The effect of temperature and headspace on the determination of ethanol in post-mortem blood specimens: A South African perspective

By

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(Submission for MSc Med research dissertation)

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10 April 2019

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Masters in Forensic Medicine and Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

The financial assistance of the National Research Foundation (NRF), Health and Welfare Sector Education and Training Authority (HWSETA) and the National Institute for Occupational Health (NIOH) a division of the National Health Laboratory Services (NHLS) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF, HWSETA, NIOH or NHLS.

10 April 2019

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ABSTRACT

The Forensic Chemistry Laboratories in South Africa have, between the year 2014 and 2017, endured a lot of media scrutiny surrounding a backlog of specimens for blood alcohol and toxicology analyses and the poor environmental and storage conditions in which these specimens are kept. Many studies have been performed on the stability of alcohol in blood, since environments are not standard, to gain a better understanding on whether the backlog issues significantly impact on the integrity of the blood-alcohol concentration (BAC) results by evaluation of conditions, especially variables such as temperature and headspace. The aim of this study was therefore, to assess the stability of ethanol concentrations in post-mortem blood specimens by evaluating temperature (room and refrigerator) and headspace (4mL and 8mL) variables at 3 months and 6 months respectively. Blood from 119 decedents was collected, analysed and subjected to the varied volumes and storage conditions. Blood alcohol was determined and quantified using a G1888 Headspace Auto sampler (Agilent Technologies®) coupled to a 6890N Agilent® Gas Chromatography instrument utilising a Flame Ionization Detector on an Agilent HP-Innowax® column. A general decrease in alcohol concentration was observed over a storage period of 6 months regardless of the storage temperature, whilst headspace was found to have no significant effect on the BAC results. It is, therefore, important that Forensic Pathologists, investigators and scientists are aware of factors such as temperature and headspace and consider them when interpreting blood alcohol results from a post-mortem environment.

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ABBREVIATIONS AND NOMENCLATURE

%Bias	Percentage bias of the measurement
ADH	Alcohol dehydrogenase
ALDH2	Aldehyde dehydrogenase 2
BAC	Blood Alcohol Concentration
CO_2	Carbon dioxide
CRM	Certified Reference Material
CYP2E1	Cytochrome P450 2E1
DF	Degrees of Freedom
FAEEs	Fatty acid ethyl esters
FCL	Forensic Chemistry Laboratories
FPM	First Pass Metabolism
GC	Gas chromatography
H_2O	Water
H_2O_2	Hydrogen peroxide
He	Helium
HSGC-FID	Headspace gas chromatography with flame-ionisation detection
IQR	Inter quartile range
JFPS MLL	Johannesburg Forensic Pathology Services and Medico legal Laboratory
K_2EDTA	Dipotassium ethylene diamine tetra acetic acid
LOD	Limit of Detection
LogP	Logarithm of the Partition coefficient of a molecule between an aqueous and lipophilic phase
LOQ	Limit of Quantification
n	Number of participants/analysis
MBA	Motor Bicycle Accident
MVA	Motor Vehicle Accident
NAD+	Nicotinamide Adenine Dinucleotide
NMISA	National Metrology Institute of South Africa
pKA	Measurement of acid strength
PM	Post-mortem
PVA	Pedestrian Vehicle Accident
QC	Quality Control
ROS	Reactive Oxygen Species
SANAS	South African National Accreditation System
SOP	Standard Operating Procedure
$U_{precision}$	Imprecision of the measurement
U_{purity}	Uncertainty surrounding the purity of the standards
USA	United States of America
VH	Vitreous Humour
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

Alcohol (ethanol) is one of the leading abused compounds in the world making it the most universally tested for and analysed drug in a forensic setting. Determination of alcohol in blood (better known as the blood alcohol concentration (BAC)) is very important in a post-mortem setting, as alcohol consumption is often suspected in an unnatural death (Pontes *et al.*, 2009). Unnatural deaths (such as; motor vehicle accidents, violent crimes, suicide, fatal mishaps and drowning) are deaths which may result from an external force, any physical factors, medical negligence or incorrect treatment; sudden or unexpected death and/ or if it is suspected that an individual has died under the influence of a chemical substance and must be subjected to a medico legal autopsy (National Health Act no 61, 2003).

Designated pathologists and dissectors at the various medico legal laboratories within the country perform these medico legal autopsies. The primary role of these medico legal laboratories is to conduct medico legal investigation on all unascertained or unnatural deaths which are regulated by the The National Health Act No 61 of 2003. The Johannesburg Forensic Pathology Services Medico Legal Laboratory (JFPS MLL) provides services for the greater Johannesburg metropolitan area (see Figure 1) and serves at the site of study for this dissertation.

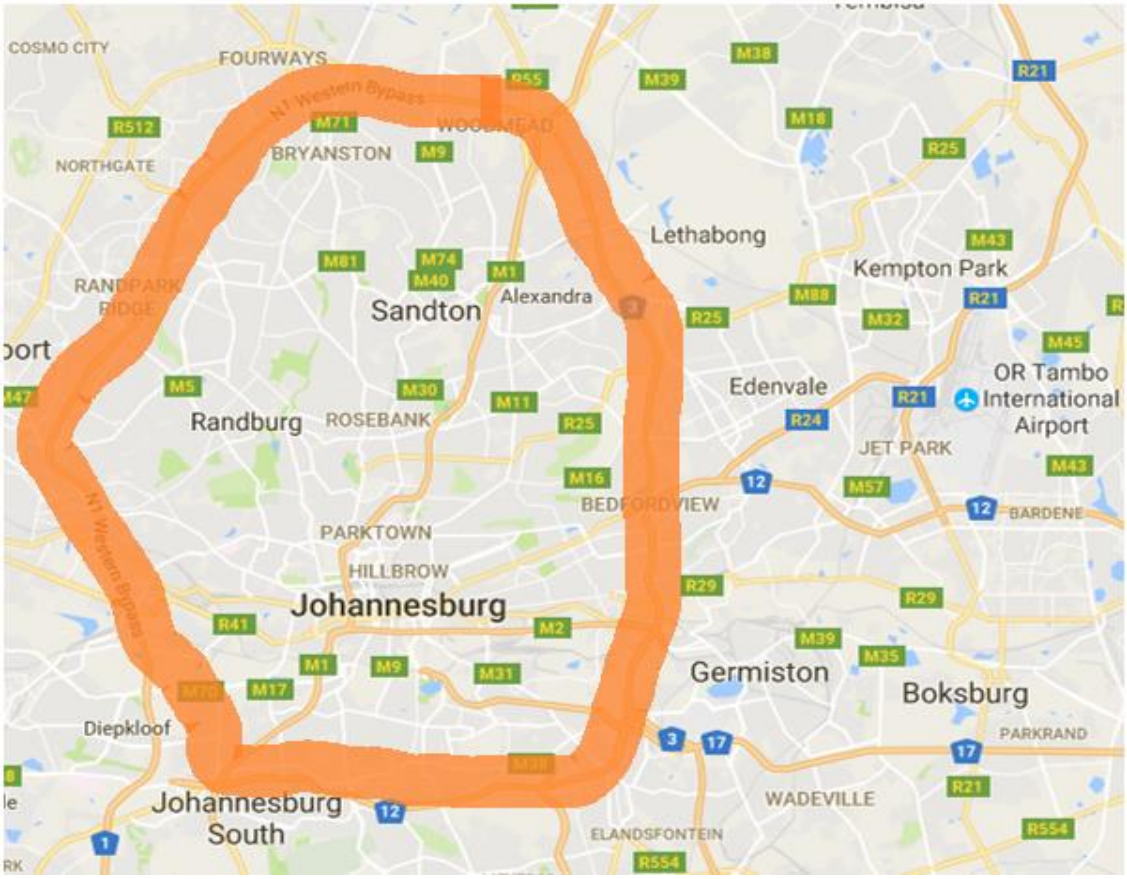

Figure 1: Map of general areas falling within the jurisdiction of the JFPS MLL

To determine if alcohol was primarily responsible or contributed to the death of an individual, accurate ethanol concentration should be determined as this will assist in the determination of guilt and accountability (Winek and Paul, 1983). Blood is the most commonly collected biological specimen (ante-mortem and post-mortem) used for toxicological and alcohol analysis (Olsen and Hearn, 2003). According to South African media reports, a backlog of 60 000 blood specimens which are ante- and post-mortem in origin (toxicology and blood alcohol analysis) exist at the South African National Department of Health (NDOH) Forensic Chemistry Laboratories (FCL) as of February 2014 (Serrao, 2014).

More than 10 000 of these specimens are reportedly for blood alcohol testing alone. The delays in testing produce a significant amount of uncertainty about the integrity of the results obtained after testing, primarily from allegations on the lengthy periods elapsed prior to analysis as well as the environmental storage conditions. Conditions such as the storage temperatures of the specimens are key to the integrity of results (Serrao, 2014). The public media reported these findings in 2016; two years later, there have been few signs of improvement of conditions at these laboratories, although, the aim is to reach a general turnaround time for reporting blood-alcohol results within three months (Serrao 2016).

There is also no standard operating procedure (SOP) within the medico legal laboratories of South Africa regarding the collection of biological samples, as is the case in the JFPS MLL. The Forensic Officers rely on the instructions of pathologists when collection biological specimens or rely solely on their internal training received.

Since no policy or SOP is standard in the mortuaries, own discretion on type of biological specimen, volume of biological specimen and location of biological specimen is followed. This lack of standardisation can lead to varying results at Forensic Chemistry Laboratories, to which these samples are sent for testing. These reasons ultimately suggests that the integrity of the results of analytical test results of these specimens potentially remains compromised due to samples being unsuitable for analysis (Serrao, 2016); which could have an effect on legal and insurance proceedings and an emotional impact on families.

Many studies have been performed on the stability of alcohol in blood under various environmental conditions. The environmental conditions (sampling conditions in post and ante-mortem), specimen containers (glass or plastic), volume of specimen collected (to ensure limited physical air space above the specimen in the collection container, known as headspace) and storage conditions (temperature) are not standard within each country; hence specifically evaluating these aspects and the determination of the stability of ethanol in post-mortem blood under local conditions, enable a better understanding as to whether the backlog issues and the lack of procedures within the JFPS MML mentioned previously are significantly impacting on BAC results and reporting to Pathologists and the Justice system.

The aim of this study is, therefore, to assess the stability of alcohol (ethanol) concentrations in post-mortem blood specimens by evaluating temperature and specimen-container headspace variables with respect to time, under conditions specific to the South African environment.

The hypothesis is that temperature and headspace will have a significant effect on the total ethanol concentration with respect to time.

This research and the data generated will seek to inform the medico legal fraternity e.g., pathologists, scientists and attorneys on the variability of post-mortem alcohol results and the effect these variables such as temperature and headspace may have on the alcohol concentrations.

This research also sets out to highlight the effect of not having standard operating procedures (SOP's) within the South African mortuaries when collecting specimens for BAC and toxicological testing.

Applicable key terms for each chapter will be emphasised at the start of each chapter, however the most important key terms that are utilised throughout this study are the following:

'Alcohol'

Ethanol or Ethyl alcohol is a primary alcohol, as the alkyl moiety is an ethyl ($-CH_2CH_3$) group. Ethanol is mostly referred to as "alcohol" (Wade, 2014).

'Blood alcohol concentration (BAC)'

The quantity of alcohol in a person's blood stream, often expressed in terms of percentage or more common in gramps per 100 millilitres of blood (McDonald, 2018)

'Headspace'

Headspace refers to the amount air above the blood specimen within a sample container (Sutlovic, Versic-Bratincevic and Definis-Gojanovic, 2014).

‘Headspace gas chromatography with flame-ionisation detection (HSGC-FID)’

The gold standard technique of alcohol determination in blood is performed using headspace gas chromatography with flame–ionisation detection (HSGC-FID). This method is standard for the determination of alcohol levels in ante-mortem and post-mortem specimens (Tisicone *et al.*, 2013).

Chapter 2 will be used to outline the literature surrounding the chemistry of ethanol, the reasons for testing BAC and the variables affecting the BAC. Chapter 3 then explains the study design and the methodology used to address the aim and the objectives of this study. The results generated and critical evaluation of the results will be explained in Chapter 4 and Chapter 5 respectively. Chapter 6 will include the limitations surrounding the sampling and analysis part of this study as well as include future recommendations for further research. Chapter 7 provides the final concluding paragraph.

CHAPTER 2: LITERATURE REVIEW

This chapter outlines the literature surrounding the chemistry of alcohol, the physiological effects of alcohol consumption, the reasons and methodology surrounding the testing of alcohol in blood and the variables affecting the blood alcohol concentration (BAC).

The applicable key terms used in this chapter are the following:

'Ethanol'

Ethanol, or better known as alcohol is a primary alcohol with an ethyl ethyl (-CH2CH3) group (Wade, 2014).

'Hydrophilic'

A compound or molecule that is attracted to water (H2O) (Larri, 2016)

'Pharmacokinetics'

Area of pharmacology that describes the movement of a drug within the human body by looking at aspects such as absorption, distribution, metabolism and excretion (Kent, 2012)**.**

'Vitreous Humour'

Vitreous humour (VH) is the fluid between the retina and the lens within the globe of the eye (Collins, 2016).

‘Post-mortem kit (PM kit)’

Polystyrene container containing a 20 mL McCartney bottle, adhesive labels, document FCL003, as well as on document FCL004 which is the instruction information sheet for the usage of the kit and all seals for the collection of biological fluid for BAC determination.

‘Internal standard’

A known concentration of a compound that behaves similarly to the compound of interest, are added to all unknown samples (O’Neal *et al.*, 1996)**.**

‘In vitro’

Latin term that means “within the glass”, which in this dissertation will refer to within a 20 mL McCartney bottle contained within a PM kit.

‘Method validation’

The process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use (Huber, 2010).

2.1. ALCOHOL

Alcohol is the most popular drug of choice worldwide, and the various perspectives surrounding alcohol are addressed in this section.

2.1.1. Chemistry

Alcohols are a class of organic compounds that are recognised by the presence of one or more of the hydroxyl (-OH) groups attached to a carbon atom of an alkyl group. Alcohols are classified into three groups namely: primary, secondary and tertiary. The most typical forms of alcohols are ethanol and methanol. Ethanol is mostly referred to as “alcohol” (Wade, 2014). Ethanol or Ethyl alcohol (Figure 2) is a primary alcohol, as the alkyl moiety is an ethyl ($-CH_2CH_3$) group.

Ethanol is commonly found in alcoholic beverages and can be tested for in a variety of biological specimens (Wade, 2014). Ethanol has hydrophilic and lipophilic properties. The lipophilic properties allow alcohol to pass through the gastrointestinal tract into the blood stream through passive diffusion, therefore requiring no energy to move across cell membranes.

The hydrophilic properties contribute to its complete solubility in water (Wongchanapai, 2008 and Kent, 2012).The physical properties of Ethanol are given in Table 1.

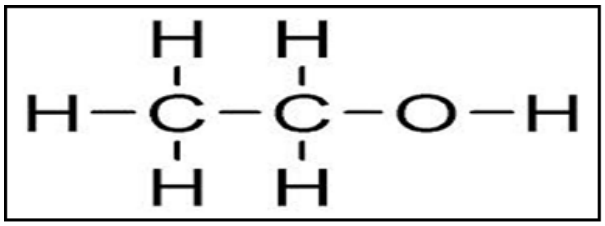

Figure 2: Chemical structure of Ethanol (Alcohol)

Table 1: Properties of Ethanol (National Center for Biotechnology Information, 2015)

Molecular formula	C_2H_6O
Molecular weight	46.07g/mol
Boiling point	78.29 °C
Density	0.79 g/cu cm
LogP	-0.31
pKa	15.90 (at 25°C)

2.1.2. Alcohol Formation in Alcoholic beverages

Alcohol is formed when the starches in wheat, rye and barley (grains) are broken down to sugar maltose and then converted to glucose through an enzymatic action. This process takes place in yeast where the yeast cells utilise glucose and convert it to alcohol (Figure 3) and form the fundamental step in the preparation of alcoholic beverages; such as beer and malt (Held, 2012)

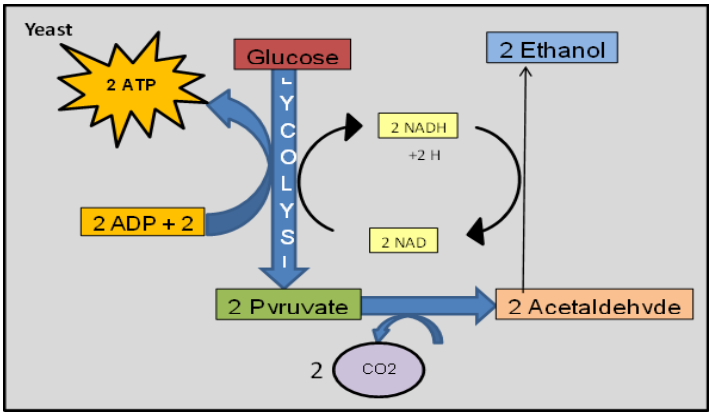

Figure 3: Alcohol Fermentation in yeast for production of alcoholic beverages (adapted from Held, 2012)

2.1.3. Pharmacokinetics

The various aspects of pharmacokinetics that include administration, absorption, distribution, metabolism and elimination will be addressed in this section.

2.1.3.1. Administration

People consume alcohol for various reasons such as part of the socialising processes or to relieve stress (Carpenter and Hasin, 1998). The most common route of administration is oral e.g. drinking alcohol (Kent, 2012), however other routes such as inhalation, transdermal, injection and given as an enema does occur on occasion (Harm Reduction for Alcohol, 2015). Inhalation occurs with the assistance of an inhalation device, which results in quicker intoxication without feeling sick the following day.

Alcohol can be injected; this remains extremely dangerous due to the result of potential alcohol poisoning from the influx of a large concentration of ethanol to the blood circulation. An enema has recently been reported at social parties in America but this, too, remains a dangerous route of administration due to a lack of enzymes to metabolise the alcohol in the large intestine and rectum. Alcohol can also be absorbed through the skin but is not a preferred method for intoxication occasion due to the slow absorption rate and this method is not practical to result in a state of alcohol inebriation or intoxication (Harm Reduction for Alcohol, 2015).

2.1.3.2. Absorption

When alcohol is taken orally, the primary routes of absorption are in the stomach and the small intestine (Kent, 2012). The absorption rate in the small intestine is faster than the absorption rate in the stomach due to the large surface area.

This large surface area is primarily due to finger-like projections called villi (Harm Reduction for Alcohol, 2015). The stomach does not have these finger-like projections therefore resulting in a lower absorption rate of alcohol (Harm Reduction for Alcohol, 2015).The presence of food in the stomach can also contribute to the slower absorption rate since the presence of food in the stomach prevent the valve between the stomach and small intestine, called the pyloric valve, from opening. This prevents gastric emptying form taking place and reduces the absorption rate in the stomach (Kent, 2012). There are various other inherent and external factors that affect alcohol absorption within an individual, which can be subdivided into the following categories:

I. Factors that result in a decrease in absorption rate of alcohol

Any food that is present in the stomach will reduce the absorption rate of alcohol due to the delay in gastric emptying as well as the type of food such as oily and fatty foods like meat, French fries and bread plays a role in lowering the absorption rate. These types of foods take longer to exit the stomach and therefore lowering the absorption rate (Harm Reduction for Alcohol, 2015). Some properties of alcohol that cause irritation to the stomach lining leading to haemorrhage or paralysis of the smooth muscles within the stomach which therefore can also result in a decrease in the rate of absorption (Cederbaum, 2012).

II. Factors that result in an increase in absorption rate

The concentration of alcohol affects the concentration gradient between the stomach and the circulatory system, which means that the higher the concentration of alcohol in the drink, the greater the concentration gradient. A concentration gradient is defined here as an area such as the stomach containing high number of alcohol molecules that will move (diffuse) to an area such as the circulatory system with a lower number of alcohol molecules. This process ultimately increases the absorption rate (Harm Reduction for Alcohol, 2015).

The type of alcoholic beverage also increases the absorption rate because alcoholic beverages served with carbonated mixers result in a faster absorption, since carbonated mixers result in an increase in stomach pressure, that ultimately speeds up the absorption rate in the blood stream (Cederbaum, 2012 and Kent, 2012). Blood flow at the site of absorption is another factor that reduces the absorption rate since it maintains the concentration gradient through effectively removing alcohol from the site of absorption (Cederbaum, 2012). The last factor that decreases the absorption rate is whether the alcoholic beverage is consumed as a single quantity of alcohol rather than various smaller quantities. This can produce higher absorption due to the concentration gradient being higher than smaller amounts of alcohol (Cederbaum, 2012).

2.1.3.3. Distribution

Ethanol routinely passively diffuses into the blood circulatory system if not subjected to first pass metabolism. First pass metabolism is a process whereby a concentration of a drug like ethanol is reduced through the action of absorption before it enters the circulatory system of the individual, but can still enter the circulatory system through the venus portal system (Bath-Hextall, 2013). Ethanol is then distributed by blood through the body until the concentration reaches equilibrium (Kent, 2012).

Some factors that can affect alcohol distribution include: blood flow (as the distribution will be more effective in areas where blood supply is very good e.g. the brain); water content of the body (because the higher the water content, will result in a higher distribution rate (Cederbaum, 2012)) and lastly the sex of the individual. Females ingesting the same amount of alcohol as their male counterparts will result, on average, in a higher blood alcohol concentration (BAC) because females have a smaller blood volume and lower water content due to the presence of more adipose tissue (Cederbaum, 2012 and Kent, 2012).

The composition of the body is an important factor to consider when the volume of distribution (V_D) is determined (Cowan *et al.*, 1996).V_D is a term that is used to refer to the total body water that is required to allow for equal distribution of a drug such as ethanol throughout the entire body which has a direct impact on the BAC. The volume of distribution (V_D) for ethanol is directly proportional to the lean body mass (Cowan *et al.*, 1996). The V_D for men and women is 0.69 L/Kg and 0.63 L/Kg respectively.

The difference can be attributed to women possessing relative more adipose tissue, therefore, having a smaller body mass and volume of blood, hence resulting in a lower V_D and therefore potentially a higher BAC when ingesting the same amount of ethanol as a male of similar stature and build (Cowan *et al.*, 1996).

2.1.3.4. Metabolism

Metabolism of ethanol occurs primarily through oxidative and nonoxidative pathways, generally in the liver and stomach.

Oxidative pathways (refer to Figure 4 for a schematic representation) of alcohol metabolism consist of primarily three pathways including first pass metabolism. These pathways involve the activity of enzymes such as Alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase. First pass metabolism (FPM) of alcohol which occurs soon after absorption, takes place mainly in the stomach, with the oxidation process facilitated by ADH.

Acetaldehyde is then oxidised to form acetate through the action of ADH (Wongchanapai, 2008) and is finally oxidised to carbon dioxide (CO_2) and water (H_2O) (Kent, 2012). This reaction involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD+), which is reduced by two electrons to form NADH.

A secondary metabolic pathway (microsomal oxidation system) also accounts for a rise in BAC metabolism rate, but to a much lesser degree contributing only 0.065% of the BAC metabolism rate (Wongchanapai, 2008). This pathway occurs in the liver but uses microsomal cytochrome P450 (CYP) 2E1 for oxidation to occur (Wongchanapai, 2008). Oxidation takes place of the enzyme Catalase.

This process requires hydrogen peroxide (H_2O_2). Acetaldehyde is then metabolised mainly by aldehyde dehydrogenase 2 (ALDH2) from acetate and NADH, which leads to an increase of ROS, reactive oxygen species (Zakhari, 2006).

A third metabolic pathway accounts for ethanol (2-3%) to be reduced to acetaldehyde and water through the heme enzyme catalyst (Catalase) (Wongchanapai, 2008). The non-oxidative pathways comprise of two systems. These pathways are not as prevalent as the oxidative pathways but do occur and their products are of relevance. Fatty acid ethyl esters (FAEEs) and phosphatidyl ethanol are products of these two pathways respectively (Zakhari, 2006).

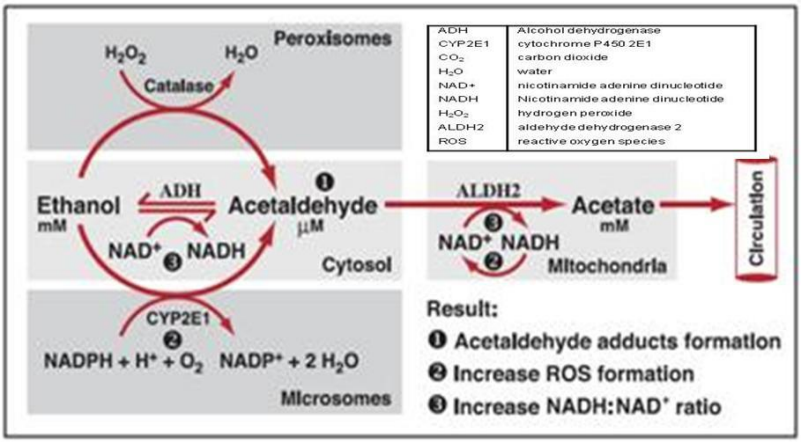

Figure 4: Alcohol metabolism in the human liver and peripheral tissues (Zakhari, 2006)

2.1.3.5. Elimination

Alcohol is eliminated primarily through urine, sweat and breath (Kent, 2012). The majority (90%) of alcohol is eliminated through metabolism (by ADH), whilst the remaining 10% remains unchanged as ethanol and is excreted in the urine (Kent, 2012). Elimination by ADH is dependent on the availability of NAD^+, and therefore can be limited to only eliminate approximately 15g of ethanol per hour (Kent, 2012).

The secondary oxidation pathway only accounts for 10% of the elimination process. This pathway, however, produces a very low rate of elimination at very low levels of blood alcohol concentrations (BAC's) and higher elimination rates at higher blood alcohol concentrations (Kent, 2012). The third oxidation pathway also contributes to alcohol elimination, however to a much lesser degree e.g. 2% (Kent, 2012).

The rate at which the alcohol elimination occurs is dependent on various factors namely; age, sex, race, whether food is present in the stomach at the time of consumption, biological rhythms such as genetics, alcoholism, exercise, presence of drugs and the BAC (Cederbaum, 2012 and Kent, 2012) and can be subdivided into the following classes

I. Factors resulting in higher alcohol elimination rates

The sex of an individual has an effect on alcohol elimination rate. Women are prone to have a higher elimination rate than men due to their body mass. Women tend to have smaller body masses and therefore can eliminate alcohol much faster (Cederbaum, 2012 and Kent, 2012). The race of an individual may increase the elimination rate of alcohol. According to Cederbaum (2012), studies suggest that the alcohol elimination rate is higher in native American individuals compared to Caucasian individuals and can be attributed to the difference in liver masses of different race populations. However, Cederbaum (2012) also states that this information is limited and that further research is required regarding the race of an individual and elimination rates of alcohol.

Food that is present in the stomach at the time of alcohol consumption also increases the ethanol elimination rate since liver blood flow is intensified thereby increasing the amount of ADH required for metabolism and ultimately elimination (Cederbaum, 2012). Exercise also leads to an increase in the elimination rate since exercise elevates the body temperature thereby increasing the ADH activity resulting in faster elimination.

At high BAC's above 0.02 g/100mL, the metabolic rate becomes faster and generally follows a zero order kinetic metabolism, which means that elimination will occur at a constant rate irrespective of the concentration. As the BAC increases (higher than 0.06 g/100mL) the rate also increases due to the higher activity of CYP2E1 (Kent, 2012).

II. Factors resulting in higher alcohol elimination rates

The age of an individual influences the elimination rate. In foetuses, the enzymes ADH and CYP2E1 are not formed and therefore this will decrease the elimination of alcohol. This is, therefore, the reason why a pregnant woman should not drink during pregnancy as foetuses are unable to eliminate alcohol thereby accumulating high levels of alcohol leading to foetal alcohol syndrome (Cederbaum, 2012).

The genetics of an individual influence the rate of elimination, due to some races for example, some Asians, not having a form of aldehyde dehydrogenase isoenzyme; this ultimately results in an increase in acetaldehyde resulting in an improper metabolism of alcohol which results in symptoms such as nausea and vomiting (Kent, 2012).

Alcoholism decreases the elimination rate as it causes damage to the liver e.g. cirrhosis of the liver and alcohol liver syndrome hepatitis and the last factor to decrease the elimination rate is drugs of abuse, that contain compounds of pyrazoles and isobutyramide, which inhibit ADH activity will decrease alcohol elimination resulting in a high BAC (Cederbaum, 2012).

2.1.4. Physiological effects of alcohol consumption

Alcohol consumption (whether acute or chronic) can have damaging effects on the five major organs e.g. heart, liver, pancreas, brain and kidneys (Caba, 2014). Alcohol has a significant effect on the brain; however, it has to reach the brain first. After absorption, and when the amount of alcohol consumed is higher than the elimination rate, alcohol can reach the brain (Schwartz Bloom *et al.*, 2017).

