A REVIEW OF THE TECHNIQUES FOR THE FORENSIC INVESTIGATION AND DIFFERENTIATION OF HUMAN BLOOD AND DECOMPOSITION FLUID STAINS

By

Rachel Lee Anderson

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in

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Principle Supervisor: Dr Gavin Turbett
Academic Supervisor: Associate Professor James Speers

Semester 2, 2016
Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: Rachel Lee Anderson
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# Table of Contents

Title Page................................................................. i
Declaration........................................................................ ii
Acknowledgements ................................................................ iii
Table of Contents................................................................ iv

## Part One

**Literature Review ........................................................... 1-89**

Title Page ........................................................................ i
Abstract ............................................................................. ii
Table of Contents ................................................................ iii
List of Figures ....................................................................... v
List of Tables ........................................................................ vii
List of Abbreviations............................................................ viii

1. **Introduction ............................................................... 1**
2. **Post-mortem Decomposition ........................................ 4**
   2.1 Early Post-mortem Changes........................................ 4
   2.2 Mammalian Decomposition........................................ 5
   2.3 Inhibitory Effects on Decomposition ......................... 10
      2.3.1 Saponification............................................. 10
      2.3.2 Mummification........................................... 11
   2.4 Summary.................................................................. 12
3. **Macromolecule Degradation ........................................ 13**
   3.1 Carbohydrate Degradation......................................... 13
   3.2 Protein Degradation................................................ 14
   3.3 Lipid Degradation................................................... 16
   3.4 Summary............................................................... 18
**Part Two**

**Manuscript** .................................................................................................................. 1-46

1. **Introduction** ............................................................................................................. 5

2. **Mammalian Post-mortem Decomposition** .......................................................... 6

3. **Macromolecule Degradation** ............................................................................... 9
   3.1 Carbohydrate Degradation ................................................................................. 9
   3.2 Protein Degradation .......................................................................................... 10
   3.3 Lipid Degradation .............................................................................................. 11

4. **Decomposition Fluid** ............................................................................................ 13
   4.1 Physical Properties of Decomposition Fluid .................................................. 13
   4.2 Chemical Properties of Decomposition Fluid ................................................ 14

5. **Blood Evidence** .................................................................................................... 23
   5.1 Biological Properties of Blood ......................................................................... 23
   5.2 Chemical Changes Associated with Blood Degradation ............................... 24
   5.3 Biological Screening for Blood ......................................................................... 25

6. **Differentiation of Blood and Decomposition Fluid** ............................................. 27
   6.1 Visual Examination ............................................................................................ 27
   6.2 pH Measurement ................................................................................................ 27
   6.3 Presumptive Testing for Blood .......................................................................... 28
   6.4 Spectroscopic Techniques ................................................................................ 30
   6.5 Analysis of Volatile Organic Compounds ....................................................... 32
   6.6 Genomics ............................................................................................................. 33
      6.6.1 mRNA-based Analysis .............................................................................. 34
      6.6.2 miRNA-based Analysis ............................................................................ 35
      6.6.3 Epigenetic Analysis ................................................................................... 35
   6.7 Proteomics ........................................................................................................... 36

7. **Conclusions** ............................................................................................................ 37

**References** .................................................................................................................. 39
Part One · Literature Review

The Differentiation of Human Blood and Decomposition Fluid Stains
Abstract

An important aspect of forensic science is the detection and identification of biological fluids at a crime scene (Virkler and Lednev 2009). The determination of the type and origin of a biological sample can yield valuable information that supports a link between the criminal act and donor, which in turn may assist in the reconstruction and sequencing of a crime scene (An et al. 2012). A body and therefore any associated biological stains may not be located for a period of time, during which the decedent will begin to decompose. Blood and decomposition fluid stains have been reported to be visually similar (Comstock 2014) and therefore, it is important to determine the source of the stain. The presence of blood would suggest an injury has occurred before or shortly after death, whereas decomposition fluid is produced as a part of the naturally occurring decomposition process. Several approaches including visual examination, pH measurements, presumptive testing for blood, spectroscopic techniques, the analysis of volatile organic compounds, genomics, and proteomics may provide potential methods of biological stain identification and differentiation (Harbison and Fleming 2016; Stuart 2013; Virkler and Lednev 2009). However, there are associated limitations to these methods. This dissertation reviewed the effectiveness of these methods, which then informed the development of a proof-of-concept study to assess if the technique of microfluidic proteomics by protein electrophoresis can identify potential differences between blood and decomposition fluid stains. When compared to conventional techniques, microfluidic devices offer many advantages including improved efficiency, a decrease in sample and reagent consumption, and automation (Li 2015). The potential results obtained from the proposed study design will assist in enhancing the knowledge base surrounding the differentiation of blood and decomposition fluid stains.
# Table of Contents

Title Page ................................................................................................................. i
Abstract ....................................................................................................................... ii
Table of Contents ....................................................................................................... iii
List of Figures .............................................................................................................. v
List of Tables ............................................................................................................. vii
List of Abbreviations ................................................................................................. viii

1. **Introduction** ....................................................................................................... 1

2. **Post-mortem Decomposition** ....................................................................... 4  
   2.1 Early Post-mortem Changes .......................................................................... 4
   2.2 Mammalian Decomposition ......................................................................... 5
   2.3 Inhibitory Effects on Decomposition ............................................................. 10  
      2.3.1 Saponification ......................................................................................... 10
      2.3.2 Mummification ....................................................................................... 11
   2.4 Summary .......................................................................................................... 12

3. **Macromolecule Degradation** .................................................................... 13  
   3.1 Carbohydrate Degradation ........................................................................... 13
   3.2 Protein Degradation ..................................................................................... 14
   3.3 Lipid Degradation .......................................................................................... 16
   3.4 Summary .......................................................................................................... 18

4. **Decomposition Fluid** ................................................................................... 19  
   4.1 Research Investigating the Physical Properties of Decomposition Fluid ....... 19
   4.2 Research Investigating the Chemical Properties of Decomposition Fluid ....... 21
   4.3 Summary .......................................................................................................... 35

5. **Blood Evidence** ............................................................................................. 37  
   5.1 Biological Properties of Blood ..................................................................... 37  
      5.1.1 Blood Components .................................................................................. 38
   5.2 Chemical Changes Associated with Blood Degradation ............................... 39
List of Figures

Chapter 2
Figure 2.1. Overview of saponification (Bardale 2011).................................11
Figure 2.2. Overview of mummification (Bardale 2011).................................12

Chapter 3
Figure 3.1. Overview of carbohydrate degradation (Adapted from Dent, Forbes and Stuart 2004)..............................................................14
Figure 3.2. Overview of protein degradation (Dent, Forbes and Stuart 2004)........15
Figure 3.3. Overview of lipid degradation (Adapted from Dent, Forbes and Stuart 2004)..................................................................................17

Chapter 4
Figure 4.1. Chromatogram showing compounds produced on day 24 of the pork rasher trial. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: trimethylacetic acid (internal standard), 4: butyric acid, 5: isovaleric 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, and 14: linoleic acid (Swann et al. 2010)................................................................................28
Figure 4.2. Chromatogram showing compounds produced on day 26 from Piglet 3. Numeric order of compounds: 1: acetic acid, 2: propanoic acid, 3: isobutyric acid, 4: trimethylacetic acid (internal control), 5: butyric acid, 6: isovaleric acid, 7: valeric acid, 8: isocaproic acid, and 9: 2-piperidone (Swann et al. 2010)..................................................................................29
Figure 4.3. Chromatogram showing compounds produced on day 6 during the adult pig trial. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: trimethylacetic acid (internal control), 4: butyric acid, 5: isovaleric acid, 6: valeric acid, 7: 4-methylvaleric acid, 8: caproic acid, 9: phenol, 10: 2-piperidone, 11: indole, 12: phenylacetic acid, 13: phenylpropionic acid, and 14: oleic acid. The peak marked ‘x’ is an unidentified peak (Swann et al. 2010).................................................................................................................30
**Figure 4.4.** Electropherogram at optimised running conditions identifying in numeric order: 1: tryptamine, 2: tyramine, 3: neutral, 4: tryptophan, 5: tyrosine, and 6: phenylalanine. Peaks marked with an asterisk are unidentified components (Swann, Forbes and Lewis 2010c) .................................................. 34

**Chapter 5**

**Figure 5.1.** Composition of blood. Liquid blood is comprised of 55% intercellular material (plasma) and 45% formed cellular elements (erythrocytes, leucocytes, and platelets) (Li 2015) ........................................................................ 38

**Figure 5.2.** Oxidative processes in haemoglobin. A) Oxidative processes occurring *in vivo*. B) Oxidative processes occurring *in vitro* (Adapted from Bremmer et al. 2011) .......................................................... 40

**Figure 5.3.** Sequence of steps involved in the ABACard® HemaTrace® immunochromatographic assay 1. Combining of a mobile monoclonal anti-human haemoglobin antibody with human haemoglobin 2. Migration of the mobile antibody-antigen complex 3. Formation of antibody-antigen-antibody sandwich 4. Positive result indicated by a pink precipitin line in the test and control areas of the assay (Johnston, Newman and Frappier 2003) .................................................................................................................................. 45

**Chapter 6**

**Figure 6.1.** Approaches taken for RNA profiling (Lee n.d.) ............................................... 59

**Figure 6.2.** Co-extraction of RNA and DNA from a crime scene stain enables body fluid identification and STR profiling (Butler 2012) ............................................................................. 60

**Chapter 7**

**Figure 7.1.** Experimental design sketch in which the decomposing carcasses are suspended ................................................................................................................. 71

**Figure 7.2.** Experimental design sketch in which the decomposing carcasses are placed on top of a galvanised mesh platform ..................................................... 71

**Figure 7.3.** Overview sketch of the decomposition research site and approximate measurements .......................................................................................................................... 72
List of Tables

Chapter 2

Table 2.1. Stages, features, and visual references of the common decomposition stages adapted from Comstock (2014). Intervals adapted from Pless, Worrell and Clark (1997). Note: All stages are highly variable and intervals are approximate.................................................................9

Chapter 7

Table 7.1. Potential methods for the differentiation of blood and decomposition fluid and the associated limitations.................................................................68
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADD</td>
<td>Accumulated Degree Days</td>
</tr>
<tr>
<td>ALS</td>
<td>Alternative Light Source</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>Attenuated Total Reflectance Infrared Spectroscopy</td>
</tr>
<tr>
<td>CDI</td>
<td>Cadaver Decomposition Island</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>ESI Q-TOF</td>
<td>Electrospray Ionisation Time of Flight</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty Acid Methyl Ester</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionisation Detection</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>Hb</td>
<td>Deoxyhaemoglobin</td>
</tr>
<tr>
<td>HbO₂</td>
<td>Oxyhaemoglobin</td>
</tr>
<tr>
<td>HC</td>
<td>Hemichrome</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionisation</td>
</tr>
<tr>
<td>Met-Hb</td>
<td>Met-haemoglobin</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro Ribonucleic Acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>NRN</td>
<td>Ninhydrin Reactive Nitrogen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid Phase Microextraction</td>
</tr>
<tr>
<td>STR</td>
<td>Short Tandem Repeat</td>
</tr>
<tr>
<td>TD-GCMS</td>
<td>Thermal Desorption Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile Organic Compound</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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</table>
An important aspect of forensic investigations is the detection and identification of biological fluids (Virkler and Lednev 2009). The determination of the type and origin of a biological sample can yield valuable information that may support a link between the criminal act and donor, which in turn may assist in the reconstruction and sequencing of a crime scene (An et al. 2012). Blood is one of the most frequently encountered and significant types of physical evidence associated with the forensic investigation of violent crime and death (James, Kish and Sutton 2005). Blood identification is central to many forensic investigations including sexual assault, aggravated assault, burglary, and homicide. The presence and evaluation of blood evidence can be crucial in establishing identity and the manner of deposition, as well as confirming or refuting statements from the person or persons of interest (Gefrides and Welch 2011).

A body and therefore any associated biological stains may not be located for days or weeks after death, during which time the decedent will begin to decompose. A thorough forensic examination of a decedent is required in part for the exclusion or documentation of injuries that may have contributed to or caused death. However, the examination of putrefied remains can be technically difficult to conduct and the interpretation of findings can be complicated by the modification and loss of tissue (Byard et al. 2006). In addition to the degenerative changes occurring throughout the process of decomposition, remains may also be subjected to animal and insect activity, environmental factors, and elements of the weather, all of which will further complicate post-mortem examinations. Putrefactive changes may obliterate markers of identity or create artificial lesions that
mimic or distort the features of actual ante-mortem injury (Dolinak, Matshes and Lew 2005). Furthermore, suspicions of inflicted injury may arise due to the purging of fluids both in the early and later stages of decomposition (Byard 2014). The presence of blood would suggest that an injury has occurred before or shortly after death, whereas decomposition fluid is generated from the naturally occurring process of decomposition. Subsequently, ante-mortem injury should be recognised and differentiated from artefacts of putrefactive decay (Dolinak, Matshes and Lew 2005). Despite the development of advanced adjuncts to traditional autopsies, such as magnetic resonance imaging and computerised tomography, the artefacts induced by autolysis and putrefaction may still require interpretation. As such, it must be recognised that the process of decomposition and its associated changes may confuse or complicate post-mortem evaluations (Byard and Tsokos 2013).

Biological stains can be detected and identified by a number of physical and chemical methods, which utilise the inherent properties of the biological evidence (Gefrides and Welch 2011). Chemical, immunological and protein catalytic activity tests, microscopy, and spectroscopic methods are current techniques used for forensic biological fluid identification (An et al. 2012). Decomposition fluid is a complex chemical mixture with associated microbial organisms, insect life, and other debris, consequently making it a challenging matrix to study (Swann, Forbes and Lewis 2010a). However, the presence of specific components and the unique composition of each biological fluid form the basis of its identification (Virkler and Lednev 2009). An experimental design that investigates the decomposition process in the presence and absence of blood evidence, or provides a comparison between the properties of aged decomposition fluid and blood may assist in
determining whether these fluids can be differentiated. This, in turn, may offer supplementary information to the post-mortem examination of highly decomposed remains, and provide probative information so that investigative processes are not misinformed and that resources and expenditure are not misallocated.
CHAPTER TWO · POST-MORTEM DECOMPOSITION

Death results from the accumulation of foreign substances or from the reduction of living matter due to the dissolution of organic tissues (Forbes and Carter 2016). Perper (1993) defined death as the irreversible cessation of the brain, circulatory, and respiratory abilities. ‘Dying’ and ‘being killed’ are the two broad classifications of the cause of death for vertebrates. Death that results from old age or illness is referred to as dying, whereas death that results from predators, enemies, external forces, or accidents relates to the process of being killed (Forbes and Carter 2016). This chapter discusses the process of mammalian decomposition that ensues following the cessation of life, irrespective of the cause of death.

2.1 Early Post-mortem Changes

Soon after the cessation of heart function, the body goes through the ‘mortis triad,’ which includes algor mortis, livor mortis, and rigor mortis (Janaway, Percival and Wilson 2009). Algor mortis is the post-mortem cooling of the body (Bardale 2011). The internal body temperature will begin to approximate the ambient temperature following death, as the body ceases to regulate its internal temperature (Goff 2009). The gravitational settling of blood due to the cessation of the heart circulating oxygenated blood is referred to as livor mortis (Forbes and Carter 2016). A reddish-purple discoloration will result in the dependent areas of the body due to the settling of the blood. Rigor mortis causes a rigidity or stiffening of the muscles, which results primarily due to the depletion of adenosine triphosphate (ATP). After death, the consumption of ATP continues, however, the production of this molecule ceases. The myosin and actin filaments in the muscles become
complexed in the absence ATP, which represents the onset of rigor mortis. The gradual release of rigor will occur due to the process of decomposition (DiMaio and DiMaio 2001).

2.2 Mammalian Decomposition

Decomposition of mammalian soft tissue is a continuous post-mortem process in which the soft tissues of the body disintegrate, eventually leading to partial or complete skeletonisation (Stuart 2013; Goff 2009). The process of decomposition is often divided into a series of stages and depending on the geographic region and the author, the number of stages has varied between one and nine (Goff 2009). Decomposition is commonly categorised into stages to aid investigators in estimating the post-mortem interval and to assist in describing the decomposition process (Comstock 2014). Despite differences in the number of stages, the process of decomposition is often broadly categorised into pre- and post-skeletonisation. The pre-skeletonisation classification can be subcategorised into four additional stages including fresh, bloated, decay, and dry (Stuart 2013). According to Goff (2009), the division of the decay stage into active and advanced decay stages is the most common modification to the classification of death. The rate at which decomposition occurs is highly variable between all carcasses, however the sequence of decay remains relatively consistent (Comstock 2014). Table 2.1 illustrates the common stages, features, and intervals of the decomposition process and provides a visual reference for each stage.

The process of decomposition may be evident microscopically soon after the cessation of life as the cellular chemistry begins to fail, yet may not be visible macroscopically (Forbes and Carter 2016; Dix and Graham 2000). Decomposition involves the two parallel
processes of autolysis and putrefaction (DiMaio and DiMaio 2001). The first identifiable process occurring during the fresh stage of decay is autolysis, which is the enzymatic destruction of the soft tissues of the body (Forbes and Carter 2016; Swann, Forbes, and Lewis 2010a). Autolysis is a chemical process that is slowed by cooler conditions, accelerated by heat, and stopped by the inactivation of enzymes or by freezing (DiMaio and DiMaio 2001). This process occurs first in the tissues that have a high enzyme and water content and can be observed macroscopically by loosening of the epidermis from the dermis, which is referred to as skin slippage and post-mortem blisters known as bullae (Spencer 2013; Bardale 2011). The superficial vessels become stained by intravascular haemolysis, which produces a visible reticulated pattern under the skin, commonly referred to as ‘marbling’ (Spencer 2013; Dix and Graham 2000). Autolysis is thought to occur due to decreased oxygen levels, which results in a decrease in the intracellular pH (Bardale 2011). Cell membrane integrity is compromised by changes in the pH and nutrient-rich fluids are released as a result of cellular membrane destruction. The released nutrient-rich fluids serve as energy and food sources for micro-organisms, facilitating putrefaction (Zhou and Byard 2011; Vass 2001).

Putrefaction is the destruction of the soft tissues caused by the action of fungi, bacteria, and protozoa (Vass et al. 2002). The release of lipid, carbohydrate, and protein by-products during autolytic degradation fuels the proliferation of these organisms (Forbes and Carter 2016). The beginnings of putrefaction can be observed during the bloated stage of decomposition (Swann, Forbes and Lewis 2010a). Greenish discolouration of the skin, which results due to the formation of sulphaemoglobin in the settled blood, is often the first visible sign of putrefaction (Vass et al. 2002). The breakdown of host cells by both
aerobic and anaerobic endosymbionts, as well as the subsequent production of gases including carbon dioxide, sulphur dioxide, hydrogen, hydrogen sulphide, methane, and ammonia cause the anatomically spacious body parts, such as the abdomen, genitals, and face to expand. An increase in the internal pressure due to the build-up of volatile gases and other products of catabolism, eventually results in the purging of fluid and gases from the natural orifices of the body (Bemelmans 2015; Vass et al. 2002). Tracheobronchial foam and bloody fluid may be discharged from the nostrils and mouth as air is expelled as a result of increasing pressure in the chest, caused by the formation of gases in the abdomen (Saukko and Knight 2016; Pinheiro 2006). Putrefactive changes are dependent predominantly on the prior state of health of the decedent and the environmental conditions (Perper 1993).

