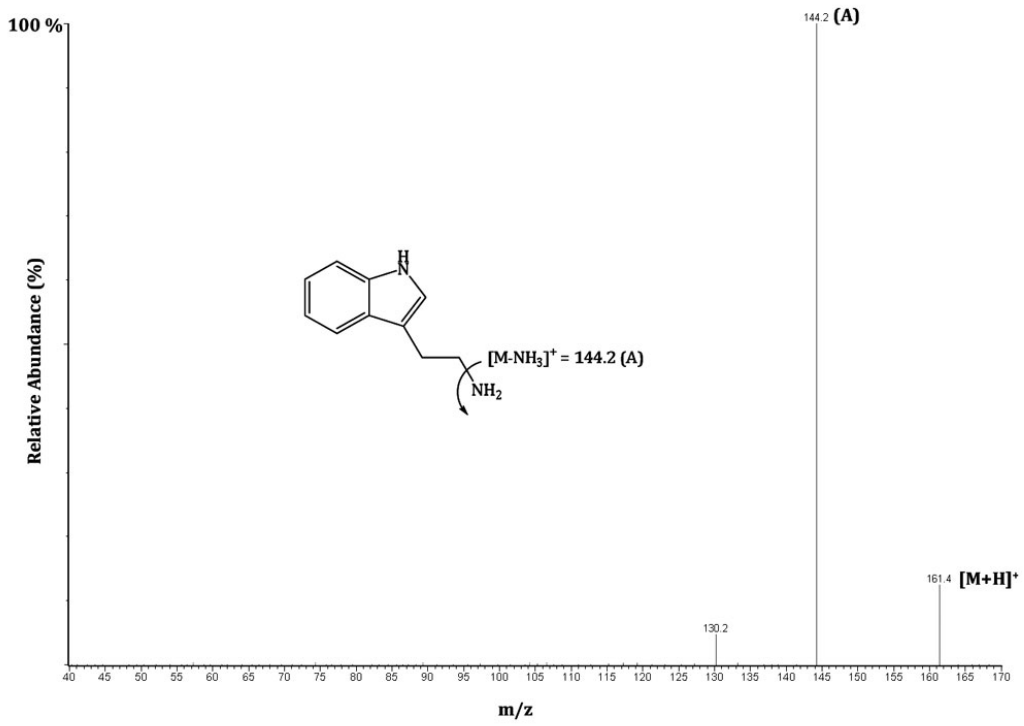


FIGURE 5.14. LC-ESI-MS OF TRYPTAMINE SHOWING PRODUCTION OF DAUGHTER IONS.

The most intense peak in the mass spectrum of an aliphatic amine, such as putrescine, arises from α-cleavage [147]. In the case of putrescine, this is represented by a peak at m/z 72.4 (Figure 5.15), indicating the loss of the primary amine group. As was the case with tryptamine, the identification of putrescine in decomposition fluid only required the production and monitoring of one daughter ion.

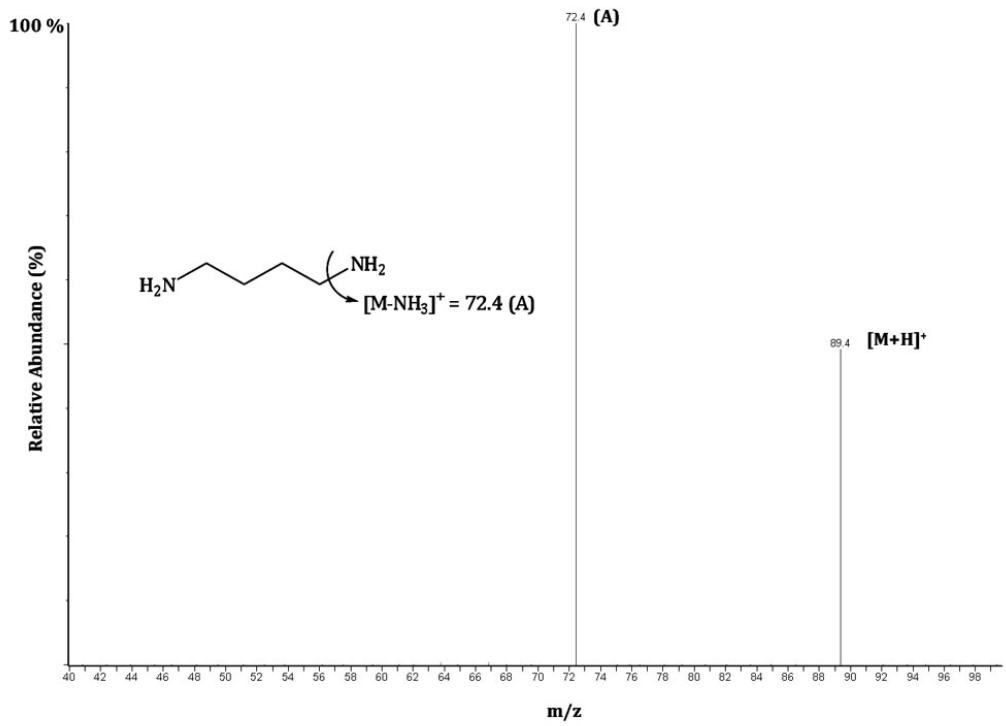


FIGURE 5.15. LC-ESI-MS OF PUTRESCINE SHOWING PRODUCTION OF DAUGHTER IONS.

For indole, the molecular ion peak (m/z 118) is the base peak. The loss of HCN to produce m/z 91 is the most stable product ion [247]. In this case, there is transfer of a proton to the fragment ion. This was confirmed in the optimization of product ions for the analysis of decomposition fluid, where this transition was the most dominant. A less dominant peak would be expected to be produced at m/z 89, corresponding to loss of H_2CN [247], however, this was not seen in the fragmentation produced by LC-ESI-MS. Further fragmentation gives $C_5H_3^+$, corresponding to m/z 65, although this transition was not required for the identification of indole in decomposition fluid. Figure 5.16 shows the formation of the fragment ion for indole.