Alcohol is transported to all organs through arteries. The arteries branch into capillaries and these capillaries ultimately lead to the cells of the brain that are called neurons. Neurons communicate with another through electrical impulses and chemical signals, which are ultimately responsible to carry information across the various neurons that are required for normal cognitive function and brain functioning (Schwartz Bloom *et al.*, 2017).

The brain is protected by a blood-brain barrier which is defined as a membrane that separates the blood from the cerebrospinal fluid and prevents any harmful and foreign substances to reach the neuron, except for alcohol (Schwartz Bloom *et al.*, 2017).

Alcohol can easily be transported through the capillaries and due to its lipophilic properties, can then easily diffuse across the blood-brain barrier to reach the neurons (Schwartz Bloom *et al.*, 2017). Once in the brain it prevents the generation of new electrical impulses and slows down the chemical signals and produces various intoxication effects such as poor judgement, loss of balance and impaired speech neurons (Schwartz Bloom *et al.*, 2017).

The alcohol intoxication levels of an individual are measured through a unit that is called blood alcohol concentration (BAC). The BAC is generally expressed as a percentage or grams of alcohol per 100 millilitres of blood(Ehmke, du Toit-Prinsloo and Saayman, 2014). The BAC is measured in the blood of an individual because the absorption rate is fast and it is the gold standard procedure. This process, however, requires a medical practitioner or nurse to collect blood in appropriate collection containers in order to send samples to the laboratory for analysis.

Since this process is not practical for rapid determination of a legal amount of alcohol in a person's system (e.g. a traffic officer needs to determine if a driver of the car is over the legal limit allowed for alcohol in their system, when the driver was stopped), calibrated breathalysers are used to determine ethanol in breath. Alcohol in breath is directly proportional to alcohol in blood (Schwartz Bloom *et al.*, 2017) and can be a rapid and alternative indication of alcohol intoxication. In a case where a person be suspected of driving under the influence of alcohol on South African roads, a breathalyser test is conducted. This involves a suspected driver blowing in to a breathalyser device, which will give an alcohol concentration in the breath. Should this result exceed 0.24 mg/1000mL, the person is detained and subjected to a blood alcohol test (South African National Road Traffic Act 93 of 1996 and News 24, 2012).

As the BAC of individual increases, e.g. an individual absorbs alcohol at a faster rate than it is eliminated result in further intoxicating effects (Dubowski, 1980, Kaestle et al., 2018 and Schwartz Bloom et al., 2017) that are highlighted in table 2.

Table 2: Physiological stages and symptoms of alcohol intoxication (Dubowski, 1980)

Blood alcohol concentration (g/100mL)	Stage	Symptom
0.01-0.05	Subclinical	• Visually the individual seems normal • Can only be detected through specialised tests
0.03-0.12	Euphoria	• Euphoria • Increased self-confidence • Poor judgement • Little control and attention • Information processing is affected • Sensory-motor skills are affected
0.09-0.25	Excitement	• Emotionally not stable • Complete loss of judgement • Lack of balance • Vision and perception, comprehension and memory are affected • Vomiting and Drowsiness
0.18-0.30	Confusion	• Vertigo • Dysphoria • Emotional states are exaggerated • Disturbances of vision • Apathy • Lethargy
0.25-0.40	Stupor	• Inertia • Sleep • Individual cannot stand or walk
0.35-0.50	Coma	• Unconsciousness • Lack of reflexes
0.45 and up	Death	• Death from respiratory arrest

2.2. ALCOHOL CONSUMPTION

Alcohol is the most abused substance in South Africa. Over 6 billion litres of alcohol is consumed in South Africa per annum with an average of 9 to 10 litres per annum per adult (Ehmke, du Toit-Prinsloo and Saayman, 2014 and Writer, 2016). The reasoning behind consumption and the importance of testing blood alcohol concentrations will be explained in this section.

2.2.1. General Alcohol Consumption in Litres per Capita

South Africa is one of the top 20 of countries globally that consumed the most alcohol per capita for the year 2015. South Africans had consumed 11, 5 litres of alcohol per capita and had tied in 19th place with Poland (Writer, 2016).

Other countries included on this list are Russia (in 4th place with 14, 5 litres of alcohol per capita), Australia (in 8th Place with 12.6 litres per capita), and the United Kingdom (in 12th place with 12.0 litres per capita). It has been reported that South Africans consume an estimated 60 grams of alcohol in one session within a 30 day period, as most of the alcohol consumers identified were binge drinkers (Writer, 2016). The World Health Organisation (2014)(WHO) (2014) estimated that globally, 6.2 litres of alcohol are consumed per annum by individuals aged 15 year and older. The World Health Organisation 2014 also concluded that males are the most likely to consume alcohol due to cultural reasons or sociological reasons (Wilsnack *et al.*, 2010).

2.2.2. Reasons for Alcohol Consumption

People consume alcohol for various reasons and these are generally motivated by an individual's social environment and personal incentives (such as avoiding a difficult but negative situation or increasing the courage to perform in a positive situation) (Rodriguez, Knee and Neighbors, 2014). Individual incentives are generally motivated due to alcohol's "mood enhancing" and "tension reducing" effects (Cox and Klinger, 1988; Rodriguez, Knee and Neighbors, 2014).

People consume alcohol in the hope that these effects will ultimately change how they would react and feel in certain situations, such as coping with stress or coping with negative relationship events (such as an end of a relationship or a fight with a partner) (Rodriguez, Knee and Neighbors, 2014).

Environmental incentives can consist of many factors that influence an individual to consume alcohol. One of these factors includes the social circle of friends that an individual has and a person may drink to be accepted by their social peers (Rodriguez, Knee and Neighbors, 2014). A study conducted on the drinking patterns of young individuals (aged 15 to 25 year) in Great Britain confirms this since their findings also attributes the social approval by peers as the main reason for drinking alcohol (Herring, Bayley and Hurcombe, 2014). Other reasons for alcohol consumption stem mainly from the socio economic statuses as well as the nature of the childhood experiences these young individuals had (e.g. if they were raised in an environment where parents drank alcohol, the children will be likely to drink alcohol) (Herring, Bayley and Hurcombe, 2014).

2.2.3. The importance of Blood Alcohol Concentration (BAC) determination

Many work places enforce official company policies that prohibit the use of alcohol on their premises or coming to work intoxicated. These companies generally use these policies to ensure health and safety in the workplace (Jordaan, 2014). These policies are generally put in place in work places such as the mining and construction companies who operate dangerous machinery (Jordaan, 2014).

Companies have certain interventions and measurements in place to prevent any injury from happening due to alcohol intoxication. This includes programmes to train and educate employees on the dangers of being intoxicated at work (Anderson, 2010). Other companies go as far as having mandatory breathalysers installed at the work premises and test all employees before they enter the work premises, for example in the mining and construction companies (Anderson, 2010).

Countries including South Africa have placed restrictions on the limit on alcohol consumption and then being allowed to drive a motor vehicle. Some countries such as Estonia, Georgia, Lithuania, Peru, Portugal and Russia have a standard alcohol in blood limit for the experienced driver but go as far to have different alcohol in blood limits for inexperienced drivers and professional drivers (Wren, 2003) (Table 3 is a list blood alcohol limits for countries worldwide). The reason for this variance in legal limitations of allowed levels of alcohol in a person blood is directly that there is no international standard on driving under the influence of alcohol.

Countries must thus rely on their own mortality statistics, attitudes to alcohol usage, religious beliefs and legislative policies (Wren, 2003).

Table 3: Blood alcohol concentration limits within countries around the world (Wren, 2003)

Blood Alcohol Concentration (g/100 mL)	Countries
0.00	Armenia, Azerbaijan, Bahrain, Czech Republic, Hungary, Jordan, Kyrgyzstan, Mali, Romania, Saudi Arabia, Slovak Republic, Uzbekistan, and UAE
0.01	Albania
0.02	Estonia, Norway, Poland, Sudan, Sweden
0.03	China, Georgia, India, Japan, Moldova, Turkmenistan
0.04	Belarus, Lithuania
0.05	Argentina, Australia, Austria, Belgium, Bosnia Herzegovina, Bulgaria, Costa Rica, Croatia, Cyprus, Denmark, Finland, France, Germany, Greece, Iceland, Israel, Italy, Latvia, Macedonia, Monaco, Namibia, Netherlands, Portugal, Russia, Serbia, Slovenia, South Africa, South Korea, Span, Switzerland, Taiwan, Thailand, Turkey, Yugoslavia
0.06	Peru
0.08	Belize, Brazil, Canada, Chile, Ecuador, Fiji, Ghana, Ireland, Jamaica, Luxembourg, Malaysia, Malta, Mauritius, New Zealand, Puerto Rico, Singapore, Tanzania, Uganda, United Kingdom, USA, Zimbabwe
0.10-0.15	Swaziland

The South African National Road Traffic Act 93 of 1996 states the allowable blood alcohol limit is 0.05g / 100 mL for all drivers of motor bicycles and light motor vehicles and 0.02 g/100mL for professional drivers of heavy motor vehicles (bus, taxi, lorry etc.).

According to Legg (2015), 50% of all road accidents in South Africa can be attributed to individuals with a BAC over the current legal limit. This is one of highest figures with Canada having 34%, the United States 31% and India and China with only 5% and 4% respectively (McCarthy, 2016). Recently discussions in South Africa have been conducted to amend the current legislation on the legal driving under the influence limit of blood alcohol concentration to 0 g/100mL, which would result in "zero booze legislation" (Legg, 2015).

The amendment to the new bill has been drafted and submitted to Parliament for processing; however, the outcome of this amendment has not yet been announced (Legg, 2015). Currently, the Forensic Chemistry Laboratory (FCL's) will report concentrations of alcohol in the blood over the analytical test method limit of quantitation e.g. the lowest alcohol concentration that can be determined analytical (Peters, Drummer and Musshoff, 2007; Pontes *et al.*, 2009). The FCL operates under the jurisdiction of the National Department of Health (responsible for all chemical analysis of post and ante-mortem blood alcohol analysis and toxicology tests for medico legal purposes) and operates under the mandate of the South African Criminal Procedure Act 51 of 1977 and the National Health Act no 61 2003.

There are currently four (Cape Town, Johannesburg, Pretoria and Kwa-Zulu Natal) FCL's in the country capable of performing blood alcohol analysis, however, only one of these laboratories (e.g. Cape town) is fully accredited for blood alcohol analysis with the South African National Accreditation System (SANAS)(Serrao, 2016). The accreditation status of a laboratory can have an influence outcomes of court rulings as well as any cases subjected to chemical analysis which can then affect the credibility of the laboratory performing these tests (James, 2015).

A Forensic Analyst in the DNA department at the Cape Town Forensic Science laboratory working under the Department of Justice jurisdiction recently testified on this aspect. They testified that although the laboratory is not accredited for DNA testing, the results are still reliable. They also testified that it is not required by law to be accredited (Petersen, 2017). Even though by law it is not required for a Forensic Laboratory to be accredited for a specific test, accreditation does cover areas such as conformity assessment in accordance with good laboratory practices that is mandatory according to the Accreditation for Conformity Assessment Calibration and Good Laboratory Practice Act 19 of 2006 in the South African law system.

Quantification of BAC in individuals that are affected by these harmful or fatal consequences (Mandić-Radić, Džingalašević and Luković, 2007 and Pontes *et al.*, 2009) is important since it is often the determining factor in insurance claims that must be processed and paid out in the event of car accidents. Another determining factor is when an employee is being dismissed from his or her place of employment, or being sent to prison especially in cases where a person had become medically impaired or died (Ehmke *et al.*, 2016). Another factor is in legal implications where one would argue that criminal acts were committed due to intoxication of (Perry, Doroudgar and Van Dyke, 2017).

2.3. SPECIMENS COLLECTED FOR ALCOHOL ANALYSIS

Various specimens are utilised as part of a medico legal process to assess the presence of alcohol in a body at the time of autopsy such as blood, urine and vitreous humor (Kerrigan, 2013). The choice of a biological specimen for alcohol determination from a post-mortem individual is dependent on the condition of the body as the analytical results may only be as accurate as the sampling of the specimen that occurred (Kugelberg and Jones, 2007).

2.3.1. Blood

Blood is the preferred biological specimen of the collection as an extensive list of analytical methods are available using blood as a matrix (ante-mortem and post-mortem) as well as an established technique correlating BAC and the physiological states of inebriation and intoxication (Kerrigan, 2013). Blood from a living patient (ante-mortem) is collected from a venipuncture from the antecubital region on the arm of the subject. After the area on the skin has been wiped using non-alcoholic antiseptic wipes, blood is collected using a needle and an collection tube, containing dipotassium ethylene diamine tetra acetic acid (K_2EDTA) which serves as an anticoagulant, and sodium fluoride which serve as a preservative (Kerrigan, 2013). Blood from a dead victim (post-mortem) is collected from the femoral vein in the groin area. This is the best location for blood collection as it is less exposed to the post-mortem redistribution phenomena (Kerrigan, 2013).

The femoral vein is exposed after dissecting the flesh around it with a scalpel. The vein is then normally clamped on both sides. Using a 30mL syringe with a wide bore needle the femoral vein is punctured and blood is collected into the syringe (Millo, Jaiswa and Behera, 2008; Dinis-Oliveira *et al.*, 2010). This is the recommended method a by TIAFT -The International Association of Forensic Toxicologists (2008) recommendations on sample collection. The blood is then transferred into the specimen collection container such as a McCartney bottle (a 20mL glass bottle with Teflon lined screw lid) containing sodium fluoride and potassium oxalate as preservatives (Kerrigan, 2013).

Blood is the preferred specimen of choice within the JFPS MLL, and is generally collected from the femoral vein. However, when blood volume is not available, scooping blood out of the chest cavity does occur and therefore results in uncertainty surrounding the quality of the results sent to the Forensic Chemistry Laboratories.

2.3.2. Urine

Urine collected from living patients are collected in preservative-free plastic containers and is mainly utilised for the determination of alcohol for qualitative purposes. The benefits of analysing a urine specimen is that it is a very easy matrix to work with because of its very high water content (Jones, 1992). However, it cannot be used to provide quantitative alcohol results due to various factors that can influence the interpretation of results e.g. clearance volume, pH, metabolism of the individual and the health of the individual (whether the individual suffered from diabetes or not) (Kerrigan, 2013). These factors can often lead to false positive reading of ethanol in urine (Kerrigan, 2013).

Urine from deceased victims is collected as it is less likely to be subjected to contamination through bacteria, unlike blood, due to the protection that the urinary bladder offers as opposed to the major veins that are more likely to be contaminated through trauma (Jones, 1992; Collison, 2005).

A hypodermic needle and syringe are used to collect a urine specimen from the bladder of a deceased victim during the autopsy process. This method has its limitations in that if the abdominal wall is damaged by puncturing either through trauma or during the autopsy process, it thereby allows other bodily fluids to contaminate the urine specimen. Another limitation includes the availability of urine during autopsy, as trauma to the body can result in the bladder rupturing, releasing all urine (Kerrigan, 2013).

Urine is often collected for toxicological testing; however, it is not collected for alcohol testing within the JFPS MLL. Urine was not collected as alternative specimen in this study because the primary role was to replicate the procedures followed by the JFPS MLL as closely as possible.

2.3.3. Vitreous Humour

Vitreous humour (VH) is the fluid between the retina and the lens within the globe of the eye. The fluid is generally colourless and very viscous. VH is an optimal specimen to use for toxicological analysis and blood alcohol analysis as it is not in contact with blood or any other bodily fluids, therefore eliminating the potential issues of contamination occurring through post-mortem changes and decomposition (Collins, 2016). VH can only be collected as a post-mortem specimen. This process is done using a hypodermic syringe and inserting it into the globe of the eye and analysed along with blood from post-mortem cases, to provide a comparison of the various alcohol levels (Kerrigan, 2013).

Different matrices comparisons will allow for accurate interpretation of alcohol concentrations. It is routine practice during autopsy to sample both specimens in order to determine the ethanol level within each. VH is chosen mainly because it is isolated from other organs and protected by the orbit of the eye, which limits the exposure to post-mortem redistribution and to internal bacteria action (Kerrigan, 2013).

The ratio of VH alcohol concentration (VHAC) to BAC is normally determined for the interpretation of accurate alcohol concentration levels in a victim (Honey *et al.*, 2005). In these ratios, the VH alcohol concentration generally is higher than the BAC, which indicate alcohol consumption before death.

Should the BAC exceed the VHAC, it is often a good indicator that no alcohol consumption took place antemortem and the alcohol formation in the blood specimen can be attributed to purely a post-mortem artefact e.g. formation of ethanol through bacterial action (Honey *et al.*, 2005). The major limitation of collecting VH is the fact that it can only be taken in a post-mortem setting (Kerrigan, 2013).

Even though the literature above states that VH is a good alternative and complementary matrix to collect and assess for alcohol determination, this is not standard practice within the JFPS MLL. In this study, no VH was collected, in order to, as closely as possible, follow and replicate the current procedures within the JPFS MLL.

2.4. VOLUME OF SPECIMEN COLLECTION

A prescribed minimum quantity of blood is required for the accurate and reliable determination of ethanol in the blood (Olsen and Hearn, 2003; Kerrigan, 2013). An inadequate amount of blood collected can lead to evaporation and oxidation of ethanol within the specimen container, which will have an effect on the quantification results (Kerrigan, 2013). This is because of the low volume of specimen that is placed in a much larger container.

The TIAFT -The International Association of Forensic Toxicologists (2011) Laboratory guidelines, recommend that sample volume should be sufficient enough to perform the desired test and to allow for re-analysis if required. The sample volume collected should be of a sufficient amount ensuring there is a limited physical air space above the specimen in the collection container. TIAFT (2008) recommends a 20% air space volume. This phenomenon is known as the headspace variable on the determination of alcohol. Table 4 provides a list of recommended volume of blood specimens required for the accurate determination of ethanol concentration (both antemortem and post-mortem)(Kerrigan, 2013).

Table 4: Recommended types of specimen and volumes required for ethanol analysis (Kerrigan, 2013)**.**

Specimen	Volume required (millilitres)
Ante-Mortem	
Blood Urine	10-20 25-100
Post-mortem	
Blood Urine Vitreous Humour	10-20(If available) All that is available All that is available

The current procedures followed by the JFPS MLL are not standard. Dissectors collect approximately 4 mL (one standard collection tube) or 8 mL (two standard collection tubes) of blood, whereas the guidelines (FCL004 in Appendix 5) within the collection containers advice that 15 mL of post-mortem blood should be collected. This therefore leaves a total headspace volume of 11 mL and 7 mL respectively. VH and urine are not routinely collected specimens in the JFPS MLL.

2.5. SPECIMEN COLLECTION CONTAINERS

According to Kugelberg and Jones, (2007), tamper-proof and air-tight containers should be used when collecting specimens for the determination of alcohol. These containers should be tightly closed with limited air spaces available between the container and the lid. It has become problematic to use plastic containers for specimen collection.

The plasticisers present in these collection containers and the rubber-lined lids may possibly interfere with the results of the various toxicological tests. This occurs primarily by analytes interacting chemically and physically bonding to the inside lining of the container, which then resulted in the recommendation and usage of glass containers instead. Glass containers are preferred when sampling specimens for volatile detection such as ethanol, as it also decreases the chances of any loss through evaporation (Kerrigan, 2013). With the introduction of glass containers, however, it also posed a greater risk to those handling the containers, as the chances of breakages occurring increased during handling and transport of specimens.

The general guidelines are that these glass containers should therefore be in a suitable storage container that will prevent leaks, in case of breakage (Skopp and Von Meyer, 2004). Polystyrene boxes are used in a standard post-mortem blood alcohol kit (PM kit) utilised in the South African setting to address this issue (Kerrigan, 2013). In other countries such as the USA, post-mortem specimens are sent in cardboard containers, which have supporting material (sponge) to keep the containers in place and to prevent leakage. This however is dependent on the regulations and stipulations of various States of the USA. An example of a sample management biological system is the PM kit utilised by the State of Alabama in the USA. The post-mortem blood and urine are collected in plastic screw top containers containing relevant preservative. The kit is then sealed with tape and transported to the Forensic Chemistry Laboratory in Alabama for the BAC analysis to take place (Alabama Department of Forensic Science, 2017) (Figure 5).

Figure 5: Post-mortem specimen collection kit used in Alabama, USA (Alabama Department of Forensic Science, 2017)

A, Plastic containers with preservative for blood and urine collection. **B**, supporting material (sponge) to keep plastic containers in place during transportation. **C**, sealed containers and box ready for transportation to the laboratory.

The glass bottles, called McCartney bottles, in use by South African mortuaries are manufactured using inert glass and Teflon lined lids which ultimately limits the chemical interferences in these containers (Kerrigan, 2013).

In a post-mortem environment, the pathologist would often collect specimens in a primary collection vessel e.g. test-tube and then transfer the specimen to the clean bottle or test tube located within the specific post-mortem kit, to minimise contamination of the external surfaces of the testing specimen collection containers (Burton and Rutty, 2010).

In the JFPS MLL, the forensic pathologist mostly follows the same practice at autopsy. A collection of blood occurs first into a 4mL test tube by the forensic dissector, and it is subsequently transferred into a McCartney bottle. The McCartney bottle is a 20 mL glass bottle with Teflon lined screw lid. Each McCartney bottle contains 0.32 mg of sodium fluoride. This is approximately 2% weight by volume within a 20 mL glass bottle, which is recommended by the international standard (TIAFT -The International Association of Forensic Toxicologists, 2008), which acts as the preservative in the container. The McCartney bottle also contains potassium oxalate which acts as the anticoagulant in the blood specimen (Ehmke, du Toit-Prinsloo and Saayman, 2014) (refer to Figure 6).

The McCartney Bottle displays an adhesive label (refer to Figure 6), which once all details are completed, provides a duplicate on the bottle, where after the adhesive duplicate can be removed and placed on the polystyrene packaging once the container is sealed. This is to maintain the mandatory chain of custody regulations of the specimen. According to Millo *et al.,*(2008), the required concentration of potassium oxalate and sodium fluoride are 10 mg/mL and 30 mg/mL respectively.

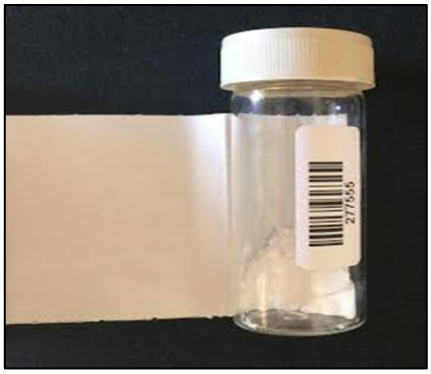

Figure 6: McCartney Bottle which contains sodium fluoride and potassium oxalate as preservatives used in South African Medico legal Mortuaries

The McCartney bottle is then placed into the provided sanctioned and standardised post-mortem blood alcohol testing kit. These kits are sealed by two cable tie type seals on either side of the polystyrene packaging (refer to Figure 7). Each post-mortem kit has its own unique identifying number. The identifying number for the specimen can be traced on all the contents of the kit (McCartney bottle, adhesive labels, document FCL003 (Appendix 5), as well as on document FCL004 (Appendix 5) which is the instruction information sheet for the usage of the kit and all seals).

The fully labelled and sealed polystyrene containers should then be stored at 4°C until delivery to the laboratory. These samples should then be transported (as per recommendation) in a cool container, such as a cooler bag, via car or motorbike to the laboratories for analysis (Ehmke, du Toit-Prinsloo and Saayman, 2014), however these recommendations are not always followed.

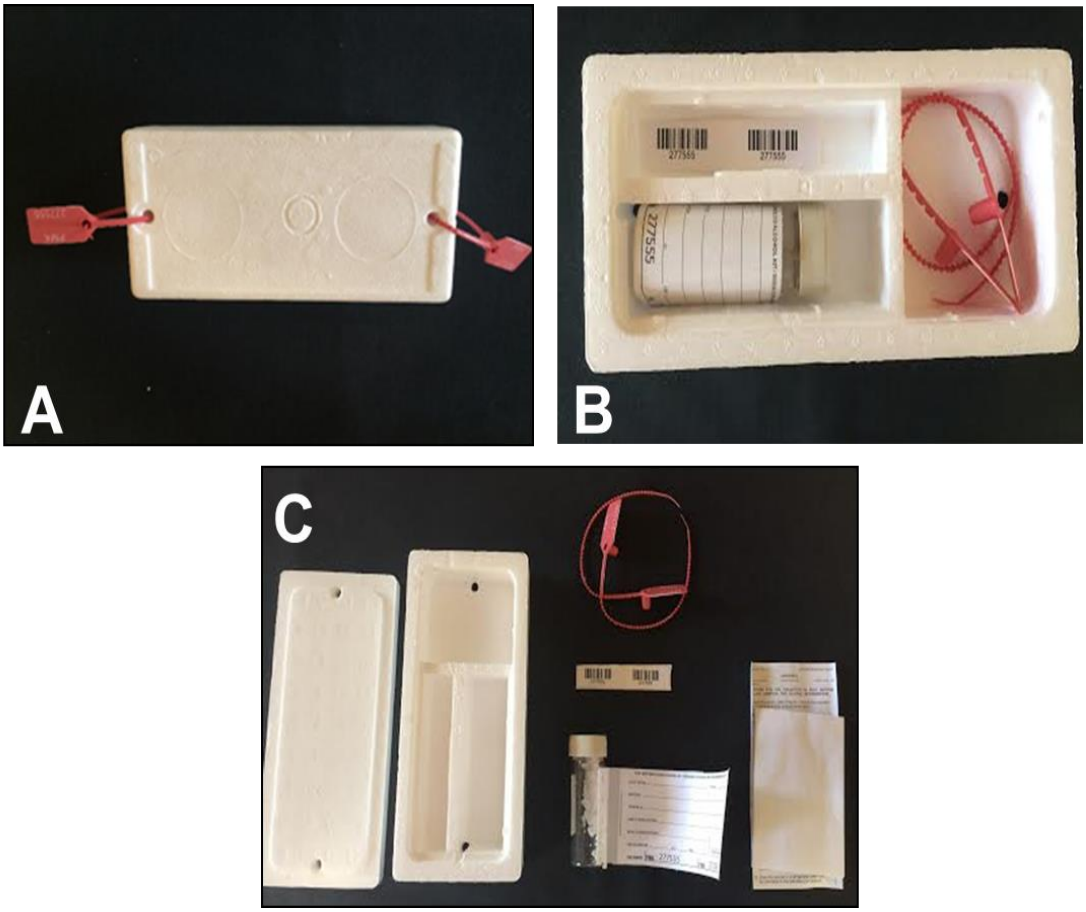

Figure 7: Post-mortem blood alcohol collection kit used in South African Medico legal Mortuaries.

A, the polystyrene container with seals that are broken upon opening kit. **B**, the kit on the inside after seals have been broken and lid and inserts have been removed. **C**, contents of kit, which consists of new seals, the McCartney bottle with preservatives, extra labels, document FCL003 and document FCL004.

2.6. DETERMINATION OF ALCOHOL IN BLOOD

Forensic pathologists require the amount of alcohol in a victim to be determined in order to provide the extent and potential contribution made by alcohol in their death (Winek and Paul, 1983)

Various factors such as the time between death and sample collection, the decomposition stage of the body, the history of the circumstances surrounding the death and the types of trauma to the body, all play a role in the eventual interpretation of the BAC result. The pathologist interpreting the data must, therefore, consider these factors before making a final determination as to the level of intoxication of the victim, its contribution towards their cause of death as well as supportive evidentiary information relating to the manner of death (Kugelberg and Jones, 2007). The authors also highlight that that the sampling method plays a significant role on the results of a test and whenever possible additional specimens (VH or urine) should be collected for comparison reasons to aid in the accuracy of results.

2.6.1. Method of Analysis

The gold standard technique of alcohol determination in blood is performed using headspace gas chromatography with flame–ionisation detection (HSGC-FID). This method is standard for the determination of alcohol levels in antemortem and post-mortem specimens because the HSGC-FID method is known for its accuracy, sensitivity, relative specificity characteristics and ease of operation (Tisicone *et al.*, 2013).

The protocol employs an internal standard method, which utilizes an internal standard for the dilution of the blood specimens prior to analysis. Tertiary Butanol (t-butanol) and Isopropanol (n-propanol) are the preferred choices of internal standard, however, t-butanol is normally used for post-mortem blood specimens as it is not a naturally occurring alcohol found in post-mortem blood and therefore eliminates the potential of interference during analysis (O'Neal *et al.*, 1996).

In addition, acetone is used as a reference quality control material because it is a known biomarker for other states of intoxication and diseases such as ketoacidosis (strong indicator for diabetes mellitus) which can be incorrectly interpreted as indicative of alcohol consumption antemortem. Isopropanol and acetaldehyde also serve as reference quality control materials, as they are known by-products of microbial action and detection of these products could indicate microbial action and not necessarily alcohol consumption (Kugelberg and Jones, 2007).