The active decay stage begins following the purging of gases and fluids (Vass 2001). Chemical constituents will continue to be degraded and released and as such, putrefaction continues throughout the active stage of decomposition (Swann, Forbes and Lewis 2010a). At this point in the decay cycle, insect activity is prominent, there are a significant number of anaerobic and aerobic bacteria present, and electrolytes are rapidly leaching out of the body (Vass et al. 2002). The rupturing of the skin accelerates degradation by allowing additional access to the corpse by arthropods, micro-organisms, and scavengers (Hau et al. 2014). Ultimately, the process of decomposition will result in liquefaction of the soft tissues (Rust and Buis 2015). A cadaver decomposition island (CDI) may form due to the release of cadaveric material (Carter, Yellowlees and Tibbett 2007).
The final stage of the decomposition process is the dry stage (Swann, Forbes and Lewis 2010a). The surface tissues will collapse, dry, and darken to assume a leathery texture and residual tissues and organs will shrink and desiccate following the ‘wet’ stages of decomposition. The body may eventually progress to skeletonisation depending on the post-mortem interval and the environmental conditions in which decomposition has occurred (Dix and Graham 2000).

The process of death is dynamic and the timeframe in which death occurs can be highly variable (Forbes and Carter 2016). There are a variety of intrinsic and extrinsic factors that govern the process of decomposition. Intrinsic factors include the weight and age of the decedent, mechanical injury, drugs and toxins, and ante-mortem medical conditions, whereas extrinsic factors include weather conditions such as humidity, temperature and sun exposure, animal predation and insect accessibility, the substrate on which decomposition took place, and protective coverings (Hau et al. 2014). Pinheiro (2006) stated that no two decomposition processes are alike, as no two individuals are alike. As such, it is important to note that the process of decomposition occurring under varying conditions cannot always be accurately described by the classifications currently published in the literature. The characterisation of stages is complicated by conditions that cause differential decomposition or by those that alter the process and rate of decomposition (Comstock 2014).
Table 2.1. Stages, features, and visual references of the common decomposition stages adapted from Comstock (2014). Intervals adapted from Pless, Worrell and Clark (1997). Note: All stages are highly variable and intervals are approximate.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Characteristic Features</th>
<th>Visual Reference</th>
<th>Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>Macroscopic changes are minimal</td>
<td></td>
<td>Zero to seven days</td>
</tr>
<tr>
<td></td>
<td>Associated with the mortis triad (Algor, livor, and rigor mortis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloat</td>
<td>Accumulation of gases and inflation of the anatomically spacious body parts, such as the abdomen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marbling is visible in the limbs and ventral areas</td>
<td></td>
<td>Initiated generally within 48 hours of death</td>
</tr>
<tr>
<td></td>
<td>Post-mortem bullae</td>
<td></td>
<td>Generally lost by the seventh day but has been recorded as late as day 13</td>
</tr>
<tr>
<td>Active Decay</td>
<td>Prominent insect activity</td>
<td></td>
<td>Occurs between the second and eighth day after death</td>
</tr>
<tr>
<td></td>
<td>Strong odours and release of cadaveric material</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exposed bone becomes discoloured and leathery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced Decay</td>
<td>Minimal soft tissue remains</td>
<td></td>
<td>Initiated at least one week after death</td>
</tr>
<tr>
<td></td>
<td>Skin discolouration</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skin exhibits rigidity and thickness decreases</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluids are no longer purging and begin to dry out</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Odours become less intense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry/Skeleton</td>
<td>Small patches of skin, bones, and teeth remain</td>
<td></td>
<td>Several weeks to months after death</td>
</tr>
</tbody>
</table>
2.2.1 Inhibitory Effects on Decomposition

Certain environmental conditions can halt the chemical processes of decomposition that result in the dissolution of soft tissue (Forbes and Carter 2016). Outlined below are the preservation processes of saponification and mummification.

2.2.2 Saponification

Saponification is process of adipocere formation, which results from the hydrogenation and hydrolysis of adipose tissue (Figure 2.1) (Pinheiro 2006). Adipocere is characterised as deposits of a greasy wax-like substance that are yellow-white in colour. When adipocere binds with sodium from interstitial fluids, a hard and crumbly composition will form. In contrast, when adipocere binds with potassium from the breakdown of cell membranes, a soft, paste-like complex will be evident (Vass 2001). The formation of adipocere is influenced by multiple factors including temperature, air flow, moisture, the place and media of disposal, and the presence of clothing (Bardale 2011). Adipocere formation can be variable, as it may be present in discrete regions of the cadaver or may form over the entire body (Forbes and Carter 2016). Once formed, adipocere can preserve remains for extended periods of time (Dent, Forbes and Stuart 2004). It has been suggested that a reduced pH in conjunction with the altered microbial environment is the primary reason for tissue preservation (Forbes and Carter 2016).
2.2.3 Mummification

Mummification is a process of artificial or natural conservation and is formed by the desiccation or dehydration of the tissues (Pinheiro 2006; Vass 2001). The remaining skin becomes dry, dark in colour, and assumes a leather-like texture, while the soft tissues begin to decompose beneath the dry and hardened skin (Dix and Calaluce 1999). Similar to adipocere formation, mummification may only be present in discrete regions or may be observed over the entire body (Forbes and Carter 2016). The formation of mummification is dependent on atmospheric conditions, air movement, and the physical size of the cadaver (Bardale 2011). Mummification most commonly occurs in dry, hot environments or in areas that have very low humidity, such as deserts or arctic regions (Vass 2001; Dix and Calaluce 1999). In these regions, the decomposition process is slowed as the soft tissues dehydrate and the effect of larval and bacterial activity wane (Forbes and Carter 2016).
The body may remain preserved for many years once in a state of mummification (Dix and Calaluce 1999). Figure 2.2 illustrates an overview of the mummification process.

![Figure 2.2. Overview of mummification. Source: Bardale 2011.](image)

**2.3 Summary**

The chemical processes of autolysis and putrefaction will typically occur in a predictable order, yet within a variable timeframe. The impact of the surrounding environment on the biological and chemical processes of decay, as well as the location in which death occurred will strongly influence the post-mortem process of decomposition. When biological activity is suppressed or eliminated, the degree of decay or preservation will be dictated by environmental conditions. To better understand the complex biological and chemical processes of decomposition, continued research across different ecozones is required (Forbes and Carter 2016).
CHAPTER THREE · MACROMOLECULE DEGRADATION

The building blocks of biological specimens are the four main macromolecules: nucleic acids, carbohydrates, proteins, and lipids. These macromolecules are broken down into their structural components including amino acids, phosphate, fatty acids, glucose, and sugars by complex reactions associated with the process of decomposition (Vass et al. 2002).

3.1 Carbohydrate Degradation

Polysaccharides are broken down into component sugars during early decomposition by the action of micro-organisms. Fungi can decompose sugars into organic acids including citric, glucuronic, and oxalic acids. Bacteria are responsible for the degradation of sugars into organic acids, such as pyruvic and lactic acids under aerobic conditions. The sugars may then be further degraded into water and carbon dioxide. Under anaerobic conditions, bacteria can breakdown sugars into acetic, butyric, and lactic acid. Bacterial carbohydrate fermentation may produce hydrogen, hydrogen sulphide, and methane gases, as well as butanol and ethanol (Stuart 2013). Figure 3.1 illustrates the process of carbohydrate degradation that occurs during decomposition.
3.2 Protein Degradation

The degradation of proteins into their component amino acids occurs as a result of bacterial enzyme activity, and is referred to as proteolysis (Stuart 2013). The rate of proteolysis varies depending on bacterial action, environmental conditions, such as moisture and temperature (Dent, Forbes and Stuart 2004), and on the protein type (Stuart 2013). Proteins of epithelial and neuronal tissues typically degrade first, while hard tissue proteins, such as keratin and collagen are more resistant to degradation. The process of decomposition results in the breakdown of proteins into amino acids, peptones, polypeptides, and proteoses (Dent, Forbes and Stuart 2004). Continuing proteolysis can result in the production of gases including ammonia, carbon dioxide, hydrogen sulphide,
and methane, as well as phenolic compounds, such as indole and skatole (Figure 3.2). The amino acids produced as a result of protein degradation can undergo: deamination, which produces ammonia; decarboxylation, which results in the formation of cadaverine, indole, putrescine, tryptamine and tyramine, and the production of carbon dioxide; and desulphurhydration, which can yield ammonia, hydrogen sulphide, pyruvic acid, and thiols (Stuart 2013).

Figure 3.2. Overview of protein degradation.
3.3 Lipid Degradation

Adipose tissue is comprised of approximately 60 to 85% lipids. Triglycerides make up 90 to 99% of the lipid composition of adipose tissue, with cholesterol esters, diglycerides, and phospholipids present in smaller amounts. Lineoleic, oleic, palmitic, and palmitoleic acids are the major fatty acids present in adipose tissue (Stuart 2013). Hydrolysis of the neutral fat of decomposing remains can yield fatty acids, which may subsequently undergo oxidation or hydrogenation (Dent, Forbes and Stuart 2004). Following death, saturated and unsaturated fatty acids are produced as intrinsic tissue lipases hydrolyse lipids. The environmental conditions in which decomposition occurs will govern the nature of the reaction. In an anaerobic environment, the mixture of saturated and unsaturated fatty acids will undergo hydrogenation and further hydrolysis, yet in an aerobic environment, oxidation of the unsaturated fatty acids can occur due to atmospheric oxygen, fungi, and bacteria (Stuart 2013). Hydrogenation of linoleic, oleic, and palmitoleic acids yields oleic, stearic, and palmitic acids, respectively (Dent, Forbes and Stuart 2004). Oxidation will produce peroxide bonds initially, with ketones and aldehydes the final products of this process. As the decomposition process continues, the concentration of fatty acids increases, whereas the concentration of neutral lipids decreases (Stuart 2013). At this stage, small amounts of hydroxy-fatty acids may also be formed. The process will continue, provided there is sufficient enzymes and water available, until the original adipose tissue is reduced to a mass of fatty acids or until no neutral fat remains (Dent, Forbes and Stuart 2004).

Fatty acids and glycerol breakdown yielding shorter-chain saturated fatty acids and eventually water and carbon dioxide (Dent, Forbes and Stuart 2004). Volatile fatty acids
(VFAs) are short-chain fatty acids (C₂–C₅), which may be useful products of decomposition as the concentration of particular VFAs, such as butyric, isobutyric, isovaleric, valeric, and propionic acids may be correlated with the rate of decomposition (Stuart 2013). Figure 3.3 illustrates the process of lipid degradation that occurs during decomposition.

**Figure 3.3.** Overview of lipid degradation. Adapted from: Dent, Forbes and Stuart 2004.
3.4 Summary

The chemical processes of decomposition are complex and result in the dissolution of soft tissues and identifiable changes to carbohydrates, proteins, and lipids. An understanding of the changes to the biological macromolecules that occur during decomposition can provide potentially valuable information concerning the post-mortem interval and the nature of death (Stuart 2013).
CHAPTER FOUR · DECOMPOSITION FLUID

Cellular compounds and structures are broken down and liquefied as the processes of autolysis and putrefaction take place (Janaway, Percival and Wilson 2009). Decomposition fluid is the liquid produced by the degradation of the organs and soft tissues of the body (Rust and Buis 2015). The fluid released as a result of decomposition is chemically complex and is often associated with microbial organisms, insect life, and other debris. Consequently, decomposition fluid is a challenging matrix to study (Swann, Forbes and Lewis 2010a). It is important to note that much research investigating the physical and chemical properties of decomposition fluid has been conducted using domestic pig (Sus domesticus) carcasses due to ethical issues associated with the use of human cadavers (Schoenly et al. 2006). Pig carcasses are considered an acceptable analogue for human cadavers because of their similarity to human torsos in hair coverage, weight, fat to muscle ratio, physiology, and biochemistry (France et al. 1992).

4.1 Research Investigating the Physical Properties of Decomposition Fluid

Comstock (2014) conducted outdoor research trials in Ontario, Canada in the spring-summer months to investigate the physical and chemical properties of decomposition fluid. Trial 1 served as a preliminary study, whereas Trials 2, 3, and 4 were conducted with the purpose of carrying out the research objectives. In each replicate trial, three carcasses were exposed to, and three were protected from insect activity. The carcasses in the insect exclusion group were further subdivided into ‘partially excluded’ and ‘completely excluded’ based on whether insects gained localised access to the carcasses. Prior to laboratory analyses, observations were made on the overall consistency and colour of the
collected samples. For all carcass groups, the decomposition fluid was initially deep red in colour and predominantly blood. Once insects gained access to the inclusion and partial exclusion carcasses, the decomposition fluid samples became more viscous, turned brown, and contained sediment and other debris. Samples collected from the complete exclusion carcasses changed colour throughout decay from red-burgundy to brown, yet remained liquid and non-viscous. The colour changes observed in the fluid samples collected from the inclusion and partial exclusion carcasses may have been attributed to maggot waste and liquefied organic material. However, micro-organism induced breakdown of cadaveric material may have been responsible for the colour change exhibited in the samples collected from the complete exclusion carcasses. It is unknown whether different experimental parameters would alter the appearance of the samples, as there is limited published research that explicitly examines the physical appearance of decomposition fluid (Comstock 2014).

pH measurements of collected decomposition fluid samples indicated that prior to insect colonisation, the pH values and trends were similar during early decomposition between each of the carcass groups. However, significant differences were observed following insect colonisation. During Trial 2, only partial results were obtained as maggot masses inhibited sample collection and as such, a trend in pH could not be established. Following the onset of the active decay stage during Trial 3, the pH from the inclusion group rapidly became more alkaline. However, on the final collection day the pH of the fluid from all experimental groups was approximately 7.7. Between days three and seven of Trial 4, the pH from the inclusion group was significantly greater ($p < 0.05$) than each of the exclusion groups. Comstock (2014) attributed the diverging pH trends to maggot activity. As maggots
feed, they release high levels of ammonia in their exudates (Turner 2005), which can raise the pH if converted to ammonium. The lack of maggot activity and subsequent lack of ammonium ions may have accounted for the why the completely excluded carcasses did not display a sharp increase in pH (Comstock 2014).

In addition to visual examination and pH measurements, Comstock analysed the conductivity in decomposition fluid samples. During the fresh and bloated stages, all experimental carcass groups displayed a general increase in conductivity, yet trends diverged between the groups once insects began feeding on the carcasses. Measurements decreased and remained low for the remainder of the collection period in each of the partial exclusion and inclusion groups, while measurements remained significantly higher in samples collected from the complete exclusion carcass. Comstock (2014) opined that the low levels observed in the inclusion and partial exclusion groups may have been attributed to maggot activity, which either allowed the inherent bacteria access to the released ions or contributed bacteria. In summary, the differences observed in the decomposition fluid properties between each experimental carcass group were attributed to insect colonisation and the feeding activity of the insects.

4.2 Research Investigating the Chemical Properties of Decomposition Fluid

Identifying the compounds present in decomposition fluid and determining the timing of their production are the first steps to understanding decomposition chemistry. A non-chromatographic approach to the chemical studies of decomposition involved the investigation of ninhydrin-reactive nitrogen (NRN) (Swann, Forbes and Lewis 2010a). In 2008, Carter, Yellowlees and Tibbett proposed that there would be a significant increase in
NRN concentrations in soils associated with cadaver decomposition. Juvenile rats (*Rattus rattus*) were used as model cadavers and were buried in one of three contrasting soil sites in Queensland, Australia. Following a sequential and destructive sampling regime, nitrogenous compounds were removed from the soil samples that were collected from beneath the decomposing remains. Ninhydrin reagent was added to the samples and absorbance was read at 570 nanometres using visible spectrophotometry. When compared to the control samples, the cadaver burial samples demonstrated a 1.4 to 2.2-fold increase in NRN. Despite results suggesting that the decomposition process of non-human cadavers released a significant concentration of NRN, the authors noted that the study lacked species specificity and that elevated concentrations of NRN may have been associated with other organic resources, such as plant litter and faecal matter. In addition, the authors identified that further research investigating the effect of burial depth, cadaver mass, time, clothing, and the diffusion of NRN in soils was needed.

Van Belle, Carter and Forbes (2009) examined surface and burial decomposition scenarios of porcine remains using the NRN methodology proposed by Carter, Yellowlees and Tibbett (2008). In addition, the study also investigated the lateral diffusion of NRN. It was hypothesised that there would be a positive correlation between decomposition progression and grave soil NRN concentrations and an inverse relationship between sampling distance and the concentration of NRN. The study followed a sequential destructive collection regime, whereby two carcasses were exhumed after a period of three, six, or 12 months’ burial. Soil samples were collected once a month for six months from the edge and centre of the gravesites and from the walls and base of the graves at the time of exhumation. To investigate surface decomposition scenarios, five swine
carcasses were placed on the soil surface. Soil samples were collected from different regions beneath the carcasses and from the control site over a period of 97 days. Results indicated that during the initial two months of burial, soil collected from the centre of the graves contained significantly greater ($p < 0.05$) concentrations of NRN and that this period corresponded with the liquefactive stages of decomposition. The surface trial results indicated that the highest concentrations of NRN were detected during the early to later post-mortem period. In addition, it was determined that the lateral influx of NRN was minimal. The authors identified that the vertical and lateral extent of decomposition fluids was dependent on the soil texture and size of the cadaver.

Further research by Carter et al. (2009) investigated the use of the NRN technique in the reconstruction of a disturbed outdoor death scene. Soil samples were collected from seven sampling sites, including a control site. The determination of NRN concentrations followed the methodology outlined by Carter, Yellowlees and Tibbett (2008). Results indicated that the presumptive primary decomposition area had significantly greater concentrations ($p < 0.001$) of NRN when compared to the other sampling sites, thus providing a strong indicator as to the area where most of the body had decomposed. However, NRN may be released from other sources including plant material and as such, an elevated level of NRN in soils is not necessarily indicative of human decomposition (Carter et al. 2009).

The above research has demonstrated that various nitrogen-containing compounds are released into the surrounding environment during the process of decomposition. The results highlighted the potential for NRN to be used for the detection of clandestine burial
sites and for determining the original site of decomposition for surface deposited remains subject to animal predation or post-mortem relocation (Van Belle, Carter and Forbes 2009). However, to provide the level of chemical knowledge needed to understand the fundamentals of decomposition chemistry, more selective analytical techniques are required. Separation science techniques are currently being researched with the aim of providing quantitative data that will supplement and expand the knowledge base concerning decomposition chemistry (Swann, Forbes and Lewis 2010a).