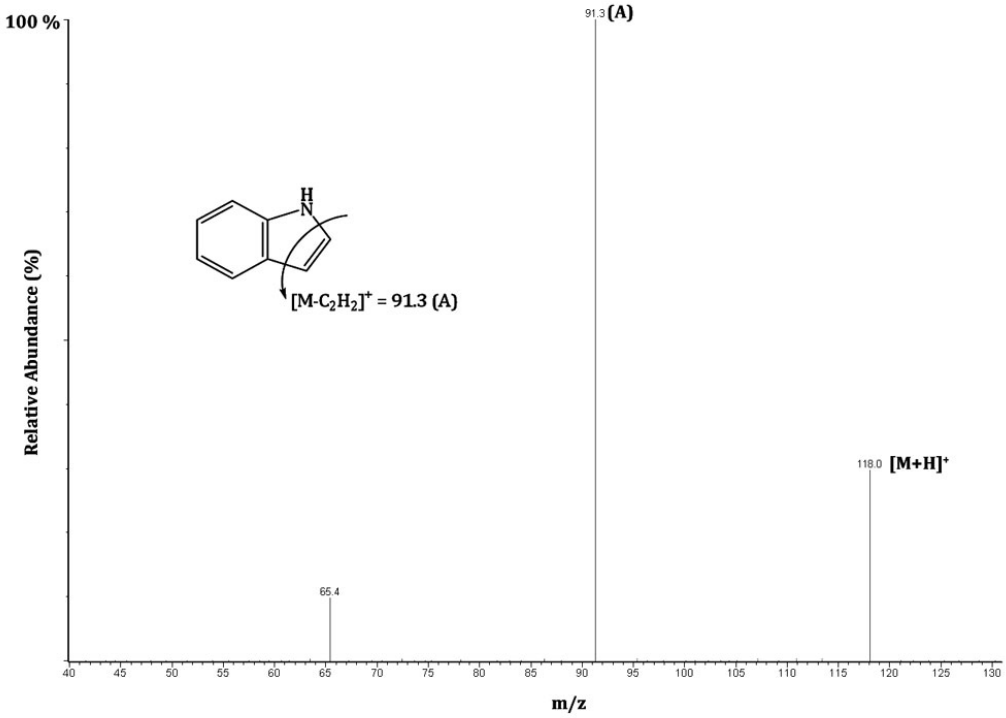


FIGURE 5.16. LC-ESI-MS OF INDOLE SHOWING PRODUCTION OF DAUGHTER IONS.

5.5 CONCLUSIONS

Liquid chromatography-electrospray-mass spectrometry proved to be a very powerful analytical tool for the detection and identification of amino acids and amines in decomposition fluid. The sensitivity and selectivity of LC-ESI-MS operated in MRM mode enabled potential trends to be identified for compounds that had lacked detection with previous instrumentation. The developed methods also enabled the identification of compounds that had the same m/z values but different pre-cursor ions, similar retention times but different product ions, and compounds with the same precursor and product ion(s) but different retention times.

The 'dilute and shoot' approach adopted to investigate the matrix effect proved to be successful and reliable. Different dilution factors of pure decomposition sample with varying injection volumes was successful in showing that minimal matrix effects were experienced by the analytes present in the sample matrix.

Given the simplicity of the method developed, the possibility exists for large-scale investigations to investigate both biogenic amines and amino acids in large-scale field trials. No sample preparation was required for relatively 'dirty' samples and results showed good sensitivity and selectivity, allowing high sample throughput in a minimum amount of time.

CHAPTER 6 – CONCLUSIONS AND FUTURE WORK

This thesis has outlined the significance of continuing research into the area of decomposition chemistry. The issues faced by researchers into this area as well as the complexities involved in setting up a field trial have been discussed. Chapters 3 – 5 have described, in detail, various analytical methodologies to characterise the compounds produced during the decomposition process. The developed analytical separations have enabled a more detailed picture of the decomposition process to be constructed.

While early studies using analytical separations focused on estimation of postmortem interval through measurement of specific chemical species, these to date have been unsuccessful. In all likelihood a single chemical test will not be accurate enough to determine postmortem interval [85]. If the potential of a chemical method to determine postmortem interval is to be realised, there is a need to develop harmonised analytical protocols through properly constituted collaborative trials.

In addition to the specific conclusions at the end of each chapter, suggestions for future work are discussed below:

6.1 Practical considerations

The set up of the field trials established the importance of the source of the fluid in decomposition studies. Stillborn piglets, whilst having being used to simulate neo natal remains in decomposition studies, produced little to no decomposition fluid that could be used for analysis and thus the subsequent establishment of trends in decomposition fluid.

Ongoing research into this field will require the use of a larger sample set, thus requiring a larger field trial area. Given the variability seen in the rate of decomposition for a small sample set, such as the ones used in this research, the opportunity to use a greater number of carcasses would be advantageous. The need for replicate experiments using the same number of carcasses in the same or similar locations during the same time frames provides the opportunity for

replicate experiments. This would need to be conducted over several years during different seasonal periods to observe trends at particular times of the year. This would also allow data comparison across years.

Whilst it is clear that insect activity, particularly maggots, has a considerable effect on the rate of decomposition, the contribution of the feeding maggots to the abundance of compounds produced is still yet to be explored.

6.2 INSTRUMENTAL CONSIDERATIONS

6.2.1 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Analysis by GC-MS confirmed that compounds produced at locations with different climatic conditions were very similar in both identity and relative abundance. The significance of this similarity is that a method using a measured scientific parameter that targets a specific compound(s) has the potential to be developed. This would be extremely advantageous, enabling comparative studies between research groups that would be less susceptible to error than methods that rely on examiner experience and are thus subjected to bias. This can be significant when applied to real forensic cases where the estimation of PMI is an important determinant.

The nature of the decomposition fluid and the associated debris limits the ability to collect a truly representative sample. Also, the volatile nature of the compounds detected by GC-MS, limits a totally quantitative field study being conducted. Rather than being quantitative, a qualitative overview of the compounds produced can provide an insight into the timing of compounds present rather than abundance. VFAs are notoriously difficult to analyse in their free form. To improve detection by GC-MS, a solution to this would be the development of methods that include preparation of fatty acid methyl ester or butyl ester derivatives.

This approach would also help avoid the introduction of large volumes of water into the GC. Typically, measures are taken to eliminate water from samples that

are required to be analysed by GC-FID or GC-MS as the stationary phase is water-soluble. However, in the case of this research, every attempt was made to replicate the previous work of Vass, who utilised aqueous samples to establish potential trends in the compounds found in decomposition fluid.

The higher molecular weight of the derivatives may improve reproducibility as well as the limit of detection. This would have to be balanced against the potential sample loss, analyte loss (particularly for highly volatile analytes or those present in trace amounts) as well as a significant increase in sample preparation time. In addition the development of methods utilising multi-dimensional chromatography may improve the chromatographic resolution.