2.6.2. Principle of HSGC-FID

Chromatography is a technique whereby separation of compounds or molecules occurs based on how they partition between two phases as they elute through the system. This ultimately results in the separation of a mixture by virtue of different affinities of the components of the mixture for each phase (Harvey, 2016).

The principle of gas chromatography (GC) (refer to Figure 8) involves a volatile sample (either a liquid or a gas) injected into a carrier gas which serves as a mobile phase and should generally be an inert gas such as Helium (He). A continuous flow of mobile phase transfers the sample onto an analytical column, that can be a packed-or a capillary-column, where the components within the sample mixture are separated, based on their selective partitioning (affinities) between the stationary and mobile phases. In GC, since the mobile phase is an inert gas, the separation depends primarily on the affinity of each component for the stationary phase. The stationary phase, also known as the analytical column, is placed in a temperature-controlled oven to volatilize the sample (Chromacademy 2016 & Harvey 2016). In this study, an Agilent HP-INNOWax® column was chosen; the stationary phase is polyethylene glycol (PEG) packing that works well for the separation of alcohols and other polar molecules. The manufacturer has many different columns specifically designed for alcohol analysis, including this column. Another study has also used this column for ethanol separation in biological matrices (Kaya *et al.*, 2010). The Analytical Services laboratory also successfully uses this stationary phase for the proficiency-testing scheme for ethanol analysis.

Once compounds of interest are separated, the components of the sample are transferred to a detector the choice of which depends on some physical or chemical property of the analytes. A plot of the detector signal/response, as a measure of amount of analyte against time is recorded as a chromatogram on a data system. The detector signal is related to the concentration of the chemical in the detector and it must be calibrated with known standards. This is then used to interpret and print the results for reporting purposes.

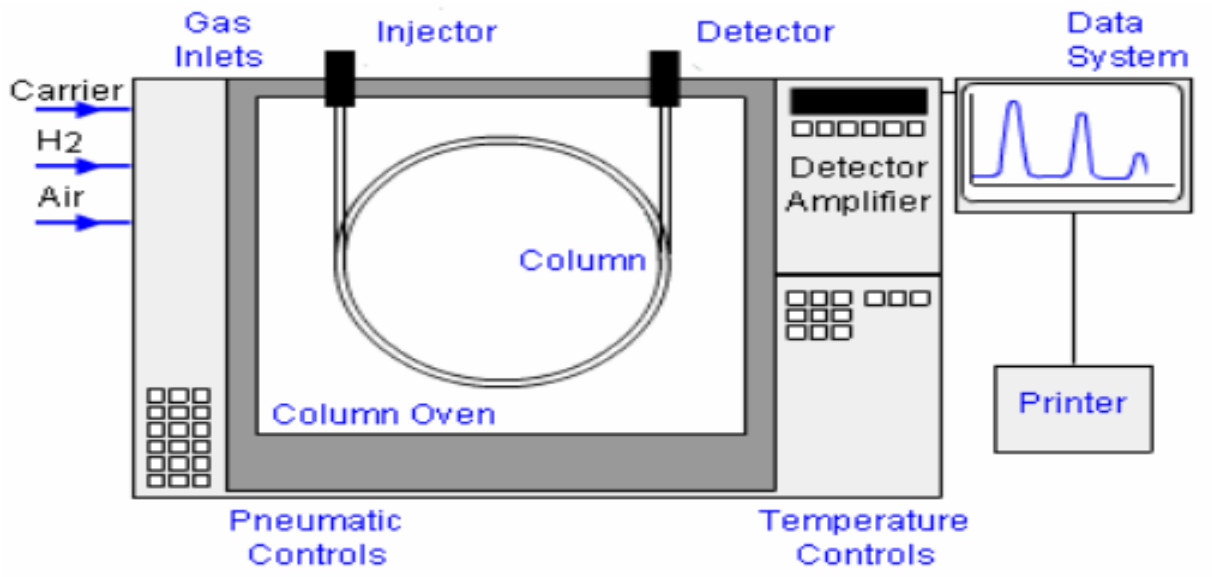

Figure 8: Schematic diagram to illustrate the basic components of Gas Chromatography (Chromacademy, 2016)

Headspace sampling is the preferred method, as it limits overloading and fouling the analytical column with blood-matrix materials that do not fully volatilize or breakdown at the temperature of operation; such material would cause blockages and contamination of the column. The technical principle of headspace sampling involves the abstraction of e.g. ethanol (boiling point of 78.29 °C) and t-butanol (boiling point of 82.2°C), from a complex sample matrix blood. It is then extracted into a gaseous headspace above the liquid specimen after it is heated in an oven, thereby allowing the volatiles in the blood samples to be efficiently partitioned into the headspace gas volume from the liquid matrix sample.

Depending on the boiling point of the compounds and the headspace oven temperature, volatile organic compounds from the sample matrix will selectively partition between the gaseous headspace and the liquid blood matrix. Also, the limited sample preparation required makes the headspace technique a simple and convenient method to use (Shan *et al.*, 2011). A known aliquot of the gas in the headspace is extracted and injected into a Gas Chromatography (GC) system for the separation of the volatile components. The alcohol is detected and quantified using a Flame Ionization Detector (FID). The FID is a very sensitive mass-flow detector that is used mainly for the detection of volatile hydrocarbons and carbon containing compounds (Chasteen, 2000).

The HSGC-FID is therefore known for its accuracy, sensitivity, wide linear range, stability and ease of use (Shan *et al.*, 2011).

2.6.3. Method Validation of HSGC-FID

In this section, the principle behind method validation will be described. This section will also address the method validation parameters used and the acceptance criteria for each parameter.

2.6.3.1. Principle of Method Validation

Any method that is devised for routine/regular use should be initially validated. The validation of an analytical method is required to eliminate the issues surrounding the over reporting and under reporting of results as well as false positives (Peters, Drummer and Musshoff, 2007). The method for the determination of ethanol in blood was thus validated prior to its application for this project.

The Analytical Services Laboratory at the National Institute for Occupational Health (NIOH) is an accredited facility and therefore has agreed to the terms and conditions set out by the South African National Accreditation System (SANAS) to provide accredited test methods according to SANAS standards ISO15189:2012 and ISO17025:2015.

The ISO17025 standard describes the general guidelines for all testing and calibrating laboratories, whilst the ISO15189 standard in the general guidelines for medical laboratories. These guidelines and requirements serve as a method of accurately controlling and monitoring laboratories to be sure that these laboratories are producing accurate results (Penders and Verstraete, 2006). Test methods must meet the requirements for analysis. These methods are accompanied by standard operating procedures (SOP), a validation protocol as well as a validation program set up for compounds tested in the relevant matrixes.

2.6.3.2. Method Validation Parameters

The South African National Accreditation System (SANAS) 2016 guideline TG 41-02 the recommended guidelines for the verification and validation of methods in forensic chemistry and the South African National Accreditation System (SANAS) 2012 guideline TG 07-01 the technical guidelines for the validation of methods used by chemical laboratories in the food water and related industries, stipulates the parameters below, for method validation for quantitative analysis. In this thesis, all defined parameters are as validated for the method described.

I. Selectivity

Selectivity refers to how the method can measure and differentiate between the desired compound, internal standard(s) and other compounds that may be present within a specimen/matrix (Pontes *et al.,* 2009 & Peters *et al.,* 2007).

The interfering alcohol-based compounds often found in blood specimens (ante-mortem and post-mortem) are acetaldehyde, 2-propanol, 1-propanol and acetone (Boumba *et al.*, 2012). Good peak resolutions and differentiation between ethanol, internal standard and other possible interfering compounds (acetone, acetaldehyde and 2-propanol) must be evaluated for a selective method (European Medicines Agency (EMEA), 2011). Different matrices can have interfering compounds and is controlled matrices are recommended (Huber, 2010). In the case of post-mortem blood, produces a lot of variability and ante-mortem blood was therefore chosen to provide a controlled matrix for blank matrix studies to determine selectivity.

II. Linearity (Calibration model)

Linearity refers to a specific and given range within the analytical process, to obtain a result or value where the signal response is directly proportional to the concentration of the desired compound (Huber, 2010).

A visual interpretation and correlation coefficient is generally used to determine linearity (Huber, 2010 and South African National Accreditation System (SANAS), 2016), however linearity has to be proven through statistical analysis to determine linearity. This is done through standardised residual plots and a randomised scatter around the zero line concludes a linear model. (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013).

III. Limit of Detection and Limit of Quantification

Limit of detection (LOD) is the lowest concentration of the desired compound that can be distinguished from the absence of that compound (a blank value) with a stated confidence level; whilst the limit of quantification (LOQ) refers to the lowest concentration that can be determined with an acceptable level of repeatability, precision and trueness (Belter, Sajnóg and Barałkiewicz, 2014; Peters *et al*., 2007 and Pontes *et al*., 2009). One of the methods of calculating the LOD is to use the mean of the blank readings with the addition of three times the standard deviation of the blank readings and LOQ was determined using the mean of the blank readings with the addition of 10 times the standard deviation of the blank readings (Huber, 2010).

IV. Accuracy (Bias)

Accuracy is referred to how close the concentration determined, is to the theoretical concentration of a compound (Pontes *et al*. 2009 & Peters *et al*. 2007). Certified reference materials are often used and it is recommended that the experimental mean value should be within 15% of the actual value of a known concentration (Huber, 2010). Accuracy can also be expressed by a percentage of analyte recovered and a recovery percentage of 80%-120% is generally accepted (United Nations Office on Drugs and Crime, 2009). Results that produce CV % below 10% is accepted and deemed accurate as this was recommended by various guidelines on method validation acceptance criteria used in a forensic setting (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013).

V. Precision

Precision verifies how varied the determinations of the concentrations of a specific compound can be after it is determined through repeated measurements (Pontes *et al*. 2009 & Peters *et al*. 2007). Intra-day precision refers to the repeatable measurements within the same laboratory, same interment conditions but different operators, whereas Inter-day precision is usually the same operator using the same operating system but repeatable measurements are compared between different days. Both these types of person assays are important to determine if the method is precise (Pontes *et al*. 2009 & Peters *et al*. 2007).

Precision is usually expressed as CV % (South African National Accreditation System (SANAS), 2016). Results that produce CV % below 10% is accepted and deemed precise as this was recommended by various guidelines on method validation acceptance criteria used in a forensic setting (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013)

VI. Repeatability and Reproducibility

Repeatability is how close results are when they are repeated under the same instrumental and environmental conditions and is done by the same analyst, whilst reproducibility refers to how close results are when an analysis is repeated on the same instrumental and environmental conditions, but is done so by two different analysts (South African National Accreditation System (SANAS), 2012).

The results were accepted when the difference between the highest quality control result and the lowest quality control result was less than the standard deviation of repeatability and reproducibility respectively multiplied by a factor of 2.8 (95% confidence level) (National Institute for Occupational Health, 2015).

VII. Matrix effects

This parameter is used to determine the effect that the matrix (blood or water) can have on the signal response of a compound (Pontes *et al*. 2009 & Peters *et al*. 2007). A true evaluation of matrix effects is recommended but not always possible, as in the case with post-mortem blood (Society of Forensic Toxicologists, 2006). It is recommended that the sample matrix of interest be evaluated without the addition of any standards and with that of known concentrations (Society of Forensic Toxicologists, 2006). Post-mortem blood is known for its variability, and therefore the use of ante-mortem blood and purchased ethanol standards in blood (this is traceable to SI units) to demonstrate matrix effects.

VIII. Carry Over

Carry over is used to determine the amount of a compound that can be detected after a certain concentration of a sample is injected onto the headspace–gas chromatography system that could possibly alter the determination of that specific compound in a subsequent analysis (Pontes et al. 2009 & Peters et al. 2007). Carry over percentage was less than 20% of the lowest calibration standard response and less than 5 % of the IS amount at this concentration (European Medicines Agency (EMEA), 2011).

IX. Ruggedness

Ruggedness refers to the reproducibility of results from measurements when slight changes in the environmental or experimental conditions were made (South African National Accreditation System (SANAS), 2012). The results were accepted when the difference between the highest quality control result and the lowest quality control result was less than the standard deviation of ruggedness measurements multiplied by a factor of 2.8 (95% confidence level) (National Institute for Occupational Health, 2015).

X. Measurement of Uncertainty

All measurements come with a certain amount of error. This error is represented as a quantity that is added and subtracted from a measurement value to determine if the measurement is intended for its purpose. This quantity is referred to as the uncertainty of measurement (Farrance and Frenkel, 2012).

2.7. VARIABLES AFFECTING THE BAC

Alcohol breakdown or formation after death is always an aspect to consider when interpreting the BAC (Kugelberg and Jones, 2007). Athanaselis, Stefanidou and Koutselinis, 2005 provide three primary factors to consider with analysing BAC results in post-mortem specimens. The first factor, alcohol consumption accounts for the overall BAC. The second factor can be attributed to post-mortem production due to bacterial activity or post-mortem redistribution. The last factor can be attributed to the combination of both antemortem consumption and post-mortem productions. The factors listed below, may have a significant bearing on BAC.

2.7.1. Factors that can affect the BAC post-mortem before sampling of specimens occurs

In a South African setting, victims that have died of unnatural causes are first assessed by the South African Police Services, then the Forensic Pathology Services are called in to collect and transport all bodies to the various mortuaries bodies are tagged and placed into fridges until autopsy can take place. It is during autopsy that biological specimens can be taken for further laboratory testing, as in the case where blood is collected for alcohol analysis. This time from presumed death until autopsy is therefore not controlled and can cause certain variations in the BAC that should be taken into consideration.

The factors that can affect the BAC within the body of the diseased are the following:

2.7.1.1. Bacteria

A person is considered dead when all bodily and metabolic functions stop (Keyes, 2016). This starts the process of decomposition. Decomposition occurs in five stages: early stage, bloated stage, active decay, advanced decay and dry stage (Keyes, 2016).

The early stage is a normally identifiable pink-white appearance on the body, and Livor Mortis. Blood alcohol analysis can only be undertaken accurately if the specimen is taken from a body in the early stages of decomposition. Any specimen taken from a victim in a more advanced stage of decomposition may not provide accurate data related to alcohol levels since BAC levels can be elevated or depleted depending on the type of bacteria present or due to the post-mortem redistribution of bodily fluids (Huckenbeck 2006 & Cook *et al*. 2000). Bacteria utilize the glucose and other starches found within the blood and causes the body to produce alcohol which is a by-product of the enzymatic activity of bacteria and may, therefore, alter the BAC (Sutlovic, Versic-Bratincevic and Definis-Gojanovic, 2014).

The post-mortem formation of alcohol from bacteria is dependent on the species of bacteria and environmental conditions, as these will have an effect on the decomposition stage when the body is found (Honey *et al.*, 2005). The environmental conditions associated with the post-mortem production of alcohol through bacteria include the temperature, humidity and location of where the body was found (in water or open field) (Kerrigan, 2013) and the manner of death, whether it was violent death or not (Johnson *et al.*, 2004).

A violent death is normally associated with severe trauma to the body, providing bacteria from external sources access to the internal compartments of the body or providing the internal bacteria access to other compartments of the body in the case of damaged organs; for example in a car crash a victims abdomen is severed whereby the bacteria found commonly in the stomach maybe exposed to the liver and kidneys (Plueckhahn, 1968).

In this study, blood specimens were only collected from bodies within the early stages of decomposition, therefore adhering to literature and minimising the risk of bacteria altering the BAC.

2.7.1.2. Post-mortem Redistribution

Post-mortem redistribution is the process known to cause a change in drug concentrations in a post-mortem environment, due to the movement of drugs (in our case, alcohol) from surrounding tissues into biological fluids (Cook, Braithwaite and Hale, 2000). The rate at which this movement takes place, is influenced by the type of drug and the time between death and autopsy (post-mortem interval) (Cook, Braithwaite and Hale, 2000). Post-mortem distribution can generally occur in two ways; through diffusion and through trauma. Diffusion relies on the permeability of cell membranes as well as the concentration gradient associated with various cells and organs (Pounder and Jones, 1990). Diffusion normally occurs in the direction to an organ or bodily fluid with a high concentration of an organ or body fluid with low concentration (Butzbach, 2010).

Diffusion of alcohol is affected by certain factors such as the concentration of the alcoholic drink, how much alcohol was consumed before death and was food consumed with alcohol (Kugelberg and Jones, 2007).

These factors will affect the concentration gradient, distribution rate and the ante-mortem absorption rate (Kent 2012 & Cederbaum 2012). When a body has been subjected to trauma ante-mortem (e.g. motor vehicle accident) or the stomach was accidentally ruptured during the autopsy, redistribution can take place (Pounder and Jones, 1990). It is therefore very important that all events during an autopsy are documented and all information of the death of a victim is recorded and noted when sent to the laboratory for analysis so that the correct interpretation of BAC results can take place (Kugelberg and Jones, 2007).

2.7.2. Factors that can have an influence on the BAC within the blood specimen after collection

Factors that will affect the BAC *in vitro* are the following:

2.7.2.1. Bacteria

Although many bacteria species, mentioned above, can produce ethanol as a by-product of their metabolism, it is *Candida albicans* that are primarily responsible for the ethanol production (Plueckhahn, 1968).

Studies have shown that prevention of microbial alcohol formation can occur when blood specimens are properly preserved with sodium fluoride (Ferrari, Triszcz and Giannuzzi, 2006). The fluoride inhibits the enzyme enolase. This enzyme plays an important part in the fermentation process of bacteria, yeast and fungi to form an alcohol (Held, 2012). Many stability studies have been done on this variable. From the literature it can be ultimately concluded that bacterial activity can be limited with the effective amount of sodium fluoride within the collection container. As it was discussed in section 2.5, that the PM kits used in South Africa contain the recommended amount of sodium fluoride, this variable was therefore not assessed.

2.7.2.2. Temperature

Environmental temperature can influence the BAC, depending on sampling and storage of the specimens prior to analysis (Honey *et al.*, 2005). The study performed by (Wongchanapai, 2008) identified a larger loss (up to 19% at a temperature at 26,7˚C) in alcohol concentration in blood stored in post-mortem blood at room temperature (26.4˚C) compared to those stored at refrigerator temperature (4˚C) and freezer temperature (-20˚C). The study conducted by Shan *et al*., (2011) showed a loss in alcohol concentrations in blood at both room temperature and at 4°C, which could be due to the storage time of 13 to 39 months. Tsokos (2006) indicated that some experiments which included storing samples at different temperatures and for different storage times produced little or no effect on the BAC whilst others (Tsokos, 2006) showed an average loss of 4.1%. They attributed this loss to catalase (an enzyme that catalyses the oxidation of toxins such as alcohol and reduces the BAC) activity within the collected blood sample.

A study conducted by Wongchanapai (2008) in evaluating the stability of ethanol in blood under various experimental conditions showed that BAC can increase by up to twice the initial measured BAC, when there is a high glucose content within the specimen. The authors recommended further studies that the glucose content within post-mortem samples be taken into consideration when evaluating BAC. Sutlovic *et al*., (2014) observed that BAC results could also increase due to concentrations of other drugs such as methadone and diazepam being present in the blood specimen. In conclusion, these studies have recommended that post-mortem blood which is submitted for alcohol analysis should be stored at 4°C.

2.7.2.3. Headspace

Headspace refers to the amount air above the blood specimen within a sample container (Figure 9). It is a factor to consider when evaluating BACs. Studies by both Smalldon and Brown, (1973) as well as Olsen and Hearn, (2003) indicated that the loss of alcohol in stored blood samples is dependent on the volume of air (headspace) above the blood specimen, as the alcohol evaporates when the specimen container is opened. Sutlovic *et al*., (2014) mentioned that whilst sodium fluoride prevents bacteria from producing alcohol, it does not affect the oxidation process that may occur due to the presence of a large headspace. Oxidation is the process where a compound such as alcohol loses electrons and increases in valence state. This reaction occurs naturally and commonly in the presence of oxygen. Alcohol is commonly oxidised to acetaldehyde under enzymatic conditions. It is then that a decrease in BAC can be seen (McMurry and Simanek, 2007).

According to Wongchanapai(2008), the general loss of ethanol in stored blood samples can be attributed to the oxidation process that occurs especially when the stored blood samples were exposed to oxygen. The literature, however, does not provide any solution to prevent oxidation during the preservation process of blood specimens. According to Tsokos (2006), other studies have indicated contradictory results on the various factors that influence the BAC.

However, headspace has very little effect if an appropriate volume of blood is collected and stored in a collection tube, as the amount of headspace is therefore minimal (Sutlovic, Versic-Bratincevic and Definis-Gojanovic, 2014). Mandić-Radić *et al*., (2007) and Dinis-Oliveira *et al*. (2010) recommended that ideally there should be no headspace above the blood specimen in the collection tube.

In South Africa, however, this is not practically possible as a sampling of the blood from a body experiences certain practical difficulty which include a large post-mortem interval; number of victims managed and assessed per day; conditions of victims as well as the sampling technique employed, which all potentially limits the amount of blood sampled for testing.

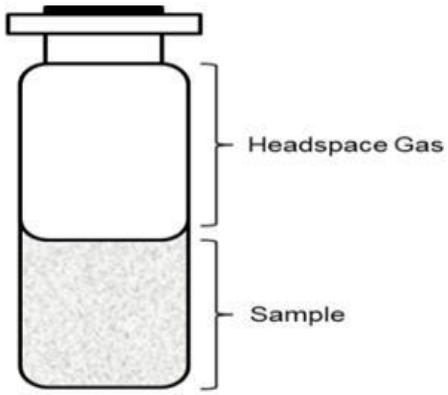

Figure 9: Illustration of the concept of headspace

2.7.2.4. Storage time

Alcohol production is also influenced by the period that post-mortem blood specimens are stored. According to Sutlovic *et al*., (2014), as the storage time increases, so the BAC decreases. The studies on post-mortem blood by Wongchanapai(2008) showed no effect on BAC for up to at 35 days at 26, 7 ˚C, whereas Winek and Paul(1983) showed no effect for up to 6 months, although, their studies were conducted on antemortem blood. Sutlovic *et al*., (2014) evaluated the stability of BAC over a longer storage time of 191 days at -20˚C in post-mortem blood, which resulted in a reduced BAC level after storage. According to Kerrigan (2013), the average losses of ethanol in post-mortem blood specimens were 4 g/100mL over a period of three year and 3,3 g/100mL over 6.75 year at room temperature.

2.7.2.5. Evaporation

The study conducted by Tsokos (2006) stated that research on the loss of alcohol can be attributed to evaporation of alcohol that takes place in cases when specimen containers remain open for long periods of time prior to analysis and if specimen tubes or bottles are not securely sealed. Evaporation can result from any of the following; temperature, the surface evaporation area of the specimen and the total amount of air circulation surrounding the specimen container.

The opening of specimen containers should, therefore, be limited and kept to a minimum in order to limit the loss of ethanol. According to Mandić-Radić *et al.,* (2007) evaporation is less likely to be attributed to ethanol loss in stored blood specimens, but can rather be due to oxidation due to the volume of headspace above the specimen in the container.

2.8. RATIONALE OF THE STUDY

The Forensic Chemistry Laboratories have made media headlines surrounding sample handling and storage conditions of specimens. It has been stated that specimens are kept at incorrect temperatures e.g. specimens are kept in refrigerators at 10°C and higher (Serrao, 2016). Other statements surrounding the sample handling, analysis and storage are also questionable (Raphaely, 2011; Serrao, 2016 and Serrao, 2017). The lack of standard operating procedure followed by the JFPS MLL on blood collection for blood alcohol analysis is also of concern since literature supports the fact that headspace could possibly influence the BAC.

The integrity and the validity of the samples are therefore uncertain and hence lead to the importance of performing a study under local Johannesburg environmental conditions that will address these concerns.

This study will therefore address the variables such as temperature, headspace with respect to storage times. The work that is undertaken and the data that is generated can be made available to government officials (e.g. the laboratory and the pathologists) to provide evidence as to what effects these variables may have on specimens that are tested up to 6 months after sampling.

2.9. AIMS AND OBJECTIVES

The aim of this study was to assess the stability of alcohol (ethanol) concentrations in post-mortem blood specimens under temperature and headspace variables with respect to time and under environmental conditions specific to Johannesburg, South Africa.

The study objectives were:

1. To determine the effect of temperature at ± 4˚C and at ± 22˚C (room temperature) on the blood alcohol concentration in post-mortem blood specimen over a period of 6 months.
2. To determine the effect of headspace (at blood volumes of 4mL and 8mL respectively in post-mortem collection containers) on the blood alcohol concentration in post-mortem blood specimen over a period of 6 months.

CHAPTER 3: METHODS AND MATERIALS

This study was a prospective, longitudinal descriptive and comparative study. The study was performed and can be described in five parts (as summarised in figure 10).

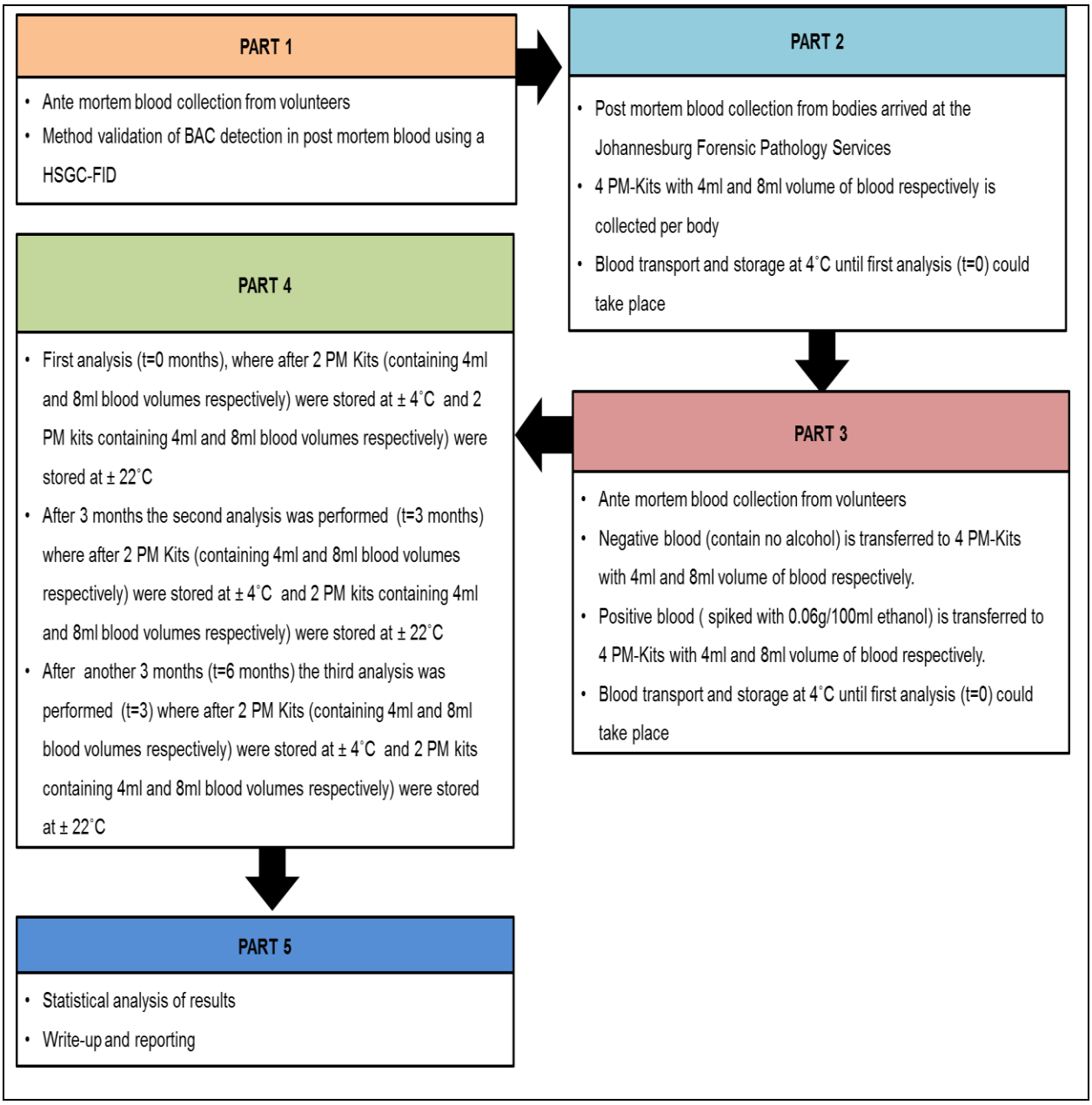

Figure 10: Flow diagram representing the entire experimental process that was followed

The following relevant key terms were utilised in this chapter:

‘Method Validation’

The process that is followed to ensure that a method correct for the intended (Huber, 2010)

‘External quality controls’

Two ethanol in aqueous standards that were purchased from NMISA. The external quality controls concentrations are 0.02 g/100mL and 0.05 g/100mL respectively. Each control standard is traceable to SI units.