Individual decomposition products may be separated by analytical techniques including gas chromatography (GC), capillary electrophoresis (CE), and liquid chromatography (LC) (Stuart 2013). Early studies researching the chemistry of decomposition focused on small organic molecules, such as volatile fatty acids ($C_2$ – $C_5$) (Swann, Forbes and Lewis 2010a), which are short-chained carboxylic acids produced by microbial-induced reductive and oxidative reactions of lipids. Volatile fatty acids (VFAs) can remain biologically active and detectable in crime scene soil samples for considerable lengths of time (Tuller 1991).

An early application to the investigation of VFAs was by Tuller (1991), who used a field portable gas chromatograph (microFast GC$^2$) and mass spectrometer (MS) to identify VFAs in soil samples collected from cemetery graves in Duz, Kosovo and a mass grave in Knin, Croatia. Only one of the seven soil samples collected from the Duz village cemetery contained detectable levels of VFAs, which included isobutyric and valeric acid. From the Knin mass grave, isovaleric acid was present in Sample 2, while isobutyric and isovaleric acids were detected in Sample 3. Tuller identified that similar to the Duz sample, co-elution with the solvent prevented the detection of the targeted fatty acids (butyric,
propionic, and valeric acid). However, several other fatty acids including capric, lauric, myristic, oleic, palmitic, and stearic acids were identified in Samples 1, 2, and 3 from the Knin mass grave. These fatty acids were not detected in the soil controls and as such, it was likely that these fatty acids were associated with decomposing remains. Tuller proposed that prior to discovery, the VFAs produced during decomposition leached from the remains, thus accounting for the lack of detectable VFAs in the samples collected from the Duz cemetery. Moreover, VFA detection may have been affected by the time between burial, exhumation and sampling, differences in the rate of decomposition, the soil structure and content, exposure to the environmental elements, or the process of air drying the samples (Tuller 1991).

Research conducted by Vass et al. (1992) analysed five microbially produced VFAs (isobutyric, n-butyric, isovaleric, n-valeric, and propionic acid), as well as various anions and cations (ammonium, calcium, chloride, magnesium, potassium, and sulphate) using GC. Gas chromatography has typically been used to analyse VFAs (Swann, Forbes and Lewis 2010a), as this technique enables the separation of mixtures and quantification of individual components (Stuart 2013). Volatile fatty acids were acidified with formic acid and analysed using packed column GC with flame ionisation detection (FID). Soil samples were collected from randomly selected areas beneath seven unclothed, unautopsied, and unembalmed cadavers. The sampling area was defined between the shoulders and pelvis and each area was sampled only once. The initial results of the study indicated that ante-mortem body weight was an important variable, as ratios of fat and muscle tissue and in turn, the concentration of VFAs is unique to each individual. In addition, the moisture content of the soil was also considered an important variable in the determination of VFA
concentrations. Once an individual’s weight and the moisture content of the soil were taken into account, the VFA concentrations were the same for any given total of accumulated degree days (ADD), regardless of the season in which the cadaver began to decompose or the physical features of the cadaver. Only seven of the 16 ions investigated \((\text{C}^1, \text{Ca}^2, \text{K}^+, \text{Mg}^{2+}, \text{Na}^+, \text{NH}_4, \text{SO}_4^2)\) were reproducible between subjects and stable in the environment. As with VFA concentrations, the anion/cation concentrations were the same for any given total ADD, irrespective of the subject or season. The authors recognised that there was a correlation between VFA production and specific stages of decomposition, which was in part attributed to the sequential decomposition of proteins and carbohydrates.

In 2013, von der Lühe et al. investigated the suitability of cholesterol and coprostanol as biomarkers of decomposition fluid within a soil matrix. According to the authors, cholesterol and coprostanol are potentially valuable compounds for the detection of decomposition fluid in soils because of their origin and chemical structure, which provides stability against degradation over time. A total of four domestic pig carcasses were buried in shallow graves; two of which were exhumed after three months and the remaining two exhumed after six months. Cholesterol and coprostanol were extracted from soil samples that had been collected from seven different sampling positions and solid phase extraction (SPE) was used to purify the samples before analysis using gas chromatography-mass spectrometry (GC-MS). The control in the study was the phytosterol \(\beta\)-sitosterol, as it was assumed to remain stable between the treated and control soils. Results indicated that the three-month-old graves had higher concentrations of cholesterol and coprostanol relative to the control. It was proposed that the cholesterol was derived from the soft tissue of the...
pig carcasses and that the release of decomposition fluid contributed to the concentrations of coprostanol in the soil samples collected from beneath the pig carcasses. There was no significant difference in sterol concentrations between the control and treatment soils after six months’ burial. The most likely explanations for this observation according to von der Lühe et al. included aerobic degradation, sorption, transformation, and transport away from the depositional source. However, further research investigating the potential influence of soil properties on these sterols is required.

A preliminary study conducted by Swann et al. (2010) aimed to advance the knowledge and understanding of the chemical components of decomposition fluid in the absence of a soil matrix. The preparation phase involved simple aqueous dilution and filtration of decomposition fluid samples collected from pork rashers (belly pork), stillborn piglets, and adult pig carcasses followed by GC-MS analysis. To provide a comparison between compounds detected in decomposition fluid samples, the study was conducted using a similar experimental design in two distinct locations, Ontario, Canada and Perth, Western Australia. Method development identified that an increase in final column temperature improved detection and resolution. Subsequently, several previously unreported compounds that were eluting at the later stages of analysis were identified. The first trial of the study examined pork rashers (belly pork) in eight collection cups, of which four were covered by muslin wrap and the remaining four exposed. The rashers were placed in the ambient environment during early autumn. For the duration of the study, no decomposition fluid was produced from the covered samples. However, decomposition fluid samples were collected between days three and 24 of the trial from the exposed rashers. Compounds produced during the pork rasher trial, as indicated by chromatogram
(Figure 4.1), included the following acids: 2-piperidone, acetic, butyric, isovaleric, linoleic, myristic, palmitic, palmitoleic, phenylacetic, phenylpropanoic, propionic, oleic, stearic, and trimethylacetic acid (internal standard).


To represent a more realistic model of decomposition, further trials were conducted with four stillborn piglets and two adult pig carcasses. As with the pork rasher trial, the piglets were both protected and exposed, yet the trial was conducted in winter. The first decomposition fluid samples were collected from the exposed piglets on day 14. According to the authors, the delay in fluid production may have been attributed to the daily temperature variations. For the piglets exposed to insect activity, sampling continued until day 47. Decomposition fluid was only produced from the protected piglets between days
Variability in fluid production between the piglets highlighted the complex nature of the decomposition process and the need for a larger sample set. Compounds produced from Piglet 3 on day 26, as indicated by chromatogram (Figure 4.2), included the following acids: acetic, propanoic, isobutyric, trimethylacetic (internal standard), butyric, isovaleric, valeric, isocaproic, and 2-piperidone.

![Figure 4.2. Chromatogram showing compounds produced on day 26 from Piglet 3. Numeric order of compounds: 1: acetic acid, 2: propanoic acid, 3: isobutyric acid, 4: trimethylacetic acid (internal control), 5: butyric acid, 6: isovaleric acid, 7: valeric acid, 8: isocaproic acid, and 9: 2-piperidone. Source: Swann et al. 2010.](image)

Two whole pig carcasses were studied in Ontario, Canada during summer (July, 2007). Fluid collection commenced on day five of the trial and concluded on day 14 when maggot and insect activity had ceased and only skeletal remains and mummified skin were evident. Similar compounds ranging from short chain acids to long chain acids and cyclic compounds were detected in the pork rasher and adult pig trials. Previously undetected indole and phenol compounds were also identified (Figure 4.3). Long chain fatty acids in
addition to 2-piperidone, isocaproic acid, phenylacetic acid, and phenylpropionic acid were the main compounds identified in this study (Swann, Forbes and Lewis 2010a). In contrast to the research conducted by Vass et al. (1992), the VFAs identified did not exhibit a trend with accumulated degree days (ADD). To ensure valid quantitative results, further research trials and continued analytical method developments are required (Swann et al. 2010).

Figure 4.3. Chromatogram showing compounds produced on day six during the adult pig trial. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: trimethylacetic acid (internal control), 4: butyric acid, 5: isovaleric acid, 6: valeric acid, 7: 4-methylvaleric acid, 8: caproic acid, 9: phenol, 10: 2-piperidone, 11: indole, 12: phenylacetic acid, 13: phenylpropionic acid, and 14: oleic acid. The peak marked ‘x’ is an unidentified peak. Source: Swann et al. 2010.

Further analyses conducted by Swann, Forbes, and Lewis (2010b) on the above experimental design identified that isovaleric and propionic acids were detected on all collection days during the pork rashers trial. Analysis of the data indicated that until day 15,
acetic, isobutyric, and propionic acids demonstrated an increasing trend, however, a decreasing trend followed until the trial was complete. Similarly, isovaleric and butyric acids followed an increasing trend, reaching a maximum at day 15, yet appeared to have a cyclic nature. The acids tended toward a second minimum value on day 16 and then increased again until the end of the trial on day 24. It was proposed that a common source produced the short chain acids, thus accounting for the initial increasing trend. However, the cyclic trends of isovaleric and butyric acid may be indicative of a new source of production during decomposition. During this trial, six long chain fatty acids including 9-hexadeconoic, linoleic, myristic, oleic, palmitic, and stearic acids were also identified. These long chain fatty acids appeared to follow an eight-day cycle and as with the target short chain acids, reached a maximum on day 15. The authors tentatively related these observations to the adipose content of the pork rashers, the feeding cycle of the maggots, and the level of fly activity.

Analysis of the piglet trial data conveyed that there were no clear trends in the compounds found in decomposition fluid for Piglets 2 and 3. There was an increasing trend in acetic acid over an eight to 10-day period exhibited by Piglet 1; however, there was a decrease in the level of acetic acid produced during the trial. It was documented that the validity of the trends observed in the fluid samples collected from Piglet 4 was difficult to justify because of inconsistencies in sample collection. Results from the pig trial indicated that the target short chain acids, with the exception of isobutyric acid, passed through a maximum on day six. A second minimum was noted on day 10, which was followed by an increase until the end of the trial on day 14, thus suggesting the acids appeared to follow a cyclic trend. However, the theory was not confirmed as fluid was not collected on day 16, which would
have allowed for the completion of a second cycle. The long chain fatty acids (linoleic, oleic, palmitic, and stearic) detected in this trial demonstrated an increasing trend, with each acid reaching a maximum on day 14. This study identified considerable variation in the abundance and type of compounds present in decomposition fluid, thereby highlighting chemical complexity of this sample.

In 2014, Comstock conducted three replicate trials analysing decomposition fluid samples produced from six domestic pig carcasses. In each replicate trial three carcasses were exposed to, and three carcasses were protected from insect activity. The insect exclusion group was further subdivided into ‘partially excluded’ and ‘completely excluded’ based on whether insects gained access. Samples were analysed using GC-MS following a fatty acid methyl ester (FAME) extraction method. Fluid collection during Trial 2 was inhibited during the active and advanced stages of decomposition for the inclusion carcasses. Consequently, overall trends could not be accurately deduced, as samples were not collected on all days. Results from the partial exclusion carcasses indicated that the amount of linoleic acid decreased, while the amount of cis-oleic, myristic, palmitoleic, and stearic acids exhibited an overall increase, thus suggesting that hydrogenation may have occurred. During the later stages of decomposition, the differences in fatty acid concentrations between the carcass groups indicated that the short chain fatty acids may have been degraded or consumed faster than the longer chain fatty acids. During Trial 3, differences were observed during the insect activity stages between the inclusion and exclusion carcasses in the amount of linoleic, myristic, palmitic, and palmitoleic acids. During the late stages of decay, differences in the amount of linoleic, myristic, and palmitic acids were observed between the carcass groups. In Trial 4, the amount of myristic and
stearic acids from the inclusion group decreased overall, yet exhibited an increase in samples collected from the partial and complete exclusion carcasses. The amount of palmitic acid increased at various stages of decomposition for all carcasses groups, while the amount of linoleic acid decreased. Differences in the amount of myristic, stearic, and trans-oleic acids differed between the exclusion groups during the localised tissue removal stage.

The dominant fatty acids present in porcine and human soft tissue including linoleic, myristic, oleic, palmitic, palmitoleic, and stearic acids were detected in all decomposition fluid samples. However, the fatty acids detected in each trial did not display consistent trends and inconsistencies were observed in the amount of fatty acids detected throughout the stages of decomposition. During the active decay stages, there were significant differences in the concentration of fatty acids between all carcass groups. When compared to the exclusion groups, the amount of linoleic, myristic, and palmitoleic acids was greater in the samples collected from the inclusion group, yet there was a decrease in the amount of palmitic acid. The results from Comstock’s study suggested that insects may consume fatty acids or accelerate the rate of degradation, thereby decreasing or increasing the amount of fatty acids detected in decomposition fluid samples.

Compounds that are not suitable for GC analysis because of thermal instability can be analysed using capillary electrophoresis (CE) (Stuart 2013). This technique offers the potential for rapid and highly efficient separations of complex chemical mixtures (Stuart 2013; Swann, Forbes and Lewis 2010c). Swann, Forbes and Lewis (2010c) used a simple capillary zone electrophoresis method for the determination of amino acids and biogenic
amines in decomposition fluid collected from porcine remains. The authors expected that amino acids and biogenic amines would be present in decomposition fluid, due to the biodegradation pathways of carbohydrates, proteins and lipids. To optimise separation, a multivariate chemometric approach was used to improve total analysis time and resolution. Subsequent to a screening design, a central composite design using total analysis time and peak resolution as response factors was employed. Phenylalanine, tryptamine, tryptophan, tyramine, and tyrosine were all identified by spiking and migration time. However, further research is required to elucidate the identity of a number of unidentified peaks observed in the electropherogram (Figure 4.4).

As with CE, high performance liquid chromatography (HPLC) can be used to study thermally unstable or potentially non-volatile decomposition products (Stuart 2013). Swann, Busetti and Lewis (2012) utilised liquid chromatography-electrospray ionisation-
tandem mass spectrometry (LC-ESI-MS/MS) operated in multiple reaction monitoring mode to analyse porcine decomposition fluid. This selective and sensitive analytical method assisted in the semi-quantitative determination of 19 amino acids and biogenic amines. Infusion experiments enabled refinement of the MS tuning parameters and an optimised LC method was applied to decomposition fluid samples. It was concluded that direct injection of 1:10 diluted samples or small volumes (0.1 to 1 μl) of undiluted samples provided the best sample introduction onto the LC-MS/MS. This approach reduced preparation time, conserved samples for future analyses, and lead to minimal matrix effect. The selected analytical approach enabled the identification of compounds that had previously lacked detection using alternative techniques and allowed potential trends to be identified. Results indicated that over the course of the field trial, tyramine, indole, and putrescine displayed a general increasing trend and that both tryptophan and L-phenylalanine compounds were present in all fluid samples. Despite not indicating potential trends individually, together the compounds displayed a cyclic trend. The sum of amino acid concentrations appeared to follow a 14-day cyclic trend, in which the amino acids passed through maximums on days 13 and 33. As these results have been tentatively established, further investigation exploring different experimental parameters, such as soil matrices and a larger sample set is required (Swann, Busetti and Lewis, 2012).

4.3 Summary

Analytical separation techniques have enabled the study of mammalian soft tissue decomposition and various by-products, including decomposition fluid (Swann, Forbes and Lewis, 2010a). The above studies have primarily focused on providing various methodologies and techniques for estimating post-mortem intervals. However,
determining what compounds are present in decomposition fluid will assist in providing a greater understanding of decomposition chemistry and in turn, provide important information needed for the differentiation of this sample and blood evidence.
CHAPTER FIVE - BLOOD EVIDENCE

Blood is one of the most frequently encountered and significant types of physical evidence associated with the forensic investigation of violent crime and death. The nature and circumstances of violent crimes often produce a variety of bloodstains that, when carefully evaluated and studied with respect to their distribution and geometry, can provide important information, which may assist with determining the sequence of events. The accurate interpretation of blood evidence has proved critical in a number of cases where the manner of death must be resolved. An understanding of the properties of blood is necessary for the interpretation and study of bloodstain evidence (James and Eckert 1998). This chapter will introduce the biological properties of blood, explain the degradation of blood outside of the human body, and discuss possible biological screening processes.

5.1 Biological Properties of Blood

Blood is a liquid form of connective tissue, comprised of 55% intercellular material (plasma) and 45% formed cellular elements. The plasma portion of blood consists mostly of water, as well as inorganic salts, proteins, and other substances, whereas the cellular component consists of erythrocytes, leucocytes, and platelets (Figure 5.1). The primary functions of blood as it circulates through the body include defence against foreign material and infection, transport of carbon dioxide, oxygen, waste products, nutrients and hormones, and heat distribution (James, Kish and Sutton 2005).
5.1.1 Blood Components

Erythrocytes, also called red blood cells (RBC), comprise approximately 98.5% of the total cellular portion of liquid blood (Reynolds 2008). The small size (approximately 7.5 μm in diameter) and geometric shape of RBCs makes them efficient in their transportation role. Normal RBCs take the shape of a biconcave disc, which simultaneously increases the surface-to-volume ratio to maximise carbon dioxide and oxygen transport and allows the cell to be small enough to pass through the capillaries (James, Kish and Sutton 2005). A plasma membrane provides a mature RBC with resilience and flexibility (Reynolds 2008). Mature human RBCs do not have a nucleus and as such, lack nuclear DNA. An important source of DNA for forensic analyses is the nucleated leukocyte or white blood cell (WBC). Leukocytes are the second population of component cellular material and can be divided into granulocytes, which include basophils, eosinophils and neutrophils, or agranulocytes,
which consist of monocytes and lymphocytes. The primary function of leukocytes is to digest pathogens and fight infection, aiding in the regulation of the immune system. The smallest cellular constituents of blood are platelets, which are approximately 2 to 4 μm in diameter (Saladin 2007). Platelets assist in haemostasis through thrombus formation and coagulation (Reynolds 2008). The remaining 55% of blood is comprised of plasma, which is a transparent, pale yellow fluid. The constituents of plasma include 90% water, 7% plasma proteins, mainly albumin, globulin and fibrinogen, 2% nutrients and 1% inorganic salts. Plasma is responsible for transporting electrolytes, hormones and nutrients, (James, Kish and Sutton 2005), as well as maintaining fluid balance and regulating pH and body temperature (Brown and Davenport 2012).

5.2 Chemical Changes Associated with Blood Degradation

Many forensic presumptive tests for blood rely on the peroxidase-like properties of haemoglobin (Vincini 2010). This oxygen carrying protein is the main component of RBCs. Haemoglobin consists of four haem subunits, each of which having one iron atom that can bind an oxygen molecule. There are differences in the conversion kinetics between various haemoglobin derivatives when comparing in vivo and in vitro environments. Haemoglobin molecules are mainly present in two forms inside a healthy human body: saturated with oxygen, oxy-haemoglobin (HbO₂) or without oxygen, de-oxyhaemoglobin (Hb) (Bremmer et al. 2012). HbO₂ can be oxidised into met-haemoglobin (met-Hb), which is incapable of binding oxygen. HbO₂ contains the ferrous (Fe²⁺) form of iron, whereas met-Hb contains the ferric (Fe³⁺) form of iron (Doty, McLaughlin and Lednev 2016). When met-Hb is formed inside the body, the NADH-dependent enzyme cytochrome-b5 reductase can reduce met-Hb back to Hb. Conversely, the transition of HbO₂ into met-Hb will no longer be reversed.
when outside of the body due to the absence of cytochrome-b5 (Smith, Marks and Lieberman 2005). Over time, met-Hb will denature to hemichrome (HC) (Marrone and Ballantyne 2009). Figure 5.2 provides a schematic representation of the oxidative processes in haemoglobin.

