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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

THE DETERMINATION OF ETHYL GLUCURONIDE AND ETHYL SULPHATE IN VITREOUS HUMOUR AS BIOMARKERS OF RECENT ALCOHOL USE IN POST-MORTEM FORENSIC CASES USING UPLC-MS/MS

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Dissertation presented in fulfilment of the requirements for the degree of Master of Science in Medicine by research, majoring in Forensic Science, in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg.

Under the supervision of:

Dr. Boitumelo Kgarebe (PhD)

Mrs. Ildiko Wainer (MSc)

May 2021

DISCLAIMER

Warning: This thesis contains figures that may be disturbing to certain individuals. Please use discretion when viewing Page 21 and Page 43.

DECLARATION

I, Gareth Riley, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine by research (Forensic Science) 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 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 HWSETA or NHLS.



Gareth Riley

18 May 2021

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ABSTRACT

Alcohol abuse is a major problem within societies all around the world. The use of alcohol contributed to 5.3% of deaths worldwide in 2016 while in South Africa, approximately 6.4% of all deaths are associated with alcohol consumption. In post-mortem investigations, the degree of alcohol intoxication is important to understand the circumstances of death. The gold standard method used to quantify alcohol intoxication analyses ethanol in blood. Despite this being a simple, accurate and specific analytical method, the interpretation and evaluation of the blood ethanol concentration (BEC) results is complicated by the behaviour of ethanol in a decedent prior to sampling. These complications are associated with the natural decomposition processes, circumstances of death, contamination and the short half-life of ethanol. The exploration into alternate biological specimens and alternate biomarkers has led to the scientific investigation of vitreous humour and analytes, such as ethyl glucuronide (EtG) and ethyl sulphate (EtS), as means to evaluate ethanol use and abuse. Vitreous humour is a simple biological matrix that is rarely contaminated by post-mortem processes. EtG and EtS are highly specific and sensitive ethanol biomarkers that are used to detect and quantify ethanol intoxication. The aim of this study was to develop and validate a method to simultaneously analyse EtG and EtS in vitreous humour using Ultra-Performance Liquid Chromatography couple with tandem Mass Spectrometry (UPLC-MS/MS) as well as to validate a method to analyse ethanol in vitreous humour using Headspace Gas Chromatography coupled with a Flame Ionization Detector (HSGC-FID). These methods were used to analyse 112 post-mortem vitreous humour samples obtained from the Johannesburg Forensic Pathology Services Medico-Legal Laboratory (JHB FPS MLL). EtG and EtS method development involved the optimization of sample preparation procedures, UPLC conditions and tandem MS conditions. The optimal sample preparation used protein precipitation with 0.1% formic acid in acetonitrile. EtG and EtS were simultaneously analysed using a UPLC-MS/MS system with a Phenyl-Hexyl column (150 mm length, 2.1 mm inner diameter and 1.7 µm particle size) and a gradient elution profile. Ethanol was analysed on a HSGC-FID system with a SUPELCOWAX® 10 GC column (30 m length, 0.25 mm internal diameter and 0.50 µm film thickness). According to European Medicines Agency (EMA) guidelines, both methods were deemed fit for purpose across the following linear ranges: ethanol (0.01-0.5 g/100mL), EtG (50–2500 ppb) and EtS (5-1000 ppb) and all analytical validation acceptance criteria were met. Of the 112 vitreous humour samples, 33 samples contained no ethanol, EtG or EtS. The remaining 79 samples had median ethanol, EtG and EtS concentration of 0.048 (0.000-0.175) g/100mL, 127.85 (54.26-932.16) ppb and 102.00 (8.44-705.25) ppb, respectively. Spearman's rank-order correlation test results suggested that, in vitreous humour, EtS is a more suitable analyte to predict ethanol consumption prior to death than EtG however, the

low correlation coefficients of 0.800 and 0.606 indicated that various other factors play a role in the relationship between ethanol and these alcohol biomarkers in vitreous humour. The pilot study results highlighted the various benefits, analysing EtG and EtS in post-mortem VH samples including to differentiate between ante mortem ethanol consumption and post-mortem formation and to clarify circumstances of death. The study also identified the short comings of solely using EtG and EtS results to determine ethanol intoxication for forensic purposes. In conclusion, the analysis of EtG and EtS in either post-mortem blood or vitreous humour samples should be considered as a routine test when pathologist request the analysis of BEC in order to differentiate between ante-mortem ethanol consumption and post-mortem formation as well as to providing additional information on the series of events prior to the death of a decedent.