‘Positive blood quality controls’

Ante-mortem blood that was donated by volunteers. This blood was spiked with a known ethanol concentration (0.05 g/100mL)

3.1. PART 1: Method Validation

The method for alcohol detection was developed using an Agilent® G1888 Headspace that is coupled to an Agilent 6890N Gas Chromatograph (GC) fitted with a Flame Ionization Detector (FID), using Agilent HP-Innowax® column. The method was the optimised and rendered fit for use by method validation (analytical conditions and column dimensions are attached in Appendix 10). The South African National Accreditation System (SANAS) 2016 guideline TG 41-02 the recommended guidelines for the verification and validation of methods in forensic chemistry and the South African National Accreditation System (SANAS) 2012 guideline TG 07-01, the technical guidelines for the validation of methods used by chemical laboratories in the food water and related industries for method validation, was utilised to provide the criteria in order to prove the analytical method for detection of ethanol in blood is fit for purpose.

All statistical tests and calculations for method validation parameters were performed in Microsoft Excel® 2007. All mathematical equations defining the method validation variables are also set out by the South African National Accreditation System (SANAS) 2016 guideline TG 41-02, the South African National Accreditation System (SANAS) 2012 guideline TG 07-01 the National Institute for Occupational Health Standard Operating Procedure (NIOH0391). In this section the method validation parameters and the process for each parameter is described. The key terms and the statistical equations used for the method validation parameters are summarised in Table 5 and described within each method validation parameter.

Table 5: Key Terms and Statistical Equations for Method Validation Parameters

KEY TERM	EQUATION
Correlation Coefficient (r)	$\frac{\sum(X_i - X_{avg})^* (Y_i - XY_{avg})}{\{\sum(X_i - X_{avg})^2 \sum(Y_i - Yavg)^2\}^{\frac{1}{2}}}$
Regression Line	$y = mx + c$
Slope (m)	$\frac{\sum(X_i - X_{avg})^* (Y_i - XY_{avg})}{\sum(X_i - X_{avg})^2}$
Intercept (b)	$Y_{avg} - bX_{avg}$
Residual Standard deviation (Standard error)	$S_{y/x} = \{\sum \frac{(Y_i - \hat{y})^2}{n-2}\}^{1/2}$
Limit of Detection (LOD)	$(Y_{LOD} - b)/m$ where $(Y_{LOD} = b + 3S_{y/x})$
Limit of Quantification (LOQ)	$(Y_{LOQ} - b)/m$ where $(Y_{LOQ} = b + 10S_{y/x})$
Coefficient of variance (CV)	$\frac{s}{\bar{x}}(100)$
Standard Deviation (s)	$\sqrt{\frac{\sum_{i=l}^{n}(X_i - \overline{x})^2}{n-1}}$
Percentage Carry over	$\frac{Mean\ of\ determined\ ethanol\ concentrations\ in\ blank\ vials}{the\ ethanol\ concentration}(100)$
Mean ($\bar{x}$)	$\frac{\sum X_i}{n}$
Uncertainty (u)	$\frac{t_{(n-1)}S}{\sqrt{n}}$
Expanded Uncertainty(U)	u^*k
% Relative Uncertainty	$\frac{U}{\bar{x}}(100)$
t_{calc}	$\frac{((\lvert experimental\ mean - true\ value\rvert)^*\sqrt{n})}{s}$
t_{crit}	Critical value for student's t-test (Hayes, 2008)
f_{calc}	$\frac{{S_1}^2}{{S_2}^2}$ (1 and 2 are in the equation so that F is always ≥1)
f_{crit}	Critical value for f-test (Hayes, 2008)

3.1.1. Materials

Flat bottom headspace (10mL volume) vials were purchased from Chemetrix (Pty) Ltd and Separations (Pty) Ltd respectively due to price changes. Headspace aluminium caps and rubber bungs were purchased from Glass Blowing Industries. The Agilent HP-Innowax® column (Length, 30m; ID, 0.250mm and film thickness, 0.50µm) was purchased from Chemetrix (Pty) Ltd. A-Grade volumetric flasks (100mL) are the property of Analytical Services, NIOH.

3.1.2. Reagents

Ethanol standards in aqueous (H_2O) of the following concentrations; 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL, were purchased from the National Metrology Institute of South Africa (NMISA). Three different lots of ethanol standards in aqueous (H_2O) of the following concentrations 0.02 g/100mL; 0.05 g/100mL and 0.2 g/100mL were purchased from the National Metrology Institute of South Africa (NMISA) and served as quality controls. Fortified matrix samples were not used though it is encouraged, it is not a stringent requirement and in BAC analyses ethanol concentration prepared in aqueous solution is more than suitable for use since these standards are traceable to SI units, and are generally used as calibration standards for quantification of ethanol in blood by the FCL laboratories, since certificates of analysis are acceptable as *prima facie* evidence in the South African Judicial systems (Marajh *et al.*, 2018).

Ethanol standards in blood matrix at the following concentrations; 0.03 g/100mL; 0.05 g/100mL and 0.2 g/100mL were purchased from Industrial Analytical. Tertiary-butanol (t-butanol) (99.7%purity), acetaldehyde (99.5% purity), 2-propanol (99.5% purity) and Acetone (99.8% purity) were purchased from Sigma Aldrich.

3.1.3. Ante-mortem Blood

Ante-mortem blood that contained no alcohol was collected from volunteers (see Appendix 4). A registered nurse collected the blood using the method set out in Section 2.3.

3.1.4. Equipment

All equipment is the property of the National Institute for Occupational Health (NIOH) Analytical Services. A-Grade volumetric flasks (100mL) were used for the preparations of 0.1% t-butanol, acetaldehyde, 2-propanol and acetone reagent preparations respectively.

These standards were then pipetted into a flat bottom headspace vial, whereby it was injected into the GC-FID system for analysis of interferences. These standards were prepared using deionised water from the Milli-Q® water EVOQUA® Ultra Clear purification system, purchased from Separations (Pty) Ltd.

A vial crimper (unknown origin but used regularly in the Analytical Services laboratory) was used to tighten aluminium crimp top caps to flat bottom headspace vials. The Hamilton Microlab® 500 series dilutor was used for sample preparation with t-butanol.

The samples were analysed on an Agilent® G1888 Headspace that is coupled to an Agilent 6890 N Gas Chromatographer (GC) that is fitted with a Flame Ionization Detector (FID), purchased from Chemetrix (Pty) Ltd.

3.1.5. Standards and Quality Controls

T-butanol was used as the internal standard in this method and is preferred since it is not a naturally occurring alcohol found in post-mortem blood; therefore eliminating the potential of any interference during analysis. The quality controls ensure verification and validation of the method. All standards and quality controls are described in this chapter.

3.1.5.1. Preparation of Internal standard and interference compounds commonly found in PM blood

The internal standard, 0.1% t-butanol, was prepared by adding 100 µL of t-butanol to a 100mL volumetric flask and filled it to the mark with deionized water. This dilution step was repeated with acetone, 2-propanol and acetaldehyde in three separate 100mL A-grade volumetric flasks respectively. These represent indicators for common interferences found in PM blood.

3.1.5.2. Preparation of Calibration Standards

Six purchased standards with known concentrations (traceable to SI units) of aqueous ethanol 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL (aqueous) were used as calibration standards. A blank (without ethanol) sample (0 g/100mL) was prepared with deionised water, in the laboratory. These standards were then diluted with t-butanol which is the internal standard.

This dilution of standards and internal standard was performed using a calibrated (calibration certificate number: SEP0822MC) Hamilton Microlab® 500 series dilutor (see Appendix 5). The dilutor was programmed to draw 500 µL of internal standard and 500 µL of sample or standard so that 1000 µL is dispensed into a 10 mL headspace vial. A rinse cycle was employed after this dispersion, by drawing 1000 µL of internal standard and dispensing the contents into a waste container. This rinse cycle was repeated two times in order to minimise any carry over issues between standards and samples.

3.1.5.3. Preparation of External Quality Control Standards

Different lot numbers of purchased ethanol standards (0.02 g/100mL, 0.05 g/100mL and 0.2 g/100mL) were used as external quality control standards. They were prepared in the same manner as the purchased standards mentioned earlier. These external quality control standards were used to perform and address method validation parameters such as accuracy, precision, repeatability, reproducibility and uncertainty of measurement.

3.1.6. Method Validation Parameters

In this section each method validation parameter and the equations that are key to each parameter will be described. The acceptance criteria for each parameter are summarised in Table 6.

Table 6: Table summarising the method validation parameters, definitions and acceptance criteria for each parameter

Parameter	Definition	Acceptance Criteria
Selectivity	Selectivity refers to how the method can measure and differentiate between the desired compound, internal standard(s) and other compounds that may be present within a specimen/matrix.	Visual observation
Linearity	Linearity refers to a specific and given range within the analytical process, to obtain a result or value where the signal response is directly proportional to the concentration of the desired compound.	Correlation coefficient (r^2) ≥ 0.9998
LOD	Limit of detection (LOD) is the lowest concentration of the desired compound that can be distinguished from the absence of that compound (a blank value) with a stated confidence level.	-
LOQ	Limit of quantification (LOQ) refers to the lowest concentration that can be determined with an acceptable level of repeatability, precision and trueness.	-
Accuracy	Accuracy is referred to how close the concentration determined, is to the theoretical concentration of a compound.	CV< 10%
Precision	Precision verifies how varied the determinations of the concentrations of a specific compound can be after it is determined through repeated measurements.	CV< 10%
Matrix Effects	This parameter is used to determine the effect that the matrix (blood or water) can have on the signal response of a compound.	Blood measurements of purchased ethanol standards should be within manufacture's range
Carry over	Carry over is used to determine the amount of a compound that can be detected after a certain concentration of a sample is injected onto the headspace–gas chromatography system that could possibly alter the determination of that specific compound in a subsequent analysis.	Carry over percentage was less than 20% of the lowest calibration standard (0.01 g/100mL) response and less than 5 % of the IS amount at this concentration (European Medicines Agency (EMEA), 2011).
Repeatability	Repeatability is how close results are when they are repeated under the same instrumental and environmental conditions and is done by the same analyst.	(Max measurement – Min measurement) $<$ $(\mathrm{SD} \times 2.8)$
Reproducibility	Reproducibility refers to how close results are when an analysis is repeated on the same instrumental and environmental conditions but is done so by two different analysts.	(Max measurement – Min measurement) $<$ $(\mathrm{SD} \times 2.8)$
Ruggedness	Ruggedness refers to the reproducibility of results from measurements when slight changes in the environmental or experimental conditions were made.	(Max measurement – Min measurement) $<$ $(\mathrm{SD} \times 2.8)$
Measurement of uncertainty	The error that is associated with all measurements is represented as a quantity that is added and subtracted from a measurement value to determine if the measurement is intended for its purpose. This quantity is referred to as the uncertainty of measurement.	-

The mean, standard deviation and coefficient of variance (CV %) was calculated for method validation parameters; accuracy, precision, reproducibility, matrix effects, carry over and uncertainty of measurement.

For each parameter the coefficient of variance was determined using:

$$\%CV = \frac{s}{\bar{x}}(100) \quad \text{.....(a)}$$

where

$$Standard\ Deviation\ (s) = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{x})^2}{n-1}} \quad \text{.....(b)}$$

and

$$Mean\ (\bar{x}) = \frac{\sum X_i}{n} \quad \text{.....(c)}$$

The following technical test parameters were also assessed as part of the method validation process:

3.1.6.1. Selectivity

Six separate flat bottom headspace vials were prepared: (i). internal standard only; (ii). Donated blood from volunteers with no alcohol in their system; (iii). 0.01 g/100mL ethanol (aqueous); (iv). 0.1 % acetone (aqueous), (v). 0.1 % acetaldehyde (aqueous) and (vi). 0.1 % 2- Propanol (aqueous). Equal volumes of these solutions were then combined and analysed in triplicate to determine the selectivity of the method over three days (in total 9 runs were assessed).

The overall chromatographic performance was assessed through visual observation and the differentiation between retention times. This included that the chromatographic peaks should have a Gaussian shape; peaks could be easily differentiated between ethanol, internal standard and other possible interfering compounds (acetone, acetaldehyde and 2-propanol) often associated with post-mortem blood. Other volatiles were not assessed as these volatiles are not often associated with post-mortem blood.

3.1.6.2. Linearity

Linearity is the result in the method where the signal response is directly proportional to the concentration of a compound at that specific signal response. The internal standard method was applied to construct a calibration curve which used tertiary butanol (t-butanol) as an internal standard (0.1 %). The Internal standard method was utilised since a ratio between the analyte (ethanol) signal and internal standard signal is used to determine the concentration of analyte. It therefore corrects the possible loss of the analyte during the sample preparation.

Linearity was also provided by the FID, since the FID is a detector d with a wide linear response range of seven orders of magnitude (10^7) (Cool and Ockendon, 2018). For this reason, a linear curve could be used for the calibration standards. Six standards with ethanol concentrations 0.00 g/100mL (blank), 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL (aqueous) were used to determine the linear range for the quantified method.

These standards were used since they cover the range of the physiological effects of alcohol at various concentrations mentioned in section 2.1.4. Calibration curves were established, using three analyses on seven different days by two different scientists (a total of 42 calibration runs), using a signal response versus area ratio (area of ethanol peak to area of internal standard peak). A linear range was determined through the close correlation between the standards used for a calibration curve. The linear range was accepted when a correlation coefficient was 0.9998 and above.

Standardised residual plots were also used to determine if the calibration curve is linear by assessing the variance of residuals across the calibration range. This will establish if the calibration model used is fit for purpose. The calibration model is linear when there is random scatter around the zero line (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013)

3.1.6.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) is the lowest concentration of a desired compound that can be detected, whilst the limit of quantification (LOQ) refers to the lowest concentration that can be determined. Seven standards (0 g/100mL, 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL) were used to establish and assess the LOD and LOQ. The LOD was determined using the mean of the blank readings with the addition of three times the standard deviation of the blank readings. The LOQ was determined using the mean of the blank readings with the addition of 10 times the standard deviation of the blank readings.

A minimum LOQ of 0.02 g/100mL was required in order to meet the legislation requirement concentration of professional drivers stated in the South African National Road Traffic Act 93 of 1996, since literature states that most deaths that occurred where alcohol could have been a contributing factor, are road traffic accidents. This study can therefore address/cover the death in which professional drivers were involved. In this study a BAC result was, therefore, considered positive when the BAC≥0.02 g/100mL and a BAC result was considered negative when the BAC<0.02 g/100mL.

The following equations were used to determine LOD and LOQ;

LOD:

$$(Y_{LOD} - b)/_{m} \quad \text{.....(d)}$$

where

$$(Y_{LOD} = b + 3Sy/_{x}) \quad \text{.....(e)}$$

and

Slope (m) is calculated by:

$$\frac{\sum(X_i - X_{avg})^{*}\,(Y_i - Y_{avg})}{\sum(X_i - X_{avg})^{2}} \quad \text{.....(f)}$$

and

Intercept (b) by:

$$Y_{avg} - bX_{avg} \quad \text{.....(g)}$$

LOQ:

$$(Y_{LOQ} - b)/_{m} \quad \text{.....(h)}$$

Where

$$(Y_{LOQ} = b + 10Sy/_{x}) \quad \text{.....(i)}$$

3.1.6.4. Accuracy (Bias)

Accuracy (Bias) is referred to how close the concentration determined, is to the theoretical concentration. Two ethanol standards (aqueous) (0.02 g/100mL; 0.05 g/100mL) were used to determine accuracy and precision by different analysts over five days, resulting in 38 results overall. Confirmation of accuracy was assessed using the student t-test at 95% confidence interval. t_{crit} is the targeted value produced by the student t-test and t_{calc} is the value that is calculated. obtained on the certificate provided by the manufacturer (NMISA). The measurement of bias (standard deviation index (SDI)) (Westgard, 2009) is used to determine if the there is any systematic difference between the result compared to the true value. Any SDI below 1 is acceptable (Westgard, 2009).

For accuracy, a student t-test was performed at the 95% confidence level, therefore:

$$t_{calc} = \frac{((|experimental\ mean - true\ value|)^{*}\sqrt{n})}{s} \quad \text{.....(j)}$$

and

t_{crit} =Critical value for student's t-test .....(k)

$$SDI = \frac{calculated\ mean - true\ value}{s} \quad \text{.....(l)}$$

3.1.6.5. Precision

Two ethanol standards (aqueous) (0.02 g/100mL; 0.05 g/100mL) were used to determine accuracy and precision by different analysts over five days, resulting in 38 results overall. An F-test at 95% confidence interval was performed to establish if there were any significant differences between the results over various days performed by the same analyst and buy two different analysts on different days. The results were deemed significant when Fcalc<Fcrit.

For confirmation of precision an F-test was performed, where:

$$f_{calc} = \frac{{S_1}^2}{{S_2}^2} \quad \text{.....(m)}$$

where

S_1^2 = variance of group 1 .....(n)

and

S_2^2= variance of group 2 .....(o)

and

f_{crit}=Critical value for f-test .....(p)

3.1.6.6. Repeatability

Repeatability is how close results are when they are repeated under the same instrumental and environmental conditions and is done by the same analyst. Repeatability was measured by analysing a set of quality control samples (n=18) in the same environmental and instrumental conditions by the same analyst. Repeatability measurement limits was calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95% of a population of results are within 1.96 standard deviation of the mean) times the square root of 2.

Repeatability was assessed using the following formula:

$$SD\ of\ repeatability\ results\ measurements\ \times 2.8 \quad \text{.....(r)}$$

The limits are accepted when the difference between the maximum and minimum results of the quality control measurements is < the SD of repeatability results measurements x 2.8.

3.1.6.7. Reproducibility

Reproducibility refers to how close results are when an analysis is repeated on the same instrumental and environmental conditions but is done so by two different analysts. Reproducibility was assessed through the repeated measurements of quality control samples under the same environmental and instrumental conditions, but by two different analysts (n=12). Reproducibility measurement limits was calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95% of a population of results are within 1.96 standard deviation of the mean) times the square root of 2.

Reproducibility was assessed using the following formula:

$$SD\ of\ reproducibility\ results\ measurements\ \times 2.8 \quad \text{.....(s)}$$

The limits are accepted when the difference between the maximum and minimum results of the quality control measurements is < the SD of reproducibility results measurements x 2.8.

3.1.6.8. Matrix Effects

This parameter was used to determine the effect that the matrix (blood or water) can have on the signal response of a compound. Purchased Ethanol standards in blood matrix of concentrations; 0.03 g/100mL; 0.05 g/100mL and 0.2 g/100mL were analysed in 5 replicate measurements over two days. The same preparations procedure for samples and quality controls in section 3.4.2 were followed.

The results determined from these prepared standards (0.03 g/100mL; 0.05 g/100mL and 0.2 g/100mL) must be within 15% of the prepared concentration. This was in accordance of the acceptance criteria provided by the certificates of the manufacturer (ACQ Science).

3.1.6.9. Carry Over

Carry over is used to determine the amount of a compound that can be detected after a certain concentration of a sample is injected onto the headspace–gas chromatography system that could possibly alter the determination of that specific compound in a subsequent analysis.

The procedure consisted of three experiments utilising the highest ethanol standard (0.5 g/100mL) injected onto a column followed by 10 blank samples (containing no ethanol). The amount of ethanol was then determined in these blank samples. Any peaks that were observed visually on the chromatogram, at the same retention time as ethanol was noted.

The acceptance criteria for a carry-over percentage was less than 20% of the lowest calibration standard (0.02 g/100mL) response and less than 5 % of the IS amount at this concentration (European Medicines Agency (EMEA), 2011).

The percentage carry-over was determined using the following equation:

$$\frac{Mean\ of\ determined\ ethanol\ concentrations\ in\ blank\ vials}{the\ ethanol\ concentration} \times (100) \quad \text{.....(q)}$$

3.1.6.10. Ruggedness

Ruggedness refers to the reproducibility of results from measurements when slight changes in the environmental or experimental conditions were made. Ruggedness was assessed by analysing ethanol quality control samples under different temperature (environmental) conditions. This was done by monitoring the temperatures on specific days and comparing the results at the maximum temperature with the results at a minimum temperature. Ruggedness measurement limits were calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95% of a population of results are within 1.96 standard deviation of the mean) times the square root of 2.

Ruggedness was assessed using the following formula:

$$SD\ of\ rugeddness\ results\ measurements\ \times 2.8 \quad \text{.....(t)}$$

The limits are accepted when the difference between the maximum and minimum results of the quality control measurements is < the SD of ruggedness results measurements x 2.8.

3.1.6.11. Measurement of Uncertainty

Expanded uncertainty (U_{exp}) is the term generally referred to when calculating the uncertainty of measurement. All contributions to this measurement such as bias, precision/imprecision, purity of the standards, volumes and equipment used should be considered. Uncertainty of measurement was done using a “top down approach” to calculate the expanded measurement of uncertainty (see appendix 5) for calculations associated with the Uncertainty of Measurement calculation. This was accomplished by calculating the percentages uncertainty for the imprecision of measurement, the uncertainty of the purity of the quality control standards, the bias of the measurements, combined uncertainty and then ultimately expanded uncertainty. Confirmation of significant Bias was assessed using the student t-test at 95% confidence interval. t_{crit} is the targeted value produced by the student t-test and t_{calc} is the value that is calculated. The method was considered significantly bias if there was a significant difference between the experimental mean and the true value ($t_{calc}<t_{crit}$).

The expanded measurement of uncertainty was calculated as follows:

The imprecision of the measurement was calculated using:

$$U_{precision} = \sqrt[2]{\frac{SD_{L1}\,(n_{L1}-1)^2 + SD_{L2}\,(n_{L2}-1)^2}{(n_{L1}+n_{L2})-2}} \quad \text{.....(u)}$$

where

L1= %CV of first level of an ethanol standard .....(v)

(QC1)

and

L2= %CV of second level of an ethanol standard .....(w)

(QC2)

The uncertainty surrounding the purity of the standards was calculated as follows:

$$U_{purity} = \sqrt[2]{\%U_{purity}} \quad \text{.....(x)}$$

where

$\%U_{purity}$ = Obtained from the manufacturer's certificate where the standards were purchased. .....(y)

The percentage bias of the measurement was calculated as follows:

$$\%Bias = \left[\frac{x_{average} - \ standard\ average}{standard\ average}\right] \times 100 \qquad \text{.....(z)}$$

The uncertainty in the mean of the quality control standards were calculated as follows:

$$U_{mean} = \frac{SD_{mean}}{\sqrt[2]{n}} \qquad \text{.....(aa)}$$

where

n=is the number of quality control standard measurements ..... (bb)

And

SD_{mean}= is the standard deviation of the average of the standard readings .....(cc)

The uncertainty in the bias of the mean was calculated as follows:

$$\%U_{bias} = \sqrt[2]{(\%U_{mean})^2 + \ (\%U_{standard})^2} \qquad \text{.....(dd)}$$

The significance of this bias was assessed using the student t test where:

$$t_{calc} = \frac{Bias}{U_{bias}} \quad \text{.....(ee)}$$

And

t_{crit} =Critical value for student's t-test .....(ff)

The combined uncertainty (U_c) was therefore calculated as follows:

$$U_{combined} = \sqrt[2]{(Bias)^2 + \left(U_{precision}\right)^2} \quad \text{.....(gg)}$$

The expanded uncertainty can therefore be calculated then for a coverage factor (k) of 2 at 95% confidence interval as follows:

$$\%U_{expanded} = k(\%U_{combined}) \quad \text{.....(hh)}$$

3.2. PART 2: POST-MORTEM BLOOD COLLECTION

The methodology surrounding the collection of blood at the JFPS MLL facility in Johannesburg will be described in this section. It should be emphasised that the collection procedure in this section, closely resembles the collection procedure that is currently performed at the JFPS MLL facility.

3.2.1. Materials

Post-mortem blood collection kits were supplied by Acacia Medical™. Blue top collection tubes (10mL volume) were purchased from PlastPro Scientific (Pty) Ltd.

3.2.2. Population

Blood specimens were collected from 119 decedents admitted to the JFPS MLL (located at 25a Hospital Street in Braamfontein) during 1 July 2015 to 31 July 2016 according to the inclusion criteria set.

3.2.3. Inclusion and exclusion criteria for specimen collection

The inclusion criteria for the sample were:

- Adult decedents (above 18 year of age).
- All decedents where the pathologist suspected the involvement of alcohol and requested an ancillary BAC test.

The exclusion criteria for the sample were:

- Decedents that were admitted to the hospital prior to death, as any medication given intravenously will influence BAC and possibly skew data analysis.
- Decedents that are in the advanced stages of decomposition e.g. bloated, decay and dry stages, as postmortem redistribution of ethanol may have occurred.
- Skeletonised decedents, as no blood will be available for collection and analysis.
- All adult decedents that meet the inclusion criteria, however, had an insufficient volume of blood for this study (volume of blood < 24mL).

3.2.4. Ethics

Full unconditional ethics clearance was granted from the Wits Human Research Ethics Committee (Clearance certificate number: M140909) (See Appendix 2). This ethical clearance includes all record reviews and sample collection as stated in the ethical clearance application. Sample collection and review of decedent records are of individuals who have been brought to the JFPS MLL due to their death being classified as an unnatural death. These individuals require a medico legal autopsy, as to establish their cause of death as per the Inquest Act 58 of 1959, therefore no consent is required for blood nor reviewing of decedent autopsy reports. The researcher also obtained permission to attend post-mortem procedures as well as gain access to the case files from the mortuary manager of the Johannesburg Forensic Pathology Service Medico legal Mortuary as well as the Head of the Division of Forensic Medicine and Pathology at the University of the Witwatersrand.

The case numbers of victims were not used, but rather assigned research numbers were used. A copy of the case number and its assigned research number was kept in a password protected file accessible only to the researcher and supervisors.

3.2.5. Blood Collection from Decedents at the JFPS MLL

The collection of specimens and the process followed by the pathologist at the JFPS MLL was as closely replicated in this study. This was done to replicate the current variability associated with the specimens sent to the FCL.

Blood from the femoral vein (located in the upper thigh and pelvic region) was collected. This process consists of making an incision in the femoral vein. The leg of the deceased was gently squeezed from the ankle in an upwards motion towards the inner thigh. Blood pooled in the incision area. Approximately 24 mL of blood was collected using three 10mL blue top collection tubes. This blood was pooled together in each vial and mixed vigorously. This blood was then transferred using a syringe into standard issue FPS mortuary sample collection bottles which utilise sodium fluoride and potassium oxalate as a preservative and stored in a standard polystyrene container. This procedure is as closely replicated as possible to the procedure followed in the JFPS MLL. In total, four post-mortem collection kits were used per decedent, where two McCartney bottles contained 4 mL (therefore a headspace volume of 11mL) of blood each and the remaining two McCartney Bottles contained 8 mL (therefore a headspace volume of 7mL) of blood each. Each McCartney bottle was mixed before it was placed in the polystyrene container before it was sealed.

The kits were then transported to the Analytical Services Laboratory in a crate and stored at 4°C until the first analysis could be performed, e.g. time 0 months. The time frame between collection and analysis was within a week and up to one month (due to the availability of the instrument). Refer to Figure 11 for the full process of sample collection.

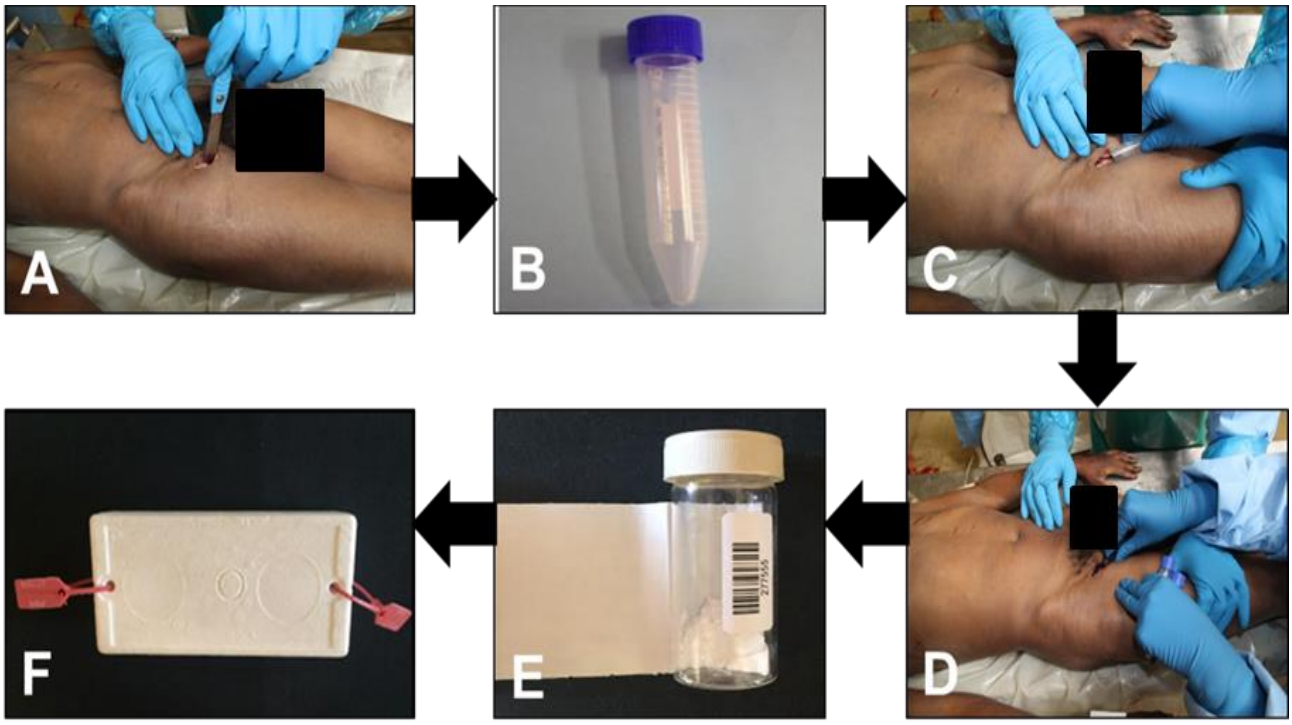

Figure 11: Process of Blood Collection at the JFPS MLL.