6.2.2 Capillary electrophoresis

The knowledge gained in combination with analytical procedures developed specifically for decomposition studies may have a wider application than forensic science. A significant concern to the food industry, particularly at the retail end of the operation is the ability to determine the shelf life of packaged meat [248]. For example, the accumulation of biogenic amines in food products is related to both food spoilage and shelf life quality. Their presence, particularly in meat products, is a health concern due to their toxicological implications [157]. Biogenic amines have the potential to act as spoilage indicators for meat products, since their formation is closely related to the presence of gram negative bacteria [156]. The potential also exists for application of the optimized CE method to the rapid determination of tryptamine and tyramine, in particular, for pharmaceutical analysis and studies into food spoilage [90, 92, 162].

Whilst CE lacked the selectivity and specificity provided by LC-MS analysis, it is still a useful screening tool for compounds such as tyrosine and tryptamine, which showed potential trends in decomposition fluid. The much shorter analysis run time, compared to LC-MS, allows high sample throughput for investigation into these compounds of interest. The introduction of a

derivatisation procedure, whilst increasing the sample preparation time, would allow a broader range of compounds to be analysed and detected.

While it was not available for this research, CE-MS would potentially offer the advantages of rapid analysis in combination with high specificity and improved detection limits.

6.2.3 Liquid chromatography-electrospray-mass spectrometry

The developed liquid chromatography-mass spectrometry method was the most powerful in terms of providing information in regards to compounds in decomposition fluid. In a similar way to GC and CE, the sample preparation was minimal, consisting of a simple filtration before direct analysis. However, in contrast to both GC and CE, the method development required for LC-ESI-MS was very straight-forward. The selectivity and specificity of LC-ESI-MS and the use of multiple reaction monitoring (MRM) allowed for the distinction between a far greater number of analytes. LC-ESI-MS also dealt with co-eluting compounds and also the absence of baseline resolution between analytes.

Investigations into the matrix effect of decomposition fluid showed that there were minimal to no effects on the analytes in question. This was an unexpected result due to the apparent complexity of the sample in question. Decomposition fluid is a very viscous fluid in itself and also contains a significant amount of associated debris that can impact upon it. This is a significant demonstration of how robust the instrument is.

The use of liquid chromatography also allowed the preliminary identification of patterns in compounds that were previously unable to be seen. Compounds such as putrescine and indole were not detected with CE. Both of these compounds showed a general increasing trend over the course of the decomposition field trials and this was only established after analysis with LC.

Following analysis, an apparent 14-day cyclic trend was established with phenylalanine and tryptophan. Although cyclic trends were identified following analysis by GC, this was with fatty acid groups which followed an 8-day trend. The trend observed for the amino acids was not seen in previous analysis, probably due to a lack of sensitivity with the instrumentation, limiting the number of days that these compounds were detected.

Although preliminary trends have been observed following analysis by LC, further replicates are required to confirm this cycling. These replicates would need to account for seasonal variation over a greater number of years as well as individual trials being conducted on a larger scale using multiple subjects.

6.3 SUMMARY

As the analysis of compounds is semi-quantitative, a trend in appearance and disappearance seems a more likely way to estimate PMI, rather than trends in concentration of compounds at a specific ADD. If the number of cycles of a compound, or class of compounds, can be monitored, an estimate of PMI may be able to be achieved. These investigations have only showed very preliminary results, and much further work is required to confirm these trends, however, the potential to combine the 8-day cycle seen in fatty acids with the 14-day cycle in total amino acid content may have the potential to aid in PMI determination. This would not be an alternative method to estimate PMI, particularly in the mid-late stages of PMI, but rather a complimentary technique to existing methods such as entomology to further strengthen the estimation of PMI for forensic investigators.

The use of a much larger carcass that would enable several cycles to be monitored would be ideal. In this case, several analytical methods could be employed to monitor the compounds produced. An indication of the number of cycles for compounds such as VFAs (monitored by GC) and amino acids (by CE-UV-Vis or LC-MS) has the potential to be related back to the initial composition of the carcass and potentially PMI.

These two classes of compounds, VFAs and amino acids, are still only a small part of the chemistry of the decomposition fluid. Investigations are thus required into other classes of compounds that may show cyclic trends.

The confirmation of biodegradation pathways is another area that requires further investigation. Whilst it is readily accepted that decomposition products are the result of the breakdown of proteins, fats and carbohydrates in the body, much of the literature presented comes from fermentation processes. Ideally, if the contribution of each degradation pathway to the decomposition process can be confirmed, it can be related back to the initial composition of the carcass. This would only strengthen the resources available to the forensic investigator.

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APPENDICES

APPENDIX 1 – ADULT PIG TRIAL – UNIVERSITY OF ONTARIO INSTITUTE OF TECHNOLOGY – JULY 2007

DAY NUMBER/DATE	AMBIENT TEMP/ RAINFALL	PIG 1	PIG 2
0 / 17.07.07	24 C	Day Zero – Set up of trial	
1 / 18.07.07	20 ℃/ 0 cm ADD: 44	No fluid or insect samples collected	No fluid or insect samples collected
2 / 19.07.07	20 ℃/ 0.25 cm ADD: 64	1st instar maggots collected from snout. No fluid, flies or beetles collected.	1st instar maggots collected from snout. No fluid, flies or beetles collected.
3 / 20.07.07	15 ℃/ 1.25 cm ADD: 79	No fluid collected. Sexton beetle collected from torso. Flies captured from upper torso.	No fluid collected. Flies collected from head region.
4 / 21.07.07	21 ℃/ 0 cm ADD: 100	No samples collected	
5 / 22.07.07	26 ℃/ 0 cm ADD: 126	Beetles collected	No samples collected
6 / 23.07.07	18 ℃/ 0 cm ADD: 144	Approx 20 mL fluid collected. Stomach organs exposed. Maggots collected from pitfall traps. Minimal fly activity	Approx 22 mL fluid collected. Apparent scavenger activity observed. Internal organs partially exposed. Maggot collection from head and pitfall trap.
7 / 24.07.07	19 ℃/ 0.05 cm ADD: 163	Active decomposition. Large maggot mass. Large pooling of fluid in pit – approx 37.5 mL collected. Significant fly activity. Maggots collected from head and rear.	Less advanced decomposition than pig 1. Smaller maggot mass. No maggots collected. Increased fly activity. Approx 30 mL fluid collected.
8 / 25.07.07	20 ℃/ 0 cm ADD: 183	Entire maggot mass. 3rd instar maggots. Mummified head. No insect samples collected. Fluid collected	Maggot mass on head and rear. Maggots collected from both areas. Corpse deflated. Fluid collected.