![Oxidative processes in haemoglobin](image)

Figure 5.2. Oxidative processes in haemoglobin. A) Oxidative processes occurring *in vivo*. B) Oxidative processes occurring *in vitro*. Adapted from: Bremmer et al. 2011.

### 5.3 Biological Screening for Blood

The development of multiple screening techniques has enabled forensic investigators to determine the type of biological material that may be present within a potential crime scene or on items that pertain to a criminal investigation. Bloodstains at a crime scene may be aged, degraded, or diluted and subsequently no longer characteristic of blood. Blood identification involves a series of steps including visual examination, presumptive and confirmatory testing, species determination, and individualisation testing. Visual examination and presumptive testing better enables the identification and collection of stains that may offer probative value to forensic investigations (Sutton 1999).
5.3.1 Presumptive Testing

Presumptive tests are a vital and viable component of scene analysis and evidence collection, particularly when the physical properties and context do not clearly indicate blood (Bevel and Gardner 2008). Upon visual identification, presumptive tests may be used to provide an indication as to what the stain may be (An et al. 2012). A positive presumptive reaction implies that the sample should be collected for further analyses, as the material tested is most likely blood (Bevel and Gardner 2008). In most presumptive assays, a colourless substrate will be oxidised in the presence of haem, typically by hydrogen peroxide (H$_2$O$_2$), causing a colour change, fluorescence, or chemiluminescence.

The most common presumptive agents include benzidine derivatives, phenolphthalein, or leucomalachite green (Li 2015). These tests are classified as catalytic tests and are based on the peroxidase-like activity of haemoglobin and its derivatives (Sutton 1999).

Benzidine was a commonly used presumptive assay, which indicated the possible presence of blood by a blue to dark blue colour change. However, benzidine and other test reagents including o-toluidine are seldom used in forensic laboratories today, as they are recognised carcinogens. The 3,3',5,5'-tetramethyl derivative of benzidine was studied as a potential replacement and continues to be utilised as a presumptive assay. Tetramethylbenzidine is the active dye in Hemastix®. Many laboratories have adopted the Hemastix® test for field use, which has a reagent treated filter paper tab at one end of a plastic strip that turns from yellow to green or blue-green in the possible presence of blood. Phenolphthalein is a simple acid-base indicator that is used in many forensic laboratories and is commonly referred to as the Kastle-Meyer test. The reaction shows a bright pink colour change as the colourless phenolphthalin is oxidised to phenolphthalein (Greenfield, Sloan and Spaulding
2014). As with phenolphthalein, leucomalachite green involves a haem-catalysed reaction and is performed under acidic conditions. In the presence of possible blood, a green colour change will result (Li 2015).

Presumptive tests may also be performed utilising organic compounds whose oxidation products have fluorescent or chemiluminescent properties. A fluorescence assay exposes an oxidised product to an alternative light source (ALS). The fluorescence is then emitted at a wavelength that is longer than the wavelength of the ALS (Li 2015). Fluorescin is an example of a fluorescence assay, in which fluorescin is oxidised to fluorescein by hydrogen peroxide. The oxidation process is accelerated by the catalytic activity of haem (Greenfield, Sloan and Spaulding 2014). A yellow-green fluorescent light may be emitted when a fluorescin-sprayed stain is exposed to a light range of 425 to 485 nanometres, thus indicating the possible presence of blood. In contrast, light is emitted as a product of a chemical reaction in a chemiluminescence assay. A common chemiluminescent reagent is Luminol, which will emit a blue-white light in the presence of possible blood. Luminol is highly sensitive, yet the chemiluminescence is short lasting and the testing must be viewed in a darkened room (Li 2015).

The above assays can cross-react with other materials and as such, are only considered presumptive for blood and not confirmatory (Bevel and Gardner 2008). Certain household cleaners and bleaches that contain hypochlorite ions, products that contain hydrogen peroxide, certain metal salts, and some plant peroxidases can catalyse the oxidation reaction, even in the absence of haem, thus producing a false-positive result. If a strong
reductant such as zinc or lithium is present in a sample, it may inhibit the oxidation reaction and produce a false-negative result (Li 2015).

5.3.2 Confirmatory Testing

Several techniques can be utilised to confirm the presence of blood including crystal, microscopic, and immunological tests, as well as spectroscopic and chromatographic methods (Virkler and Lednev 2009). Crystal assays result in the formation of distinctive crystals of haem derivatives by chemically treating a possible bloodstain. Two common crystal assays include the Takayama and Teichmann tests. A microscope can then be used to visualise the resultant crystal morphologies (Li 2015).

5.3.3 Species Determination

The aforementioned presumptive assays and microscopic examinations lack species specificity. Species determination of a stain presumptively identified as blood is necessary for determining whether the stain is of human origin. Serological techniques, including primary and secondary binding assays, form the basis of most species identification assays (Li 2015). Hexagon OBTI® and ABAcard® HemaTrace® are commercially available kits that screen for the presence of human haemoglobin by making use of the binding between an antigen and its homologous antibody (Bevel and Gardner 2008). The ABAcard® HemaTrace® test strip works by combining a mobile monoclonal anti-human haemoglobin antibody with human haemoglobin that may be present in a stain. The formation of an antibody-antigen complex will then migrate to the test area ‘T’ through an absorbent membrane (An et al. 2012). This complex will then bind to an immobile polyclonal antihuman haemoglobin antibody. The control area ‘C’ contains the immobile anti-
immunoglobulin (Ig)-antibody (Johnston, Newman and Frappier 2003). An antibody-antigen-antibody sandwich is formed when the immobilised antibody captures the mobile antibody-antigen complex. A pink precipitin line can be visualised when the human haemoglobin concentration exceeds the minimum detection level of 0.05 μg/mL. Human haemoglobin antibody-dye conjugates cannot bind to the antibody in the test area, thus providing an internal control (Reynolds 2004). Two bands will appear in the test and control areas of the assay if the test is positive for human haemoglobin (Johnston, Newman and Frappier 2003) (Figure 5.3), whereas a negative result is indicated by the visualisation of only one band in the control area (Reynolds 2004). The RSID™ test is another blood detection assay, which detects the glycophorin-A protein present in the membrane of red blood cells (Harbison and Fleming 2016). In summary, immunochromatographic assays are sensitive, specific, rapid, and practicable for field and laboratory testing (Li 2015).
Figure 5.3. Sequence of steps involved in the ABAcard® HemaTrace® immunochromatographic assay

1. Combining of a mobile monoclonal anti-human haemoglobin antibody with human haemoglobin
2. Migration of the mobile antibody-antigen complex
3. Formation of antibody-antigen-antibody sandwich
4. Positive result indicated by a pink precipitin line in the test and control areas of the assay

Determining the origin and type of biological crime scene samples can provide valuable information that may assist with supporting a link between sample donors and the criminal act and reconstructing the sequence of events surrounding the commission of a crime. For example, a bloodstain may indicate assault, physical struggle, or murder (An et al. 2012), whereas a decomposition fluid stain is essentially an artefact of the decomposition process.

6.1 Visual Examination
Sutton (1999) detailed that visual examination was the first step in bloodstain identification. Alterations in the haemoglobin as the bloodstain ages results in a visible colour change from red to reddish-brown to green and finally dark brown. The duration and sequence of the colour changes are affected by extrinsic factors including environmental conditions and the presence of micro-organisms and bacteria (James and Eckert 1998). Comstock (2014) identified that decomposition fluid also progresses through a series of colour changes from red-burgundy to brown. The similar colour changes exhibited by both fluids over time do not permit visual identification as a method of biological fluid determination and differentiation.

6.2 pH Measurement
Ante-mortem blood pH is highly regulated to remain between 7.35 and 7.45 (Donaldson and Lamont 2013). The pH of blood drops following death due to the accumulation of CO₂ from glycolysis and glycogenolysis, and the accumulation of phosphoric and lactic acid
from the breakdown of fatty acids and amino acids. With the onset of putrefaction, muscle proteins degrade, which results in the accumulation of ammonia and in turn causes the blood pH to become more alkaline (Karmakar 2010). Similarly, alkaline pH measurements of decomposition fluid samples were reported by Comstock (2014). Results indicated that prior to insect colonisation the pH values and trends were similar during early decomposition. However, significant differences in pH measurements were observed following insect colonisation, whereby pH levels became more alkaline for insect inclusion and partial insect exclusion samples. The carcasses that were completely excluded from insect activity did not display a sharp increase in pH. Despite this research focusing on the liquid form of these samples, the results still highlight the variability of pH measurements due to the influence of extrinsic factors. In addition, Karmakar (2010) noted that post-mortem blood pH does not follow a definite sequence. Consequently, pH analyses would lack the specificity required to definitively differentiate blood and decomposition fluid stains.

6.3 Presumptive Testing for Blood

In 2006, Cranstoun researched in part the effect of decomposition on presumptive biological fluid identification. Undyed fabric strips were separated into three 5 cm sections. Each section was doped with four drops of either blood, saliva, or semen and left to dry overnight. The strips were attached to the flanks of a t-shirt with 14 samples on top of and beneath three medium-sized pig carcasses, totalling 28 samples per carcass. The carcasses were placed in an open coastal sand dune area to decompose. Seven control strips were created using the same protocol and attached to a sheet of fabric, which was then connected to the weather station pole. This placement was chosen as it provided a
reasonable distance between the decomposing pig carcasses and the control strips. Samples were collected at varying intervals over an eight-week period. Post collection, the blood section of each strip was removed and tested using the Combur³ Test®E and Kastle-Meyer presumptive tests for blood. Over the 56-day collection period, four negative results were obtained using the Combur® test strips in the area of the original bloodstain and three samples tested negative using the Kastle-Meyer reagent. The control samples returned a positive result for 25 days using the Combur® test strips. In contrast, positive results could be obtained for 42 days using the Kastle-Meyer reagent, suggesting that this presumptive test was more sensitive and in turn would be a preferable technique for compromised samples.

Cranstoun identified that breakdown products of blood would be present in decomposition fluid and consequently all areas of the blood section would test positive if they had been in contact with the fluids released during decomposition. To provide a result for comparison, an area outside of the original bloodstain referred to as ‘Other’ was tested using the presumptive reagents. The samples tested from the upper side of the pig carcasses produced a stronger reaction with the original bloodstain area using the Combur® test strips, yet equally strong reactions were recorded from samples collected from the lower side of the pig carcasses. Most samples produced an equally strong reaction between the ‘Other’ test areas and the original bloodstain when using the Kastle-Meyer reagent, thus confirming the presence of blood products in decomposition fluid.

An additional 28 strips were prepared for pattern analysis using Luminol. Seven strips doped with 0.5 mL of blood were placed on top of and beneath two large-sized pig
carcasses, totalling 14 samples per pig. A cross pattern was assigned to Pig 4, whereas Pig 5 was allocated a triangular pattern. In comparison to Pig 4, visual identification of the patterns on Pig 5 was possible for a longer period of time. These results were reflected when examining the samples using Luminol, as no patterns were identified from Pig 4, yet Luminol testing was able to detect the triangular patterns attached to Pig 5 for six days after initial contact with the carcass. Subsequent to the six-day period, results had an indiscriminate chemiluminescence appearance, suggesting a reaction was occurring with the decomposition fluid (Cranstoun 2006).

In 2015, Bemelmans also assessed in part how the process of decomposition may affect presumptive screening for biological fluids. A porcine model was used to simulate human decomposition to ensure the biological fluid samples were correctly being identified as originating from the deposited stains, as opposed to the decomposing remains. Thirty microlitres of human blood was deposited on to 88 squares that had been sectioned from two cotton t-shirts. The blood samples were placed directly on top of or beneath a pig carcass or a bag of sand, which was comparable in weight and served as the control. One bloodstain was collected daily from each segment from both the cadaver and the control, yielding a total of four blood samples per day. Subsequent to visual examination at the laboratory, presumptive testing using the Kastle-Meyer reagent and ABACard® HemaTrace® confirmatory testing were performed on any areas of red-brown staining. Positive presumptive and confirmatory results were obtained for all samples collected from the top of the pig carcass and control through day 21. Similarly, positive presumptive results were returned for all samples collected from beneath the pig carcass and the control. Positive confirmatory results were obtained from beneath the pig carcass through
day 10, however results were negative thereafter. Bemelmans attributed the dilution and degradation of the biological fluid samples to the compounding effects of rainfall, varying temperatures, soil type and condition, and the products of decomposition. Positive confirmatory results were returned on days 1 to 11, 13 and 16 from samples beneath the control. Negative results were obtained for control samples collected on the remaining days.

Despite exposure to decomposition products and adverse environmental surroundings, all samples returned positive presumptive results, thus suggesting biological testing may still be possible despite exposure to harsh environmental conditions. However, it is unknown if blood products present in the decomposition fluid produced from the porcine remains contributed to the positive results. Negative results were obtained from ABAcard® HemaTrace® confirmatory testing after day 10. This data identified the potential detrimental effects of the surrounding environment on the recoverability of biological evidence. It is unknown what adverse effect blood products present in the decomposition fluid would have on confirmatory testing, as any haemoglobin present in the decomposition fluid produced by the decaying porcine remains would not have reacted with the human specific test. Had this research been conducted using human cadavers, it is possible more positive confirmatory results would have been returned. The sensitivity of ABAcard® HemaTrace® confirmatory testing may detect blood breakdown products present in human decomposition fluid samples and as such, may not be a viable option in the differentiation of blood and decomposition fluid stains. The research conducted by Cranstoun (2006) and Bemelmans (2015) highlights that conventional presumptive and confirmatory testing currently employed in the field of forensic science may not be
sufficient in conclusively determining if blood evidence is present. Differentiation of blood and decomposition fluid may require more selective analytical techniques that allow comparison of the constituents within these fluids.

In recent years, several approaches have been researched to overcome the limitations of current methods and to develop more reliable and sensitive techniques for identifying human biological fluids and thereby stains (Legg et al. 2014). Emerging approaches that may be utilised for the differentiation of blood and decomposition fluid stains include spectroscopic techniques, the analysis of volatile organic compounds, messenger- and micro-RNA expression profiles, epigenomic modifications of DNA markers, and protein biomarker detection.

6.4 Spectroscopic Techniques

Spectroscopic techniques are being researched in the field of forensic science for the detection and identification of human biological fluids. These techniques can enable identification based on the characteristic spectral signatures and are reported to be easy to use, solvents free, fast, and cost-effective. When irradiated by ultraviolet light, most biological fluids undergo absorption processes or fluorescence, which allows for the rapid and widespread detection of biological fluids. Ultraviolet-visible (UV-Vis) spectroscopy has been applied to the detection of sweat, saliva, semen, urine, and blood stains (Zapata, Gregorio and García-Ruiz 2015). Within the forensic field, UV-Vis lamps are currently known as forensic light sources or alternate light sources (ALS) (Zapata, Fernández de la Ossa and García-Ruiz 2015). However, UV-Vis spectroscopy presents several limitations including substrate interference, a lack of body fluid specificity, and false positives. As
such, this technique is more applicable for presumptive identification and exploratory purposes (Zapata, Gregorio and García-Ruiz 2015).

Promising results have been obtained from research investigating the application of infrared and Raman spectroscopy to the identification of biological fluids (Zapata, Gregorio and García-Ruiz 2015). Spectroscopic techniques utilise the characteristic spectral signatures of haemoglobin and its derivatives for blood identification (Li 2015). The infrared wavelengths and the chemical vibrations in molecules are correlated, which establishes the characteristic bands observed in Raman or infrared spectra. Both mid-wavelength and near infrared ranges are being explored and different instrumentation including attenuated total reflectance, diffuse reflectance, hyperspectral imaging, and transmittance are being investigated. Instrumentation that combines infrared spectroscopy and photography, such as hyperspectral imaging, may assist in the detection of biological stains, as this technique provides both spectral and spatial information (Zapata, Gregorio and García-Ruiz 2015).

Mathematical methods of multivariate analysis, chemometrics, and advanced statistics allow researchers to extract useful information and interpret complex spectral data, thus allowing for more accurate and comprehensive results (Muro et al. 2014). Using statistical procedures, the Raman spectral signatures of saliva, sweat, semen, vaginal fluid, and blood have been established (Zapata, Fernández de la Ossa and García-Ruiz 2015). Zapata, Gregorio and García-Ruiz (2015) concluded that both infrared and Raman spectroscopy are rapid, selective, and non-destructive techniques and as such, these methods overcome the limitations associated with UV-Vis and provide a suitable method for biological fluid
discrimination. However, to establish the spectral signature, and in turn, identify and characterise each biological fluid, further analyses and research is required (Zapata, Fernández de la Ossa and García-Ruiz 2015).

Spectroscopic techniques have also been employed for the study of decomposition products. Infrared spectroscopy can be used to identify carbohydrates, proteins, and lipids, as well as the compounds that result from macromolecule degradation. In addition, spectroscopy may be used to identify the compounds present within a sample and to observe how these compounds change with time (Stuart 2013). Comstock (2014) analysed porcine decomposition fluid samples using attenuated total-reflectance-infrared spectroscopy (ATR-IR) and GC-MS. The aim of Comstock’s research was to examine fatty acid degradation trends and to identify potential biomarkers for the estimation of post-mortem intervals. The trends observed from the spectroscopic data between the decomposition trials yielded inconsistent results. The effect of insect activity on the levels of unsaturated fatty acid C=C and saturated fatty acid C=O stretching bands is unclear and as such, it is possible that insect activity may have contributed to the inconsistent results (Comstock 2014).

There are a number of limitations associated with the use of spectroscopic techniques, for example, biological fluids do not appear as isolated substances and the substrate on which the stain is formed or absorbed can contribute to the vibrational spectra (Zapata, Gregorio and García-Ruiz 2015). The spectroscopic signal of biological fluids may also be completely masked by contaminants. This issue is further complicated by the varied composition of possible contaminants (Sikirzhyskaya et al. 2013). Moreover, decomposition fluid is a
chemically complex matrix that is thus far not completely understood. Additional comprehensive research focusing on the validation and optimisation of spectroscopic techniques for the identification of biological fluids is needed. Furthermore, research investigating substrate effect, body fluid mixtures, and test samples that more closely resemble evidence from forensic cases is required (Zapata, Gregorio and García-Ruiz 2015). Thus far, various spectroscopic methods may provide useful trend information, however to gain a more thorough understanding of decomposition chemistry and in turn to differentiate between blood and decomposition fluid stains, more selective analytical techniques may be required (Swann, Forbes and Lewis 2010a).