A, Incision is made in the femoral vein; **B**, 10mL test tube is used to collect blood; **C** and **D** Test tube is filled with blood after blood has pooled at the area of the incision; **E**, McCartney bottles are filled with the appropriate volumes of blood and placed in the polystyrene container of the post-mortem kit. **F**, Post-mortem kits are sealed and transported to laboratory.

3.3. PART 3: ANTE-MORTEM BLOOD COLLECTION AND BLOOD SPIKING THAT SERVES AS BLOOD QUALITY CONTROLS (POSITIVE AND NEGATIVE CONTROL) FOR THE PRIMARY STUDY

In this section the procedure that was followed to obtain ante-mortem blood from volunteers and the procedure for spiking this ante-mortem blood (for positive blood quality controls) is described. Although it is advisable to take the same matrix (in this case post-mortem blood) and spike the matrix with a known concentration, this could not be done. Since post-mortem blood is known for its variability, little control over the post-mortem blood can be applied. It would also have not been practical to collect post-mortem blood from victims, take the blood to the laboratory, screen for ethanol, go back to the victims and collect more blood. It was therefore decided to used ante-mortem blood for the spiked positive ethanol and negative ethanol controls. The principle of the headspace method allows for this since only the volatile sample components that have partitioned into the headspace are injected onto the column, leaving the matrix behind.

3.3.1. Materials

A-grade 50 mL measuring beaker and a grade 25mL volumetric flask (all property of NIOH). Post-mortem blood collection kits were supplied by Acacia Medical™.

3.3.2. Reagents

High Performance Liquid Chromatography Grade ethanol (>99.9%) was purchased from Sigma Aldrich (Pty) Ltd.

3.2.1. Ante-mortem Blood

Ante-mortem blood was collected from volunteers (see Appendix 4) whose blood contained no alcohol. A registered nurse collected the blood using the method set out in Section 2.3.1. This blood was used for the control measures for the experimental process for the assessment of headspace and temperature variables experiment.

3.2.2. Equipment

The PRECISA® 925M-202A analytical balance.

3.2.3. Procedure

Blood specimens that represented the positive controls consisted of donated blood from volunteers that contained no alcohol. The blood was mixed thoroughly and was pooled together in an A-grade 50 mL measuring beaker. High Performance Liquid Chromatography Grade ethanol (>99.9%) was purchased from Sigma Aldrich (Pty) Ltd and was used to prepare a 10% v/v solution with deionised water. The PRECISA® 925M-202A analytical balance was used to accurately measure 0.05g (approx. 157 µL) of the 10% solution into a calibrated A-grade 25mL volumetric flask. The flask was made up to the mark with donated blood. The volumetric flask was mixed thoroughly. This method set out by Jones and Ericsson (2016) on the preparation of spiked ethanol in blood samples was followed. After the blood was spiked it was analysed in duplicate and the mean concentration of (0.05 g/100mL) was used as the first initial analysis.

Post-mortem collection kits were then filled with the volumes of blood and stored in the conditions set out in the headspace experiment and temperature experiment respectively (as mentioned in section 3.3.7).

3.4. PART 4: ANALYSIS OF SAMPLES AT DIFFERENT STORAGE TIMES AND STORAGE AT DIFFERENT TEMPERATURES

The analysis of specimens was conducted at the Analytical Services Laboratories at the National Institute of Occupational Health (NIOH), located at 25 Hospital Street in Braamfontein. NIOH is a division of the National Health Laboratory Services (NHLS), whose main activity is to provide specialised laboratory services for diagnostic and research purposes, with its primary focus on occupational health.

3.4.1. Materials

Flat bottom headspace (10mL volume) vials were purchased from Chemetrix (Pty) Ltd and Separations (Pty) Ltd respectively due to price changes. The difference in suppliers was due to price differences. Headspace aluminium caps and rubber bungs were purchased from Glass Blowing Industries. The Agilent HP-Innowax® column (Length, 30m; ID, 0.250mm and film thickness, 0.50µm) was purchased from Chemetrix (Pty) Ltd. This column was used as it was the current column utilised at the Analytical Services Laboratory with the stationary phase primarily being polyethylene glycol, which works well for alcohol determination.

3.4.2. Reagents

Ethanol standards in aqueous (H_2O) of the following concentrations; 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL, were purchased from the National Metrology Institute of South Africa (NMISA).

Two different lots of ethanol standards in aqueous (H_2O) of the following concentrations 0.02 g/100mLand 0.05 g/100mL were purchased from the National Metrology Institute of South Africa (NMISA) and served as quality controls. Tertiary-butanol (t-butanol) (99.7%purity), acetaldehyde (99.5% purity), 2-propanol (99.5% purity) and Acetone (99.8% purity) were purchased from Sigma Aldrich.

Deionised water from the Milli-Q® water EVOQUA® Ultra Clear purification system, purchased from Separations (Pty) Ltd.

3.4.3. Ante-mortem Blood

Ante-mortem blood that contained no alcohol was collected from volunteers (see Appendix 4). A registered nurse collected the blood using the method set out in Section 2.3.1

3.4.4. Equipment

All equipment, apart from the Data Loggers, is the property of the National Institute for Occupational Health (NIOH) Analytical Services. A-Grade volumetric flasks (100mL).

A vial crimper (unknown origin but used regularly in the Analytical Services laboratory) was used to tighten aluminium crimp top caps to flat bottom headspace vials.

The Hamilton Microlab® 500 series dilutor was used for sample preparation with t-butanol. The samples were analysed on an Agilent® G1888 Headspace that is coupled to an Agilent 6890 N GC that is fitted with a Flame Ionization Detector (FID), purchased from Chemetrix (Pty) Ltd.

The temperature of storage conditions was monitored throughout the entire study using calibrated temperature data loggers from Coldchain Thermodynamics®.and refrigerator temperature thermometers (as used in the Analytical Services Laboratory). Temperature data loggers were placed in the refrigerators and in the secured room where samples were stored.

A Vortex® Genie 2 mixer from Lasec (Pty) Ltd was used for sample mixing before analysis.

3.4.5. Dilutor Verification before Sample Preparation

It is standard for the Analytical Services Laboratory to have key equipment calibrated to SI units and send for service once a year. One of this equipment is the Hamilton dilutor, however to ensure that the right volumes of solutions/reagents or samples are pipetted at the volumes that it is calibrated, a verification step is required.

The dilutor was therefore verified with the dispensing of 1000 µL water (as per the calibration certificate states) as is required by the method. This verification process was performed before each preparation of sample or standard.

A total of five empty 10mL headspace vials were initially weighed and followed by dispensing 1000 µL distilled water into each vial. The vials were re-weighed, and the actual mass of the water was determined making an adjustment for the density of water at the temperature of the experiment. A percentage was calculated from this data and a 98% and better, acceptance criterion was used before any analysis could begin.

3.4.6. Standards and Quality Controls

The procedure for the preparation of the standards and quality controls is described in this section.

3.4.6.1. Preparation of Internal standard

The internal standard, 0.1% t-butanol, was prepared by adding 100 µL of t-butanol to a 100mL volumetric flask and filled it to the mark with deionized water.

3.4.6.2. Preparation of Calibration Standards

Seven purchased standards with known concentrations (traceable to SI units) of aqueous ethanol 0.01 g/100mL, 0.02 g/100mL, 0.05 g/100mL, 0.1 g/100mL, 0.2 g/100mL and 0.5 g/100mL (aqueous) were used as calibration standards.

Method validation guidelines recommend a calibration model consisting of a minimum of four standards (points) on a calibration curve. Seven standards were chosen for this study, which covered the range of concentrations that cause severe physiological effects described in section 2.1.4. A blank (without ethanol) sample (0 g/100mL) was prepared with deionised water, in the laboratory. These standards were then diluted with t-butanol which is the internal standard. This dilution of standards and internal standard was performed using a calibrated (calibration certificate number: SEP0822MC) Hamilton Microlab® 500 series dilutor (see Appendix 5).

The dilutor was programmed to draw 500 µL of internal standard and 500 µL of sample or standard so that 1000 µL is dispensed into a 10mL headspace vial. A rinse cycle was employed after this dispersion, by drawing 1000 µL of internal standard and dispensing the contents into a waste container. This rinse cycle was repeated two times in order to minimise any carry over issues between standards and samples.

3.4.6.3. Preparation of External Quality Control Standards

Different lot numbers of purchased ethanol standards (0.02 g/100mL and 0.05 g/100mL) were used as quality control standards. They were prepared in the same manner as the purchased standards for calibration mentioned earlier. These external quality control standards were analysed after the calibration standards and blank sample, and then after every 10 samples. The results of these external quality control standards are then plotted on a Levi-Jennings Curve and the Modified Modern Westgard (Westgard, 2009) rules are applied to verify that the method used is working.

3.4.1. Post-mortem Blood Sample Preparation for analysis on HS-GC/FID

Blood, standards and quality control specimens were prepared with the addition of an internal standard for analysis. This dilution of samples and internal standard was performed using a calibrated Hamilton Micro lab 500 series dilutor. The dilutor was programmed to draw 500 µL of internal standard and 500 µL of sample or standard so that 1000 µL is dispensed into a 10mL headspace vial. Thereafter the dilutor was programmed to perform two rinsing steps which entailed drawing 1000 µL of internal standard and dispensing it into a waste container; this was repeated for the second rinsing step.

After the samples, standards or quality controls were dispensed; the vials were sealed with a rubber bung and capped tightly with the aluminium cap using a 10mL vial crimper. The samples, standards or quality controls were then mixed with a vortex mixer and placed onto the headspace tray.

3.4.2. Sample analysis on HS-GC/FID

Alcohol was determined and quantified using a G1888 Headspace Auto sampler (Agilent Technologies®) coupled to a 6890N Agilent® Gas Chromatography instrument utilising a Flame Ionization Detector on an Agilent HP-Innowax® column (analytical conditions and column dimensions are attached in Appendix 10).

The gold standard technique of alcohol determination in blood is performed using headspace gas chromatography fitted with flame–ionization detection (HS-GC/FID). The HSGC-FID is known for its accuracy, sensitivity, relative specificity characteristics and ease of operation. Each blood specimen was analysed in duplicate and the mean of these results was the reported.

Calibration standards were analysed before a batch of duplicated samples was analysed. This was done to establish if the instrument was at optimal conditions. A correlation coefficient of a minimum of 0.9998 was accepted.

Different lots of purchased ethanol (aqueous) standards (0.02 g/100mL and 0.05 g/100mL) served as external quality controls. Duplicate sets of these controls were analysed after a calibration curve was established. Results had to be within manufacturer's ranges before the calibration curve could be accepted and analysis conducted. These external quality controls were also analysed after 10 samples.

The results of these external quality control standards are then plotted on a Levi-Jennings Curve and the Modified Modern Westgard (Westgard, 2009) rules are applied to verify that the method used is working.

3.4.3. Specimen storage and time to analysis conditions

In this section the different storage conditions and the different storage volumes will be described. These conditions will address the aim and objectives of this study.

3.4.3.1. Temperature Variable Experiment

The initial measurement was considered as time zero. All PM kits (a total of four vials) were analysed, and an average of these results were used for the initial analysis at time 0= 12 to 14 days from time of collection. Further analysis of each PM Kit was done at time 3= 3months after first analysis (t=0), time 6 = 6 months after second analysis (t=3 months) (Figure 12). A victim therefore provided a total of 24mL volume of blood. The vials containing 8mL for room and fridge temperatures and the vials containing 4mL for room and fridge temperatures were compared for the temperature variable experiment.

In this experiment the volumes were kept constant, but the temperature variable changed between fridge and room temperature throughout the storage period. Two post-mortem kits containing 4 mL and 8 mL post-mortem blood respectively were stored at ± 4˚C and two post-mortem kits containing 4 mL and 8 mL post-mortem blood respectively were stored at ± 22˚C (room temperature).

3.4.3.2. Headspace Variable Experiment

In this experiment the volumes were altered between 8 mL and 4 mL at room temperature and at fridge temperature over storage period time. The same specimens in the same storage conditions that were described in the Temperature Variable experiment was used (Figure 12). The difference was however was in the comparison and management of the data. In the Headspace Variable experiment the 4 mL and 8 mL vials stored in fridge temperature was compared. The same was done for the 4 mL and 8 mL vials at room temperature.

3.4.3.3. Experimental Blood Positive and Negative Controls for Primary Study

Each experiment was conducted with spiked blood positive and blood negative controls under the same temperature and storage conditions described in the Temperature Variable and Headspace Variable experiment (Figure 12). This was done to determine if the method of analysis was accurately detecting ethanol in blood as well as to establish if similar trends were observed in each experiment.

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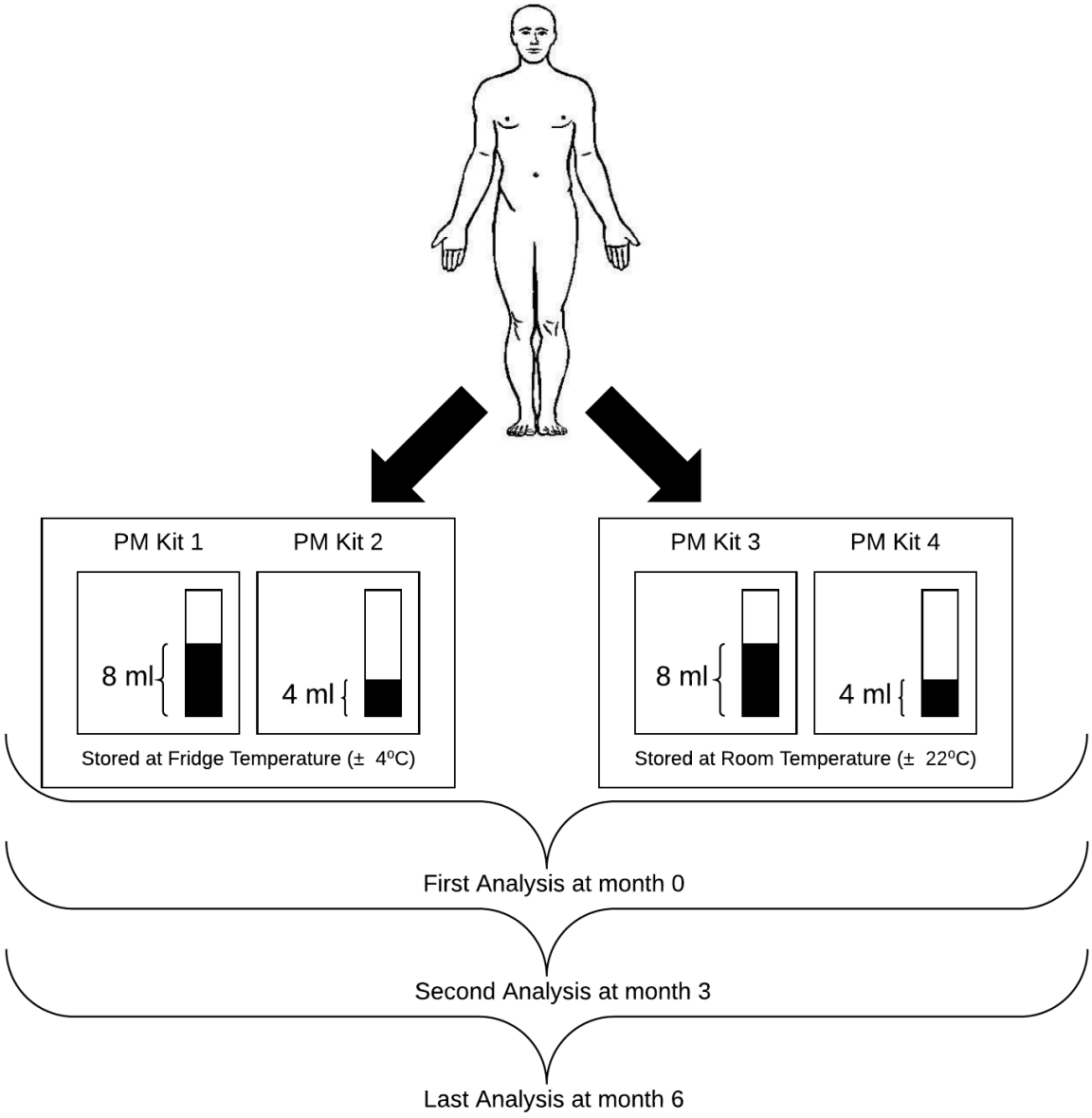

Figure 12: Figure illustrating the volumes of blood collected and the storage temperatures

3.4.4. Data Processing

Agilent® Open Lab CDS ChemStation® Edition for GC Systems C.01.05 software was used for instrument control and data processing. A calibration curve (the y-axis showing the signal to noise ratio and the x-axis the ethanol concentration) was determined using this software and the line of best fit was used to calculate the correlation coefficient. The area under the chromatographic peaks was then measured using this integration software, by manually drawing a best fit line at the base of the peak. The area ratio of ethanol to the internal standard (t-butanol) of the specimen was determined from the calibration curve.

3.5. PART 5: STATISTICAL ANALYSIS

Statistical analysis was conducted with the assistance of Mr Felix Made, Biostatistician of the Epidemiology and Surveillance section within the National Institute for Occupational health. Tables containing all positive alcohol readings were imported on the Statistical program Stata Corps® version 14.2. Since the data was not normally distributed a non-parametric Wilcoxon signed-rank test was used to compare median alcohol concentrations in blood samples after storage at 3 months and at 6 months at 95% confidence interval. This was done for both readings of alcohol concentrations at 4mL and 8mL blood volumes at different storage temperatures (temperature variation experiment).

A non-parametric Wilcoxon signed-rank test was also used to assess the difference in alcohol concentration readings between the 4mL and 8mL blood volumes (headspace variation experiment).

The percentage loss in BAC was calculated using the following formula:

$$\frac{original\ BAC\ result - the\ BAC\ result\ at 3\ or\ 6 months}{original\ BAC\ result} \times 100 \quad \text{.....(hh)}$$

A pie chart was used to illustrate the number of test with respect to their alcohol readings (positive or negative). A line graph was used to illustrate the linearity (method validation parameter) in the calibration curve.

CHAPTER 4: RESULTS

4.1. PART 1: METHOD VALIDATION

All method validation parameters referred to in this chapter were defined in section 3.6.3.2. Other key terms used in this section are the following:

'Coefficient of Variance (CV %)'

The ratio of the standard deviation to the calculated mean and is expressed as a percentage**.**

'Standard Deviation (SD)'

Quantity expressed of how much the mean of ethanol concentrations differ from the true value of that concentration.

'Standard Deviation Index (SDI)'

A measurement of bias of the difference between the mean calculated and the true value.

'Non-parametric'

Data not fitting a normal distribution.

4.1.1. Selectivity

Selectivity as defined in Section 3.6.3.2; in gas chromatography, it is predominantly a function of the type of column (the stationary phase) used. The retention times and standard deviations (SD) of the retention times for each compound are represented in Table 7. Five blank whole blood samples were also analysed to determine if any interfering peaks are present within a whole blood sample. No other interfering peaks were observed.

Table 7: Ethanol, IS and possible interfering compounds found in post-mortem blood according to ethanol analysis method

Sample Name	Retention Time (RT) (minutes)
Tertiary Butanol (IS)	1.798 ± 0.002
Ethanol	2.033 ± 0.002
2-Propanol	1.968 ± 0.007
Acetone	1.522 ± 0.004
Acetaldehyde	1.259 ± 0.001

Figure 13 illustrates the chromatograph when all compounds were combined in one vial demonstrating good resolution (chromatographic peaks are Gaussian and peaks could be easily differentiated between ethanol, internal standard and other possible interfering compounds (acetone, acetaldehyde and 2-propanol) between peaks, resulting in good resolution and selectivity.

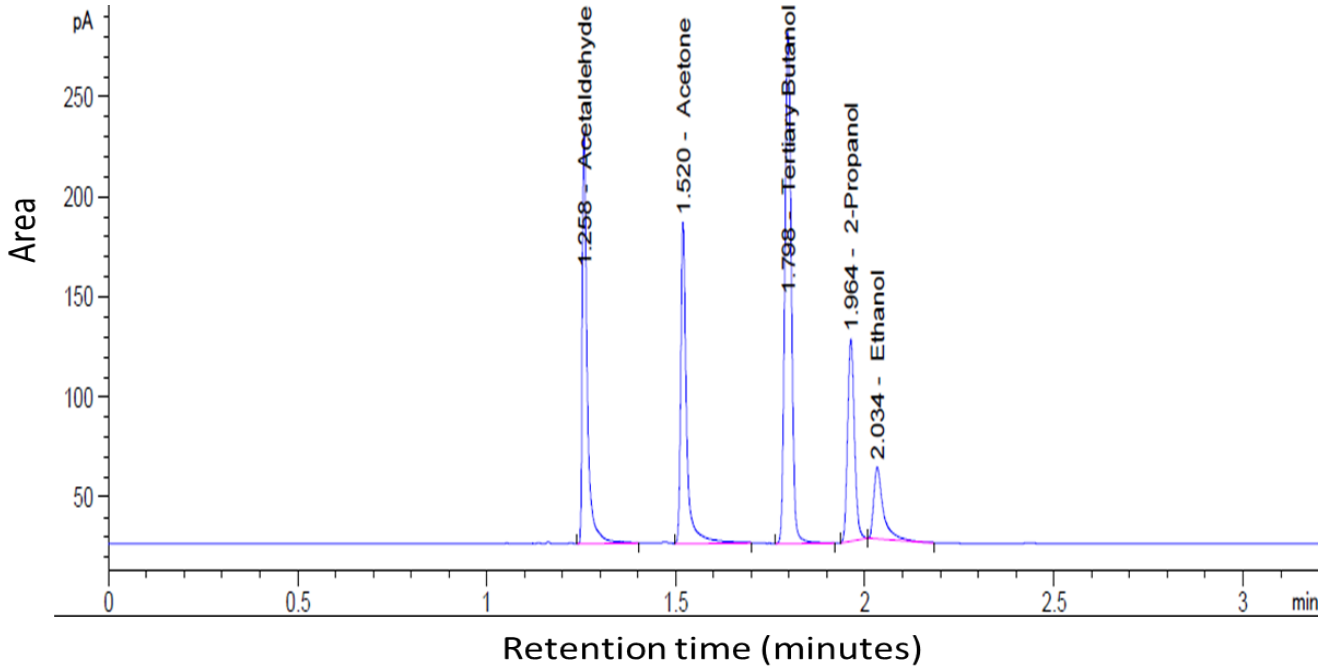

Figure 13: Chromatograph illustrating that there was no interference between the IS (t-butanol) and ethanol under the analytical conditions of the method

With high concentrations, peak tailing occurs at very high concentration and therefore shows that the method is selective and that ethanol can be differentiated from other identifying peaks at the concentration ranges/levels studied in this work.

4.1.2. Linearity

The calibration standards showed linearity in the working range of 0.02 g/100mL to 0.5 g/100mL with an average correlation coefficient of 0.9999 (Figure 14).

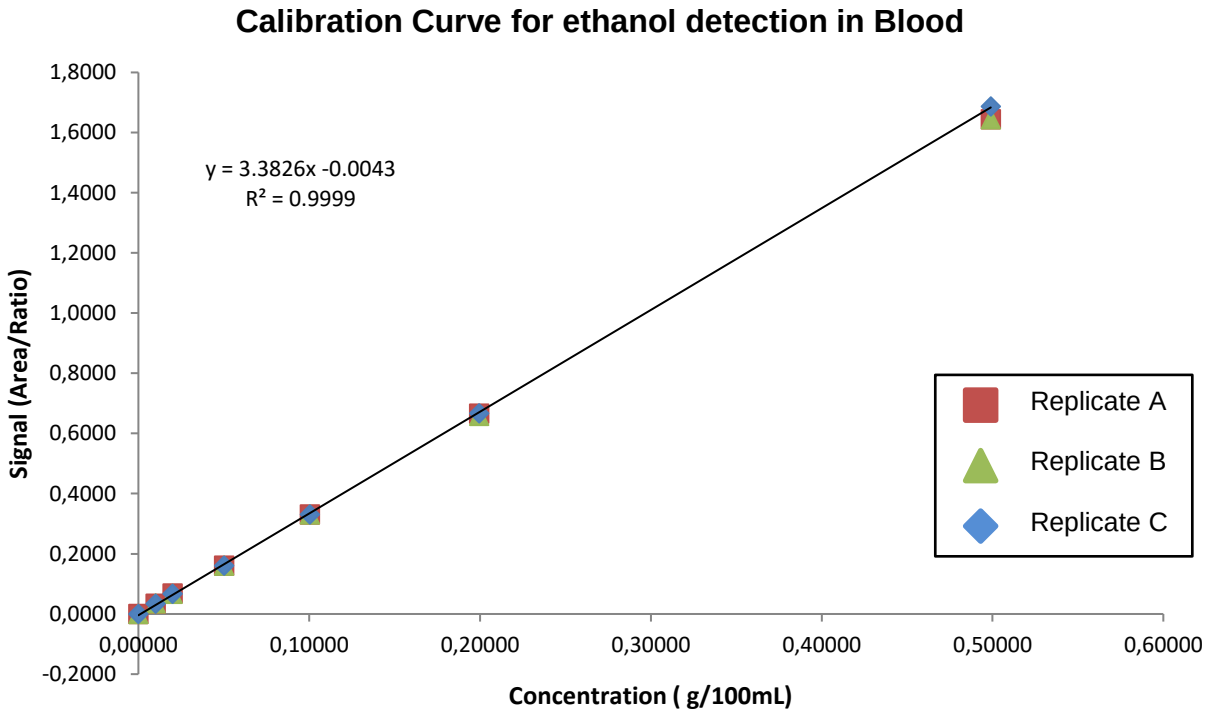

Figure 14: Calibration curves of ethanol in blood

Standardised residual plots were also used to visually detect any outliers of any calibration points, refer to residual plot accompanying the calibration curve in Figure 15.