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ABBREVIATIONS AND NOMENCLATURE

CAN	Acetonitrile
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
BEC	Blood ethanol concentration
BRB	Blood-retinal barrier
CNS	Central Nervous System
CRM	Certified Reference Material
CYP2E1	Cytochrome P450 2E1
ESI	Electrospray Ionization
EtG	Ethyl Glucuronide
EtS	Ethyl Sulphate
FA	Formic Acid
FAEE	Fatty acid ethyl esters
GABA	Gamma Aminobutyric Acid
GC	Gas Chromatography
HCl	Hydrochloric acid
HLB	Hydrophilic-Lipophilic Balance
HSGC-FID	Headspace Gas Chromatography coupled with Flame Ionization Detector
IQR	Inter quartile range
IUPAC	International Union of Pure and Applied Chemistry
JHB FPS-MLL	Johannesburg Forensic Pathology Services Medicolegal Laboratory
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MBA	Motor Bike Accident
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MVA	Motor Vehicle Accident
NAD	Nicotinamide adenine dinucleotide
NHLS	National Health Laboratory Service
NIMSS	National Injury Mortality Surveillance System
NIOH	National Institute of Occupational Health

NMISA	National Metrology Institute of South Africa
PEth	Phosphatidylethanol
PP	Protein Precipitation
PVA	Pedestrian Vehicle Accident
QC	Quality Control
RP	Reverse Phase
RSD	Relative Standard Deviation
SD	Standard Deviation
SPE	Solid Phase Extraction
SRM	Single Reaction Monitoring
UOM	Uncertainty of measurement
UPLC-MS/MS	Ultra-Performance Liquid Chromatography couple with tandem Mass Spectrometry
VH	Vitreous Humour
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

Ethanol, which is commonly referred to as 'alcohol', is a simple chemical compound consisting of two carbon atoms, six hydrogen atoms and an oxygen atom (C_2H_6O) (Wade, 2019). Ethanol's primary and most well-known use is as the intoxicating agent found in alcoholic beverages such as wine, beer and other liquor (Wade, 2019). Ethanol is one of the most commonly consumed substances in the world with approximately 43% of the population consuming ethanol every year (World Health Organization, 2018). Annually, risky and excessive ethanol use contributes, both directly and indirectly, to 5.3% of deaths worldwide (World Health Organization, 2018). In South Africa, approximately 6.4% of all deaths are associated with ethanol consumption (World Health Organization, 2018).

In post mortem medico-legal investigations, the degree of ethanol intoxication is important to help understand the circumstances and, in some situations, the manner of death of a decedent (Thierauf *et al.*, 2011). The gold standard method used to determine ethanol consumption quantifies the presence of ethanol in blood samples using a headspace gas chromatography system coupled with a flame ionization detector (HSGC-FID) (Jones, 2019b). In post mortem blood samples, the accurate interpretation of results from this method can be complicated due to post mortem redistribution, putrefaction processes and the rapid elimination of ethanol from blood (Dahl *et al.*, 2011). It is therefore of value to either utilize an alternative sample matrix, such as vitreous humour, and/or to evaluate more stable biomarkers, such as ethyl glucuronide (EtG) and ethyl sulphate (EtS).

Vitreous humour (eye fluid) is a simple matrix that consists of water (±98%), collagen fibres, electrolytes, carbohydrates and various other proteins (Bévalot *et al.*, 2016). Due to its anatomical location, vitreous humour is rarely influenced by post-mortem redistribution and putrefaction (Dinis-Oliveira *et al.*, 2010, Keten *et al.*, 2009). Furthermore, in post-mortem cases with severe trauma such as exsanguination and charring, vitreous humour may be the only reliable biological sample available for forensic analysis (Harper, 1989, Levine and Jufer, 2008).

Between 0.1 and 1.6% of consumed ethanol is metabolised in the liver to form the minor non-oxidative metabolites, EtG and EtS (Czech and Hartleb, 2002, Nanau and Neuman, 2015). These metabolites are highly specific ethanol biomarkers and can only be detected within biological samples if ethanol has been consumed (Cabarcos *et al.*, 2015). Studies have suggested that the simultaneous analysis of EtG and EtS provides a highly sensitive and specific analytical technique to detect recent ethanol use, up to 24 hours in blood and seven days in urine (Krabseth *et al.*, 2014, Elrasheed *et al.*, 2017).

The analysis of EtG and EtS, in a post mortem setting, is useful to distinguish between the consumption of ethanol prior to death and the post mortem formation of ethanol due to microbial activity (Vezzoli *et al.*, 2015). In addition, the identification of these ethanol biomarkers, in post mortem biological samples, can aid pathologists in clarifying the circumstances of death of a decedent (Sundström *et al.*, 2014).

The aim of this study was to develop and validate a method to simultaneously analyse EtG and EtS in vitreous humour using UPLC-MS/MS. In addition, a method was developed and validated to quantitatively determine ethanol in vitreous humour using HSGC-FID. These methods were used to profile the ethanol, EtG and EtS concentrations of vitreous humour samples sampled from 119 decedent received at the Johannesburg Forensic Pathology Service Medico Legal Laboratory (JHB FPS MLL).