6.5 Analysis of Volatile Organic Compounds

Over the last several years, there has been an increase in the study of biological volatile organic compounds (VOCs), their correlation to human odour, and their practical application to the fields of health science, forensic science, and policing. To understand what attracts carrion insects to decomposing remains and what elicits a response in cadaver dogs, research has focused on identifying the chemical compounds produced by decomposition (Stadler 2013). During the process of decay, different biological and chemical processes contribute to the evolution VOCs, which are odour chemicals (Perrault, Stuart and Forbes 2014). Volatile organic compounds are a focus of decomposition chemistry, as they arise from the catabolism of the main biological macromolecules. Carbohydrates produce a range of oxygenated compounds including esters, ethers, alcohols, aldehydes, and ketones, whereas proteins give rise to phosphorous and nitrogen compounds, and lipids yield oxygenated compounds, hydrocarbons, phosphorus and nitrogen (Stuart 2013). Research by Rust and Buis (2015) proposed that the scent profiles
of decomposition fluid and decomposed remains should be similar, as decomposition fluid is a liquefied version of the body. To date, results from chemical examination and comparison have indicated that the scent profiles produced from human remains and decomposition fluid are similar, with decomposition fluid training aids having approximately 70% of the compounds produced by decomposed remains (Rust and Buis 2015).

Several studies have been conducted to elucidate the VOC profile of decomposed remains. Research by Vass et al. established a Decomposition Odour Analysis (DOA) Database in 2004, which identifies and details chemicals that are released during the process of decomposition from buried human remains. Vass et al. (2004) identified eight separate classes of chemicals containing a total of 424 specific volatile compounds using thermal desorption gas chromatography mass spectrometry (TD-GC-MS). The eight classes included: acids/esters, cyclic and non-cyclic hydrocarbons, halogen, nitrogen, oxygen and sulphur containing compounds, and other/miscellaneous compounds that were thought to be related to the burial process but may have been products of decomposing vegetation (Vass et al. 2004). Continued research by Vass et al. in 2008 aimed to define the chemical fingerprint produced by volatile compounds associated with burial decomposition. Results identified eight major classes of chemicals, which now contained 478 compounds identified as semi-volatile or volatile components of the burial decomposition process.

A total of 104 VOCs were identified by TD-GC-MS in a study conducted Dekeirsschieter et al. (2009), which aimed to examine the VOC profile produced from the surface decomposition of pig carcasses in three different biotopes. The decomposition process
was categorised into five stages including fresh, bloated, active decay, advanced decay, and dry remains. In addition to identifying various VOCs, it was noted that there was a transition in the compounds produced as decomposition progressed. The fresh decomposition stage exhibited no cadaveric VOCs, however, alcohols, sulphur compounds including sulphur dioxide, dimethyldisulphide and dimethyltrisulphide, and the nitrogen containing compound, trimethylamine, were detected during the bloated stage. The active stage of decomposition produced the strongest olfactory signature and was characterised by cyclic compounds including 4-methylphenol, indole, and phenol. In addition, dimethyldisulphide and dimethyltrisulphide, and organic acids including 2- and 3-methylbutanoic acid and butanoic acid were also present. The final stage of decomposition exhibited an increase in aldehydes.

Inconsistencies in the VOC profile are evident across the published literature, which reflects variation in the sample collection and preparation, the analytical technique and instrumentation, the decomposition variables including the physical size of the cadaver, geographical location, soil type, and weather conditions, as well as the dynamicity of the VOC profile (Forbes and Perrault 2014; Perrault, Stuart and Forbes 2014; Vass 2012). Consequently, a consistent VOC profile of decomposition is still being investigated. However, research concerning the VOC profile of decomposition has provided a guideline for determining what compounds will be produced and their concentration ranges (Vass 2012).

A newly specialised unit of blood-detection canines has been introduced by law enforcement agencies in Italy, the United Kingdom, and Australia. The canines are trained
specifically to detect and locate blood evidence based on the VOC profile produced from fresh and aged blood training aids (Rust, Nizio and Forbes 2016). However, research conducted by Forbes et al. (2014) identified that distinctive VOC profiles were produced from fresh and aged blood samples and that as time passed, the VOC profile became more complex. As with decomposition odour profiling, there are inconsistencies in the type and number of compounds reported in the literature and as such, a consistent blood odour profile is lacking (Rust, Nizio and Forbes 2016). Inconsistencies in blood odour profiling may be attributed physiological differences between blood donors, which is influenced by an individual’s medication history, lifestyle, and diet, as well as sampling method, or analytical technique (Rust, Nizio and Forbes 2016; Rust and Buis 2015). Irrespective of the observed variations in the scent profiles, Rust and Buis (2015) concluded that detection dogs are still capable of detecting blood samples.

The training of blood and cadaver detection dogs using VOC profiling may assist in the location of remains and identification of blood evidence. Decomposed remains and blood have distinct scent profiles that do not closely resemble each other (Rust and Buis 2015) and as such, VOC profiling may be employed to differentiate between decomposition fluid and blood. However, this technique may not be applicable for stain identification and differentiation in the presence of human remains, as VOC profiling is predominantly utilised for detection dog training and the location of clandestine burial sites. In addition, an odour profile will consist of several chemical signatures that change with time (Vass et al. 2004) and the exact profile of either fluid is yet to be determined (Rust, Nizio and Forbes 2016; Forbes and Perrault, 2014).
Advancements in forensic genetics have led to the development of new techniques that involve the detection of messenger RNA (mRNA), micro-RNA (miRNA), and differential DNA methylation patterns. The identification and differentiation of biological fluids can be achieved due to tissue-specific RNA expression (An et al. 2012). The most frequently proposed RNA markers for blood are generally divided into proteins associated with haemoglobin and the haem biosynthesis pathway, or proteins associated with the erythrocyte membrane (Harbison and Fleming 2016). Research investigating RNA techniques has indicated that RNA is still useful for biological stain identification despite being considered less stable than DNA due to rapid destruction from digesting enzymes and its single stranded structure (Butler 2012; Juusola and Ballantyne 2003).

Biological fluids of forensic interest typically contain multiple cell types, each of which expresses a distinctive pattern of mRNA transcripts. The development and implementation of mRNA profiling is based on harnessing these multicellular transcriptomes. The identification of mRNA transcripts is related to the stability and abundance of each transcript in the cell (Harbison and Fleming 2016). Multiple RNA transcripts have been detected using real time polymerase chain reaction (PCR) (Haas et al. 2009; Nussbaumer, Gharehbaghi-Schnell and Korschineck 2006) or reverse-transcriptase-PCR (Haas et al. 2009; Juusola and Ballantyne 2007). Figure 6.1 illustrates the approaches taken for RNA profiling. Some RNA techniques have simultaneously identified semen, vaginal secretions, menstrual blood, venous blood, and saliva (Haas et al. 2009). Within the last five years, the European DNA Profiling Group organised a collaborative exercise to evaluate the reproducibility and robustness of mRNA profiling for the identification of blood. The results
indicated that 15 of the 16 participating laboratories were able to detect and isolate blood specific mRNA from dried bloodstains, thus highlighting the potential for mRNA profiling in forensic casework (Haas et al. 2011).

![Figure 6.1. Approaches taken for RNA profiling. Source: Lee n.d.](image-url)

The advantages of using mRNA based identification methods include the possibility of detecting multiple biological fluids in one multiplex reaction, simultaneous extraction of DNA and mRNA from the same stain (Figure 6.2), a greater specificity, and the potential for automation, all of which can preserve the sample and save time (Li 2015; An et al. 2012). Despite several mRNA markers having been proposed as specific, sensitive, and stable methods for forensic biological fluid determination (An et al. 2012), it is still possible that mRNA stability will be influenced by UV radiation, humidity, moisture, and heat (Zubakov et al. 2010; Zubakov et al. 2009). Other limitations associated with mRNA analysis include
difficulties in profile interpretation, the destructiveness of the technique, and that there are no validated guidelines in place for mRNA profiling interpretation (Orphanou 2015).

![Figure 6.2](image_url)  
**Figure 6.2.** Co-extraction of RNA and DNA from a crime scene stain enables body fluid identification and STR profiling. Source: Butler 2012.

An alternative option to mRNA for the identification of forensically relevant samples is miRNAs, which are a class of small non-protein coding RNA molecules (Harbison and Fleming 2016). The size and stability of miRNA highlight their potential applicability to the identification of degraded evidential samples. The use of miRNA may also be considered advantageous, as analysis can be performed using the same methodology employed in traditional RNA and DNA analysis, thereby reducing additional consumables and equipment (Orphanou 2015). However, a key limitation to the use of miRNA analysis is their specificity for both species and biological fluids, as any given target may have multiple miRNAs and a single miRNA may have multiple mRNA targets (Harbison and Fleming 2016).
According to Orphanou (2015), there has been a lack of reproducible and consistent results, which reflects the need for further research.

Epigenetic differences have been identified between biological fluids of forensic interest and as such, DNA methylation, which is an epigenetic modification, has been explored for biological fluid identification (Harbison and Fleming 2016). DNA methylation is the addition of methyl (CH₃) to the 5’-position of the pyrimidine ring of cytosine in CpG dinucleotides (Orphanou 2015; An et al. 2012). Bisulphite sequencing or the use of a methylation-sensitive/dependent restriction enzyme followed by PCR are the predominant methods for detecting methylation (Harbison and Fleming 2016). Research conducted by Frumkin et al. (2011) identified the benefits of DNA methylation as a method of forensic biological fluid identification. The research demonstrated that DNA methylation can be multiplexed with existing DNA protocols, thereby reducing the need for specialist training or additional equipment and reagents, and that the assay requires small amounts of input DNA. An et al. (2012) also identified that for the characterisation of biological fluids, DNA methylation based methods could be a valuable technique, yet for forensic casework application further validation studies with more markers would be required. In concordance, Orphanou (2015) detailed that to ensure the validity of the techniques, further research is required and that until all key body fluids have tissue specific methylated DNA loci and differential methylation patterns that do not overlap, DNA methylation is not an appropriate tool for routinely identifying biological fluids.

A complexity of forensic analyses is that many tissues and body fluids are mixtures of different cell types. The presence of other cell types has the potential to dilute marker
responses and in turn reduce signal strength. With DNA methylation markers, the presence of other cell types will level out the hyper- or hypo-methylation status in a cell type, thus reducing the discriminatory value of the methylation status. In addition, biological stain characterisation poses interpretational challenges when employing the above assays (Sijen 2015). At present, the application of these techniques may not be possible for the differentiation of blood and decomposition fluid stains, however, these particular assays should be considered when developing new approaches.

6.7 Proteomics

Among the promising approaches to biological stain identification is the use of protein biomarkers (Legg et al. 2014). Each biological fluid has a unique protein signature, which is attributed to the different proteins within the sample, or to the different combinations and relative abundance of proteins (Prinz et al. 2011). Protein analysis of biological samples involves the separation, identification, and characterisation of proteins (Kennedy 2001). Post-translational modification in different tissues allows for the diversity of potential targets. This, in conjunction with the stability of many proteins, highlights the advantage of protein biomarkers for the identification of biological stains (Legg et al. 2014).

Yang et al. (2013) and Van Steendam et al. (2013) aimed to identify protein biomarkers for biological fluid identification using mass spectrometry (MS) based technology. Research conducted by Yang et al. (2013) attempted to define multiple markers for saliva, semen, and blood using liquid chromatography matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry. At a greater than, or equal to 95% confidence interval, results identified 59 unique proteins in blood. Relative to each body fluid, the alpha (α) and beta
(β) subunits of haemoglobin were considered the most abundant and distinct markers for blood. Van Steendam et al. (2013) analysed cotton-tipped swabs of nasal secretions, saliva, urine, faeces, semen, vaginal secretions, menstrual blood, and venous blood using electrospray ionisation time of flight (ESI Q-TOF) mass spectroscopy. A decision tree based on the most prominent and specific proteins present in biological matrices was constructed to validate the use of MS for the determination of the samples. In concordance with research conducted by Yang et al. (2013), Van Steendam et al. (2013) identified the same specific proteins within blood. It is important to note however, that the procedure utilised by Van Steendam et al. was time-consuming and that not all laboratories may be equipped with the required technology and as such, this approach may not yet be appropriate for routine analyses.

Research conducted by Legg et al. (2014) aimed to identify and isolate candidate high-specificity protein biomarkers for the reliable identification of trace quantities of urine, seminal fluid, vaginal fluid, menstrual fluid, peripheral blood, and saliva. Samples were obtained from five unrelated females and five unrelated males. All samples were extracted and analysed using a three-phase 2D high performance liquid chromatography (HPLC)-based biomarker discovery initiative. A list of 29 candidate protein biomarkers was proposed for the target fluids being investigated based on the three-pronged comparative proteomic strategy. The proposition that these proteins would have utility as reliable biomarkers of their target body fluids was supported by: the combination of consistent identification by multiple strategies within the current research, information on tissue functionality and expression from the proteomic literature, and confirmation in independently compiled proteomic databases. The authors detailed that although larger-
scale validation studies are still required and that comprehensive forensic validation was beyond the scope of the research, the potential utility of the candidate biomarkers identified was still encouraging. The identification of reliable protein biomarkers commonly encountered in evidentiary stains has the potential to assist in the development of a unified multiplexed approach to forensic biological fluid identification (Legg et al. 2014).

6.8 Summary
Several approaches have been investigated in recent years with the primary aim of developing more reliable and sensitive strategies for the identification of biological fluids, which in turn will assist in overcoming the limitations associated with current identification techniques (Legg et al. 2014). These approaches include spectroscopic techniques, volatile organic compound analysis, genomics, and proteomics. These techniques have the potential to expand the field of biological fluid identification and with time and development, to assist in the differentiation of blood and decomposition fluid stains.
This dissertation detailed the physical and chemical properties of decomposition fluid and blood. However, research pertaining to the explicit differentiation of these fluids is currently lacking. Several techniques and methodologies have been evaluated for their potential use and effectiveness in the differentiation of blood and decomposition fluid stains. Table 7.1 outlines the potential techniques discussed and the associated limitations. The following research proposal is a proof-of-concept study outline, which aims to assess if the technique of microfluidic proteomics by protein electrophoresis can identify a potential biosignature that will enable the differentiation of blood and decomposition fluid. The results obtained from the proposed study design may assist in enhancing the knowledge base surrounding the differentiation of these fluids. The objectives for this research design are four-fold. The first objective is to document the stages of decomposition for all carcasses. The second objective is to examine the physical properties of decomposition fluid produced by non-injured and injured carcasses. The third objective is to analyse the fluids produced during decomposition using the presumptive Kastle-Meyer reagent. The final objective is to analyse the protein profiles of the fluids to determine if there are differences between blood and decomposition fluid stains and to compare these results with the results obtained from the Kastle-Meyer testing.

One can draw conclusions from the literature on potential techniques that may enable the identification of a stain next to a decomposing body as blood or decomposition fluid, however much remains to be investigated. For example, Comstock (2014) hypothesised that decomposition fluid would consist of released plasma, intra- and extracellular, and
interstitial fluids. This information, in conjunction with the fact that blood is comprised of 55% plasma, suggests that it is plausible that differences in the protein profiles may be observed between blood and decomposition fluid stains. Research by Ebah (2012) supports the hypothesis that the protein profiles of decomposition fluid and blood will vary. Ebah (2012) in part, analysed the proteomic and metabolomic profiles of samples collected from uremic subjects. For proteomic analysis, paired plasma and interstitial fluid samples were obtained from two patients with renal dysfunction. The samples were analysed using GC-MS and liquid chromatography with tandem mass spectrometry (LC-MS/MS) after electrospray ionisation (ESI) for small metabolites. LC-MS/MS was also used to digest and analyse protein samples. In all samples, 357 protein peaks were identified with molecular weights between 5 and 527 kDA. Results identified 44 proteins in all interstitial fluid samples, yet these proteins were not present in the plasma samples. Based on peak spectral counts, several other proteins were found to be more abundant in interstitial fluid samples than the plasma samples. Irrespective of the subjects, the results from this study are promising for the research aims, given that decomposition fluid is a liquefied version of the body (Rust and Buis 2015) and that the largest constituent of blood is plasma (Reynolds 2008).

Chapter 6 identified possible methods that may be used for the differentiation of blood and decomposition fluid stains. However, the associated limitations of these techniques suggest that they are not currently viable options for the application of differentiation. As such, the proposed study design will utilise the techniques of proteomics through protein electrophoresis by microfluidics. Microfluidic devices can perform biochemical reactions and analyses and when compared to conventional techniques, offer many advantages such
as improved efficiency, which is attributed to the high surface area to volume ratio of the system, and automation. Also, sample and reagent consumption is decreased due to the required nanolitre reaction volume range (Li 2015). Some methods that may allow for the differentiation of blood and decomposition fluid are binary, however, protein analysis of the samples does not require that the identity of target molecule(s) are known in advance. This is advantageous as a certain combination of molecules and the relative abundance of those target molecules may provide the biosignature required for the differentiation of the samples. If differences between the protein biosignatures of blood and decomposition fluid can be demonstrated, then the identity of these proteins could then be investigated.
Table 7.1 Potential methods for the differentiation of blood and decomposition fluid and the associated limitations.

<table>
<thead>
<tr>
<th>Potential Methods for the Differentiation of Blood and Decomposition Fluid Stains</th>
<th>Associated Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual Examination</td>
<td>A similar colour change exhibited by both fluids does not permit visual identification as a reliable method of differentiation.</td>
</tr>
<tr>
<td>pH Measurement</td>
<td>Insect colonisation has a variable effect on the pH measurements of decomposition fluid samples. In addition, post-mortem blood pH does not follow a definite sequence. Consequently, pH analyses would lack the specificity required to definitively differentiate these fluids.</td>
</tr>
<tr>
<td>Presumptive Tests for Blood</td>
<td>Decomposition fluid does not exist in isolation and is likely to contain blood products, thus compromising the interpretation of results.</td>
</tr>
<tr>
<td>Spectroscopic Techniques</td>
<td>There is a need for comprehensive research focusing on the validation and optimisation of spectroscopic techniques for the identification of biological fluids. Further research investigating spectral interference from the substrate and contaminants, body fluid mixtures, and test samples that more closely resemble forensic samples is required.</td>
</tr>
<tr>
<td>Volatile Organic Compounds</td>
<td>The exact odour profile of each fluid is yet to be elucidated and volatile organic compound profiling is predominantly utilised for detection dog training and the location of clandestine burial sites. As such, this method may not be applicable for stain identification when remains are present.</td>
</tr>
<tr>
<td>Genomics</td>
<td>The presence of other cell types has the potential to dilute marker responses, thereby reducing signal strength, and to reduce the discriminatory value of the methylation status.</td>
</tr>
<tr>
<td>Proteomics</td>
<td>Further validation studies are required and the technique may not currently be appropriate for routine analysis.</td>
</tr>
</tbody>
</table>
7.1 Proposed Experimental Design, Sampling, and Analysis

A minimum of four medium-sized pig carcasses will be utilised for the research objectives. Vass (2001) identified that fluids and gases will be released from the natural orifices of the body during decomposition. To provide a focal point for sample collection and to observe the diffusion of the released fluids, each pig carcass will be suspended within a small animal cage (Figure 7.1). At the bottom of the cage will be a plain white tile that has been washed with neutral soap to avoid possible cross-reactions with the Kastle-Meyer reagent. The tile will be placed within a removable tray for ease of sampling and documentation. An alternative methodology is to lay the pig carcasses on top of a galvanised mesh platform within the small animal cages (Figure 7.2). The galvanised mesh platform will allow the decomposition fluids to drain away from the carcasses and will limit possible false-positive Kastle-Meyer reactions by reducing the potential for rust.