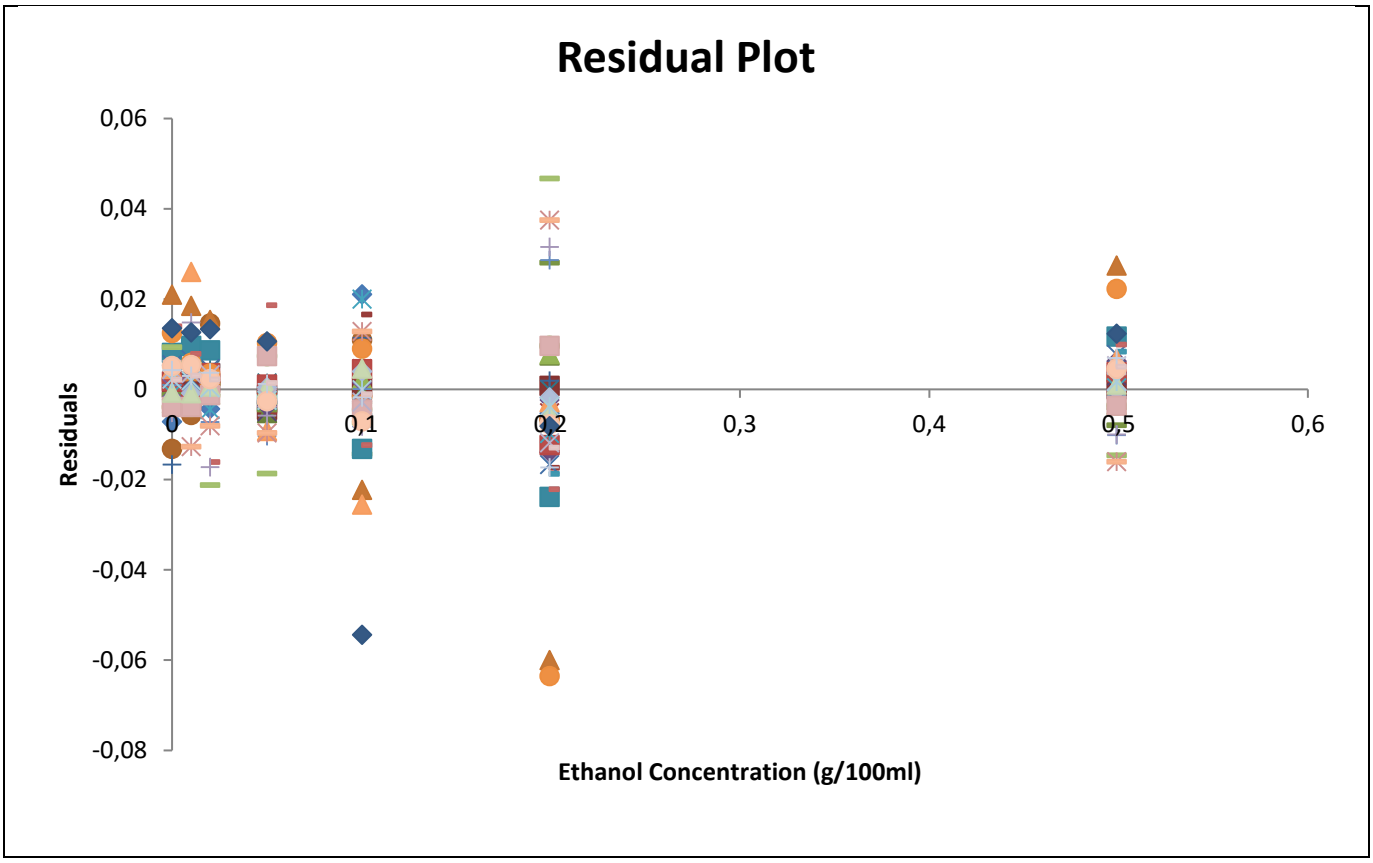

Figure 15: Residual plot of the calibrations of all 42 replicates

Since the residual plot showed random scatter around the zero line with no outliers that had to be statistically viable to eliminate and therefore the method is linear. All calibration curves for the completed on various days, by two different analysts are represented in Appendix 7. The average recovery of 100% was determined and all calibration data (slope, intercept and correlation coefficient) of each calibration curve that was analysed by two different analysts are represented in Appendix 7.

4.1.3. LOD and LOQ

The results were determined from the calibration curves utilising the equations set out in sections 3.1.6.3 (i.e. regression statistics). Regression statistics were used to determine the LOD and LOQ and an example of these statistics are represented in table 8. An LOD of 0.01 g/100mL and LOQ of 0.02 g/100mL respectively were obtained at the correlation coefficient of 0.9998.

Table 8: Regression Statistics Summary

Multiple R	0.9999
R Square	0.9999
Adjusted R Square	0.9999
Standard Error	0.0021
Observations	7

ANOVA

	df	SS	MS	F	Significance F
Regression	1	0.1922	0.1922	43831.835	0.0000
Residual	5	0.0000	0.0000		
Total	6	0.1922			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-0.0001	0.0010	-0.1083	0.9179	-0.0027	0.0024	-0.0027	0.0024
X Variable 1	0.9996	0.0048	209.3605	0.0000	0.9873	1.0119	0.9873	1.0119

LOD	0.01
LOQ	0.02

4.1.4. Accuracy

The results of all quality controls and certified reference materials produced recovery mean percentages between 99% and 102% and fall in an acceptable criteria range which is ± 15% of reference values (true values). This was verified using the t-test of the mean values obtained for each quality control (table 9).

Table 9: The External Quality Control Data from Analyst 1 (n=32)

Date	QC 1 Result	QC 2 Result	Analyst
20.02.2015	0.02098	0.05032	Analyst 1
	0.01915	0.04823	
	0.01999	0.05042	
23.02.2015	0.21150	0.04862	
	0.01999	0.05113	
	0.02098	0.05150	
24.02.2015	0.02030	0.05381	
	0.02085	0.05148	
25.02.2015	0.02109	0.04961	
	0.02109	0.04636	
	0.02246	0.04971	
27.02.2015	0.01966	0.04970	
	0.01992	0.04961	
	0.01997	0.05211	
	0.02035	0.05279	
	0.02056	0.05003	
Mean	0.02041	0.05034	
Average Recovery (%)	102	99	
CV%	5	4	
SD	0.001	0.002	
SDI	0.074	-0.100	

Table 10: The External Quality Control Data from Analyst 2 (n=44)

Date	QC 1 Result	QC 2 Result	Analyst
04.03.2015	0.02016	0.05089	Analyst 2
	0.02016	0.05092	
	0.02211	0.05011	
	0.02032	0.05125	
	0.02076	0.04991	
	0.02191	0.05183	
	0.01733	0.05465	
	0.02151	0.05196	
16.03.2015	0.01733	0.05087	
	0.02065	0.05037	
	0.02155	0.05085	
17.03.2015	0.02098	0.05087	
	0.02071	0.05037	
	0.01733	0.05085	
	0.02062	0.04969	
	0.02124	0.04899	
18.03.2015	0.02146	0.05285	
	0.02172	0.05266	
	0.02155	0.05285	
	0.02030	0.05013	
	0.02117	0.05013	
	0.01733	0.04920	
Mean	0.02037	0.05101	
Average Recovery (%)	102	100	
CV%	8	3	
SD	0.002	0.001	
SDI	0.066	0.008	

Table 11: Accuracy at 95% of measurements between different analysts at different days

Day	Analyst	Experimental mean (g/100mL)	True Value (g/100mL)	Standard deviation	t_{calc}	t_{crit}	Accuracy at 95%
1	1	0.02041	0.01999 ± 0.0005	0.001	1.71	2.042	Yes
	2	0.02037		0.002	1.31	2.042	Yes
2	1	0.05034	0.05008 ± 0.000652	0.002	2.21	2.042	Yes
	2	0.05101		0.001	0.22	2.042	Yes

4.1.5. Precision

The intra- day precision results produced CV% (%RSD) in the range of 2 to 8% for both analysts on respective days (refer to table 12 and table 13).

Table 12: Intra-day (between runs) precision results for Analyst 1 (n=5)

Date	QC 1 Result	QC 2 Result	Analyst
27.02.2015	0.01966	0.04970	Analyst 1
	0.01992	0.04961	
	0.01997	0.05211	
	0.02035	0.05279	
	0.02056	0.05003	
Mean	0.02009	0.05085	
Average Recovery (%)	100.261	99.718	
CV%	2	3	
SD	0.0004	0.0015	

Table 13: Intra-day (between runs) precision results for Analyst 2 (n=5)

Date	QC 1 Result	QC 2 Result	Analyst
17.03.2015	0.02098	0.05087	Analyst 2
	0.02071	0.05037	
	0.01733	0.05085	
	0.02062	0.04969	
	0.02124	0.04899	
Mean	0.02018	0.05016	
Average Recovery (%)	101	98.365	
CV%	8	2	
SD	0.0016	0.0008	

The inter- day (between days) precision results produced CV% (%RSD) in the range of 3 to 7% for both analysts on respective days (refer to table14 and table 15).

Table 14: Inter-day (between days) precision results for Analyst 1 (n=8)

Date	QC 1 Result	QC 2 Result	Analyst
20.02.2015	0.02098	0.05032	Analyst 2
	0.01915	0.04823	
	0.01999	0.05042	
27.02.2015	0.01966	0.04970	
	0.01992	0.04961	
	0.01997	0.05211	
	0.02035	0.05279	
	0.02056	0.05003	
Mean	0.02007	0.05040	
Average Recovery (%)	100	99	
CV%	3	3	
SD	0.0006	0.0014	

Table 15: Inter-day (between days) precision results for Analyst 2 (n=14)

Date	QC 1 Result	QC 2 Result	Analyst
04.03.2015	0.02016	0.05089	Analyst 2
	0.02016	0.05092	
	0.02211	0.05011	
	0.02032	0.05125	
	0.02076	0.04991	
	0.02191	0.05183	
	0.01733	0.05465	
	0.02151	0.05196	
18.03.2015	0.02146	0.05285	
	0.02172	0.05266	
	0.02155	0.05285	
	0.02030	0.05013	
	0.02117	0.05013	
	0.01733	0.04920	
Mean	0.02056	0.05138	
Average Recovery (%)	103	101	
CV%	7	3	
SD	0.0015	0.0015	

An F-test at 95% confidence interval was performed to establish if there were any significant differences between the results represented in table 16. An F-test at 95% confidence interval was performed to establish if there were any significant differences between the results over two days performed by two different analysts and the results between 2 different analysts and are represented in table 16 and table 17.

Table 16: Confirmation of precision of tests between two different analysts

Precision between Analysts	F_{calc}	F_{crit}	Fcalc<Fcrit
Analyst 1 and Analyst 2 (QC 1)	**0.05**	**0.16**	**Yes**
Analyst 1 and Analyst 2 (QC 2)	**3.37**	**6.39**	**Yes**

Table 17: Confirmation of precision of tests between different days

Analyst	Day	F_{calc}	F_{crit}	Fcalc<Fcrit
Analyst 1 (QC 1)	20.02.2015 vs 27.02.2015	6.37	6.94	Yes
Analyst 1 (QC 2)	20.02.2015 vs 27.02.2015	1.44	19.25	Yes
Analyst 1 (QC 1)	03.03.2015 vs 18.03.2015	1.23	3.97	Yes
Analyst 2 (QC 2)	03.03.2015 vs 18.03.2015	1.25	3.97	Yes

4.1.6. Repeatability

The results of the repeatability studies are represented in table18 and table 19.

Table 18: Summary of the results from 9 replicate analyses (3 results in each replicate set) of two analysts over the period of three days (n=18)

Date	QC 1 Result	QC 2 Result	Analyst
20.02.2015	0.02098	0.05032	Analyst 1
	0.01915	0.04823	
	0.01999	0.05042	
23.02.2015	0.01999	0.04862	
	0.02115	0.05113	
	0.02267	0.05150	
25.02.2015	0.02109	0.04961	
	0.02109	0.04636	
	0.02246	0.04971	
max-min	0.00268	0.00328	
2.83*SD	0.00319	0.00448	

Table 19: Summary of the results from 10 replicate analyses (5 results in each replicate set) of two analysts over the period of three days (n= 20)

Date	QC 1 Result	QC 2 Result	Analyst
17.03.2015	0.02098	0.05087	Analyst 2
	0.02071	0.05037	
	0.01733	0.05085	
	0.02062	0.04969	
	0.02124	0.04899	
18.03.2015	0.02146	0.05285	
	0.02172	0.05266	
	0.02155	0.05285	
	0.02030	0.05013	
	0.02117	0.05013	
max-min	0.00439	0.00386	
2.83*SD	0.00441	0.00407	

Repeatability results show a difference between the max and min value is less than 2.8 x SD between the same analyst.

4.1.7. Reproducibility

The results of the reproducibility studies are represented in table 20.

Table 20: Summary of the results from two replicate analyses (3 results in each replicate set) of two analysts on different days (n= 12).

Date	QC 1 Result	QC 2 Result	Analyst
20.02.2015	0.02098	0.05032	Analyst 1
	0.01915	0.04823	
	0.01999	0.05042	
16.03.2015	0.01733	0.05087	Analyst 2
	0.02065	0.05037	
	0.02155	0.05085	
max-min	0.00422	0.00265	
2.83*SD	0.00427	0.00276	

Reproducibility results show a difference between the max and min value is less than 2.8 x SD between two analysts.

4.1.8. Matrix Effects

The concentration results determined from the commercially purchased ethanol (0.03 g/100mL; 0.05 g/100mL and 0.2 g/100mL) in blood reference materials and the purchased ethanol (0.02 g/100mL; 0.05 g/100mL and 0.2 g/100mL) in aqueous solution reference materials all lay within the 15 % range. All statistical criteria were met (refer to table 21).

Table 21: Summary of the results within range of the true values of the certified reference materials in blood and aqueous matrix respectively (n= 20 results for each concentration)

Matrix	Concentration (g/100mL)	True Value (g/100mL)	True Value Tolerance Range (g/100mL)	Experimental Mean results (g/100mL)	Experimental Mean results Within Manufacturer range
Blood	0.03	0.03 ± 0.0005	0.0255-0.0355	0.0311±0.004	Yes
	0.05	0.0 5± 0.0005	0.0450-0.0550	0.0480±0.004	Yes
	0.20	0.20 ± 0.0010	0.1902-0.2102	0.1902±0.010	Yes
Aqueous	0.02	0.01999 ± 0.0005	0.01994-0.02049	0.0201±0.001	Yes
	0.05	0.05008 ± 0.000652	0.04942-0.05073	0.0506±0.001	Yes
	0.20	0.19947 ± 0.0022	0.20167-0.19727	0.1993±0.010	Yes

4.1.9. Carry Over

A carry over of less than 1 % was determined during the method validation process and results are represented in table 22.

Table 22: Carry-over results of 10 blank samples directly after a high concentration (0/5 g/100mL) value (n=10)

Date	Sample	Concentration (g/100mL)
20.02.2015	0.5 g/100mL (Aqueous)	0.503
	Blank vial	0.003
	Blank vial	0.003
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Blank vial	Not Detected
	Mean (blank values only)	0.0005
	SD	0.0011
	% carry over	0.09%

The carry over results were deemed acceptable since it fits the acceptance criteria by not exceeding 20 % of the signal of the lowest calibration standard (0.02 g/100mL).

4.1.10. Ruggedness

Ruggedness measurements were assessed at 20.9°C and 23°C respectively as these were the temperatures recorded as the lowest temperature and highest temperature during the method validation period.

Table 23: Summary of the results from 2 replicate analyses (3 results in each replicate set) of two analysts on different days.

Temperature	Date	QC 1 Result	QC 2 Result
23.0°C	**20.02.2015**	0.02098	0.0503
		0.01915	0.0482
		0.01999	0.0504
20.9°C	**23.02.2015**	0.01809	0.0486
		0.02115	0.0511
		0.02098	0.0515
max-min		0.00306	0.0029
2.8xSD		0.00345	0.0037

Ruggedness results show a difference between the max and min value is less than 2.8 x SD between two days at different temperature variations.

4.1.11. Measurement of Uncertainty

Table13 represents the uncertainty of measurement parameters to ultimately obtain a result for the combined uncertainty of measurement for the current analytical alcohol method. All calculations are in Appendix 6.

Table 24: Summary of the Uncertainty of Measurement Parameters

Uncertainty of Measurement Parameter	Result
$U_{precision}$	2.23%
U_{purity}	0.33%
$\%Bias$	0.002%
U_{mean}	1.58%
$\%U_{bias}$	1.38%
$U_{combined}$	2.31%
$\%U_{expanded}$	4.63%

The method was evaluated using all the variables according the South African National Accreditation System (SANAS) 2016 guideline TG 41-02, the South African National Accreditation System (SANAS) 2012 guideline TG 07-01 the National Institute for Occupational Health Standard Operating Procedure (NIOH0391). All validation criteria were met and the method was deemed fit for purpose for the determination of ethanol in blood.

4.2. PART 4 AND PART 5: ALCOHOL RESULTS

Blood from 119 decedents was collected, analysed and subjected to the outlined volumes and storage conditions as per experimental conditions mentioned in section 3.3.7. Blood from 36 (30%) decedents tested positive for alcohol (BAC≥0.02 g/100mL) (refer to Figure 16).

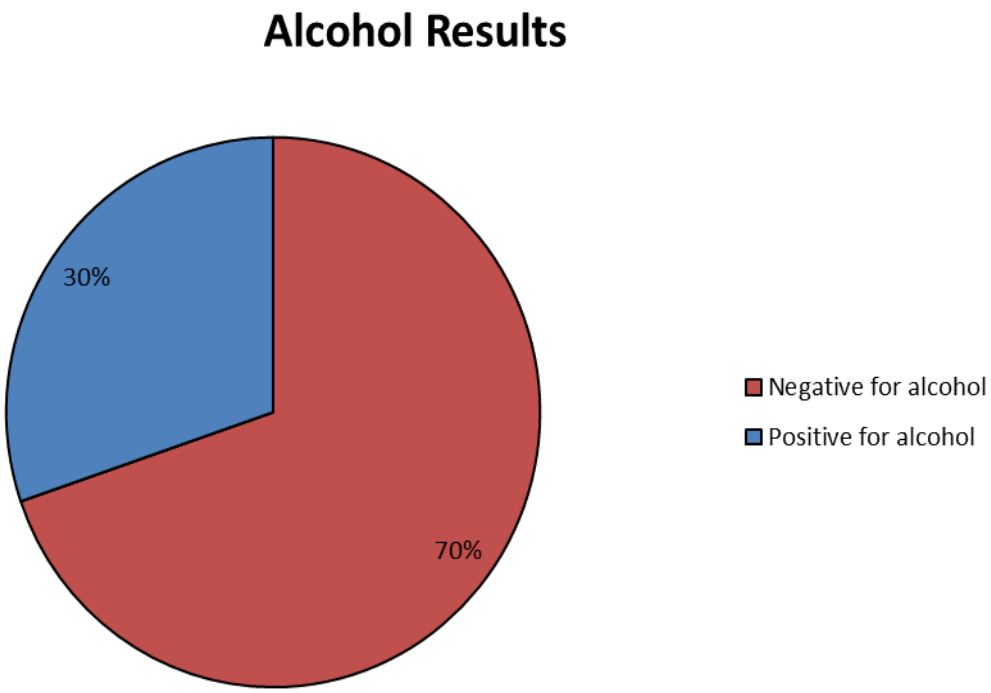

Figure 16: Percentage of alcohol results of blood from 119 decedents collected at the JFPS MLL

4.3. THE EFFECT OF STORAGE TEMPERATURE ON BAC IN POST-MORTEM BLOOD SPECIMENS

The evaluation of temperature variable experiment is shown in this section. Results for the specimens were kept at constant volumes (4mL and 8mL respectively) at refrigerator (fridge) temperature (average temperature 4.9°C) and room temperature (average temperature 23°C).

One result was excluded from the temperature analysis (assigned research number 56), although the BAC results showed similar trends, this specimen had an original BAC result of 1.24 g/100mL, which is very high for any living individual.

4.3.1. Negative alcohol results for the entire period of study at the different headspace and temperature variables

From the 119 decedents, 83 (70%) of the BAC results were negative (BAC<0.02 g/100mL). The BAC of these specimens (volumes at 4mL and 8mL) remained unchanged for the entire evaluated storage period of 6 months.

All of these specimens, except for five, original BAC result at t=0 months were analysed between 12 to 14 days from time of collection. These five samples were analysed within 30 days (1 month) of sample collection due to the instrument breakdown that occurred within this period. These results were negative (BAC<0.02 g/100mL).

4.3.2. Positive alcohol results of the 4mL volume at room and fridge temperatures over the established storage period

Table 24 displays the positive (BAC>0.02 g/100mL) BAC of the 35 (29%) decedents, collected at the JFPS MLL, at a volume of 4mL over a storage period of 6 months at fridge and room temperatures respectively.

All of these specimens, except for two, original BAC result at t=0 months were analysed between 12 to 14 days from time of collection. These two samples were analysed within 30 days (1 month) of sample collection sample collection due to the instrument breakdown that occurred within this period. These results are represented in the tables at specimen with assigned research number 30 and 35.

The overall trend that was observed for the BAC results at fridge temperature, over the period of 3 to 6 months is a general decrease (loss) in BAC results. A similar trend for the BAC results at room temperature over the period of 3 to 6 months was also observed.

A non-parametric Wilcoxon signed-rank test showed that temperature had a significant ($p<0.0001$) effect on BAC over the storage period of 6 months. It was also noted that there was a higher average percentage loss in BAC at room temperature than at fridge temperature at the 3 months and 6 months mark.

Table 24: The positive BAC results and the % Loss of BAC over the stipulated period of 6 months for 4mL volume PM Kits stored at room and refrigerator temperatures

Assigned research number	Original BAC result (g/100mL)	Fridge Temperature (Average Temperature of 4.9°C)				Room Temperature (Average Temperature 23°C)			
		3 months BAC result (g/100mL)	% Loss in BAC	6 months BAC result (g/100mL)	% Loss in BAC	3 months BAC result (g/100mL)	% Loss in BAC	6 months BAC result (g/100mL)	% Loss in BAC
Control	0.05	0.05	0	0.05	0	0.05	0	0.05	0
9	0.03	0.03	0	< LOQ	100	0.02	33	0.02	33
10	0.18	0.17	6	0.10	44	0.15	17	0.10	44
11	0.03	0.03	0	0.03	0	< LOQ	100	< LOQ	100
12	0.12	0.04	67	0.02	83	0.06	50	0.04	67
13	0.12	0.09	25	0.02	83	0.07	42	0.07	42
14	0.06	0.03	50	0.03	50	0.04	33	< LOQ	100
17	0.17	0.05	71	0.05	71	0.08	53	0.07	59
19	0.09	0.08	11	0.03	67	0.03	67	< LOQ	100
20	0.17	0.14	20	0.08	54	0.09	49	0.06	66
22	0.12	0.12	0	0.02	83	0.07	42	0.06	50
27	0.02	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100
30	0.24	0.22	8	0.04	83	0.18	25	0.03	88
35	0.04	0.03	25	0.03	25	< LOQ	75	< LOQ	100
36	0.03	0.02	33	< LOQ	100	0.02	33	< LOQ	100
45	0.11	0.05	55	0.04	64	< LOQ	91	0.04	64
47	0.18	0.13	28	0.10	44	0.16	11	0.08	56
55	0.22	0.13	41	< LOQ	100	0.16	27	0.11	50
57	0.35	0.06	83	0.06	83	0.20	43	0.15	57
61	0.19	0.11	42	0.06	68	0.11	42	0.09	53
62	0.32	0.02	94	< LOQ	100	< LOQ	97	< LOQ	100
65	0.05	0.04	20	0.03	40	0.04	20	< LOQ	100
66	0.07	0.03	57	< LOQ	100	0.05	29	0.05	29
71	0.03	0.02	33	< LOQ	100	0.03	0	< LOQ	100
76	0.03	0.03	0	0.01	67	0.03	0	< LOQ	67
79	0.10	0.07	30	0.06	40	0.08	20	0.04	60
80	0.13	0.07	46	0.07	46	0.08	38	0.07	46
82	0.13	0.06	54	0.03	77	0.03	77	0.02	85
87	0.06	0.06	0	0.06	0	0.04	33	0.03	50
89	0.12	0.04	67	0.03	75	0.03	75	< LOQ	100
90	0.10	0.04	60	0.04	60	0.02	80	0.02	80
93	0.14	0.14	0	0.07	50	0.13	7	0.06	57
96	0.04	0.04	0	0.04	0	0.02	50	< LOQ	100
101	0.16	0.14	13	0.13	19	0.11	31	0.11	31
109	0.12	0.04	67	< LOQ	100	0.09	25	0.00	100
116	0.13	0.11	15	0.07	46	0.09	31	0.00	100
Median	**0.12**	**0.05**	**41***	**0.03**	**66***	**0.05**	**43***	**0.03**	**68***

Non-parametric Wilcoxon sign rank test results: p=<0.0001

***Average % Loss in BAC**

4.3.3. Positive alcohol results of the 8mL volume at room and fridge temperatures over the established storage period

Table 25 displays the positive (BAC>0.02 g/100mL) BAC of the 35(29%) decedents, collected at the JFPS MLL, at a volume of 8mL over a storage period of 6 months at fridge and room temperatures respectively.

The overall trend for the PM kits with an 8mL volume at fridge temperature, over the period of 3 to 6 months is a general decrease (loss) in BAC results. A similar trend for the BAC results at room temperature over the period of 3 to 6 months was also observed. A non-parametric Wilcoxon signed-rank test showed that temperature had a significant ($p<0.0001$) effect on BAC over the storage period of 6 months. It was also noted that there was a higher average percentage loss in BAC at room temperature than at fridge temperature at the 3 month and 6 month mark.

In summary the BAC results at refrigerator, temperature proves to have a lower percentage loss in BAC, when compared to the BAC results stored at room temperature. The statistical analysis support this by showing that temperature does have a significant effect on the BAC.

Table 25: The positive BAC results and the % loss of BAC over the stipulated period of 6 months for 8mL volume PM Kits stored at room and refrigerator temperatures

Assigned research number	Original BAC result (g/100mL)	Fridge Temperature (Average Temperature of 4.9°C)				Room Temperature (Average Temperature 23°C)			
		3 months BAC result (g/100mL)	% Loss in BAC	6 months BAC result (g/100mL)	% Loss in BAC	3 months BAC result (g/100mL)	% Loss in BAC	6 months BAC result (g/100mL)	% Loss in BAC
Control	0.05	0.05	0	0.05	0	0.05	0	0.04	20
9	0.03	0.02	33	0.02	33	0.02	33	< LOQ	67
10	0.18	0.13	28	0.11	39	0.11	39	0.09	50
11	0.03	0.02	33	0.02	33	< LOQ	100	< LOQ	100
12	0.12	0.12	0	0.12	0	0.04	67	0.03	75
13	0.12	0.08	33	0.05	58	0.12	0	0.08	33
14	0.06	0.04	33	0.02	67	0.04	33	0.04	33
17	0.17	0.13	24	0.10	41	0.17	0	0.02	88
19	0.09	< LOQ	100	< LOQ	100	0.03	67	0.03	67
20	0.17	0.16	6	0.08	53	0.11	35	0.09	47
22	0.12	0.04	67	0.04	67	0.03	75	0.02	83
27	0.02	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100
30	0.24	0.23	4	0.06	75	0.24	0	0.06	75
35	0.04	0.03	25	< LOQ	100	0.03	25	< LOQ	100
36	0.03	0.03	0	0.02	33	0.02	33	0.02	33
45	0.11	0.10	9	0.05	55	0.03	73	0.03	73
47	0.18	0.11	39	< LOQ	100	0.18	0	< LOQ	100
55	0.22	0.18	18	0.02	91	0.19	14	0.06	73
57	0.35	0.20	43	0.12	66	0.25	29	0.15	57
61	0.19	0.13	32	0.09	53	0.11	42	< LOQ	95
62	0.32	0.02	94	< LOQ	100	0.02	94	< LOQ	100
65	0.05	0.05	0	0.05	0	0.04	20	0.04	20
66	0.07	0.07	0	0.03	57	0.04	43	0.04	43
71	0.03	0.03	0	0.03	0	0.03	0	< LOQ	100
76	0.03	0.03	0	< LOQ	100	< LOQ	100	< LOQ	100
79	0.10	0.02	80	0.02	80	0.07	30	0.05	50
80	0.13	0.12	8	0.12	8	0.12	8	0.11	15
82	0.13	0.09	31	0.03	77	0.08	38	0.08	38
87	0.06	0.06	0	0.04	33	0.04	33	0.03	50
89	0.12	0.04	67	0.03	75	0.03	75	0.03	75
90	0.10	0.03	70	0.03	70	0.06	40	0.04	60
93	0.14	0.13	7	0.11	21	0.12	14	0.10	29
96	0.04	0.02	50	< LOQ	100	0.02	50	< LOQ	100
101	0.16	0.13	19	0.13	19	0.13	19	0.13	19
109	0.12	0.03	75	< LOQ	100	0.03	75	0.03	75
116	0.13	0.09	31	0.09	31	0.11	15	0.10	23
Median	**0.12**	**0.06**	**35***	**0.03**	**57***	**0.03**	**39***	**0.03**	**63***
Non-parametric Wilcoxon sign rank test results: p=<0.0001									
***Average % Loss in BAC**									

4.4. THE EFFECT OF HEADSPACE ON BAC IN POST-MORTEM BLOOD SPECIMENS

The results for the headspace variable experiment are shown in this section. Results for the specimens with volumes at 4mL and 8mL and were kept at a constant refrigerator (fridge) temperature (average temperature 4.9°C) and room temperature (average temperature 23°C) was used.

4.4.1. Positive alcohol results of the 4mL and 8mL volume at fridge temperature over the established storage period

Table 26 displays the positive (BAC>0.02 g/100mL) BAC of the 35(29%) decedents, collected at the JFPS MLL, at a volume of 4mL and 8mL respectively over a storage period of 6 months at fridge temperatures.

The results show that the highest percentage loss was within the 4 mL (headspace volume of 11 mL) volume PM Kits when compared to the 8 mL (headspace volume of 7 mL). This trend was also observed in the same volumes at room temperature.