CHAPTER 2: LITERATURE REVIEW

2.1. ALCOHOL (ETHANOL)

Alcohols are a class of organic compounds that have various uses (Wade, 2019). This section will discuss the chemistry, pharmacokinetics, use and abuse of alcohols with special reference to ethanol.

2.1.1. Chemistry

Alcohols are a class of organic compounds that are characterized by one or more hydroxyl groups (-OH) attached to a carbon atom of a hydrocarbon chain (Wade, 2019). The general formula for an alcohol is R-OH where R represents the hydrocarbon portion of the molecule (Wade, 2019). Alcohols are one of the most common organic compounds and can be used as an ingredient in alcoholic beverages, to make perfumes, as a fuel or as a solvent (Wade, 2019, Vitz *et al.*, 2019).

The two most common forms of alcohol are methanol and ethanol (Wade, 2019). Ethanol, which is commonly referred to as 'alcohol', is a simple chemical compound consisting of two carbon atoms, six hydrogen atoms and an oxygen atom (C_2H_6O) (Refer to Figure 2.1) (Wade, 2019). Besides the use of ethanol in fuels, toiletries, pharmaceuticals and for sterilization, its primary and most well-known use is as the intoxicating agent found in alcoholic beverages such as wine, beer and other liquor (Wade, 2019). In alcoholic beverages, ethanol is formed when yeast is combined with water and sugar in a fermentation reaction (Vitz *et al.*, 2019).

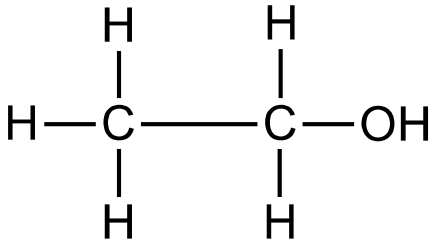


Figure 2.1: The chemical structure of Ethanol (CH_3CH_2OH) (Wade, 2019)

2.1.1.1. Physical properties of ethanol

Ethanol in its purest form is a flammable, volatile, colourless liquid that is highly polar and amphipathic, has both lipophilic and hydrophilic properties (National Center for Biotechnology Information, 2019, Ferreira and Willoughby, 2007). The hydrophilic nature allows ethanol to be highly soluble in water and other organic solvents (Ferreira and Willoughby, 2007). The physical properties of ethanol are highlighted in Table 2.1.

Table 2.1: The physical properties of ethanol (National Center for Biotechnology Information, 2019)

Physical property	Value
Molecular weight	46.069 g/mol
Density	0.79 g/cm^3
pH	~7
Boiling point	78.2 °C
Melting point	-114.1 °C
Stability	Stable under recommended storage conditions

2.1.2. Use and Abuse

Ethanol use, in alcoholic beverages, is a large part of cultural, religious and social practices worldwide (World Health Organization, 2018). It is used as part of celebrations, relaxation and social interactions (Cowan *et al.*, 2016). Ethanol consumed in moderation can have beneficial health effects which include reducing the risk of cardiovascular disease, relieving stress and contributing to a healthy digestive system (Grønbæk, 2009). However, ethanol is rarely consumed in moderation (World Health Organization, 2018).

The dependence-producing properties of ethanol has resulted in it being one of the most commonly consumed substances in the world which is often excessively consumed and abused (World Health Organization, 2018, Jones, 2011). Ethanol is also a psychoactive substance that, upon consumption, acts primarily on the central nervous system and alters brain functioning (Cowan *et al.*, 2016). These properties have caused ethanol consumption, especially abuse, to have an adverse effect on societies worldwide (World Health Organization, 2018).

Ethanol abuse is one of the world's leading risk factors for morbidity, disability and mortality (World Health Organization, 2018). According to the 2018 global status report on alcohol and health by the World Health Organization, ethanol consumption contributed to more deaths worldwide (~5.3%, 3 million) than diseases such as tuberculosis, HIV/AIDS and diabetes (World Health Organization, 2018). Nearly 30% of these deaths were associated with intentional or unintentional injuries such as interpersonal violence and road injuries (World Health Organization, 2018). A further 18% of these deaths were associated with digestive diseases such as liver cirrhosis and pancreatitis (World Health Organization, 2018).

The 2018 global status report on alcohol and health by the World Health Organization stated that, in 2016, on average, 9.3 L of pure ethanol was consumed by each person in the South African population (World Health Organization, 2018). This was a decrease from the 2010

statistics whereby the ethanol per capita consumption was 10.5 L (World Health Organization, 2018). This report further highlighted that the ethanol per capita consumption for ethanol drinkers was approximately 30 L since only a third of the South African population reported consuming ethanol in 2016 (World Health Organization, 2018). These statistics ranked South Africa into the top ten ethanol consuming countries in the world which was led by Tunisia, ethanol drinkers consumed 33.4 L of ethanol per capita (Chelwa and Walbeek, 2019, World Health Organization, 2018).