When examining the stages and rates of decomposition, the cumulative effects of temperature and insect activity must be a significant consideration. At various stages of decomposition, insects and flies will be attracted to the decaying remains depending on the volatile compounds produced and the associated odours that are emitted (Comstock, 2014). Comstock (2014) identified that the physical and chemical composition of decomposition fluid is affected by insect activity. To reduce the number of variables, access by insects will be limited by encasing the carcasses in protective netting. To avoid an increase in humidity and temperature, the protecting netting will permit airflow. Access to the carcasses by crawling insects may also be minimised by applying petroleum jelly around the base of the cages.
Once the pig carcasses are in position, post-mortem stab wounds will be inflicted to generate blood flow for half of the experimental carcasses. Observations will be documented and photographically recorded on each experimental day. Observations are to note the physical appearance of the carcass and any fluids produced. In addition, all fluid sampling days will be documented and recorded. Kastle-Meyer testing will be performed on the fluids produced by all carcasses, as per the reagent protocol. At the laboratory, all swabs will be analysed using protein electrophoresis by microfluidics.
Figure 7.1. Experimental design sketch in which the decomposing carcasses are suspended.

Figure 7.2. Experimental design sketch in which the decomposing carcasses are placed on top of a galvanised mesh platform.
7.2 Decomposition Research Facility

The field trial area will be located at the Murdoch Veterinary Farm, 90 South St, Murdoch, Western Australia, Australia, 6150. Within the field trial area are two caged structures (Figure 7.3). The study will be performed in the east caged structure, as it will protect the carcasses from larger predators and scavengers. In addition, this structure has a covered section that will protect the pig carcasses from direct sunlight, which may dry the carcasses and the fluids, and from potential rainfall, which may dilute the fluids and alter the decomposition process. While extrinsic factors, such as rainfall, direct sunlight or predation could realistically be encountered at an outdoor crime scene, a key goal of this present study is to limit such variables.

**Figure 7.3.** Overview sketch of the decomposition research site and approximate measurements.
7.3 Experimental Subjects

Decomposition fluid samples will be collected from four pig carcasses. As outlined previously, pig carcasses are considered an acceptable analogue for human cadavers because of their similarity to human torsos in hair coverage, weight, fat to muscle ratio, physiology and biochemistry, and they avoid the ethical issues associated with the use of human cadavers (Schoenly et al. 2006; France et al. 1992). The pig carcasses will be obtained from Nambeelup Piggery, Mandurah and euthanised humanly in accordance with animal ethics. The carcasses will be received fresh and wrapped in plastic for transportation to the research facility. In attempt to decrease variability in results, all carcasses will be of the same gender and approximate weight. As previously mentioned, post-mortem stab wounds will be inflicted on two of the four carcasses to generate blood flow. Raymond, Smith and Liesegang (1996) compared fresh human blood with fresh and aged porcine blood and concluded that it was valid to use porcine blood in place of human blood for educational and research purposes, as the human and porcine blood showed remarkable similarity. At the completion of the research, the pig carcasses will be disposed of in a manner that complies with ethics approval.

7.4 Weather Data

Weather loggers will be placed within the research site and will be set to record the temperature at five-minute intervals. Data from the Murdoch University Weather Station will also be recorded for comparative purposes. To account for temperature data, elapsed time will be documented as accumulated degree days (ADD), which represent heat energy units available to sustain chemical and biological processes required for decomposition. Using ADD allows research that scores the decomposition process to be more reliably
compared between environments, seasons, and years, as the temperature data is accounted for (Megyesi, Nawrocki and Haskell 2005). At the completion of the fieldwork, the weather data will be transferred to a computer for analysis.

7.5 Adaptations to Study Design

The two primary factors affecting the rate of decay include temperature and insect activity. Continued and more extensive research within this field may attempt to utilise the above experimental design in different environmental conditions and in the presence and absence of insect activity, to determine more thoroughly how these factors impact the differentiation of blood and decomposition fluid. Other factors that influence the rate of decomposition include trauma to the body, body size and weight, and the substrate on which decomposition takes place (Hau et al. 2014). The above experimental design can again be adapted to study how age, gender, or various other methods of inflicted trauma potentially influence the physical and chemical properties of decomposition fluid and in turn, the differentiation of this fluid and blood. In addition, different substrates such as soil, carpet, bedding material, or linoleum may be used to assess potential substrate interference.
Determining the type and origin of a biological sample can yield valuable information that will supplement forensic casework (An et al. 2012). As previously detailed, blood is one of the most frequently encountered and significant types of physical evidence associated with the forensic investigation of violent crime and death (James, Kish and Sutton 2005). The identification of possible blood evidence is a critical component to an investigation in which the ante-mortem events of the decedent are unknown. A body and therefore any associated biological stains may not be located for a period of time after death, during which the decedent will begin to decompose, thereby limiting information available concerning the events prior to death (Cranstoun 2006). It is important to determine whether a stain observed next to decomposing remains is blood or decomposition fluid, as blood would suggest an injury has occurred before or shortly after death, whereas decomposition fluid is naturally released during decomposition.

Continued research conducted by a number of authors including Vass et al. and Swann, Forbes, and Lewis in the field of decomposition chemistry has attempted to elucidate the physical and chemical properties of decomposition fluid. However, inconsistencies are evident across the literature concerning the compounds present throughout the various stages of decay and the potential trends exhibited by these compounds, thus highlighting the chemical complexity of decomposition fluid. This, in conjunction with literature suggesting that decomposition fluid stains closely resemble the physical characteristics of bloodstains and the presence of blood breakdown products in decomposition fluid
(Cranstoun 2006), complicates the interpretation and determination of stains located next to decomposed remains and poses significant challenges for the forensic investigators.

Despite their usefulness, current visual, enzymatic, immunological, and catalytic presumptive and confirmatory assays are substantially impacted by environmental insults, may suffer limitations associated with specificity and sensitivity, and may only be applicable to a subset of samples (Frumkin et al. 2011). Several approaches have been investigated in recent years with the primary aim of developing more reliable and sensitive strategies for the identification of biological fluids (Legg et al. 2014). However, research that pertains to the explicit differentiation of blood and decomposition stains is currently lacking. It is possible to draw conclusions from the literature on potential techniques that may enable identification and differentiation, however, much work remains to be completed the area of biological fluid identification and in the field of decomposition chemistry. For complete characterisation of fluid produced in both the presence and absence of soil matrices under field and controlled conditions, further validation studies and development of analytical methodologies is required. To enable direct comparison of results, there is also a need to establish harmonised analytical protocols (Swann, Forbes and Lewis 2010a).

In the field of forensic science, there is a need for an unbiased, universal, and specific method that can identify and differentiate biological fluids (Van Steendam et al. 2013). Continued research into the differentiation of blood and decomposition fluid stains will assist in the development of a technique that meets these requirements and will also provide valuable information to determine the series of events prior to death. This will
better enable post-mortem investigations of remains that are in the advanced stages of decomposition, and may provide probative information so that investigative processes are not misinformed and that resources and expenditure are not misallocated.
Future analyses may seek to focus more comprehensively on the known properties of decomposition fluid. For example, further studies may aim to identify the presence or absence and relative proportions of fatty acids, amino acids, and various metabolites in both blood and decomposition fluid. These elements have been addressed in the literature and thereby provide an opportunity for development and advancement. In addition, development and alteration of the proposed experimental parameters with regard to season, location or technological platform may provide additional information concerning the composition and degradation of these fluids and may assist in determining how these factors potentially influence differentiation.

However, it is important to first further investigate the physical and chemical properties of decomposition fluid and blood and how these properties change with time. Collaborative research within the forensic disciplines and a more comprehensive understanding of the basic properties of these fluids and how they degrade will better enable their differentiation and application to stain identification. With enhancements in technology, it may be possible with time that blood and decomposition fluid stains can be differentiated by volatile organic compounds for casework involving outdoor scenarios or by spectroscopic, genomic, and proteomic techniques. Once a more thorough understanding has been established, future research should seek to conduct analyses using human samples. This will help to ensure that any differences identified in porcine samples accurately reflect those that would be observed in human cadavers and in turn forensic investigations.
References


Bemelmans, Elena Alice. 2015. "Effects of Decomposition on the Recoverability of Biological Fluid Evidence". Master of Science, Boston University School of Medicine.


Reynolds, Mark. 2008. "Bloodstain Size, Shape and Formation". Ph.D, The University of Western Australia.


Spencer, Jessica Rose. 2013. "Defining Postmortem Changes in Western Montana: The Effects of Climate and Environment on the Rate and Sequence of Decomposition Using Pig (Sus Scrofa) Cadavers". Master of Arts in Forensic Anthropology, The University of Montana.


The Differentiation of Human Blood and Decomposition Fluid Stains: A Review
Abstract

An important aspect of forensic science is the detection and identification of biological fluids, as this may support a link between a criminal act and donor and assist in the reconstruction and sequencing of a potential crime scene. A body and therefore any associated biological stains may not be located for a period of time, during which the decedent will begin to decompose. Decomposition fluid and blood have been reported to be visually similar, and therefore, it is important to determine the source of the stain. The presence of blood would suggest an injury has occurred before or shortly after death, whereas decomposition fluid is a by-product of the naturally occurring decomposition process. This review evaluates various techniques including visual examination, pH measurement, presumptive testing for blood, spectroscopy, the analysis of volatile organic compounds, genomics, and proteomics for their potential use and effectiveness in the differentiation of blood and decomposition fluid stains.

Key Words: Forensic science, decomposition chemistry, forensic biology, decomposition, macromolecule, decomposition fluid, blood, biological fluid, pH, presumptive testing, spectroscopy, volatile organic compounds, genomics, proteomics.
Contents

1. Introduction ............................................................................................................. 5
2. Mammalian Post-mortem Decomposition ......................................................... 6
3. Macromolecule Degradation .............................................................................. 9
   3.1 Carbohydrate Degradation .............................................................................. 9
   3.2 Protein Degradation ....................................................................................... 10
   3.3 Lipid Degradation ......................................................................................... 11
4. Decomposition Fluid .......................................................................................... 13
   4.1 Physical Properties of Decomposition Fluid .................................................. 13
   4.2 Chemical Properties of Decomposition Fluid ............................................... 14
5. Blood Evidence .................................................................................................... 23
   5.1 Biological Properties of Blood ....................................................................... 23
   5.2 Chemical Changes Associated with Blood Degradation ............................... 24
   5.3 Biological Screening for Blood ..................................................................... 25
6. Differentiation of Blood and Decomposition Fluid .......................................... 27
   6.1 Visual Examination ....................................................................................... 27
   6.2 pH Measurement .......................................................................................... 27
   6.3 Presumptive Testing for Blood ...................................................................... 28
   6.4 Spectroscopic Techniques ............................................................................ 30
   6.5 Analysis of Volatile Organic Compounds .................................................... 32
   6.6 Genomics ....................................................................................................... 33
      6.6.1 mRNA-based Analysis ........................................................................... 34
      6.6.2 miRNA-based Analysis .......................................................................... 35
      6.6.3 Epigenetic Analysis ................................................................................ 35
   6.7 Proteomics ...................................................................................................... 36
7. Conclusions .......................................................................................................... 37

References .................................................................................................................. 39
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADD</td>
<td>Accumulated Degree Days</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>Attenuated Total Reflectance Infrared Spectroscopy</td>
</tr>
<tr>
<td>CDI</td>
<td>Cadaver Decomposition Island</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty Acid Methyl Ester</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>Hb</td>
<td>Deoxyhaemoglobin</td>
</tr>
<tr>
<td>HbO₂</td>
<td>Oxyhaemoglobin</td>
</tr>
<tr>
<td>HC</td>
<td>Hemichrome</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionisation</td>
</tr>
<tr>
<td>Met-Hb</td>
<td>Met-haemoglobin</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro Ribonucleic Acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>NRN</td>
<td>Ninhydrin Reactive Nitrogen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>STR</td>
<td>Short Tandem Repeat</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile Organic Compound</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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</tbody>
</table>
1. Introduction

An important aspect of forensic investigations is the detection and identification of biological fluids at a crime scene. Blood is one of the most frequently encountered and significant types of physical evidence associated with the forensic investigation of violent crime and death. The presence and evaluation of blood evidence can be crucial in the reconstruction and sequencing of a crime scene, supporting a link between the criminal act and sample donor, and confirming or refuting statements from the person or persons of interest.

A body and therefore any associated biological staining may not be located for days or weeks after death, during which time the decedent will begin to decompose. A thorough forensic examination of a decedent is required in part for the exclusion or documentation of injuries that may have contributed to or caused death. However, the examination of putrefied remains can be technically challenging, and the interpretation of findings can be complicated by the modification and loss of tissue. In addition to the degenerative changes occurring throughout putrefactive decay, remains may be subject to insect activity, animal predation, and varying environmental factors, all of which will further complicate post-mortem examinations. Putrefactive changes may obliterate markers of identity or create lesions that mimic or distort the features of actual ante-mortem injury. Furthermore, suspicions of inflicted injury may arise due to the purging of decomposition fluids. The presence of blood would suggest that an injury has occurred before or shortly after death, whereas decomposition fluid is released during the naturally occurring process of decomposition. Consequently, ante-mortem injury should be recognised and differentiated from artefacts of putrefactive decay. Despite the development of advanced
adjuncts to traditional autopsies, such as magnetic resonance imaging and computerised tomography, the artefacts induced by putrefaction may still require interpretation and can complicate post-mortem evaluations.\textsuperscript{6}

Current techniques used for forensic biological fluid identification include immunological, chemical and protein catalytic activity tests, microscopy, and spectroscopic methods.\textsuperscript{1,3} Decomposition fluid is a chemically complex mixture with associated micro-organisms, insect life, and other debris, thus making it a challenging matrix to study.\textsuperscript{9} However, the presence and relative abundance of specific compounds, and the unique composition of each biological fluid can form the basis of its identification.\textsuperscript{1} The following review summarises the properties of decomposition fluid and blood and evaluates the potential use and effectiveness of various techniques for the differentiation of these fluids. Identifying the source of a stain as decomposition fluid or blood may offer supplementary information to the post-mortem examination of highly decomposed remains, and provide probative information so that investigative processes are not misinformed and that resources and expenditure are not misallocated.

2. Mammalian Post-mortem Decomposition

Decomposition of mammalian soft tissue is a continuous post-mortem process.\textsuperscript{10} Circulatory stasis and the ensuing anoxic environment give rise to a series of biochemical reactions, which lead to the failure of homeostatic mechanisms and the gross morphological changes associated with the observable stages of decay.\textsuperscript{11} Decomposition is often broadly categorised into pre- and post-skeletonisation. The pre-skeletonisation classification can be subdivided into four stages including fresh, bloated, decay, and dry.\textsuperscript{12}
Defining the process of decomposition into stages can assist investigators in estimating the post-mortem interval, and in describing the complex biochemical reactions associated with death.\textsuperscript{13}

Decomposition involves the two parallel processes of autolysis and putrefaction.\textsuperscript{14} The first identifiable process occurring during the fresh stage of decay is autolysis, which is the enzymatic breakdown of the soft tissues.\textsuperscript{9,15} Autolysis is a chemical process that is slowed by cooler conditions, accelerated by heat, and stopped by the inactivation of enzymes or by freezing.\textsuperscript{14} This process progresses more rapidly in tissues that have a high water and enzyme content, and can be observed macroscopically by epidermal sloughing and subcutaneous blisters.\textsuperscript{16} The superficial vessels become stained by intravascular haemolysis, which produces a visible reticulated pattern under the skin, commonly referred to as ‘marbling’.\textsuperscript{17,18} Autolysis is thought to occur due to reduced oxygen levels, which causes a decrease in the intracellular pH.\textsuperscript{19} The anoxic and acidic environment, in conjunction with unregulated enzyme activity, results in cellular membrane dissolution and the release of nutrient-rich fluids, which facilitates putrefaction.\textsuperscript{20}

The beginnings of putrefaction can be observed during the bloated stage of decomposition. Putrefaction is the breakdown of the soft tissues caused by the action of fungi, bacteria, and protozoa.\textsuperscript{9} The release of lipid, carbohydrate, and protein by-products during autolytic degradation encourage the proliferation of these micro-organisms, and the increasingly fluid state of the tissues facilitates bacterial transmigration.\textsuperscript{15,21} The formation of sulphaemoglobin in the settled blood results in a greenish discolouration of the skin, which is often the first visible sign of putrefaction.\textsuperscript{16} Micro-organisms produce
various gases including hydrogen sulphide, methane, and more complex volatile organic compounds, which accumulate within the respiratory and intestinal tracts causing tissue distension.\textsuperscript{15} An increase in the internal pressure due to the build-up of volatile gases and other products of catabolism eventually results in the purging of fluid and gases from the natural orifices of the body.\textsuperscript{16,22} Putrefactive changes are dependent primarily on the prior state of health of the decedent and the environmental temperature.\textsuperscript{23}

Active decay begins following the purging of gases and fluids.\textsuperscript{20} Chemical constituents will continue to be degraded and released and as such, putrefaction continues throughout active decay.\textsuperscript{9} At this point in the decomposition process, insect activity is prominent, there is a significant number of anaerobic and aerobic bacteria present, and electrolytes are rapidly leached out of the body.\textsuperscript{16} Liquefaction of the soft tissues will result from continued decomposition,\textsuperscript{18} and a cadaver decomposition island (CDI) may form due to the release of cadaveric material.\textsuperscript{24} The final stage of decomposition is the dry stage.\textsuperscript{9} Following the phases of ‘wet decay’ the surface tissues collapse, dry, and darken to assume a leathery texture and residual organs shrink and desiccate. The body may eventually progress to skeletonisation depending on the post-mortem interval and the surrounding environmental conditions.\textsuperscript{18}

The process of decomposition is dynamic and the timeframe in which it occurs can be highly variable.\textsuperscript{15} Intrinsic factors that govern decomposition include ante-mortem medical conditions, the weight and age of the decedent, and the nature and extent of trauma. Extrinsic factors including moisture, temperature, animal predation and insect accessibility, the manner of disposition, and protective coverings also influence the sequence and
duration of decomposition.\textsuperscript{19,25} It is important to note that the process of decomposition occurring under varying conditions cannot always be accurately described by the classifications currently published in the literature. The characterisation of decompositional stages is complicated by conditions that cause differential decay or by those that alter the process and rate of decomposition.\textsuperscript{13}

### 3. Macromolecule Degradation

The four major classes of biological macromolecules include nucleic acids, carbohydrates, proteins, and lipids. These macromolecules are broken down into their structural components by the complex reactions associated with the process of decomposition.\textsuperscript{16}

#### 3.1. Carbohydrate Degradation

Polysaccharides are broken down into component sugars during early decomposition by the action of micro-organisms. Fungi can breakdown sugars into organic acids including citric, glucuronic, and oxalic acids. Bacteria are responsible for the degradation of sugars into pyruvic and lactic acids under aerobic conditions, which can be further degraded into water and carbon dioxide. Under anaerobic conditions, bacteria can breakdown sugars into acetic, butyric, and lactic acid. Bacterial carbohydrate fermentation may produce hydrogen, hydrogen sulphide, and methane gases, as well as butanol and ethanol\textsuperscript{12} (Figure 3.1).
3.2. Protein Degradation

The degradation of proteins into their component amino acids occurs as a result of bacterial enzyme activity and is referred to as proteolysis. The rate of proteolysis varies depending on bacterial action, moisture, temperature, and on the protein type. Proteins of epithelial and neuronal tissues typically degrade first, while hard tissue proteins, such as keratin and collagen, are more resistant to degradation. The process of decomposition results in the breakdown of proteins into amino acids, peptones, polypeptides, and proteoses. Continuing proteolysis can lead to the evolution of gases including ammonia, carbon dioxide, hydrogen sulphide, and methane, and the production of the phenolic compounds, indole and skatole (Figure 3.2). The amino acids produced as a result of protein degradation can undergo: deamination, which produces ammonia;
decarboxylation, which results in the formation of cadaverine, indole, putrescine, tryptamine, tyramine, and carbon dioxide; and desulphurhydralation, which can yield ammonia, hydrogen sulphide, pyruvic acid, and thiols.\textsuperscript{12}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.2.png}
\caption{Overview of protein degradation.\textsuperscript{26}}
\end{figure}

3.3. Lipid Degradation

Following death, saturated and unsaturated fatty acids are produced as intrinsic tissue lipases hydrolyse lipids.\textsuperscript{12,26} As decomposition continues, the concentration of fatty acids increases, whereas the concentration of neutral lipids decreases. In an anaerobic environment, saturated and unsaturated fatty acids will undergo hydrogenation and
further hydrolysis. In an aerobic environment, oxidation of the unsaturated fatty acids can occur due to atmospheric oxygen, bacteria, or fungi. Oxidation of lipids will initially yield peroxide bonds, with ketones and aldehydes the final products of this process. Fatty acids and glycerol degrade yielding shorter chain saturated fatty acids and eventually water and carbon dioxide. Figure 3.3 illustrates the process of lipid degradation that occurs during decomposition.