Table 26: The positive BAC results and the % loss of BAC over the stipulated period of 6 months for the 4mL volume and 8mL volume PM Kits stored at refrigerator temperature

Assigned research number	Original BAC result (g/100mL)	3 months				6 months			
		4mL PM kit BAC result (g/100mL)	% Loss in BAC	8mL PM kit BAC result (g/100mL)	% Loss in BAC	4mL PM kit BAC result (g/100mL)	% Loss in BAC	8mL PM kit BAC result (g/100mL)	% Loss in BAC
Control	0.05	0.05	0	0.05	0	0.05	0	0.05	0
9	0.03	0.03	0	0.02	33	< LOQ	100	0.02	33
10	0.18	0.17	6	0.13	28	0.10	44	0.11	39
11	0.03	0.03	0	0.02	33	0.03	0	0.02	33
12	0.12	0.04	67	0.12	0	0.02	83	0.12	0
13	0.12	0.09	25	0.08	33	0.02	83	0.05	58
14	0.06	0.03	50	0.04	33	0.03	50	0.02	67
17	0.17	0.05	71	0.13	24	0.05	71	0.10	41
19	0.09	0.08	11	< LOQ	100	0.03	67	< LOQ	100
20	0.17	0.14	20	0.16	6	0.08	54	0.08	53
22	0.12	0.12	0	0.04	67	0.02	83	0.04	67
27	0.02	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100
30	0.24	0.22	8	0.23	4	0.04	83	0.06	75
35	0.04	0.03	25	0.03	25	0.03	25	< LOQ	100
36	0.03	0.02	33	0.03	0	< LOQ	100	0.02	33
45	0.11	0.05	55	0.10	9	0.04	64	0.05	55
47	0.18	0.13	28	0.11	39	0.10	44	< LOQ	100
55	0.22	0.13	41	0.18	18	< LOQ	100	0.02	91
57	0.35	0.06	83	0.20	43	0.06	83	0.12	66
61	0.19	0.11	42	0.13	32	0.06	68	0.09	53
62	0.32	0.02	94	0.02	94	< LOQ	100	< LOQ	100
65	0.05	0.04	20	0.05	0	0.03	40	0.05	0
66	0.07	0.03	57	0.07	0	< LOQ	100	0.03	57
71	0.03	0.02	33	0.03	0	< LOQ	100	0.03	0
76	0.03	0.03	0	0.03	0	< LOQ	67	< LOQ	100
79	0.10	0.07	30	0.02	80	0.06	40	0.02	80
80	0.13	0.07	46	0.12	8	0.07	46	0.12	8
82	0.13	0.06	54	0.09	31	0.03	77	0.03	77
87	0.06	0.06	0	0.06	0	0.06	0	0.04	33
89	0.12	0.04	67	0.04	67	0.03	75	0.03	75
90	0.10	0.04	60	0.03	70	0.04	60	0.03	70
93	0.14	0.14	0	0.13	7	0.07	50	0.11	21
96	0.04	0.04	0	0.02	50	0.04	0	< LOQ	100
101	0.16	0.14	13	0.13	19	0.13	19	0.13	19
109	0.12	0.04	67	0.03	75	< LOQ	100	< LOQ	100
116	0.13	0.11	15	0.09	31	0.07	46	0.09	31
Median	**0.12**	**0.05**	**41***	**0.06**	**35***	**0.03**	**66***	**0.03**	**57***

***Average % Loss in BAC**

4.4.2. Positive alcohol results of the 4mL and 8mL volume at room temperature over the established storage period

Table 27 displays the positive (BAC>0.02 g/100mL) BAC of the 35 (29%) decedents, collected at the JFPS MLL, at a volume of 4mL and 8mL respectively over a storage period of 6 months at room temperature.

The results shows that the highest percentage loss was within the 4 mL (headspace volume of 11 mL) volume PM Kits when compared to the 8 mL (headspace volume of 7 mL).This trend was also observed in the these volumes at room temperature.

Table 27: The positive BAC results and the % Loss of BAC over the stipulated period of 6 months for the 4mL volume and 8mL volume PM Kits stored at room temperature

Assigned research number	Original BAC result (g/100mL)	3 months				6 months			
		4mL PM kit BAC result (g/100mL)	% Loss in BAC	8mL PM kit BAC result (g/100mL)	% Loss in BAC	4mL PM kit BAC result (g/100mL)	% Loss in BAC	8mL PM kit BAC result (g/100mL)	% Loss in BAC
Control	0.05	0.05	0	0.05	0	0.05	0	0.04	20
9	0.03	0.02	33	0.02	33	0.02	33	0.01	67
10	0.18	0.15	17	0.11	39	0.10	44	0.09	50
11	0.03	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100
12	0.12	0.06	50	0.04	67	0.04	67	0.03	75
13	0.12	0.07	42	0.12	0	0.07	42	0.08	33
14	0.06	0.04	33	0.04	33	< LOQ	100	0.04	33
17	0.17	0.08	53	0.17	0	0.07	59	0.02	88
19	0.09	0.03	67	0.03	67	< LOQ	100	0.03	67
20	0.17	0.09	49	0.11	35	0.06	66	0.09	47
22	0.12	0.07	42	0.03	75	0.06	50	0.02	83
27	0.02	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100
30	0.24	0.18	25	0.24	0	0.03	88	0.06	75
35	0.04	< LOQ	75	0.03	25	< LOQ	100	< LOQ	100
36	0.03	0.02	33	0.02	33	< LOQ	100	0.02	33
45	0.11	< LOQ	91	0.03	73	0.04	64	0.03	73
47	0.18	0.16	11	0.18	0	0.08	56	< LOQ	100
55	0.22	0.16	27	0.19	14	0.11	50	0.06	73
57	0.35	0.20	43	0.25	29	0.15	57	0.15	57
61	0.19	0.11	42	0.11	42	0.09	53	< LOQ	95
62	0.32	< LOQ	97	0.02	94	< LOQ	100	< LOQ	100
65	0.05	0.04	20	0.04	20	< LOQ	100	0.04	20
66	0.07	0.05	29	0.04	43	0.05	29	0.04	43
71	0.03	0.03	0	0.03	0	< LOQ	100	< LOQ	100
76	0.03	0.03	0	< LOQ	100	< LOQ	67	< LOQ	100
79	0.10	0.08	20	0.07	30	0.04	60	0.05	50
80	0.13	0.08	38	0.12	8	0.07	46	0.11	15
82	0.13	0.03	77	0.08	38	0.02	85	0.08	38
87	0.06	0.04	33	0.04	33	0.03	50	0.03	50
89	0.12	0.03	75	0.03	75	< LOQ	100	0.03	75
90	0.10	0.02	80	0.06	40	0.02	80	0.04	60
93	0.14	0.13	7	0.12	14	0.06	57	0.10	29
96	0.04	0.02	50	0.02	50	< LOQ	100	< LOQ	100
101	0.16	0.11	31	0.13	19	0.11	31	0.13	19
109	0.12	0.09	25	0.03	75	0.00	100	0.03	75
116	0.13	0.09	31	0.11	15	0.00	100	0.10	23
Median	**0.12**	**0.05**	**43***	**0.03**	**39***	**0.03**	**68***	**0.03**	**63***

***Average % Loss in BAC**

4.4.3. Significance testing between the BAC results at the tested headspace volumes (4mL and 8mL)

The data is not normally distributed and therefore a non-parametric Wilcoxon signed-rank test was used to determine if there was a significant difference in BAC results between 4mL and 8mL volumes, at fridge and room temperature, over a storage period of 3 and 6 months respectively (refer to table 28). The median and the probability values (p-values), are shown in table 20.

Table 28: Significant test of alcohol concentration between 4 mL and 8 mL volumes

		Blood volume		
Storage Temperature	**Months**	**4 mL**	**8 mL**	**P-Values**
Fridge (Average Temperature of 4.9°C)	**3 median**	0.05	0.06	0.43
	6 median	0.03	0.03	0.08
Room (Average Temperature of 23°C)	**3 median**	0.05	0.03	0.07
	6 median	0.03	0.03	0.43

A non-parametric Wilcoxon signed-rank test showed no significant figures (p-value>0.05) for the headspace variable on BAC results.

CHAPTER 5: DISCUSSION

The aim of this study was to assess the stability of alcohol (ethanol) concentrations in post-mortem blood specimens evaluating temperature and headspace variables with respect to time, under environmental conditions specifically in Johannesburg, South Africa. Much research has been done on the stability of alcohol in stored blood specimens, but limited studies are available in South Africa.

Due to the increasing uncertainty around the storage and collection conditions around blood specimens sent for toxicological and blood alcohol analysis and the lack of standard operating procedure on collection of biological specimens within the JFPS MLL, this study set out to determine the scientific technical impact of these factors (temperature and headspace) on the interpretation of the results. Before these factors could be addressed however, the analytical method utilised had to be developed, optimised and validated; only then could specimen collection and analysis commence.

The following key terms were utilised in this section:

‘Blood alcohol concentration (BAC)’

The quantity of ethanol in the volume of blood that is expressed in grams per 100 millilitres.

5.1. METHOD VALIDATION

A validation of an analytical method is required to eliminate the uncertainties surrounding over-reporting and under-reporting of results as well as the prevention of false positives (Peters, Drummer and Musshoff, 2007). The method validation parameters that were assessed are as follows:

5.1.1. Selectivity

The interfering compounds often found in blood specimens (ante-mortem and post-mortem) are acetaldehyde, 2-propanol, 1-propanol and acetone (Boumba *et al.*, 2012). This method demonstrated good peak resolutions between all possible interfering compounds (acetone, acetaldehyde and 2-propanol). This method can, therefore, be deemed selective for the detection of ethanol and tertiary-butanol (internal standard) (refer to Figure 13). No other interfering peaks in the whole blood samples were observed and the method can therefore be deemed selective (Huber, 2010).

5.1.2. Linearity

The results from the calibration standards showed excellent linearity in the range of 0.02 g/100mL to 0.5 g/100mL as given by the correlation coefficients that were 0.9998. Standardised residual plots showed scattered randomly around the zero line with no outliers that had to be statistically viable to eliminate and therefore the method is considered linear (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013).

5.1.3. LOD and LOQ

An LOD of 0.01 g/100mL and LOQ of 0.02 g/100mL respectively were obtained at the correlation coefficient of 0.9998 on the calibration curve. These analytical cut-off points were required in order to meet the legislative requirements of the South African National Road Traffic Act 93 of 1996 whereby the allowable blood alcohol limit is 0.05 g / 100 mL for all drivers of motor bicycles and light motor vehicles and 0.02 g/100mL for professional drivers of heavy motor vehicles.

Literature showed that the most common death associated with alcohol was that of motor vehicle accidents (World Health Organisation, 2014). The LOD and LOQ in this study could therefore meet the legislative requirements. However, the sample set in this study had very little samples collected from motor vehicle accidents. The reason for this was the limited volume of blood available in these types of deaths due to severity of trauma.

5.1.4. Accuracy

The results produced recovery mean percentages between 99 % and 102 % and fall in an acceptable criteria range which is ± 15 % of reference values (true values)(Huber, 2010; Peters *et al*., 2007 and National Institute for Occupational Health, 2015). The CV% are below 10% which is the recommended guideline to assess bias within in method validation (United Nations Office on Drugs and Crime, 2009). Confirmation of accuracy was assessed using the student t-test at 95% confidence interval. t_{crit} is the targeted value produced by the student t-test and t_{calc} is the calculated value.

The method was considered accurate if there was no significant difference between the experimental mean and the true value ($t_{calc}<t_{crit}$) obtained on the certificate provided by the manufacturer (NMISA). The results adhere to these criteria and therefore the method is considered accurate.

5.1.5. Precision

The precision results produced CV% in the range of 2 to 8 %.The acceptance criteria state good precision should be below 10% is accepted and deemed precise as this was recommended by various guidelines on method validation acceptance criteria used in a forensic setting (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013).

The results adhere to these acceptance criteria and therefore indicate good precision. An F-test at 95 % confidence interval was performed to establish if there were any significant differences between the results over two days performed by the same analyst.

5.1.6. Repeatability

Repeatability measurement limits was calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95 % of a population of results are within 1.96 standard deviation of the mean) times the square root of 2 (Westgard, 2009).

5.1.7. Reproducibility

Reproducible measurement limits were calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95 % of a population of results are within 1.96 standard deviation of the mean) times the square root of 2 (Westgard, 2009).

5.1.8. Matrix effects

The amounts determined at the CRM' s (0.03 g/100mL; 0.05 g/100mL and 0.2 g/100mL) prepared in blood lie within the 15 % of the true value, which are the acceptance criteria provided by the certificates of the manufacturer (ACQ Science $_{GMBH}$) of these certified reference materials (refer to table 21). Hence the blood matrix has no significant effect on the analytical method and the results can be considered accurate. Although, this method is shows that blood has no significant effect on the method, it does pose a challenge when analysing post-mortem blood. Post-mortem blood can be more viscous, since it contains more water, can be clotted, the pH can be more acidic and haemolysis of cells could have occurred on a microscopic level (Kerrigan, 2013).

To some extent, post-mortem blood should not have a significant effect because this matrix is not directly injected into the GC-FID system, due to the headspace auto sampler that is used. Since the headspace technique is based on the principle of Henry's law, whereby "the amount of a given gas dissolved in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid" (McShane, 2010).

It is with this reason that the sample stays behind in the vial whilst the volatiles are injected on the chromatographic column (Zhu and Chai, 2005) and therefore limits the matrix effect to some extent.

5.1.9. Carry Over

A carry over of less than 1 % was determined (refer to Table 22). This falls within the acceptance criteria in that it is less than 20 % of the lowest calibration standard (0.02 g/100mL) response and lower than 5 % of the IS amount at this concentration (European Medicines Agency (EMEA), 2011).

5.1.10. Ruggedness

Ruggedness was calculated by using the standard deviation of the results of the quality control measurements and multiplying it with the factor 2.8. This factor is derived from 1.96 (95 % of a population of results are within 1.96 standard deviation of the mean) times the square root of 2 (Westgard, 2009). At different varying temperature, this method produced results within the acceptance criteria and can be deemed rugged for any future analysis.

5.1.11. Measurement of Uncertainty

According to Eurachem/CITAC guide: Quantifying Uncertainty in Analytical Measurement (2000), the measurement of uncertainty should demonstrate measurements that show the accuracy and validity of the quantified result. The measurement of uncertainty is depended on the needs of the clients and what the laboratory is capable to achieve (Gullberg, 2012). The uncertainty of measurement needs to be considered when there are limits attached to the specific measurement e.g. certain limits are associated with driving under the influence or a zero alcohol tolerance at the work place.

The uncertainty of measurement therefore needs to be taken into account when interpreting the results. The expanded uncertainty of measurement for this method was 4.6 %. For this study, the limitations were that BAC results were quantified at BAC≥ 0.02 g/100mL, but a sample was considered positive at BAC≥0.01 g/100mL. This expanded uncertainty was taken into consideration on decedents that had BAC results at these thresholds.

The measurement of uncertainty should always be taken into consideration especially in BAC's that are on the threshold of legal limits (BAC at 0.02 g/100mL and 0.05 g/100mL), therefore the measurement of uncertainty around these limits could result in an individual being statistically sober and therefore have a greater impact on any legal proceedings or insurance claims.

Various authors have used HSGC-FID for the determination of ethanol in blood, since it is the gold standard technique. The CV% produced by Pontes *et al.*, (2009) and Kaya *et al.*, (2010) were 10 % to 11 % and 8 % to 15 % respectively. This method produced similar results. Both studies also showed good linearity (R^2>0.99) and selectivity between ethanol and the internal standards (Pontes *et al.*, 2009 and Kaya *et al.*, 2010). Percentage recoveries for this study were also in line with the studies done by Pontes *et al.*, (2009) and Kaya *et al.*, (2010) and were in the range of 80% to 110%. To meet the low LOD's and LOQ's, the linearity of the calibration curve must be achieved with a correlation coefficient greater than 0.9998.

Purchased quality control standards at 0.02 g/100mL and 0.05 g/100mL in aqueous solution were used to ensure that quality measures are adhered to throughout the analytical method. Purchased aqueous quality control standards can be used as this method demonstrated that no matrix interference occurred. Low measurements of uncertainties were attained to provide certainty surrounding the accuracy of results.

Since this method adhered to all acceptance criteria it resulted in the method to be deemed fit for purpose in the determination of ethanol in blood.

5.2. ALCOHOL RESULTS

The results showed a positive blood alcohol concentration in 30% of the sample size that was collected. A positive result is generally a result that is higher than the analytical test method limit of detection (LOD) (Pontes *et al.,* 2009 & Peters *et al.,* 2007), currently any alcohol result above the LOD are shown to be positive for alcohol, however the result can only be quantified when the result is above the LOQ of the method. In this study, the LOQ was 0.02 g/100mL and LOD was 0.01 g/100mL.

The positive results in this study shows good comparison with literature, since a study done in Pretoria showed similar results in that 47% of their sample size (1455 decedents) (Ehmke, du Toit-Prinsloo and Saayman, 2014). Another study done on self-reported alcohol use and binge drinking in South Africa, in that 30% of South Africans reported that they consume alcohol (Vellios and van Walbeek, 2018).

5.3. THE EFFECT OF STORAGE TEMPERATURE ON BAC IN POST-MORTEM BLOOD SPECIMENS

In the sample size, 83 (70 %) specimens had a negative BAC result. The results from these specimens remained unchanged throughout the entire storage period of 6 months at the different temperatures. This was observed in both 4mL volume and 8 mL volume specimens respectively. A study completed by Sutlovic *et al.,* (2014) of blood alcohol stability in post-mortem blood samples showed similar results in that there was no change in the BAC, when the BAC results were negative (BAC<LOQ).

Their study however, showed different trends in BAC results that were positive (BAC≥LOQ) (Sutlovic, Versic-Bratincevic and Definis-Gojanovic, 2014). Since so much variability is observed during the sampling of the post-mortem specimens at the JFPS MLL, it cannot accurately be stated that at the time of collection the BAC was below the linear limit of quantification. There is very little control over how these blood specimens are handled and treated during the autopsy process. The blood is also exposed to air which in turn can cause a loss in BAC (Richardson, 2000). To eliminate this uncertainty of alcohol is present in blood and if it was present due to ante-mortem can only be confirmed if other complimentary specimens are collected in conjunction with post-mortem blood such as vitreous humor (Kerrigan, 2013).

From table 24 it was observed that at the 4mL volume, 9 (25.7 %) BAC results of decedents at fridge temperature and five BAC results at room temperature, had a percentage BAC loss at 3 months compared to the original BAC result, however no further BAC percentage loss was observed at 6 months. From table 25 it was observed that at the 8mL volume, 9 (25.7 %) BAC results of decedents at fridge temperature and 10 (28.5%) BAC results at room temperature, had a percentage BAC loss at 3 months compared to the original BAC result, however no further BAC percentage loss was observed at 6 months. There was no trend observed to state that a greater percentage loss was observed in results of BAC>0.05 or that a greater percentage loss was observed in results of BAC<0.05, however the same sporadic percentage losses were observed in other studies done by Olsen and Hearn, (2003) and Sutlovic, Versic-Bratincevic and Definis-Gojanovic, (2014).

It can therefore be concluded that a decrease in BAC observed in post-mortem blood is not dependent on the concentration of BAC which is also confirmed by Olsen and Hearn, (2003).

Tsokos (2006) confirm that studies to determine the effect of storage time and temperature have shown changes in BAC results; however they confirmed that one study showed no significant changes in BAC results in samples that had high concentrations (BAC>0.19 g/100mL), but their study consisted of ante-mortem blood samples and was only stored for 12 weeks. The positive blood control samples used in this study was that of ante-mortem blood samples. These samples produced similar results in the study done by Tsokos (2006) in which the percentage change was observed over the storage period at different temperatures.

This just confirms that ante-mortem and post-mortem are different (Kerrigan, 2013) and true comparison between post-mortem and ante-mortem blood cannot be made, since ante-mortem whole blood and post-mortem blood are very different matrix wise, due to the bacterial composition and post-mortem redistribution effects found in post-mortem blood (Dubowski *et al.,* 1997 and Kerrigan 2013).

Using post-mortem samples as controls, also poses a challenge since post-mortem samples vary from decedent to decedent, therefore pooling post-mortem samples and spiking them with alcohol cannot provide a true evaluation of alcohol in post-mortem blood, since these specimens can vary a lot.

Ideally, the only way to overcome this variability is to assess each decedent case individually. This is done where the specimen from a decedent is collected, spiked with known concentrations of alcohol for the calibration model, spiked with known concentrations of alcohol to provided method controls and ultimately quantifying the alcohol concentration (Gergov *et al.*, 2015).

This would ultimately result in more volume of blood required from each decedent, as well as more time on sample preparation is required (Gergov *et al.*, 2015) which will result in longer turn-around–time (TAT). This is practically not possible to do in South Africa due to the backlog of samples, as well as the volume of post-mortem blood available.

The remaining 26 (74%) positive BAC results in table 24 and 25 showed a different trend to the trend mentioned above in that there was a significant loss in BAC in both specimens stored at fridge and room temperatures and for both volumes over the established storage period. The study conducted by Wongchanapai (2008) confirm this trend loss, as the BAC in their study decreased significantly by 67% over a storage period of 90 days (3 months). In their study, they confirmed that BAC results of samples stored at fridge temperature would start showing a significant decrease after 14 days, versus 7 days for samples stored at room temperature.

Hence, specimens that were stored at fridge temperature (4°C) were more stable than specimens stored at room temperature were. Wongchanapai (2008) also concluded that the greatest loss of alcohol concentration in loss was observed at 90 days.

The results in this study are in agreement with observations made in post-mortem blood samples by studies Olsen and Hearn, (2003); Sutlovic, Versic-Bratincevic and Definis-Gojanovic, (2014) and Wongchanapai (2008), in that post-mortem blood specimens stored at fridge temperature are more stable (have a lower percentage BAC loss) than the specimens stored at room temperature; however a significant decrease is observed over time regardless of the temperature. Many authors (Kocak *et al.*, 2015; Shan *et al*., 2011; Smalldon & Brown 1973; Kugelberg & Jones 2007; Wongchanapai 2008; Winek & Paul 1983 and Olsen & Hearn 2003) attribute this loss of ethanol to oxidation processes occurring in the blood specimen vessels, which they confirm is temperature dependent. Oxidation could have possibly played a role to the ethanol loss in this study since the specimens were in contact with air at sampling during autopsy and at re-opening of samples for analysis at the various times. Prevention of this type of alcohol loss is inevitable and should be taken into consideration, especially in cases where extreme alcohol loss was observed (percentage loss of 80 % and higher).

Re-analysis of specimens is not uncommon in a laboratory. This can be due to many factors such as high concentrations and control values that are not accepted during an analytical run and therefore this should be noted in a forensic report since it can possibly have an effect on the BAC as shown in this study, but further studies addressing this aspect is required.

All positive BAC results showed a percentage loss in BAC over time regardless of the storage temperature. This therefore affects the accuracy and interpretation of the results of specimens that are stored for too long before analysis as well as the media scrutiny in the temperatures that these specimens are kept in. Inaccuracy of results can therefore affect the interpretation of the cause of death of an individual, as the death of an individual could have been the direct or indirect cause of alcohol but will not be reflected. In turn, this might influence legal proceedings and insurance claims e.g. claims are not paid out. The decrease can also have an effect in that the direct or indirect cause of alcohol of alcohol could possibly have caused the death of a person, however the results will not reflect this.

5.4. THE EFFECT OF HEADSPACE ON BAC IN POST-MORTEM BLOOD SPECIMENS

The effect of the headspace variable was determined in this study. From the results and statistical analysis, it was determined that the headspace variable had no statistical significant effect on the BAC in this study. This contrasts with what is mentioned in literature.

Studies from Mandić-Radić *et al.,* (2007), Sutlovic *et al.,* (2014), Tsokos (2006) and Wongchanapai (2008) showed BAC loss over their established storage periods (14 days, 6 months, 2 days to 12 weeks and 6 months respectively) and all attributed the decrease to oxidation due to the amount of air volume (headspace) above the blood sample volume.

Not all these studies, however, specifically tested for this variable in isolation, but rather in conjunction with other variables such as storage temperature, storage time and addition of preservatives. Only one study, did, however, evaluated this variable alone. Olsen & Hearn (2003) reported results of BAC of post-mortem blood collected in different volume containers. They concluded that the sample containers with less headspace provided greater stability than the larger sample containers with more headspace. Even though statistically, in this study, headspace did not have an effect on BAC results, the average percentage BAC loss that was observed in the 4mL volume specimen was higher when it was compared to the 8mL volume specimen.

Tsokos (2006) mentions that evaporation can also have an effect on BAC even though some authors such as Mandić-Radić *et al.,* (2007) have disregarded this effect. Tsokos (2006) stated that evaporation could play a role in the opening of bottles for analysis of the same sample after an initial analysis was performed at an earlier time (re-analysis). This was observed in especially those that have a large headspace above the sample volumes.

Another factor to consider with re-analysis of samples is that the blood volume in the sample container decreases and therefore the headspace above the sample concomitantly increases. Since reanalysis from the specimen vessels was performed in this study, these factors cannot be ruled out completely.

These factors could have attributed to the greater percentage loss in BAC observed in the 4mL volume specimens compared to the percentage BAC losses in 8mL volume specimens. However, to accurately state, that headspace had a significant contribution to the decrease in BAC; further investigation of this variable in isolation is required.

5.5. ANOMALY (SAMPLE 56) THAT WAS FOUND

As there are no specific guidelines in place for mortuaries on sample collection and transportation, attention to the proper collection and sampling of biological fluid is often not a priority and can lead to incorrect sampling techniques, e.g. collecting pooled blood from body cavity or collecting blood from the femoral artery. Some contamination may occur in the specimen due to the rupturing of the bladder and the concomitant mixing of fluids. According to Dubowski (2006), any alcohol concentration higher that 0.40 g/100mL would result in death. Since no other factors surrounding research decedent #56 death was observed, a possible cause for the extremely high BAC results seen in this case (BAC result 1.125 g/100mL) could be attributed to microbial action within the body from the time of death to the time of sample collection during autopsy (Kerrigan, 2013).

CHAPTER 6: LIMITATIONS AND RECOMMENDATIONS

In this chapter, the limitations surrounding this study and future recommendations will be described.

6.1. LIMITATIONS

The limitations regarding the sampling procedure and the sample analysis will be described in this section and are as follows:

6.1.1. Limitations in sampling

A major limitation of this study was the availability of the required blood volumes. Many bodies were excluded from sample collection due to the limited volume of blood available after a pathologist had collected blood as part of their ancillary testing procedure.

Variability in sample collection is inevitable since no standard operation procedure exist within the JFPS MLL.

Another limitation was that the sample collection was only from one mortuary, therefore decedents that this mortuary received was from a certain area of Johannesburg, therefore resulting in a localised decedent pool.

The number of decedents (35) that had a positive BAC was low, as a larger number of positive BAC results can show a better statistical portrayal and other possible trends.

Decedents had unknown history of alcohol consumption that accompanied each decedent due to the lack of proper administration (filling in reports).

6.1.2. Limitations in testing

Specimens of 119 decedents were reopened for each analysis and the effect of these re-opening cycles could not be ruled out completely.

It is recommended, where possible, that the alcohol test should be analysed on two separate chromatographic systems under different methodologies, which is what the state FCL units conduct. This could not be done, as the NIOH was limited to only one instrument.

In this study, other technical variables and the influence thereof on the BAC result could not be ruled out, since the level of control could not be applied to all variables such as oxidation and evaporation.

6.2. RECOMMENDATIONS

It is important to understand the influence of any one variable on BAC by limiting the influence of other variables. Hence, it is recommended that only one variable be analysed at a time. Further temperature and headspace studies should be conducted independently rather than simultaneously. This research has also provided a basis to evaluate other variables such as; concentration of preservative and storage time and their effect on alcohol stability in a South African perspective.

Future research is also recommended to determine if oxidation in isolation influences BAC, since many authors attribute it to their loss in BAC.

The possibility of analysing additional biological specimens (e.g. vitreous humor and urine) for alcohol concentrations could also be investigated. Additional matrices can be collected in conjunction with a blood specimen and analysed for alcohol accordingly to provide certainty surrounding the accuracy of the BAC results.

Guidelines and standard operation procedures on sample collection, transport and storage in a South African perspective, which can be utilised by the Forensic Officers at the JFPS MLL, could also be investigated and be introduced.

CHAPTER 7: CONCLUSION

Many studies have been performed on the stability of alcohol in blood, which included the following; sampling conditions in post and ante-mortem environments, type of specimen containers, type of preservatives in specimen containers, headspace and storage temperatures. Since environments are not standard, evaluating these aspects especially regarding variables such as temperature and headspace to determine the stability of ethanol in post-mortem blood under local conditions. This will afford better insight to the conditions cited in South African media reports about delayed testing of blood specimens for alcohol testing by state facilities and hence to the integrity surrounding these results.

In order to evaluate the BAC obtained in blood specimens, the validation of a test method is vital to ensure accuracy surrounding the results in order to eliminate the issues surrounding the over reporting and under reporting of results as well as false positives. The current method proves to be quick (less than 3 min per analysis), easy to apply, accurate and fit for purpose. However, certain criteria must be obtained before analysing specimens e.g. a correlation of at least 0.9998 in order to achieve LOD and LOQ of 0.01 g/100mL and 0.02 g/100mL respectively. Any deviation would result in a larger LOD and LOQ and hence potentially cause inaccurate reporting of lower BAC's. The measurement of uncertainty should always be taken into consideration especially in BAC's that are on the threshold of legal limits (BAC at 0.05 g/100mL). Uncertainty of measurement in this study was 12 % and since most BAC results were above or below these legal limits resulted in little or no impact.