In South Africa, due to the large consumption volumes, deaths associated with ethanol consumption contribute to approximately 6.4% of annual deaths (World Health Organization, 2018). This is the third largest contributor to annual deaths in South Africa (World Health Organization, 2018). In addition, ethanol consumption is associated with thousands of disabilities (e.g. paraplegia) and disorders (e.g. alcohol-use disorder) per year in South Africa (World Health Organization, 2018). Along with the contribution to death and disabilities, it is estimated that 10-12% of South Africa's GDP per year is spent on costs associated with ethanol abuse (Matzopoulos *et al.*, 2013). This includes health care costs, labour costs and damage to state property (Matzopoulos *et al.*, 2013).

2.1.3. Physiological effects of ethanol consumption

Both acute and chronic ethanol use affects numerous physiological systems within the body including the cardiovascular system, the nervous system, the hepatic system and the gastrointestinal system (Brick, 2012). The effects of chronic ethanol consumption can lead to liver diseases, heart diseases, cancer and in some instances death (Heier *et al.*, 2016).

Ethanol is a central nervous system (CNS) depressant (Costardi *et al.*, 2015). Upon entering the brain, ethanol acts on different neurotransmitters including gamma-aminobutyric acid (GABA) and glutamate (Costardi *et al.*, 2015). GABA, the main inhibitory neurotransmitter of the CNS, is stimulated by ethanol which results in symptoms such as motor incoordination, sedation, relaxation, slurred speech and loss of inhibitions (Costardi *et al.*, 2015, Brick, 2012). Glutamate is inhibited by ethanol which results in symptoms such as reduced cognitive functions, attention deficit, impaired sleep cycles and ethanol blackouts (Costardi *et al.*, 2015).

The acute physiological effects of ethanol consumption depend on the amount of ethanol circulating through the blood (refer to Table 2.2) (Cowan *et al.*, 2016, Dubowski, 2006).

These physiological effects alter the behaviour of individuals and increases the likelihood of people engaging in illegal, risky behaviours including drunken-driving, drug abuse and sexual or physical assault (Jones, 2019a, Freeman and Parry, 2006). These risky behaviours further contribute to the mortality and morbidity statistics associated with ethanol use (Jones, 2019a).

Table 2.2: The physiological effects associated with increasing ethanol blood concentrations (Dubowski, 2006)

Blood ethanol concentration (g/ 100mL)	Stage of ethanol intoxication	Symptoms*
0.01-0.05	Subclinical	• No noticeable symptoms • Special tests can detect impairment
0.03-0.12	Euphoria	• Increased self-confidence • Decreased inhibitions • Elation, sociability and talkativeness • Reduced attention, judgment and control • Some sensory-motor impairment
0.09-0.25	Excitement	• Emotional instability • Loss of judgment • Impairment of perception, memory and understanding • Impaired balance, slurred speech, vomiting and drowsiness
0.18-0.30	Confusion	• Disorientation, mental confusion and vertigo • Exaggerated emotions • Increased pain threshold • Increased muscular incoordination • Lack of interest and lethargy
0.25-0.40	Stupor	• Approaching the loss of motor functions • Decreased response to stimuli • Inability to stand or walk • Vomiting • Lack of control of excretory functions • Impaired consciousness
0.35-0.50	Coma	• Complete unconsciousness or coma • Impairment of circulation and respiration • Possible death
0.45+	Death	• Death from respiratory arrest

*These symptoms vary from person to person depending on several factors such as genetics, tolerance and medical conditions.

2.1.4. Pharmacokinetics

Pharmacokinetics is a field of pharmacology that concerns the nature and fate of xenobiotics within the human body (Tozer and Rowland, 1995, Saghir and Ansari, 2018). Xenobiotics are foreign chemical substance which do not naturally occur within a human body e.g. pesticides, therapeutic drugs, drugs of abuse and ethanol (Qadir *et al.*, 2017). The four processes involved in pharmacokinetics are categorized in general as absorption, distribution, metabolism and excretion (Tozer and Rowland, 1995). The pharmacokinetics of ethanol has been extensively studied due to the important clinical and medico-legal implications (Jones, 2011).

2.1.4.1. Absorption

Absorption is the process by which a toxin passes from the administration site into the bloodstream (Jones, 2011). Ethanol is administered dermally, via inhalation, intravenously and via ingestion (Levine and Caplan, 2003). Ethanol exposure via inhalation and dermal routes is ineffective at causing ethanol intoxication (Levine and Caplan, 2003). Intravenous administration of ethanol can cause ethanol intoxication however it is not commonly used (Levine and Caplan, 2003).