Figure 3.3. Overview of lipid degradation (adapted).
4. Decomposition Fluid

Decomposition fluid is the chemically complex liquid produced by the degradation of the organs and soft tissues of the body. The released fluid is often associated with microorganisms, insects, and other debris, thereby making it a challenging matrix to study.

Much research investigating the physical and chemical properties of decomposition fluid has been conducted using domestic pig (Sus domesticus) carcasses due to ethical restrictions associated with the use of human cadavers. Pig carcasses are considered an acceptable analogue for human cadavers because of their similarity to human torsos in hair coverage, weight, fat to muscle ratio, physiology, and biochemistry.

4.1. Physical Properties of Decomposition Fluid

Table 4.1 summarises the physical properties of porcine decomposition fluid as reported from outdoor research trials conducted in Ontario, Canada during the spring-summer months. In each replicate trial, three carcasses were exposed to, and three carcasses were protected from insect activity. The insect exclusion group was subdivided into ‘partially excluded’ and ‘completely excluded’ based on whether insects gained access.
Table 4.1. Summary of the physical properties of porcine decomposition fluid produced in the absence, partial presence, and presence of insect activity.\textsuperscript{13}

<table>
<thead>
<tr>
<th>Physical Properties of Decomposition Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
</tr>
<tr>
<td><strong>pH Measurement</strong></td>
</tr>
<tr>
<td><strong>Conductivity</strong></td>
</tr>
</tbody>
</table>

4.2 Chemical Properties of Decomposition Fluid

A non-chromatographic approach to the chemical study of decomposition involved the investigation of ninhydrin-reactive nitrogen (NRN).\textsuperscript{9} Cadaver decomposition releases nitrogenous compounds, which can be detected by reaction with ninhydrin.\textsuperscript{29,30} Carter et al.\textsuperscript{29} proposed that there would be a significant increase in NRN concentration in soils associated with mammalian decomposition. Juvenile rats (*Rattus rattus*) were buried in contrasting soil sites in Queensland, Australia. Following a sequential and destructive sampling regime and NRN analysis, results indicated that the cadaver burial samples demonstrated a 1.4 to 2.2-fold increase in NRN. Van Belle et al.\textsuperscript{30} adapted the NRN
technique to examine surface and burial decomposition scenarios of porcine remains, and to investigate the lateral diffusion of NRN. The burial trial results corresponded with the surface trial results, whereby increased concentrations of NRN were associated with the most active stages of decomposition. The lateral influx of NRN was determined to be minimal. A case study investigated the use of the NRN technique in the reconstruction of a disturbed outdoor death scene. The presumptive decomposition site had significantly greater concentrations ($p < 0.001$) of NRN when compared to the other sampling sites and as such, was concluded to represent the area in which the majority of decomposition had occurred.

This research highlights the potential for NRN to be used for the detection of clandestine burial sites, and for determining the original site of decomposition for surface deposited remains subject to post-mortem relocation or animal scavenging. However, it was noted that other organic sources release NRN and as such, an elevated concentration of NRN is not necessarily indicative of human decomposition.

Non-chromatographic methods have provided a qualitative profile of decomposition fluid, however more selective analytical techniques are required to provide quantitative data, and to offer the level of specificity needed to understand the fundamentals of decomposition chemistry. Separation science techniques including gas chromatography (GC), capillary electrophoresis (CE), and liquid chromatography (LC) may be used to analyse the products of decomposition. Early decomposition chemistry studies focused on short chain volatile fatty acids ($C_2 - C_6$), which are produced during active decomposition by a series of microbially-induced reductive and oxidative reactions of
Tuller\textsuperscript{32} analysed volatile fatty acids (VFAs) in soil samples collected from known grave and execution sites in the former Yugoslavia using a field portable gas chromatograph (microFast GC\textsuperscript{2}) and mass spectrometer (MS). Only three of the collected soil samples contained detectable levels of the targeted VFAs. However, several other fatty acids including capric, lauric, myristic, oleic, palmitic, and stearic acids were identified in the mass grave soil samples. These fatty acids were not detected in the control samples and as such, were likely to be associated with decomposing remains. It was proposed that detection of the targeted VFAs may have been affected by experimental design, co-elution with the solvent, decomposition rate, soil structure or environmental conditions. Despite these factors, the author concluded that VFAs could remain detectable in crime scene soil samples for a considerable length of time.

Vass et al.\textsuperscript{33} studied soil solutions from beneath decomposing human cadavers using GC analysis to collect data on microbially-produced VFAs and various anions and cations. Gas chromatography can be used to analyse VFAs, as the technique enables quantification of individual components and the separation of mixtures.\textsuperscript{12} Once ante-mortem weight was standardised and the moisture of the soil taken into account, the VFA and anion/cation concentrations were the same for any given total of accumulated degree days (ADD), irrespective of the subject or season in which decomposition occurred. The authors recognised that there appeared to be a correlation between VFA production and the stages of decomposition, which was attributed in part to the sequential degradation of carbohydrates and proteins.
In 2013, von der Lühe et al. investigated the suitability of cholesterol and coprostanol as biomarkers for decomposition fluid within a soil matrix. These sterols were considered valuable compounds for the detection of decomposition fluid due to their origin and stability against degradation over time. The phytosterol, β-sitosterol, was also examined as it was assumed to remain stable between the control and treated soils. Domestic pig carcasses were buried in shallow graves and exhumed after three and six months. Soil samples were analysed using gas chromatography-mass spectrometry (GC-MS). When compared with the control and six-month old graves, higher concentrations of the investigated sterols were detected in the three-month old graves. The detection of cholesterol and coprostanol was attributed to soft tissue decomposition and the release of decomposition fluid. Aerobic degradation, sorption, transformation, and transport away from the depositional source were proposed as likely explanations for the results obtained after six months of burial. The results highlight the need to investigate the influence of soil properties on the detection of decomposition products.

Preliminary research by Swann et al. aimed to determine the chemical composition of decomposition fluid in the absence of a soil matrix. The preparation phase involved dilution and filtration of decomposition fluid samples followed by GC-MS analysis. The first trial examined the fluids produced from pork rashers (belly pork) that were either exposed to or protected from insect activity. For the duration of the study, no decomposition fluid was produced from the protected samples. However, fluid was collected between days three and 24 of the trial for samples exposed to insect activity. Compounds detected during the pork rasher trial, as indicated by chromatogram are illustrated in Figure 4.1.
Additional trials were conducted with stillborn piglets and adult pig carcasses to represent a more realistic model of decomposition. For the piglets exposed to insect activity, fluid was produced between days 14 and 47, whereas the protected piglets produced decomposition fluid between days 42 and 54. Compounds produced from Piglet 3 on day 26, as indicated by chromatogram, are illustrated in Figure 4.2. Fluid collection commenced on day five of the pig carcass trial and concluded on day 14 when maggot and insect activity had ceased and only skeletal remains and mummified skin were evident. Similar compounds including short and long chain acids and cyclic compounds were detected in the pork rasher and adult pig trials. Previously undetected indole and phenol compounds were also identified (Figure 4.3). Variability in fluid production and the complex nature of the decomposition process highlighted the need for a larger sample set in both the piglet and adult pig trials.

Figure 4.1. Chromatogram showing compounds produced on day 24 of the pork rasher trial. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: trimethylacetic acid (internal standard), 4: butyric acid, 5: isovaleric acid, 6: 2-piperidone, 7: phenylacetic acid, 8: phenylpropanoic acid, 9: myristic acid, 10: palmitic acid, 11: palmitoleic acid, 12: stearic acid, 13: oleic acid, 14: linoleic acid.
Figure 4.2. Chromatogram showing compounds produced on day 26 from Piglet 3. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: isobutyric acid, 4: trimethylacetic acid (internal control), 5: butyric acid, 6: isovaleric acid, 7: valeric acid, 8: isocaproic acid, 9: 2-piperidone.²⁵

Figure 4.3. Chromatogram showing compounds produced on day six during the adult pig trial. Numeric order of compounds: 1: acetic acid, 2: propionic acid, 3: trimethylacetic acid (internal control), 4: butyric acid, 5: isovaleric acid, 6: valeric acid, 7: 4-methylvaleric acid, 8: caproic acid, 9: phenol, 10: 2-piperidone, 11: indole, 12: phenylacetic acid, 13: phenylpropionic acid, 14: oleic acid. The peak marked ‘x’ is an unidentified peak.²⁵
Further analyses conducted by Swann et al.\textsuperscript{36} on the above experimental design identified that isovaleric and propionic acids were detected on all collection days during the pork rasher trial. The data indicated that until day 15, acetic, isobutyric, and propionic acid followed an increasing trend and then a decreasing trend until the completion of the trial. Similarly, isovaleric and butyric acid followed an upward trend, reaching a maximum at day 15, yet the trend appeared to be cyclic. The long chain fatty acids detected in this trial (9-hexadecenoic, linoleic, myristic, oleic, palmitic, and stearic acid) passed through a maximum on day 15 and appeared to follow an eight-day cycle. The authors tentatively related these observations to the adipose tissue content of the pork rashers, the feeding cycle of the maggots, and the level of fly activity. Analysis of the piglet trial data conveyed that there were no clear trends in the compounds identified. Results from the pig trial demonstrated that the target short chain acids, with the exception of isobutyric acid, appeared to follow a cyclic trend, while the long chain fatty acids (linoleic, oleic, palmitic, and stearic acid) showed an increasing trend. However, continuation and confirmation of the trends could not be demonstrated due to disruptions in sampling. This study identified considerable variation in the abundance and type of compounds present in decomposition fluid, thereby highlighting the chemical complexity of this sample.

To characterise decomposition fluid produced from porcine remains in the presence, partial presence, and absence of insect activity, fluid samples were analysed using GC-MS following fatty acid methyl ester (FAME) extraction.\textsuperscript{13} The dominant fatty acids present in porcine and human soft tissue including linoleic, myristic, oleic, palmitic, palmitoleic, and stearic acids were detected in all samples. However, the fatty acids did not display consistent trends, and there was variation in the compounds produced throughout the
decay stages. These results suggest that insect activity influences the degradation and concentration of fatty acids.

Compounds that are not suitable for GC analysis because of thermal instability can be analysed using capillary electrophoresis (CE). This technique offers the potential for rapid and efficient separations of complex chemical mixtures. A capillary electrophoresis method was adapted for the determination of amino acids and biogenic amines in porcine decomposition fluid samples. There was an expectation that amino acids and biogenic amines would be present in decomposition fluid, due to the biodegradation pathways of the main macromolecules. Method optimisation was achieved using a screening design followed by a central composite design, in which total analysis time and peak resolution were response factors. Phenylalanine, tryptamine, tryptophan, tyramine, and tyrosine were all identified by spiking and migration time. However, further research is required to elucidate the identity of a number of unidentified peaks observed in the electropherogram (Figure 4.4).

**Figure 4.4.** Electropherogram at optimised running conditions identifying in numeric order: 1: tryptamine, 2: tyramine, 3: neutral, 4: tryptophan, 5: tyrosine, 6: phenylalanine. Peaks marked with an asterisk are unidentified compounds.
As with CE, high-performance liquid chromatography (HPLC) can be used to study thermally unstable or potentially non-volatile decomposition products. Liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS), operated in multiple reaction monitoring mode, was used to analyse porcine decomposition fluid for the detection of amino acids and biogenic amines. Infusion experiments enabled refinement of the tuning parameters and an optimised LC method was applied to the samples. Over the course of the trial, tyramine, putrescine, and indole displayed a general increasing trend with respect to time and temperature. Tryptophan and phenylalanine compounds were detected in all fluid samples, and despite not indicating potential trends individually, together the compounds displayed a 14-day cyclic trend. The sum of amino acid concentrations, with the exception of two sampling days, also appeared to follow this trend. However, further investigation is required to ensure the proposed method can be used for quantitative assessment.

Analytical separation techniques have enabled the study of mammalian decomposition and the associated by-products including decomposition fluid. The above studies have primarily focused on providing more reliable techniques for estimating post-mortem intervals. However, determining what compounds are present in decomposition fluid will assist in providing a greater understanding of decomposition chemistry, and in turn provide valuable information needed for the differentiation of this sample and blood evidence.
5. Blood Evidence

Blood is one of the most frequently encountered and significant types of physical evidence associated with the forensic investigation of violent crime and death. The nature and circumstances of violent crimes often produce various bloodstains that, when carefully studied with respect to their distribution and geometry, may assist with the reconstruction of a potential crime scene. The accurate interpretation of blood evidence has proved critical in a number of cases where the manner of death must be resolved. An understanding of the properties of blood is necessary for the interpretation of bloodstain evidence.39

5.1. Biological Properties of Blood

Blood is a liquid form of connective tissue comprising 55% intercellular material (plasma) and 45% formed cellular elements (erythrocytes, leukocytes, and platelets).2,40 The most abundant cellular component in blood are erythrocytes or red blood cells (RBCs). The small size (approximately 7.5 μm in diameter) and biconcave shape of erythrocytes makes them efficient in their transportation of oxygen and carbon dioxide. Erythrocytes do not have a nucleus and as such, lack nuclear DNA. An important source of DNA for forensic analyses is the nucleated leukocyte or white blood cell (WBC). Leukocytes can be divided into granulocytes (basophils, eosinophils, and neutrophils) or agranulocytes (monocytes and lymphocytes), all of which protect against pathogens and infectious micro-organisms. The smallest constituent of blood are platelets, which assist in haemostasis through platelet plug formation and coagulation.2,41 The formed cellular elements are suspended in plasma, which is responsible for transporting electrolytes, hormones and nutrients, removing waste products,2 regulating pH and temperature, and maintaining fluid balance.42
5.2. Chemical Changes Associated with Blood Degradation

Many forensic presumptive tests for blood rely on the peroxidase-like properties of haemoglobin, which is an oxygen transporting protein in erythrocytes.\textsuperscript{43} Haemoglobin consists of two alpha (α) and two beta (β) chains that are conjugated with a haem moiety.\textsuperscript{41} Inside a healthy human body, haemoglobin may be saturated with oxygen, oxyhaemoglobin (HbO\textsubscript{2}) or without oxygen, de-oxyhaemoglobin (Hb).\textsuperscript{44} Oxy-haemoglobin is a low-spin ferrous (Fe\textsuperscript{2+}) compound that can be oxidised into met-haemoglobin (met-Hb), which is a high-spin ferric (Fe\textsuperscript{3+}) protein incapable of binding oxygen.\textsuperscript{45} When formed inside the body, the NADH-dependent enzyme cytochrome-b5 reductase can reduce met-Hb back to Hb. Upon exiting the body, haemoglobin saturates with oxygen, and the transition of HbO\textsubscript{2} into met-Hb will no longer be reversed due to the decreasing availability of cytochrome-b5.\textsuperscript{44,46} Over time, met-Hb will denature to hemichrome (HC).\textsuperscript{45} Figure 5.2 provides a schematic representation of the oxidative processes in haemoglobin.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.2}
\caption{Oxidative processes in haemoglobin. A) Oxidative process occurring \textit{in vivo}. B) Oxidative process occurring \textit{in vitro} (adapted).\textsuperscript{46}}
\end{figure}
5.3. Biological Screening for Blood

The development of multiple screening techniques has enabled forensic investigators to determine the type of biological material that may be present within a potential crime scene, or on items that pertain to a criminal investigation. Bloodstains at a crime scene may be aged, degraded, or diluted and subsequently no longer characteristic of blood. Blood identification involves a series of steps including visual examination, presumptive and confirmatory testing, species determination, and individualisation testing.\textsuperscript{47} Table 5.1 details the presumptive, confirmatory, and species determination assays available for blood identification and details the associated limitations.
Table 5.1. Presumptive, confirmatory, and species determination assays available for blood identification and the associated limitations.\textsuperscript{43,48,49}

<table>
<thead>
<tr>
<th>Classification</th>
<th>Assay</th>
<th>Reaction</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presumptive</td>
<td>Tetramethylbenzidine (Hemastix\textsuperscript{®})</td>
<td>Produces a yellow to green or blue-green colour change as tetramethylbenzidine is oxidised.</td>
<td>Products containing hypochlorite ions or hydrogen peroxide, certain metal salts, and some plant peroxidases can catalyse the oxidation reaction, producing false-positive results. Strong reductants such as zinc or lithium may inhibit the oxidation reaction and produce false-negative results.</td>
</tr>
<tr>
<td></td>
<td>Phenolphthalein (Kastle-Meyer test)</td>
<td>Shows a pink colour change as the colourless phenolphthalin is oxidised to phenolphthalein.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucomalachite Green</td>
<td>Shows a green colour change as the colourless leuco base form of malachite green is oxidised.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluorescence (Fluorescin)</td>
<td>Fluorescin is oxidised to fluorescein, which emits a yellow-green light when exposed to a light range of 425 to 485 nm.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemiluminescence (Luminol)</td>
<td>The oxidation reaction of luminol produces a blue-white light.</td>
<td></td>
</tr>
<tr>
<td>Confirmatory</td>
<td>Microcrystal (Takayama and Teichmann tests)</td>
<td>Bloodstains are chemically treated forming crystals of haemochromagen or haematin. The crystal morphologies are compared to a standard using microscopy.</td>
<td>Confirmatory assays lack species specificity and are considered less sensitive than presumptive assays.</td>
</tr>
<tr>
<td>Species</td>
<td>Immunochromatographic: Identification of human haemoglobin. (Hexagon OBTI\textsuperscript{®} and ABAcard\textsuperscript{®} HemaTrace\textsuperscript{®})</td>
<td>Utilises the binding between an antigen and its homologous antibody. A pink precipitin line in the test and control areas indicates a positive result.</td>
<td>High concentrations of haemoglobin may produce false-negative results, while higher primate and ferret blood may produce false-positive results. Requires the epitope to be undamaged.</td>
</tr>
<tr>
<td>Determination</td>
<td>Immunochromatographic: Identification of Human Glycophorin A Protein (RSID\textsuperscript{™} test)</td>
<td>Detects the glycophorin-A protein present in the erythrocyte membrane.</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{43,48,49}
6. Differentiation of Blood and Decomposition Fluid Stains

Determining the origin and type of biological evidence can yield valuable information that will supplement forensic casework. A bloodstain may indicate assault, physical struggle, or murder,\(^3\) whereas a decomposition fluid stain is essentially an artefact of the naturally occurring decomposition process. Possible techniques for the differentiation of these fluids are evaluated below.