DAY NUMBER/DATE	AMBIENT TEMP/ RAINFALL	PIG 1	PIG 2
9 / 26.07.07	20 ℃/ 0 cm ADD: 203	Very deflated corpse. Mummification of head and extremities. Maggot mass on entire body – samples collected. Maggots wandering/ departing from carcass No fluid collected.	Corpse deflated. Hind mummified. Less advanced decomposition compared to Pig 1. Maggot mass on head – not collected. No fluid collected. Flies captured. Maggots observed wandering from corpse.
10 / 27.07.07	20.5 ℃/ 0 cm ADD: 223.5	Mummified corpse. Maggots present – none collected. No fly activity observed. Fluid collected.	Deflated corpse. Mummification observed. Minimal fly activity. Maggot mass from pit trap collected.
11 / 28.07.07	22 ℃/ 0 cm ADD: 245.5	No sampling	
12 / 29.07.07	24.5 ℃/ 0 cm ADD: 270	Mummified body. Skeletal limbs. Skin still present. Minimal maggots – not collected. Pupal cases in pit. No fly activity observed. Fluid collected.	Partial mummification in head and limbs. Skin across body still intact. Minimal maggots. Some fly activity. No fluid collected.
13 / 30.07.07	21 ℃/ 0 cm ADD: 291	No sampling	
14 / 31.07.07	22 ℃/ 0 cm ADD: 313	Mummified body. Skeletal limbs. No maggot activity. Fluid collected from pit	Mummification in head and limbs. No maggot or fly activity. Beetle larvae collected from pit. Carrion beetles with mites collected from pit. Fluid collected.

APPENDIX 1 - ADULT PIG TRIAL – CURTIN UNIVERSITY - SEPTEMBER 2007

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (° C)	PIG 1	PIG 2	PIG 3
31.08.2007	0	PA: 18.9, PM: 19.2, JA: 17.6 ADD: 11.57	day zero	day zero	day zero
01.09.2007	0	PA: 19.3, PM: 19.2, JA: 18.3 ADD: 23.5	no samples collected	no samples collected	no samples collected
02.09.2007	0	PA: 19.6, PM: 19.7, JA: 19.5 ADD: 36.34	slight discolouration in lower torso. Fleshfly observed - not collected.	no samples collected	slight discolouration in lower torso
03.09.2007	0	PA: 27.4, PM: 27.6, JA: 26.7 ADD: 55.55	minimal ant activity around head. Slight bloating. Minimal fly activity. Fleshfly collected.	Slight bloating. Beetles collected from head region. Minimal fly activity. Carrion beetles collected (pitfall).	Slight bloating. Minimal fly activity. Minimal maggot activity (wound) - not collected.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (° C)	PIG 1	PIG 2	PIG 3
04.09.2007	0.8	PA: 21.3, PM: 21, JA: 19.9 ADD: 72.25	Inc fly activity. Bloating. Min maggot activity in wound - head contents exposed. Egg mass in mouth.	Min fly activity. Bloating in lower torso. Egg mass in head/mouth area.	Partial bloat. Fly activity in head region. Egg mass in mouth
05.09.2007	PA: 2.2, PM: 2.4, JA: 3.0	PA: 20.3, PM: 19.4, JA: 18.6 ADD: 86.75	Inc bloat around upper limbs/head. Inc fly activity, Inc discolouration. Maggots in mouth - collected. 1 fly collected. Fluid collected	Fluid collected. Inc fly activity. Inc bloat in upper limbs/neck/head. Protruding torso (skin intact)	Inc fly activity. Fluid collected. Maggots in mouth - collected.
06.09.2007	0	PA: 22.0, PM: 22.1, JA: 21.3 ADD: 101.32	Inc fly activity on rear. Inc bloating. Egg mass - wound region. Maggots in mouth - not collected. Beetles - head region - collected.	Inc bloating. Egg mass in mouth	Maggots in mouth - not collected. Inc bloating. Beetles in mouth area - collected. Fluid collected
07.09.2007	0	PA: 18.1, PM: 17.6, JA: 16.5 ADD: 125.84	Fluid collected. Inc bloating. Sig fly activity. Maggots in mouth - collected. Flies from body collected. Inc discolouration.	Fluid collected. Inc bloating and discolouration. Sig fly activity - 2 collected. Maggots in ear - collected. Beetles in pitfall - collected	Fluid collected. No maggot collection.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
08.09.2007	PA: 23.6, PM; 21.4, JA: 28.0	PA: 19.2, PM: 18.4, JA: 18.5 ADD: 139.07	No sampling		
09.09.2007	PA: 0.8, PM: 0.2, JA: 0.4	PA: 19.8, PM; 19.8, JA: 18.8 ADD: 152.69	No sampling		
10.09.2007	0	JA: 22.1, PM; 23.5, PA: 23.9 ADD: 166.1	Mouth completely open (partial skeletal). Maggots in mouth - collected. Fluid collected. 3 flies collected (pit). 2 worms collected.	Jaw protruding. Maggots in mouth - collected. Fluid collected. 2 flies on body collected. Egg mass in joints and along back. 2 flies collected from pit.	Egg mass in snout/neck and back area. Fluid collected. Sig fly activity.
11.09.2007	0	PA: 22.1, PM: 21.5, JA: 20.9 ADD: 180.69	Sig discolouration in torso. Jaw protruding. Sig fly activity. Fluid collected	Head open. Maggot mass in head - not collected. Torso protruding - skin not burst. Fluid collected. Egg mass on front limbs.	Egg mass on head and upper limbs. Ant activity on upper limbs - collected. Flies collected. Fluid collected.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
12.09.2007	PM: 23.6, PA:21.4, JA: 23.4	PM: 17.8, PA: 18.2, JA: 17.8 ADD: 194.35	Large rainwater content in pit. Sig discolouration. Fluid collected	sig rainwater in pit (emptied). Fluid collected. Maggots under skin layer of upper torso - collected.	egg mass on snout. Large rainwater content in pit. Fluid collected. Beetles collected from snout/head. Discolouration in upper limbs.
13.09.2007	PM: 7.8, PA:8.6, JA: 6.6	PM: 17.6, PA: 17.1, JA: 17.2 ADD: 207.55	Discolouration in upper torso/shoulders. Egg mass in back/shoulder area. Fluid collected	Head collapsed/skeletal. Maggot mass in head/shoulder cavity - collected. Egg mass on back.	Sig fly activity. Maggots in mouth - collectd. Bloating in torso.
14.09.2007	PM: 0.2,	PM: 17.8, PA: 18.5, JA: 17.3 ADD: 220.52	No maggots - very large in head although dead. Black discolouration in chest and stomach.	Maggot mass near shoulder. Small amount of movement.	Egg mass on shoulder. Eye puffed open - maggots above eye - collected. No mOzzies.
18.09.2007	PM: 4, PA:3.0, JA: 8.2	PM: 19.5, PA: 19.7, JA: 18.8 ADD: 282.4	Maggots on body collected.	Not much change - head skeletonised.	Large egg mass on back