Ingestion is the most common and most effective route of ethanol administration because nearly 100% of ingested ethanol becomes pharmacologically available (Levine and Caplan, 2003). Upon ingestion, a portion of the ethanol is absorbed into the bloodstream through the surfaces of the oral cavity (Jones, 2011). The remainder of the ethanol is absorbed into the bloodstream primarily from the upper small intestine but also from the stomach (Jones, 2011, Ferreira and Willoughby, 2007, Kent, 2012). The lipophilic properties of an ethanol molecule enables the molecule to passively diffuse across cell membranes (Kent, 2012).

The rate of ethanol absorption determines the blood ethanol concentration and the associated physiological symptoms that a person experiences (Kent, 2012). Several intraindividual and interindividual factors influence the rate of ethanol absorption (refer to Table 2.3).

Table 2.3: Factors that influence the rate of ethanol absorption (Kent, 2012, Jones, 2019a)

	Effect on ethanol absorption rate	
Factors	**Increases**	**Decreases**
Intraindividual		
Empty stomach	X	
Ethanol ingestion with or after food		X
Alcohol concentration	X	
Carbonated alcoholic beverages	X	
Exercise		X
Emotion state		X
Interindividual		
Low body weight	X	
Gender- Female	X	
Smoking cigarettes		X
Drug use- drugs that cause gastric emptying (e.g., Amphetamines)	X	
Drug use- drugs that delay gastric emptying (e.g., Tricyclic antidepressants)		X

2.1.4.2. Distribution

Distribution is the process whereby toxins are transported throughout the body by the circulating body fluids (blood) to the sites of action (Clayton and Willihnganz, 2017). Subsequent to the absorption of ethanol by the stomach and small intestine, ethanol passes into the blood through the portal vein (Levine and Caplan, 2003). This ethanol containing blood is transported to the liver and passes through the hepatic vein to the right side of the heart (Levine and Caplan, 2003). The heart pumps the blood to the lungs where carbon dioxide is removed and supplemented with oxygen. The blood then returns to the heart after which it is pumped throughout the entire circulatory system (Jones, 2019a, Levine and Caplan, 2003). The distribution of ethanol into the lungs along with the high volatile nature of ethanol allows it to be identified and quantified in breath (Levine and Caplan, 2003).

As ethanol is transported throughout the circulatory system, it diffuses from the blood into all different bodily tissues and fluids in proportion to their relative water content (Begleiter and Kissin, 1996, Levine and Caplan, 2003). The tissues and fluids with a higher water content (vitreous humour, oral fluid, urine and sweat) will have a higher ethanol concentration than the

blood at equilibrium while tissues like the brain, liver and kidneys will have lower ethanol concentrations than the blood due to relatively low water contents (Jones, 2011, Levine and Caplan, 2003). There are two ways in which ethanol molecules diffuse across cell membranes and into bodily tissues (Begleiter and Kissin, 1996, Jones, 2019a). Firstly, the small molecular size of ethanol allows the free movement of ethanol through the water channels and pores of cell membranes. Alternatively, ethanol molecules can passively diffuse through the lipid bilayer of cell membranes due to the lipophilic properties of these ethanol molecules (Cederbaum, 2012, Begleiter and Kissin, 1996).

2.1.4.3. Metabolism

Metabolism is the chemical biotransformation, through specific enzymatic systems, of a toxin from one chemical form to another within a living body (Taxak and Bharatam, 2014). In general, it involves the transformation of lipophilic toxins into polar compounds that can be excreted efficiently (Taxak and Bharatam, 2014).

Gastric first pass metabolism metabolises approximately 2% of consumed ethanol (Ferreira and Willoughby, 2007). This ethanol is oxidized in the cell membrane of the stomach cells and does not actively enter the circulatory system (Cederbaum, 2012). The remaining 98% of consumed ethanol is metabolised in the liver via two types of metabolism, known as oxidative and non-oxidative metabolism (Dinis-Oliveira, 2016). Approximately 90-95% of consumed ethanol is subjected to oxidative metabolism while the remaining ethanol undergoes non-oxidative metabolism (Czech and Hartleb, 2002) (Refer to Figure 2.2).

- Oxidative metabolism

Oxidative metabolism involves two enzymes; ADH and aldehyde dehydrogenase (ALDH) (National Institute on Alcohol Abuse and Alcoholism, 2007) (Refer to Figure 2.2). Both enzymes require a vitamin-related cofactor, nicotinamide adenine dinucleotide (NAD), to accept hydrogen atoms and electrons during the reactions (Ferreira and Willoughby, 2007). Initially, ADH oxidises ethanol to acetaldehyde, a highly toxic compound that is a known carcinogen and responsible for the organ damage found in chronic alcoholics (Cederbaum, 2012, Czech and Hartleb, 2002). Acetaldehyde is subsequently oxidized by ALDH to form acetate. Acetate leaves the liver and is converted to acetyl-CoA which is oxidized in the Krebs cycle to form carbon dioxide and water (Cederbaum, 2012). When large amounts of ethanol are consumed, Cytochrome P450 2E1 (CYP2E1) and catalase oxidise ethanol to form acetaldehyde (National Institute on Alcohol Abuse and Alcoholism, 2007).