This study showed that temperature had a significant effect on BAC over the established storage period in that an overall percentage decrease was observed. It is therefore possible that the integrity of the results surrounding the specimens in the media reports is affected. Inaccuracy of results can therefore have a great impact on the interpretation of the cause of death of an individual by a pathologist, as the death of an individual could have been the direct or indirect cause of alcohol but will not be reflected, which can have multiple repercussions such as no payments of insurance claims and life policies. The headspace variable had no statistical significant effect on the BAC results, however a percentage decrease was still observed in the tested sample volumes. As well as a great impact on any legal proceedings and insurance claims. This study does however highlight the fact that there is a lack of standard operating procedure surrounding the sampling process of blood at the JFPS MLL. The results in this study can therefore be used to improve the procedures and put certain measures in place to improve the analysis of results for accurate testing and reporting of accurate results.

In conclusion, it is important that Forensic Pathologists, investigators and scientists are aware of all the above mention factors and consider everything when interpreting blood alcohol results from a post-mortem environment.

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APPENDICES

9.1. APPENDIX 1- DATA COLLECTION SHEET

Case number: | Pathologist:

Assigned number: | Date of Autopsy:

DEMOGRAPHICS

Age: | Sex: Male | Female

Ethnicity: White | Black | Indian | Coloured

Weight: | Height:

PARTICULARS SURROUNDING DEATH

Manner of death: Homicide | Suicide | Accident | Natural

History of alcohol consumption: Yes | No | NA

Date of death | Time of death

BAC Taken | Date Analysed | Report Returned

Stage of decomposition: Early Stage | Bloat Stage | Active Decay Stage | Advanced Decay Stage | Dry Stage

Mode of death:

Pathologist Confirmation of COD:

ALCOHOL ANALYSIS

Temperature Experiment

	0 months			3 months			6 months		
Date of Analysis:									
Alcohol reading:	1	2	Ave	1	2	Ave	1	2	Ave
Fridge (°C)									
Room (°C)									

Headspace experiment

	0 months			3 months			6 months		
Date of Analysis:									
Alcohol reading:	1	2	Ave	1	2	Ave	1	2	Ave
Fridge (°C)									
Room (°C)									

9.2. APPENDIX 2- ETHICS CLEARANCE CERTIFICATE

R14/49 Ms Bianca Southon

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140909

NAME: (Principal Investigator)	Ms Bianca Southon
DEPARTMENT:	Forensic Medicine and Pathology National Health Laboratory Service National Institute of Occupational Health
PROJECT TITLE:	The Effect of Temperature and Headspace on the determination of Ethanol in Post-Mortem Blood Specimens: A South African Perspective
DATE CONSIDERED:	03/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Ildi Fenyvesi and BV Kgarebe
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/10/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature Date 10/11/2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

9.3. APPENDIX 3- CONSENT FORM

University of the Witwatersrand
Faculty of Health Sciences
Division of Forensic Medicine
Forensic Pathology Service: Southern Gauteng

Adjunct Professor Jeanine Vellema
Head Clinical Department (Chief Specialist) and Head of Division
Tel: 011 - 489 1659/00
Cell: 082 777 0737
Fax: 086 658 8413
E-mail: vellema@telkomsa.net

University of the Witwatersrand
Faculty of Health Sciences
HREC

08 September 2014

TO WHOM IT MAY CONCERN

RE: RESEARCH REQUEST BY MS. BIANCA SOUTHON

Permission is hereby granted for Ms. Bianca Southon to conduct her research as proposed, at the Southern Cluster Forensic Pathology Service (FPS) Medico-legal Mortuary Facilities. She is granted access to the FPS autopsy suites to view autopsies as they are carried out as well as access to the scribe notes/ autopsy report for the purposes of her research as outlined in her proposal.
She also understands that the strictest patient confidentiality must be maintained and that all data must remain anonymous and unlinked.

Yours sincerely,

.....

Professor Jeanine Vellema
Adjunct Professor and Head of Division of Forensic Medicine & Pathology
Wits Faculty of Health Sciences
Head Clinical Department (Chief Specialist): Forensic Pathology Service – Southern Gauteng

9.4. APPENDIX 4- PERMISSION FORM FOR THE DONATION OF ANTE-MORTEM BLOOD

University of the Witwatersrand
Faculty of Health Sciences
Division of Forensic Medicine

INFORMATION SHEET AND CONSENT FORM

PROJECT TITLE:

The effect of temperature and headspace on the determination of ethanol in post-mortem blood specimens: A South African perspective

PRINCIPAL INVESTIGATOR	Ms. Bianca Southon
INVESTIGATOR CONTACT NUMBER	072 611 8527
INVESTIGATOR ORGANIZATION	Division of Forensic Medicine, University of the Witwatersrand, Johannesburg
SUPERVISORS	Ms. Ildi Fenyvesi Dr Boitumelo Kgarebe

Hello

My name is Bianca Southon and I would like to invite you to participate in the above-mentioned study. Your participation is voluntary and you may change your mind at any time. Please feel free to ask me any questions.

I am investigating the stability of alcohol (ethanol) concentrations in post-mortem blood specimens under temperature and headspace variables with respect to time, under environmental conditions specifically in South Africa. Blood specimens are prepared with the addition of an internal standard for analysis. Alcohol will be determined and quantified by Headspace Gas Chromatography utilizing a Flame Ionization Detector. This is the gold standard method for detection of ethanol in blood.

Part of my project involves the use of donated blood, which will be pooled amongst all volunteers (18 years and older), and will then be used in the following experiments:

1. Negative quality control specimens: These specimens will be subjected to temperature (4°C and 22°C) and Headspace variables (4 mL and 8 mL) over a period of 6 months with ethanol detection conducted every 3 months.
2. Positive quality control specimens: These specimens will be spiked (serve as the positive control) with a concentration of 0.05g/100 mL and be subjected to temperature (4°C and 22°C) and Headspace variables (4 mL and 8 mL) over a period of 6 months with ethanol detection conducted every 3 months.

I require two (2) EDTA tubes of blood that will be drawn by a registered pathologist or nurse to use in the project as stated above. The blood will not be used for any other purpose other than those stipulated above. Your blood will be disposed of safely once all the required tests have been conducted. Only the people involved in this study will have access to your blood and to these results. Should you want to have the results of these tests, feel free to contact me and I will be happy to provide you with the report.

You can decide in your own time whether you want to volunteer the blood specimen or not. You can also withdraw your decision to volunteer at any time without any reason. I am willing to answer any questions that you might have. Thank you for your time.

If you require any further information on the study or would like to volunteer some blood for this study, please contact Ms. B Southon (0726118527) or Ms I J Fenyvesi (0834521261).

INFORMED CONSENT FORM

PROJECT TITLE:

The effect of temperature and headspace on the determination of ethanol in post-mortem blood specimens: A South African perspective

I have understood the information (verbal and/or printed) about this study, and agree to donate specimens of blood to be pooled amongst all volunteers. This pooled blood specimen is to be utilized as a positive and negative control in order to investigate the stability of ethanol over a period of 6 months under the various conditions mentioned. Any remaining blood will be destroyed and not used for any other purpose.

I understand I have been invited to participate in this study, and I understand that my agreeing to participate is voluntary and I can withdraw at any time.

SUBJECT NAME :_____(PRINT) _____ (SIGN)

DATE: _____AGE OF PARTICIPANT: _____

……

INFORMATION PROVIDED AND WRITTEN CONSENT WITNESSED BY

_____ (PRINT) _____ (SIGN)

SAMPLE TAKEN BY_____ (PRINT) _____(SIGN)

STUDY SAMPLE NUMBER: _____

9.5. APPENDIX 5- DOCUMENTATION WITHIN THE POST-MORTEM KITS

HP05-2014DI – ITEM 21 BID RESPONSE DOCUMENT

ANNEXURE B

Document number: FCL003 Version: Revision 05 Effective Date: Kit implementation

FORENSIC PATHOLOGY SERVICES

(Pathologist to Complete in full and in duplicate or make a copy)

This form is to be used to request for the analysis of blood for the presence of ethanol and/or carboxyhaemoglobin or toxicology analysis, or eye-fluid for the presence of ethanol

Address of mortuary:

_____

_____

_____

_____

Contact person at mortuary: _____ **Telephone number:** _____

Expiry date of the kit: _____

Analysis requested:

1. A sample container containing a **blood/ eye fluid (please √)** sample with identifying marking DR/PM_____ or WC/_____/_____ and SAPS station _____ CAS_____ and re-sealed with seal number _____ is being handed in at the Forensic Chemistry Laboratory, Cape Town / Durban / Johannesburg / Pretoria by _____
2. The sample is to be analysed for the presence of **(please √)**:

Ethanol ☐ Carboxyhaemoglobin ☐ Toxicology Suspected Compound/s: ☐

_____

_____

Note to Forensic Medical Practitioner: If Ethanol and/or CO and/or Toxicology analysis is required, two/three blood samples have to be submitted, one for each analysis. Complete one form for each blood sample.

Signature of Forensic Medical Practitioner: _____ Date: _____

For laboratory use only:

Laboratory reference number or bar-coded label if issued by Laboratory: _____

Laboratory Official name and signature: _____

HP05-2014DI – ITEM 21 BID RESPONSE DOCUMENT 4

Date received (laboratory date stamp):

ANNEXURE A

Document number: PCL004 Version: Revision 04 Effective Date: – KB

Implementation:

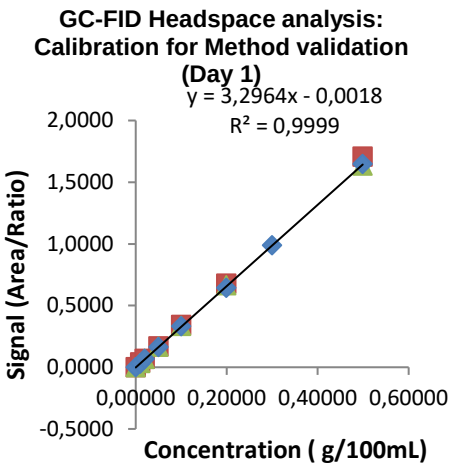
DIRECTIONS FOR THE COLLECTION OF POST MORTEM BLOOD SAMPLES FOR ALCOHOL DETERMINATION

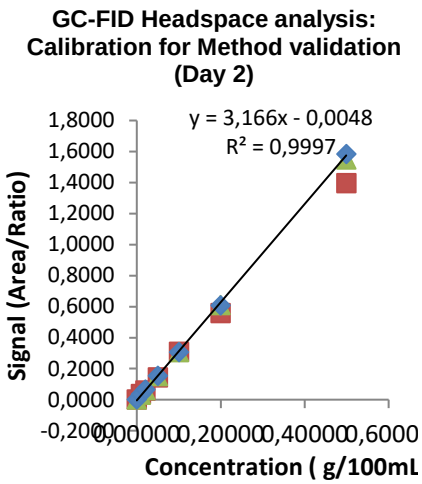
1. Check the expiry date of the kit – if the kit has expired, or is about to expire, it must not be used.
2. Break the seal and check the contents of the kit.
3. Remove glass bottle and tap the cap to shake away white powder (sodium fluoride and potassium oxalate) which may adhere to the cap, back into the bottle.
4. Collect 15 ml liquid cadaver blood from a peripheral vascular source.
5. Replace the cap firmly.
6. Immediately after filling the bottle, mix the contents by gently inverting the bottle at least ten times.
7. Complete the label. Remove the self adhesive label and fix it to the outside top of the container.
8. Put the capped bottle back into the polystyrene container. Ensure that the bottle be placed in the position that it was found.
9. Place broken first seals back into the polystyrene container. Reseal the polystyrene container with un-used seals found in the kit.
10. Secure the seals firmly without damaging the polystyrene, whilst ensuring that the plastic tubes remain in the holes that the seals are pushed through. Ensure that the seals are secured to such an extent that it does not allow for the lid to be opened wide enough to interfere with the contents.
11. Relevant accompanying documentation must not be sealed in the container that contains the sample.
12. Accompanying documentation must contain SAPS Station and CAS number.
13. Store the sample in a refrigerator until it can be submitted to the laboratory for analysis.

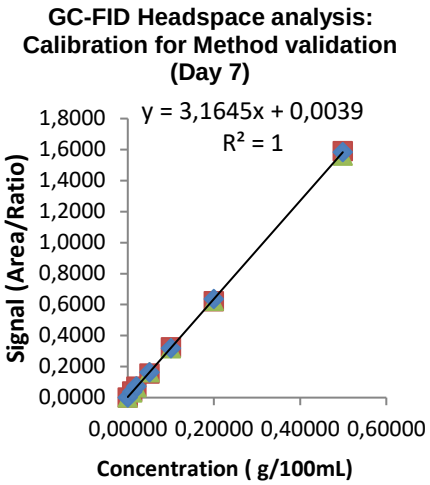
9.6. APPENDIX 6- MEASUREMENT OF UNCERTAINTY CALCULATIONS

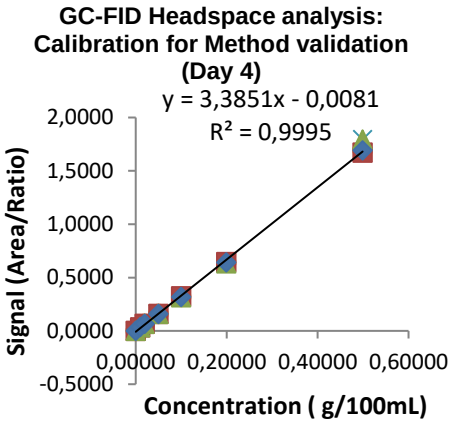
Imprecision of the measurement ($U_{precision}$)					
	QC 1 (0.02g/100 mL)	QC2 (0.05g/100 mL)			
(%RSD)²	6.572	3.104			
(%RSD)² x n(Number of readings)	249.719	117.965			
Sum of QC's	367.684				
Sum of QC's/ (Number of all readings -2)	4.969				
Sqrt (Sum of QC's/ (Number of all readings -2))	Precision	2.229 %		square	4.969
Uncertainty of the purity of stock standard (U_{purity})					
Standard (99% thus 1% uncertainty)	0.577	square	0.333 %		
		Sum	0.333 %		
Uncertainty of Dilutor					
Dilutor dispensing 1000 µL	0.027	square	0.001 %		
Percentage Uncertainty					
u%	0.578 %	Square	0.334 %		
Percentage Bias (%$Bias$)					
Average mean - standard mean/standard mean	0.048 %	square	0.002 %		
Uncertainty of the mean (U_{mean})					
CV%/sqrt(number of readings)	1.256 %	square	1.579 %		
Uncertainty of the Bias (%U_{bias})					
Sqrt(Uncertainty of the mean + uncertainty of standard)	1.383 %				
Bias significant or not					
tcalc	0.002	No significant Bias as tcalc<tcrit			
Uncertainty of Bias/Uncertainty of Precision					
	0.620 %	Above 10% and must be included			
	square	0.385 %			
Combined Uncertainty ($U_{combined}$)					
Sqrt(Bias + precision)	2.314 %				
Expanded Uncertainty (%$U_{expanded}$)					
Combined uncertainty x kfactor	4.628 %				

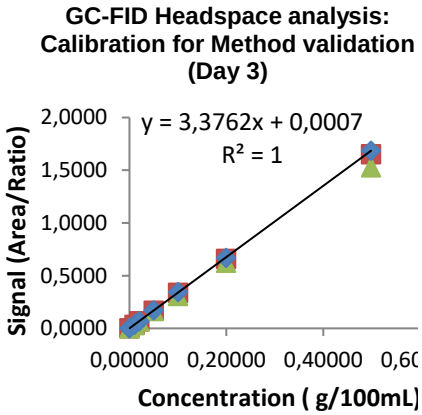
9.7. APPENDIX 7- CALIBRATION CURVES

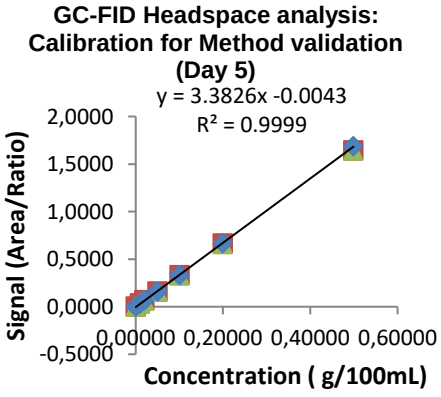


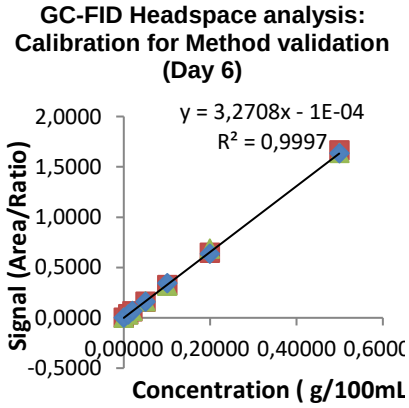


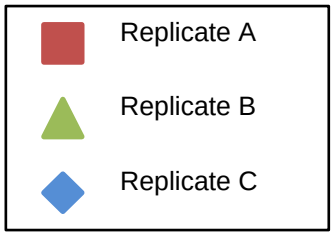




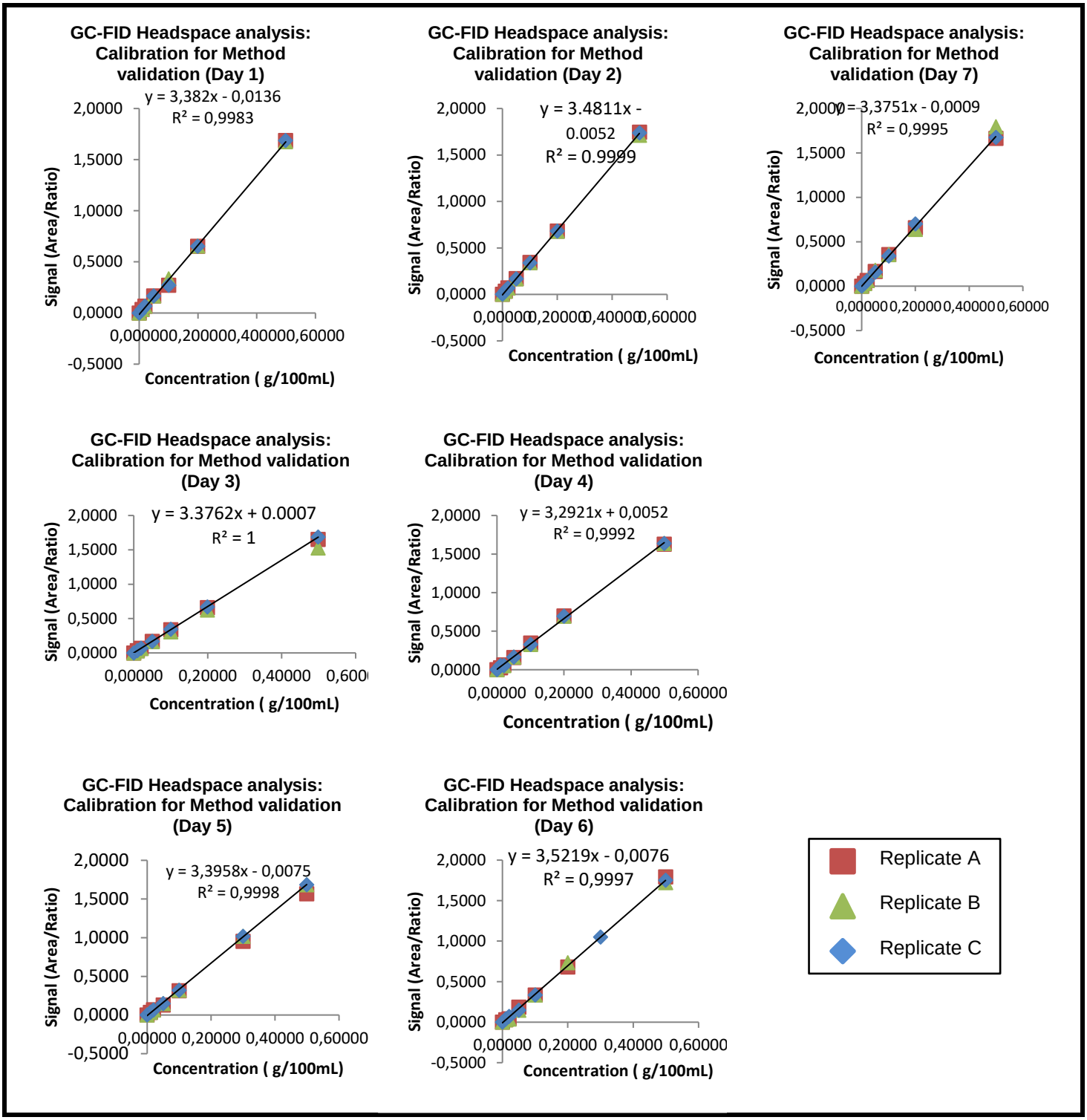








Calibration curves of ethanol concentrations versus signal response of the various calibration standards by analyst 1 over seven days



Calibration curves of ethanol concentrations versus signal response of the various calibration standards by analyst 2 over seven days

The intercept, slope, correlation coefficient and recovery of all calibration data that was analysed by two different analysts on various days (n=42).

ANALYST	DAY	REPLICATE	SLOPE	INTERCEPT	CORRELATION COEFFICIENT	RECOVERY
ANALYST 1	1	A	3.415	-0.0003	0.99998	100
		B	3.277	0.0031	0.99996	
		C	3.296	-0.0018	0.99991	
	2	A	2.787	0.0071	0.99963	100
		B	3.099	-0.0022	0.99996	
		C	3.166	-0.0048	0.99971	
	3	A	3.305	0.0021	0.99999	101
		B	3.059	0.0039	0.99988	
		C	3.376	0.0007	0.99998	
	4	A	3.348	-0.0055	0.99978	87
		B	3.586	-0.0210	0.99762	
		C	3.385	-0.0081	0.99949	
	5	A	3.298	0.0003	0.99996	97
		B	3.294	-0.0003	0.99999	
		C	3.383	-0.0043	0.99995	
	6	A	3.330	-0.0054	0.99985	100
		B	3.294	0.0012	0.99948	
		C	3.271	-0.0001	0.99970	
	7	A	3.175	0.0012	0.99985	104
		B	3.121	0.0032	0.99998	
		C	3.165	0.0039	0.99997	
ANALYST 2	8	A	3.353	-0.0043	0.99982	107
		B	3.352	-0.0021	0.99990	

		C	3.382	-0.0136	0.99827	
	9	A	3.495	-0.0044	0.99990	102
		B	3.428	-0.0023	0.99999	
		C	3.481	-0.0052	0.99991	
	10	A	3.305	0.0021	0.99999	99
		B	3.059	0.0039	0.99988	
		C	3.376	0.0007	0.99998	
	11	A	3.290	0.0039	0.99896	98
		B	3.250	0.0048	0.99981	
		C	3.292	0.0052	0.99916	
	12	A	3.155	-0.0037	0.99958	106
		B	3.223	-0.0046	0.99925	
		C	3.396	-0.0075	0.99978	
	13	A	3.602	-0.0140	0.99935	97
		B	3.498	-0.0093	0.99857	
		C	3.522	-0.0076	0.99970	
	14	A	3.344	0.0013	0.99969	100
		B	3.575	-0.0125	0.99800	
		C	3.375	-0.0009	0.99950	

9.8. APPENDIX 8- HAMILTON DILUTER CALIBRATION CERTIFICATE

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Certificate of Calibration

Certificate No:	SEP0822MC			**Customer:**	National Institute for Occupational Health
Calibration Date:	21-Oct-14				
Expiry Date:	21-Oct-15				
Manufacturer:	Hamilton				
Type:	Dual Dispenser	**Unit Ser.No:**	MD91HE4552	**Key Pad Ser.No:**	MD93HF6790
Balance:	Mettler Toledo	**Model No:**	AB204-S	**Serial No.:**	1125240738
Calibration Weight(g):		N/A		**Expiry Date:**	09-Apr-15
Calibration Certificate No:		51352			
Thermometer:	Testo	**Model No:**	Testo110	**Serial No.:**	33934468/005
Calibration Certificate No:		308-11459		**Expiry Date:**	01-Jan-16

Left Syringe Calibration

Size: 5ml(5000µl) **Type:** TLL

Weight (g):	1-5% Volume (µl): 1000	Weight (g):	5-30% Volume (µl): 2500	Weight (g):	30-100% Volume (µl): 5000
0.9976	1000.30	2.4970	2503.77	4.9868	5000.32
0.9973	1000.00	2.4866	2493.34	4.9866	5000.12
0.9976	1000.30	2.4964	2503.17	4.9867	5000.22
0.9978	1000.51	2.4868	2493.54	4.9875	5001.02
0.9980	1000.71	2.4969	2503.67	4.9872	5000.72
0.9978	1000.51	2.4868	2493.54	4.9870	5000.52
0.9976	1000.30	2.4967	2503.47	4.9870	5000.52
0.9979	1000.61	2.4868	2493.54	4.9868	5000.32
0.9981	1000.81	2.4968	2503.57	4.9866	5000.12
0.9982	1000.91	2.4978	2504.57	4.9869	5000.42
Results	Pass	Specifications	Pass	Specifications	Pass
Avg Volume:	**1000.50**		**2499.62**		**5000.43**
Std. Deviation:	**0.27**		**5.29**		**0.28**
Accuracy (%):	**0.05**	± 1.2	**-0.02**	± 1.2	**0.01**
Precision (%) :	**0.03**	≤ 0.5	**0.21**	≤ 0.5	**0.01**

Right Syringe Calibration

Size: 1ml (1000µl) **Type:** TLL

Weight (g):	1-5% Volume (µl): 250	Weight (g):	5-30% Volume (µl): 500	Weight (g):	30-100% Volume (µl): 1000
0.2499	250.58	0.4989	500.25	0.9979	1000.61
0.2494	250.08	0.4992	500.55	0.9977	1000.41
0.2498	250.48	0.4994	500.75	0.9978	1000.51
0.2498	250.48	0.4996	500.95	0.9979	1000.61
0.2493	249.98	0.4998	501.16	0.9980	1000.71
0.2499	250.58	0.4990	500.35	0.9978	1000.51
0.2497	250.38	0.4991	500.45	0.9978	1000.51
0.2498	250.48	0.4993	500.65	0.9973	1000.00
0.2494	250.08	0.4995	500.85	0.9973	1000.00
0.2496	250.28	0.4988	500.15	0.9975	1000.20
Results	Pass	Specifications	Pass	Specifications	Pass
Avg Volume:	**250.34**		**500.61**		**1000.41**
Std. Deviation:	**0.22**		**0.32**		**0.25**
Accuracy (%):	**0.13**	± 1.2	**0.12**	± 1.2	**0.04**
Precision (%) :	**0.09**	≤ 0.5	**0.06**	≤ 0.5	**0.03**

Remarks:

Water Temperature:	**23.8°C**	**Calibrated by:**	**Moyeni Chekela**
Calibration Liquid :	**Distilled H_2O**		
Water Density at 20.0°C	**0.997296**		
Water conductivity	**0.55µS/cm**		
Relative Humidity:	**28.9%RH**		

9.9. APPENDIX 9- TURNITIN REPORT

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9.10. APPENDIX 10- INSTRUMENT PARAMETERS

Instrument Parameters: Agilent G1888 Headspace that is coupled to the GC

Event Times	
GC Cycle	3.2 minutes
Vial equilibrium	10 minutes
Pressurize	0.2 minutes
Loop fill	0.2 minutes
Loop equilibration	0.05 minutes
Injection	0.5 minutes

Temperatures	
Oven	70˚C
Loop	80˚C
Transfer line	90˚C

Parameters: Agilent 6890 N GC that is fitted with a Flame Ionization Detector (FID)

Oven	
Initial temperature	70°C
Run time	3.2 minutes
Equilibrate time	0.5 minutes
Post run temperature	40°C
Post run Time	0.2 minutes

Detector	
Temperature	300°C
Carrier gas Flow	40 mL/min
Air Flow	400 mL/min
Mode	Constant makeup flow
Make-up Flow	25.0 mL/min
Makeup Gas Type	Helium

Column	
Model and Type	Agilent HP-Innowax®
Max Temperature	260˚C
Length	30 m
Diameter	0.25 mm
Film Thickness	0.50 µL
Initial Flow	2.6 mL/min
Pressure	180 kPa
Average Velocity	51 cm/sec

Injector	
Mode	Split
Initial Temperature	150°C
Pressure	150 kPa
Split Ratio	40:1
Split Flow	104 mL/min
Gas Type	Helium
Injection Volume	2 µL