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
20.09.2007	0	PM: 21, PA: 21.3, JA: 20.9 ADD: 312.54	2 new sets of maggots on neck and back - collected	Pupae casings observed in maggots collected on 07.09.2007 (Mouth). Slightly deflated	Many flies – really stiff.
21.09.2007	0	PM: 21.6, PA: 22, JA: 21 ADD: 328.82	Pig 1 mouth - pupal cases observed (07.09.2007). Fresh maggots under back leg and stomach. Gas releasing from under belly. Sig fly activity.	Pig 2 ear (07.09.2007)- pupal cases observed. Slightly deflated. No fresh maggots.	Stiff and bloated. Discolouration of chest and between hind legs. Small maggots on rear.
24.09.2007	PM: 7.8, PA:8.8, JA: 10	PM: 18.2, PA: 18, JA: 17.5 ADD: 356.27	rain water collected. Not as bloated. Deflated. Deformed	Cover off - rained on.	Still very stiff. No new maggots. Opening formed in neck.
27.09.2007	PM: 2.8, PA:2.8, JA: 2.2	PM: 20.2, PA: 19.7, JA: 19.9 ADD: 400.74	Deflated.	Large maggot mass on rear. Top half skeletonised.	Sig fly activity. Maggots under top front leg - split open.
29.09.2007	0	PM: 22, PA: 22.8, JA: 21.3 ADD: 432.45	Pig 1 (05.09.2007) - flies emerged		Pig 3 (05.09.2007) - flies emerged.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (° C)	PIG 1	PIG 2	PIG 3
01.10.2007	JA: 1.0	PM: 20.2, PA: 21.2, JA: 19.8 ADD: 462.7	deflated. Skin and hair still present. Maggots on rear - collected. Beetles on upper torso. Min fly activity. Fluid collected. Carrion beetles in pitfall. Flies emerged - Pig 1 mouth (7.9.07)	Deflated. Head skeletal. Min fly activity. Carrion beetles in pitfall - collected. Fluid collected. Maggots on rear/back leg - collected. Pig 2 - flies emerged - ear (7.9.07)	Head skeletal. Bloating in rear. Maggots in torso/front leg - collected. Sig fly activity. Fluid collected. Flies collected from body.
03.10.2007	0	PM: 22.6, PA: 23.2, JA: 21.3 ADD: 493.89	No maggot activity apparent. Minimal fly activity. Fluid collected. Rear skeletal - skin still present. Carrion beetles in pitfall - collected. Flies emerged - Pig 1 mouth (10.09.2007)	Complete deflation. No fly activity. Fluid collected. Torso bones visible. Head completely skeletal. No apparent maggot activity. Flies emerged - Pig 2 mouth (10.09.2007).	Head skeletal. Torso deflated. Maggots in upper torso - not collected. Minimal fly activity. Fluid collected.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
05.10.2007	0	PM: 24.6, PA: 25.6, JA: 23.8 ADD: 524.84	Minimal fly activity. Skin separating from carcass. Partial mummifications. Little blue beetles present. Fluid collected.	Fluid collected. Mummification. No fly activity. Flies emerged - Pig 2 head (13.09.2007)	Fluid collected. Significant fly activity. Maggot movement in belly - skin not burst. Black discolouration in stomach. Flies emerged - Pig 3 mouth (13.09.07)
08.10.2007	PM: 10.4, PA: 11.4, JA: 18	PM: 20.7, PA: 20.1, JA: 21.5 ADD: 577.07	Flies emerged - Pig 1 body (18.09.07), Pig 1 back (20.09.07), Pig 1 neck (20.09.07)	-	Flies emerged - Pig 3 head (14.09.07)
10.10.07	PM: 1.4, PA: 0.6, JA: 2.0	PM: 19.3, PA: 19.3, JA: 18.4 ADD: 606.9	Flies emerged - Pig 1 under back leg (21.09.07). Fluid collected. Sig fly activity. Deflated	Fluid collected. Mummification. Some beetle activity.	Sig fly activity. Maggots in torso - 3rd instar - collected. Fluid collected. Torso deflated.

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
12.10.07	0	PM: 24.8, PA: 25.2, JA: 23.7 ADD: 637.29	Beetle activity. Beetles in pitfatt collected. Skin and hair still present. Mummified. Fluid collected	Mummified. Fly collected. Fluid collected	Significant fly activity. Torso deflated. Upper body mummified. Hair and skin present. Head skeletal. Fluid collected. 2 flies collected.
17.10.07	0	PM: 21, PA: 22.8, JA: 20 ADD: 733.1	No fluid collected	No fluid collected	Significant fly activity. Maggots present in fluid - not collected. Fluid collected.
19.10.07	0	PM: 27.1, PA: 27.6, JA: 26.9 ADD: 765.54	Flies emerged - Pig 1 rear (1.10.07). Fluid collected	Fluid collected	Fluid collected
21.10.07	PM: 1.2, PA: 1.6, JA: 4.4	PM: 21.3, PA: 22.8, JA: 22.1 ADD: 796.14	-	-	Flies emerged - Pig 3 front leg (1.10.07)

DATE	RAINFALL (MM)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
24.10.07	0	ADD: 844.64	-	-	Flies emerged – Pig 3 torso (10.10.07)