- Non-oxidative metabolism

The non-oxidative routes of ethanol metabolism result in the enzymatic conjugation of ethanol to biological compounds such as glucuronic acid, sulphate, fatty acids and phospholipids (Heier *et al.*, 2016) (Refer to Figure 2.2). These reactions result in specific ethanol metabolites generally known as ethyl glucuronide (EtG), ethyl sulphate (EtS), fatty acid ethyl esters (FAEE) and phosphatidylethanol (PEth) (Oppolzer *et al.*, 2016). Despite non-oxidative metabolism representing a minor proportion of the total ethanol metabolism, these metabolites remain in bodily fluids and tissues for much longer than ethanol and can be detected several hours or days after ethanol exposure (Dinis-Oliveira, 2016, Heier *et al.*, 2016). More detailed information on these ethanol metabolites can be found in Section 2.2.2.

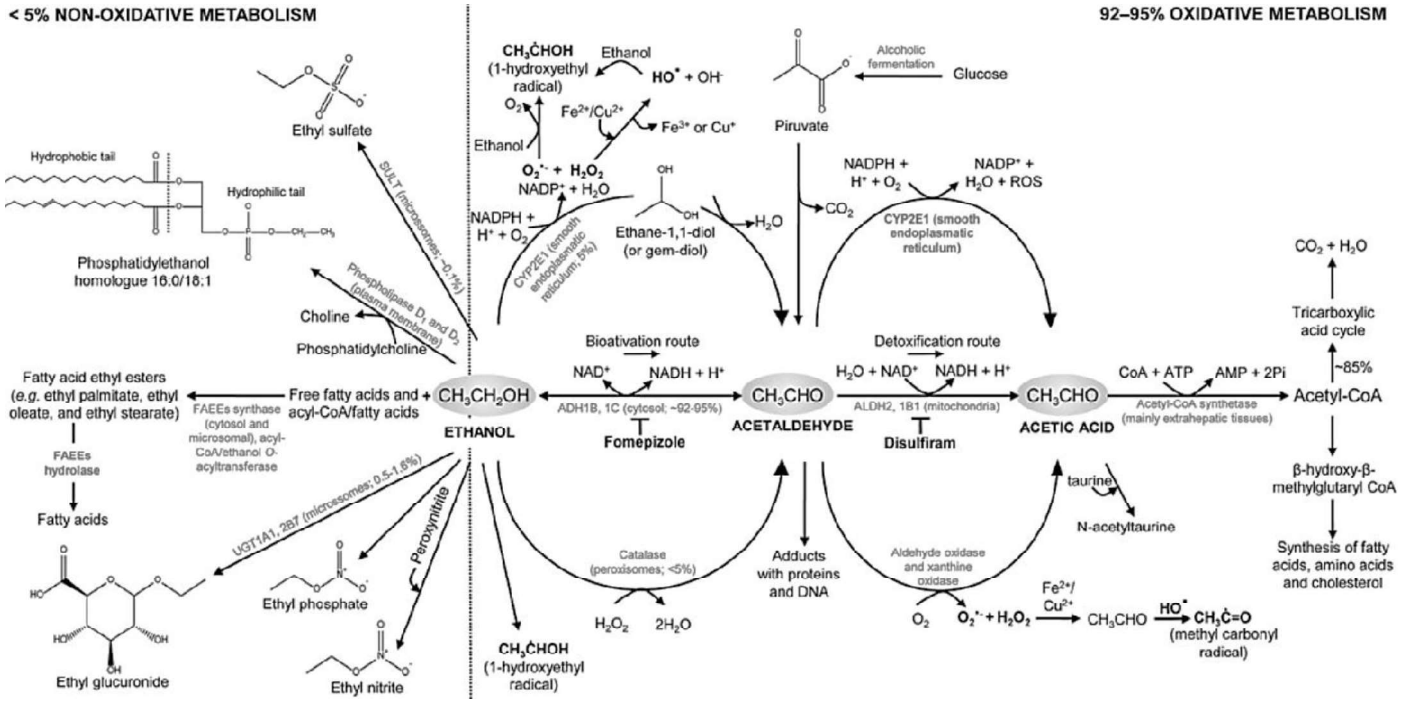


Figure 2.2: The oxidative and non-oxidative metabolism of ethanol. Adapted from (Dinis-Oliveira, 2016), with permission.