6.1. Visual Examination

Sutton\(^{47}\) detailed that visual examination was the first step in bloodstain identification. Alterations in the haemoglobin as a bloodstain ages results in a progressive colour change from red to reddish-brown to green and finally dark brown. The duration and sequence of the colour changes are affected by environmental conditions and the presence of microorganisms and bacteria.\(^{39}\) Decomposition fluid also progresses through a series of colour changes from red-burgundy to brown.\(^{13}\) The similar colour changes exhibited by both fluids over time does not permit visual identification as a method of biological fluid determination and differentiation.

6.2. pH Measurement

Ante-mortem blood has a pH of between 7.35 and 7.45.\(^{50}\) After death, the pH of blood drops due to the accumulation of phosphoric and lactic acid from the breakdown of fatty acids and amino acids, and the accumulation of CO\(_2\) from glycolysis and glycogenolysis. With the onset of putrefaction, muscle proteins degrade resulting in the build-up of ammonia, which in turn causes the blood pH to become more alkaline.\(^{51}\) Similarly, alkaline pH measurements of decomposition fluid samples were reported by Comstock.\(^{13}\) Prior to
insect colonisation pH measurements were neutral and the trends were similar between experimental groups. Measurements diverged following insect colonisation, whereby the pH levels became more alkaline. The carcasses completely excluded from insect activity did not display a sharp increase in pH. Despite this research focusing on the liquid form of these samples, the results still highlight the variability of pH measurements due to the influence of extrinsic factors. In addition, Karmakar noted that post-mortem changes in blood pH do not follow a definite sequence. Consequently, pH analyses would lack the specificity required to definitively differentiate blood and decomposition fluid stains.

6.3. Presumptive Testing for Blood

Cranstoun researched in part the effect of decomposition on the presumptive detection of biological fluids. Strips of fabric stained with saliva, semen or blood were attached to a t-shirt and placed on top of and beneath pig carcasses. The carcasses were left to decompose over an eight-week period in an open coastal dune area. Post collection, the blood section of each strip was removed and tested using the Combur® Test®E and Kastle-Meyer presumptive tests for blood. The author noted that decomposition fluid contains blood breakdown products and consequently, all areas of the bloodstain would test positive if they had been in contact with the fluids released during decomposition. As such, an area outside of the original bloodstain referred to as ‘Other’ was tested for comparative purposes. Samples from the upper side of the carcasses produced a stronger reaction with the original bloodstain area using the Combur® test strips. Equally strong reactions were recorded for samples collected from beneath the carcasses using the Combur® test strips, and for most samples using the Kastle-Meyer reagent. Additional strips were analysed using Luminol to determine whether a pattern of body fluid staining could be identified.
and distinguished from decomposition fluid staining. Pattern recognition was only possible for six days, after which results produced an indiscriminate chemiluminescence appearance, suggesting a reaction was occurring with the decomposition fluid.

Bemelmans\textsuperscript{22} also assessed in part how screening for biological fluids may be affected by the process of decomposition. A porcine model was used to simulate human decomposition to ensure the samples were correctly being identified as originating from the deposited stains, as opposed to the decomposing remains. Blood samples were placed on top of or beneath a pig carcass or a similarly weighted bag of sand, which served as the control. Presumptive testing using the Kastle-Meyer reagent and ABACard\textsuperscript{®} HemaTrace\textsuperscript{®} confirmatory testing were performed on any areas of red-brown staining. Positive presumptive and confirmatory results were obtained for all samples collected from the top of the pig carcass and control. Samples from beneath the pig carcass returned positive presumptive results, and positive confirmatory results through day 10, however, results were negative thereafter. Positive confirmatory results were only returned on days 1 to 11, 13 and 16 for samples beneath the control. The dilution and degradation of the samples was attributed to the compounding effects of rainfall, varying temperatures, soil properties, and the products of decomposition.

All samples returned positive presumptive results, thus suggesting that biological testing may still be possible despite exposure to decomposition products and environmental insults. However, it is unknown if blood breakdown products present in the decomposition fluid influenced these results. Furthermore, it is unknown what effect the constituents of decomposition fluid would have on confirmatory testing, as any haemoglobin present in
the fluids produced by the decaying porcine remains would not have reacted with the human-specific test. Had this research been conducted using human cadavers, it is possible more positive confirmatory results would have been returned. However, the sensitivity of the assay may detect the blood breakdown products present in human decomposition fluid, thereby producing false-positive results. The above research highlights that conventional biological screening may not be sufficient to conclusively determine if blood evidence is present.

In recent years, several approaches have been researched with the aim of developing more reliable and sensitive techniques that overcome the limitations of current human biological fluid identification methods. Emerging approaches that may be utilised for the differentiation of blood and decomposition fluid stains include spectroscopy, the analysis of volatile organic compounds, messenger- and micro-RNA expression profiles, epigenetic modifications of DNA markers, and protein biomarker detection.

6.4. Spectroscopic Techniques

When irradiated by ultraviolet light, most biological fluids will undergo absorption processes or fluorescence, which enables their rapid and widespread detection. Ultraviolet-visible (UV-Vis) spectroscopy has been applied to the detection of sweat, saliva, semen, urine, and blood stains. However, this technique is more applicable for presumptive identification and exploratory purposes, due to the associated limitations including substrate interference, poor selectivity and specificity, and false-positive results. Vibrational spectroscopies, such as Raman and infrared spectroscopy appear to overcome the low specificity of UV-Vis. These techniques report on the molecular vibrational
characteristics within a sample and are rapid, selective, and non-destructive.\textsuperscript{54,55} However, variability between donors and the heterogeneity of dried biological fluids contribute to the complexity of the Raman spectra and as such, advanced statistical analyses are required to build a unique spectroscopic signature.\textsuperscript{56} Mathematical methods of multivariate analysis and chemometrics allow researchers to extract useful information and interpret complex spectral data, thus allowing for more accurate and comprehensive results.\textsuperscript{55} Using statistical analyses, the Raman spectral signatures of saliva, semen, vaginal fluid, and blood have been established and species determination achieved.\textsuperscript{57-61}

Decomposition products may also be studied using spectroscopic methods. Infrared spectroscopy can be used to identify carbohydrates, proteins and lipids, and the compounds that result from the degradation of these macromolecules. In addition, spectroscopy may be used to determine the compounds within a sample and to observe how these compounds change with time.\textsuperscript{12} Porcine decomposition fluid samples were analysed using attenuated total-reflectance-infrared spectroscopy (ATR-IR) and GC-MS.\textsuperscript{13} However, the trends observed from the spectroscopic data between decomposition trials yielded inconsistent results.

Spectroscopic techniques are limited in that biological fluids do not appear as isolated substances, and the substrate on which the stain is formed or absorbed can contribute to the vibrational spectra.\textsuperscript{54} The spectroscopic signal of biological fluids may also be completely masked by contaminants, an issue further complicated by the varied composition of possible contaminants.\textsuperscript{62} Moreover, decomposition fluid is a chemically complex matrix that is thus far not completely understood. Additional comprehensive
research investigating the validation and optimisation of spectroscopic techniques is needed. To demonstrate the capabilities of spectroscopy, it is necessary to test samples that more closely resemble evidential samples from forensic casework. Various spectroscopic methods may provide useful trend information, however to gain a more thorough understanding of decomposition chemistry and in turn to differentiate between blood and decomposition fluid stains, more selective analytical techniques may be required.\textsuperscript{9}

6.5. Analysis of Volatile Organic Compounds

The complex series of biological processes and chemical reactions that occur during decomposition contribute to the evolution of odorous chemicals, referred to as volatile organic compounds (VOCs).\textsuperscript{63} These compounds arise from the catabolism of the main biological macromolecules.\textsuperscript{12} To date, results from chemical examination and comparison have shown that decomposition fluid and human remains produce similar odour profiles.\textsuperscript{27} In 2004, Vass et al.\textsuperscript{64} established the Decomposition Odour Analysis (DOA) Database, which identifies and details chemicals associated with human burial decomposition. Analysis by GC-MS identified eight major classes of chemicals containing 424 specific volatile compounds. Continued research aimed to define the volatile chemical signature released during human burial decomposition.\textsuperscript{65} Of the 478 semi-volatile or volatile compounds detected, 30 were identified as important indicators of human decomposition. However, it was acknowledged that the selected compounds were also detected in various environmental VOC profiles. Further research demonstrated that the decomposition odour profile is dynamic and that there is a transition in the compounds produced as decomposition progresses.\textsuperscript{66-68}
Decomposed remains and blood have distinct scent profiles that do not closely resemble each other\textsuperscript{27} and as such, VOC profiling may be employed to differentiate between decomposition fluid and blood. Forbes et al.\textsuperscript{69} chemically profiled human blood and identified that aged and fresh blood samples demonstrated different VOC profiles, with aged samples exhibiting increased complexity. However, further research indicated that after ageing for up to one year, the complexity was lost.\textsuperscript{70}

Variability in environmental conditions, substrate, insect activity, and the physical characteristics of the remains contribute to changes in the evolution and liberation of VOCs.\textsuperscript{64} Decomposition odour research has provided a guideline for determining what chemicals will be produced and their concentration ranges.\textsuperscript{71} However, a consistent decomposition VOC profile is still lacking, which may reflect variation in analytical methodology or decomposition variables.\textsuperscript{63} There are also inconsistencies in the type and number of volatile compounds observed in blood odour profiles, which may be attributed to physiological differences between donors, the analytical technique, or differences in the targeted compounds.\textsuperscript{70} This method may not be applicable for stain identification and differentiation, as much research remains to be conducted in the field of decomposition chemistry and VOC profiling. Moreover, VOC profiling is predominantly utilised for detection dog training and the location of clandestine burial sites.\textsuperscript{71}

\textit{6.6. Genomics}

Advancements in forensic genetics have led to the development of new techniques that involve the detection and analysis of messenger RNA (mRNA), microRNA (miRNA), and differential DNA methylation patterns.\textsuperscript{3} These tissue-specific approaches to biological fluid
identification can supplement DNA analyses to provide information regarding cell type and origin, which in turn may assist with the inference of activities.\textsuperscript{72,73} Despite being considered less stable than DNA due to ubiquitously present ribonucleases, the stability and recoverability of RNA in biological stains has been demonstrated.\textsuperscript{74}

\textbf{6.6.1. mRNA-based Analysis}

Biological fluids usually contain multiple cell types, each of which expresses a distinctive pattern of mRNA transcripts. The development and implementation of mRNA profiling is based on harnessing these multicellular transcriptomes.\textsuperscript{56} Multiple RNA transcripts have been detected using real-time polymerase chain reaction (PCR)\textsuperscript{75,76} or reverse-transcriptase-PCR.\textsuperscript{75,77} The most frequently proposed RNA markers for blood are generally divided into proteins associated with the haem biosynthesis pathway and haemoglobin, or proteins associated with the erythrocyte membrane.\textsuperscript{56} A collaborative exercise on mRNA profiling for the identification of blood demonstrated that 15 of the 16 participating laboratories were able to detect and isolate RNA from dried bloodstains, thus highlighting the potential for mRNA profiling in forensic casework.\textsuperscript{78} When compared to conventional biochemical analysis, mRNA profiling offers high specificity and sensitivity, the possibility of detecting various biological fluids in one multiplex reaction, compatibility with DNA extraction methods, and the potential for automation, all of which can preserve the sample and improve timeliness.\textsuperscript{3,48,74} Despite several mRNA markers having been proposed as specific, sensitive, and stable methods for forensic biological fluid determination,\textsuperscript{3} it is still possible that UV radiation, moisture, and heat will influence mRNA marker stability.\textsuperscript{79} Other limitations associated with mRNA analysis include difficulties in profile
interpretation, destructiveness of the technique, and that there are no validated guidelines for the consistent interpretation of mRNA analysis data.\textsuperscript{80}

6.6.2. miRNA-based Analysis

An alternative option to mRNA for the identification of evidential samples are miRNAs, which are a class of small, non-coding RNA molecules approximately 18 to 25 nucleotides in length. The tissue-specific expression pattern and small size, make miRNA less prone to environmental degradation.\textsuperscript{81} Analyses can be performed using the same methodology employed in traditional RNA and DNA analysis, thereby reducing additional consumables and equipment.\textsuperscript{80} However, a key limitation to the use of miRNA analysis is the specificity for species and biological fluids, as any given target may have multiple miRNAs and a single miRNA may have multiple targets.\textsuperscript{56} Reproducible and consistent results have not been demonstrated in the literature, which reflects the need for further research to establish appropriate candidate markers.\textsuperscript{80}

6.6.3. Epigenetic Analysis

Epigenetic differences have been identified between biological fluids of forensic interest and as such, DNA methylation, which is an epigenetic modification, has been explored for the purpose of biological fluid identification.\textsuperscript{56} DNA methylation is the addition of methyl (CH$_3$) to the 5'-position of the pyrimidine ring of cytosine in CpG dinucleotides.\textsuperscript{3,80} Bisulphite sequencing or the use of a methylation-sensitive/dependent restriction enzyme followed by PCR are the predominant methods for detecting methylation. Advantages of epigenetic analysis include co-analysis with the STR amplification and a level of sensitivity comparable to DNA profiling. However, results may be compromised by low-level samples
or samples with degraded or excess template, inhibition, or incomplete restriction, as these factors can distort the methylation ratios.\textsuperscript{56} DNA methylation based methods could be a valuable technique for the characterisation of biological fluids, yet for forensic casework application further validation studies exploring differential methylation patterns and tissue-specific methylated DNA loci are required.\textsuperscript{3,80}

Interpretational challenges are associated with the use of mRNA, miRNA, and DNA methylation assays. Variations in the environment, physiology, or genetic constitution can result in altered m(i)RNA or DNA methylation levels. The discriminatory value of markers may also be nullified or reduced by the presence of multiple sources.\textsuperscript{72} At present, the application of these techniques may not be possible for the differentiation of blood and decomposition fluid stains, however, these assays should be considered when developing new approaches.

\textit{6.7. Proteomics}

Protein biomarkers are among the promising approaches for biological stain identification.\textsuperscript{53} Proteomic analysis of biological samples involves the identification, separation, and characterisation of proteins.\textsuperscript{82} Each biological fluid has a unique protein signature, which is attributed to the specific proteins within the sample, or to the different combinations and relative abundance of proteins.\textsuperscript{83} Multiple studies have aimed to identify and isolate candidate high-specificity protein biomarkers for the reliable identification of biological fluids.\textsuperscript{49,53,84,85} Using a comparative MS-based whole proteome approach, 29 candidate protein biomarkers were proposed as highly specific indicators for trace quantities of urine, seminal, vaginal and menstrual fluid, peripheral blood, and saliva.\textsuperscript{53} In
2013, Yang et al.\textsuperscript{84} attempted to define multiple markers for saliva, semen, and blood using liquid chromatography matrix-assisted laser desorption/ionisation mass spectrometry (LC-MALDI-MS). At a greater than, or equal to 95% confidence interval, results identified 59 distinct proteins in blood. Relative to each biological fluid, the alpha ($\alpha$) and beta ($\beta$) subunits of haemoglobin have been considered to be the most abundant markers for blood.\textsuperscript{49,84,85} It has been demonstrated that protein biomarker detection using MS-based technology is reproducible, sensitive, and accurate.\textsuperscript{49} The identification of reliable protein biomarkers commonly encountered in evidentiary stains has the potential to assist in the development of a unified multiplexed approach to forensic biological fluid identification. However, it is possible candidate biomarkers may be released in non-target samples and as such, additional larger-scale studies are required.\textsuperscript{53}

7. Conclusions

The identification of possible blood evidence is a critical component to an investigation in which the ante-mortem events of the decedent are unknown.\textsuperscript{52} It is important to determine whether a stain observed next to decomposing remains is blood or decomposition fluid, as blood may suggest an injury has occurred before or shortly after death, whereas decomposition fluid is naturally released during the process of decomposition. However, research pertaining to the explicit differentiation of blood and decomposition fluid is currently lacking. Decomposition chemistry research has attempted to elucidate the physical and chemical properties of decomposition fluid. However, inconsistencies are evident across the literature concerning the compounds present throughout the various stages of decay and the potential trends exhibited by these compounds, thus highlighting the chemical complexity of decomposition fluid. This, in
conjunction with the literature suggesting that decomposition fluid contains blood breakdown products,\textsuperscript{52} poses interpretational challenges for forensic investigators and complicates the determination of the origin of staining.

Despite their usefulness, current presumptive and confirmatory assays are substantially impacted by environmental insults, may suffer limitations associated with sensitivity and specificity, and may only apply to a subset of samples.\textsuperscript{86} Several approaches have been investigated with the primary aim of developing more reliable and sensitive techniques for the identification of biological fluids,\textsuperscript{53} yet much research remains to be conducted in the fields of decomposition chemistry and forensic biology. This manuscript evaluated several methods for their potential use and effectiveness in the differentiation of blood and decomposition fluid stains. The associated limitations of these techniques suggest that they are not currently viable options for determining the origin of staining. However, protein analysis and more specifically microfluidic proteomics by protein electrophoresis, appear to hold a promising opportunity to move closer to developing a technique that may identify a potential biosignature that will enable the differentiation of these fluids.

Continued research and a more comprehensive understanding of the basic properties of these fluids and how they degrade over time, will better enable their differentiation and application to stain identification. Once a more thorough understanding has been established, future research should seek to conduct analyses using human samples. This will assist in ensuring that any differences identified in porcine samples accurately reflect those that would be observed in human cadavers and in turn forensic investigations.
References