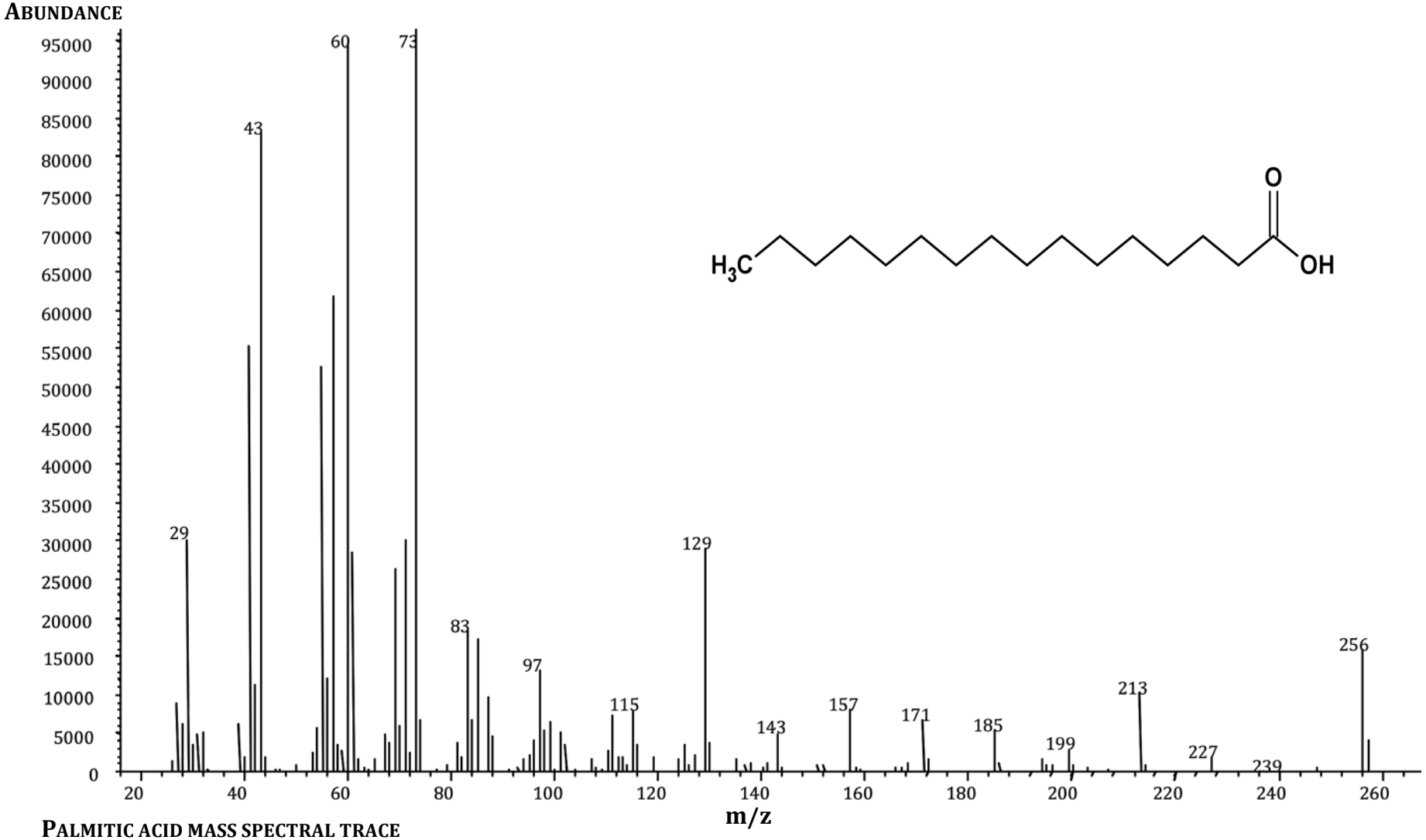
APPENDIX 1 - ADULT PIG TRIAL – CURTIN UNIVERSITY - FEBRUARY 2008

DATE (DAY)	RAINFALL (mm)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
20.02.08 (0)	0	20.05 ADD: 20.05	Day zero	Day zero. Sarcophagid fleshfly on pig carcass within 5 minutes - not collected.	Day zero.
21.02.08 (1)	0	22.08 ADD: 42.13	Bloating in torso. Intestines purged. Minimal fly activity.	Slight bloating in torso.	Bloating in torso. No discolouration.
22.02.08 (2)	0	20.78 ADD: 62.91	Ant activity on torso and rear - collected. Discolouration in torso. Further purging of intestines. Fluid collected. Beetle collected	Further bloating in torso. Slight discolouration. Ant activity in mouth.	Significant bloating and discolouration in torso. Intestines slightly purged. Ant activity on rear. Fleshfly (Sarcophagid) collected x 2.