2.1.4.4. Excretion

Excretion is the process of removing a toxin and its metabolites from the body (Lu and Xue, 2019). Excretion of ethanol from the body is controlled by the liver (Wigmore, 2014). Only 5% of consumed ethanol is excreted unchanged in urine, breath and sweat (Jones, 2019a). The remaining ethanol is metabolised and excreted as carbon dioxide, water or ethanol metabolites (Wigmore, 2014, Jones, 2019a).

Ethanol exhibits zero-order excretion kinetics at a rate of 0.01 to 0.02 g/100mL per hour (Wigmore, 2014, Jones, 2011). Zero-order kinetics imply that a constant ethanol concentration is excreted from the body per unit time (Jones, 2019a). These zero-order kinetics prevail due to the saturation of the primary ethanol metabolising enzyme, ADH, at blood ethanol concentrations of 0.02g/ 100mL (Jones, 2011). The rate of ethanol excretion is stated as a range because it depends on various factors including sex, age and digestive state (Cederbaum, 2012, Wigmore, 2014).

2.2. ETHANOL TESTING

The physiological impairment of human performance due to ethanol is determined by assessing the degree of ethanol intoxication (Wigmore, 2014). The accurate assessment of the degree of ethanol intoxication is important in clinical, occupational and medico-legal settings (Olson *et al.*, 2013). In clinical settings, the degree of ethanol intoxication is assessed in patients with suspected ethanol poisoning (Olson *et al.*, 2013). In certain occupational industries such as mining and construction, employers have implemented zero tolerance policies that advocate “coming to work free of alcohol and drugs” (Jordaan, 2014). These policies permit employers to monitor the degree of ethanol intoxication of employees before and after work in order to prevent workplace injuries and fatalities due to ethanol intoxication (Jordaan, 2014).

Medico-legal settings perform the majority of ethanol tests (Olson *et al.*, 2013). These tests are conducted by forensic toxicology laboratories for both ante mortem and post mortem medico-legal applications (Kelly and Mozayani, 2012). Ante mortem ethanol analysis is conducted to determine legal actions in cases of sexual and physical assault, driving under the influence of ethanol and monitoring offenders during probation periods (Kelly and Mozayani, 2012). For post mortem applications, the degree of ethanol intoxication is important to determine the surrounding circumstances of death in cases such as poisoning, fatal accidents, suicide and drowning (Thierauf *et al.*, 2011). The interpretation of ethanol concentrations aids a pathologist in their determination of a person’s state of intoxication and physiological impairment at the time of death (Kugelberg and Jones, 2007). This subsequently allows a pathologist to determine the potential level of contribution of ethanol to a decedent’s

death (Kugelberg and Jones, 2007). The outcome of post mortem ethanol tests also provides insurance companies with important information relating to life insurance claims especially in cases of fatal motor vehicle accidents where the driver was intoxicated (Kugelberg and Jones, 2007).

2.2.1. Ethanol testing method

The primary method used to assess the degree of ethanol intoxication, for both ante mortem and post-mortem applications, analyses the concentration of ethanol in blood samples (Kugelberg and Jones, 2007, Hunsaker *et al.*, 2015). Blood is the matrix of choice for ethanol testing because blood ethanol concentrations (BEC) are directly related to the physiological symptoms associated with ethanol intoxication; additionally the majority of publications reference BEC when reporting ethanol intoxication (Kerrigan, 2013).

2.2.1.1. Sampling

For clinical, occupational and ante mortem applications, blood is sampled from a superficial vein on the upper arm using a syringe and needle (Hunsaker *et al.*, 2015). These sampling sites are disinfected, prior to sampling, with alcohol-free swabs (Williams and Leikin, 1999). For post mortem applications, blood is preferably sampled from a femoral vein, prior to autopsy (Dinis-Oliveira *et al.*, 2010, Jones, 2019b). Femoral veins are less susceptible to contamination from post mortem changes because there are a number of anatomical valves that prevent the movement of blood from the intestines to the lower extremities (Dinis-Oliveira *et al.*, 2010). After sampling, blood samples are stored at 4°C which prevents the neoformation of ethanol from microorganisms (Cowan *et al.*, 2016). Furthermore, sodium fluoride is added to blood collection tubes to prevent microbial growth and degradation of ethanol in the sample (Cowan *et al.*, 2016).

2.2.1.2. Sample preparation

Prior to analysis, blood samples are diluted with an internal standard (Kugelberg and Jones, 2007). The dilution of the blood sample with an internal standard decreases the percent error of BEC results by compensating for interindividual matrix differences (Boswell and Dorman, 2015). Alcohols with similar chemical properties to ethanol, such as tertiary butanol and n-propanol, are used as internal standards because these alcohols undergo the same matrix effects in the blood sample as ethanol (Boswell and Dorman, 2015, Kugelberg and Jones, 2007). The use of n-propanol is limited for post-mortem samples because it is a volatile compound produced during decomposition as a product of putrefaction (Dorubet *et al.*, 2011, O'neal *et al.*, 1996). Tertiary butanol is not a by-product of putrefaction and is therefore

recommended as the suggested internal standard for ethanol analysis of post mortem samples (Dorubet *et al.*, 2011). Specialized semi-automated diluters are used to dilute ethanol samples with an internal standard in order to provide the high degree of precision required (Jones, 2019b).