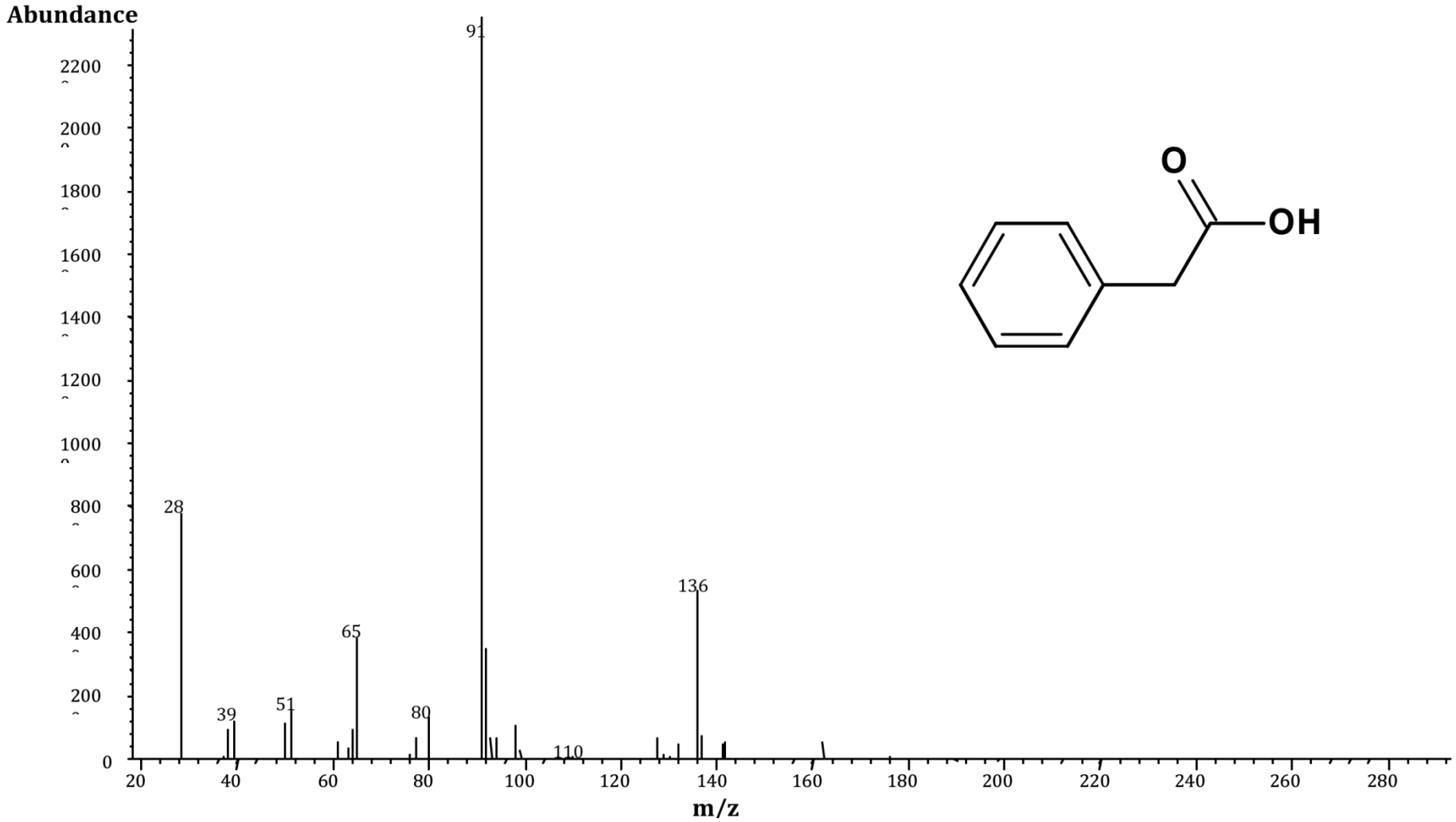
DATE (DAY)	RAINFALL (mm)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
23.02.08 (3)	0	21.75 ADD: 84.66	Bloating and partial discolouration in upper torso. Deflation in lower torso. Decreased ant activity. Fluid collected. Small maggot mass in head wound - not collected.	Intestines purged and deflated. Decreased ant activity. Minimal fly activity. Fluid collected. Increased discolouration in torso. 'Fat-like' substance on surface of fluid.	Further purging of intestines. Fluid collected. 'Fat-like' layer on fluid surface. Decreased ant activity. Bloating in upper limbs. Minimal fly activity.
24.02.08 (4)	0	24.42 ADD: 109.08	No sampling		
25.02.08 (5)	0	25.83 ADD: 134.91	Deflated. Discolouration in head region. Some ant activity on rear. Minimal fly activity - collected from head. Fluid collected. Maggot activity in fluid.	Deflated. Fly activity near head wound. Beetles on head - collected. Fluid collected. 'Fat' layer on fluid surface. Minimal ant activity. Some maggot activity in fluid.	Deflated. Bruising and discolouration on head and upper torso. Minimal fly activity. Fluid collected. 'Fat' layer on surface of fluid. Beetles on head region - not collected.

DATE (DAY)	RAINFALL (mm)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
26.02.08 (6)	0	26.90 ADD: 161.81	Partial mummification. Deflated. Minimal insect and fly activity. No visible maggot activity. Fluid collected.	Deflated. Partial mummification. No visible maggot activity. Fluic collected.	Deflated. Partial mummification. No visible maggot activity. Fluid collected.
27.02.08 (7)	0	29.47 ADD: 191.28	No sampling		
28.02.08 (8)	0	31.32 ADD: 222.6	Further mummification. No visible maggot activity. Skin split under upper left limb. No insect or fly activity visible. Fluid collected. Drowned maggots visible in fluid.	Deflated. Further mummification. No visible insect or fly activity. Fluid collected	Fluid collected. No visible fly or insect activity. Further mummification.
29.02.08 (9)	0	24.68 ADD: 247.28	Fluid collected	Fluid collected	Fluid collected.
01.03.08 (10)	0	25.35 ADD: 272.63	No sampling		
02.03.08 (11)	0	24.22 ADD: 296.85	No sampling		

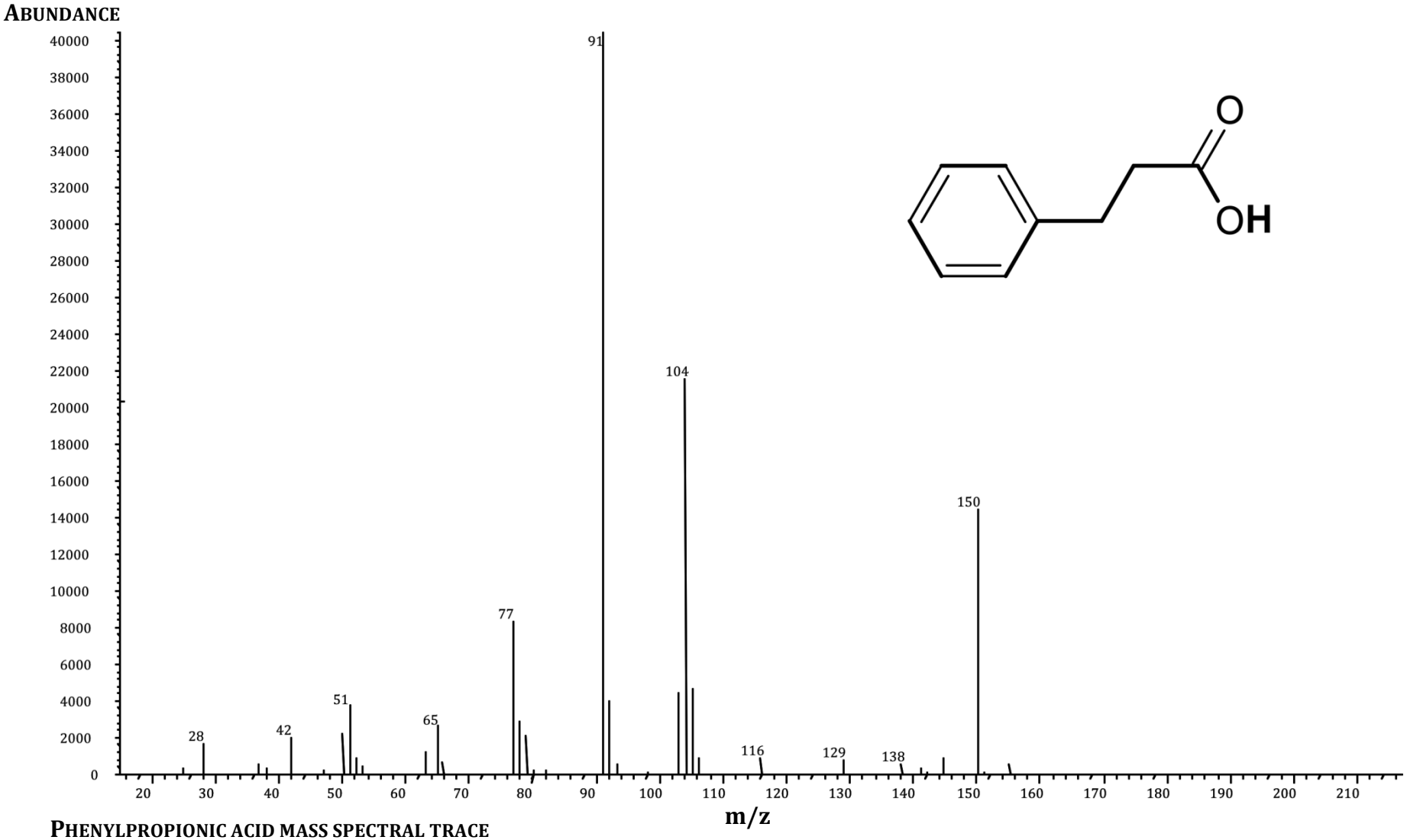
DATE (DAY)	RAINFALL (mm)	AVERAGE DAILY TEMPERATURE (°C)	PIG 1	PIG 2	PIG 3
03.03.08 (12)	0	25.53 ADD: 322.38	Fluid collected. Further mummification. No visible insect activity. Skin and hair still present.	Fluid collected. Further mummification. Ant activity on head/snout. Skin and hair still present.	Fluid collected. No visible fly or insect activity. Further mummification. Skin and hair still present.
04.03.08 (13)	0	25.3 ADD: 347.68	Fluid collected	Fluid collected	Fluid collected.
05.03.08 (14)	0	24.95 ADD: 372.63	Fluid collected.	Fluid collected.	Fluid collected.
06.03.08 (15)	0		No sampling		
07.03.08 (16)	0				



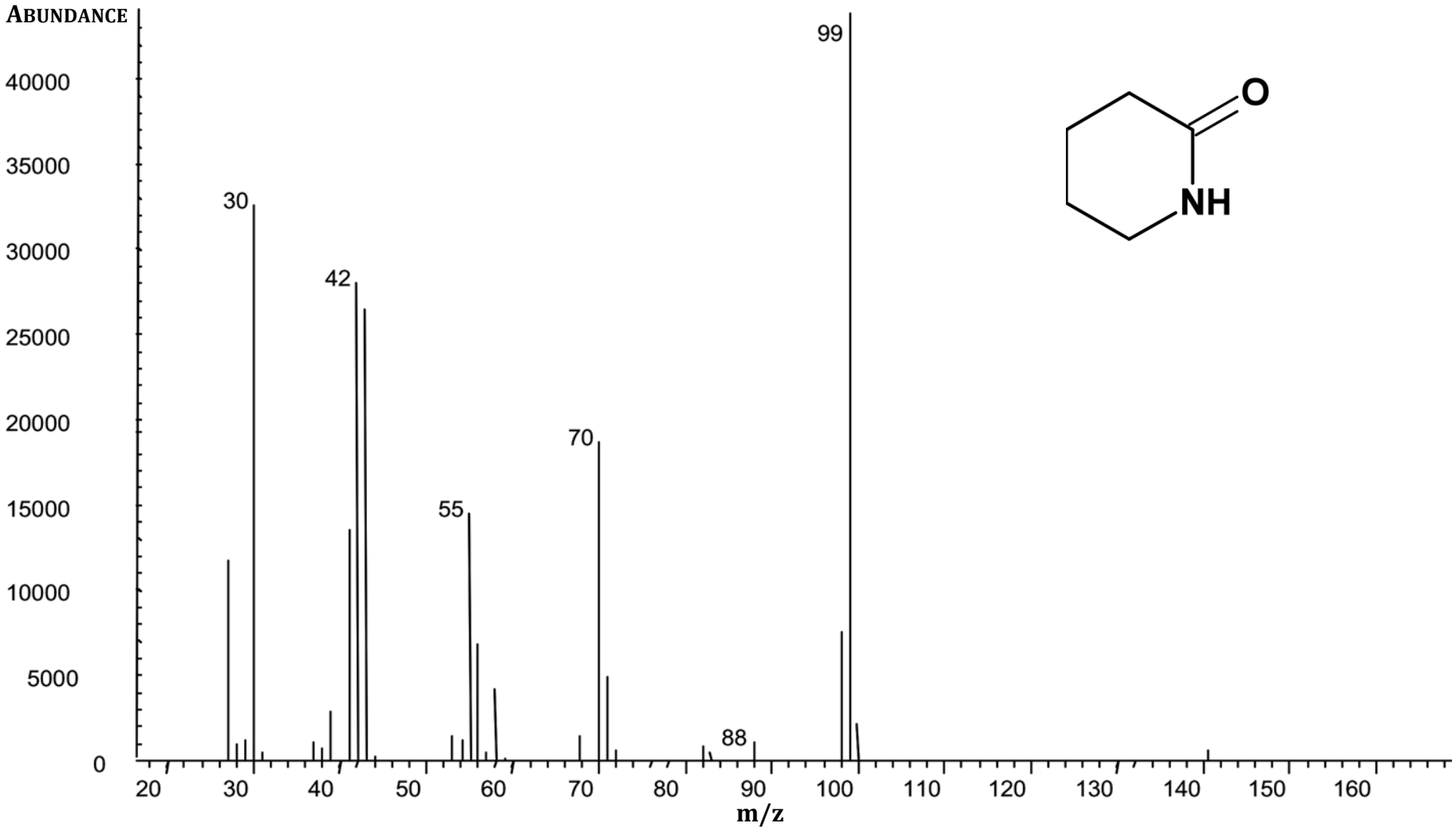
PALMITIC ACID MASS SPECTRAL TRACE



PHENYLACETIC ACID MASS SPECTRAL TRACE



PHENYLPROPIONIC ACID MASS SPECTRAL TRACE



PIPERIDONE MASS SPECTRAL TRACE