2.2.1.3. Analysis

The gold standard analytical technique used to analyse ethanol in blood as well as other biological matrices is headspace gas chromatography coupled with a flame ionization detector (HSGC-FID) (Kugelberg and Jones, 2007). This instrument involves three hyphenated analytical techniques each with a specific principle and function. The three techniques are headspace (HS), gas chromatography (GC) and flame-ionization detection (FID) (Refer to Figure 2.3).

- Headspace (HS)

HS is a sampling technique that prevents the contamination, by non-volatile sample matrix components, on the GC and FID components of the instrument (Kugelberg and Jones, 2007). The HS technique uses the principle of Henry's Law which states that at a specific temperature, the concentration of a volatile substance in a liquid is directly proportional to the concentration of the volatile in the airspace above the liquid (Kelly and Mozayani, 2012). An air-tight glass vial containing the sample is heated at a specific temperature for a definite period of time, e.g. ethanol analysis requires the vial to be heated to approximately 60 °C for a period of 10-20 minutes (Jones, 2019b). The volatile compounds are separated from the liquid sample and diffuse into the airspace above the sample. A small volume of the gas contained in the HS is then injected into the GC, by a gas-tight syringe (Kelly and Mozayani, 2012, Kugelberg and Jones, 2007).

- Gas Chromatography (GC)

GC is a chromatographic technique that separates volatilised analytes within a sample based on their physiochemical properties (Hussain and Maqbool, 2014, Jones, 2019a). Analytes are partitioned between a stationary phase and a mobile phase which consists of an inert gas such as helium (Hussain and Maqbool, 2014). The stationary phase is coated onto the inner walls of a long, narrow, coiled analytical column made of fused silica (Hussain and Maqbool, 2014, Wilson and Walker, 2010). These stationary phases consist of high-boiling-point materials, such as polymers and fluorocarbons, with varying polarities (Wilson and Walker, 2010). The selectivity of GC methods depends on the polarities of these stationary phase materials, e.g. polar stationary phases containing polyethylene glycol are used to separate polar analytes while non-polar stationary phases containing methyl-polyclones are used to

separate non-polar analytes (Wilson and Walker, 2010, Soria *et al.*, 2014). GC method selectivity also depends on temperature therefore the chromatographic column is located in a temperature programmable oven (Hussain and Maqbool, 2014).

A volatilised sample is carried through the chromatographic column by the mobile phase (Jones, 2019a). Each analyte within the sample partitions between the stationary phase and mobile phase based on their physiochemical properties, i.e., boiling point and polarity (Hussain and Maqbool, 2014, Jones, 2019a). The temperature of the oven further affects the partitioning of these analytes, e.g. an increased temperature can decrease the retention of specific analytes by the stationary phase (Hussain and Maqbool, 2014). Each analyte within the sample elutes from the column at a different time known as retention time (RT), which is dependent on the equilibrium partition coefficient (K) (Hussain and Maqbool, 2014, Kolb, 2000). This retention time is used for the identification of analytes within a sample (Hussain and Maqbool, 2014) Upon elution from the column, analytes pass over a detector that generates a signal which corresponds to the concentration of the analyte (Jones, 2019a).
For ethanol analysis, a GC column containing polyethylene glycol is recommended due to the very polar nature of the analyte (Jones, 2019b). This polar GC column coupled with an oven temperature of approximately 60°C provides the optimal selectivity for ethanol analysis (Jones, 2019b).

- Flame Ionization Detection (FID)

FID is a universal detector for organic analytes because it detects the carbon-carbon or carbon-hydrogen bonds within an analyte (Jones, 2019a). It is a very sensitive detector with a minimum detection quantity of 5×10^{-12} g/s and a linear range of up to 10^7 (Jones, 2019a). Upon elution from the chromatographic column, the column effluent (containing sample and carrier gas) enters the bottom of the FID (Hinshaw, 2005). The column effluent is mixed with hydrogen and medical air before being ignited by a flame (Soria *et al.*, 2014). The combustion of the carbon atoms in the sample forms ions which are attracted to the collector electrode (cathode) and deflected away from the polarized electrode (anode) (Soria *et al.*, 2014). Upon hitting the collector electrode plate, a current is induced that is measure by an electrometer and registered by a computer as a chromatogram (Hinshaw, 2005, Soria *et al.*, 2014). The current induced is directly proportional to the concentration of the compound (Soria *et al.*, 2014).

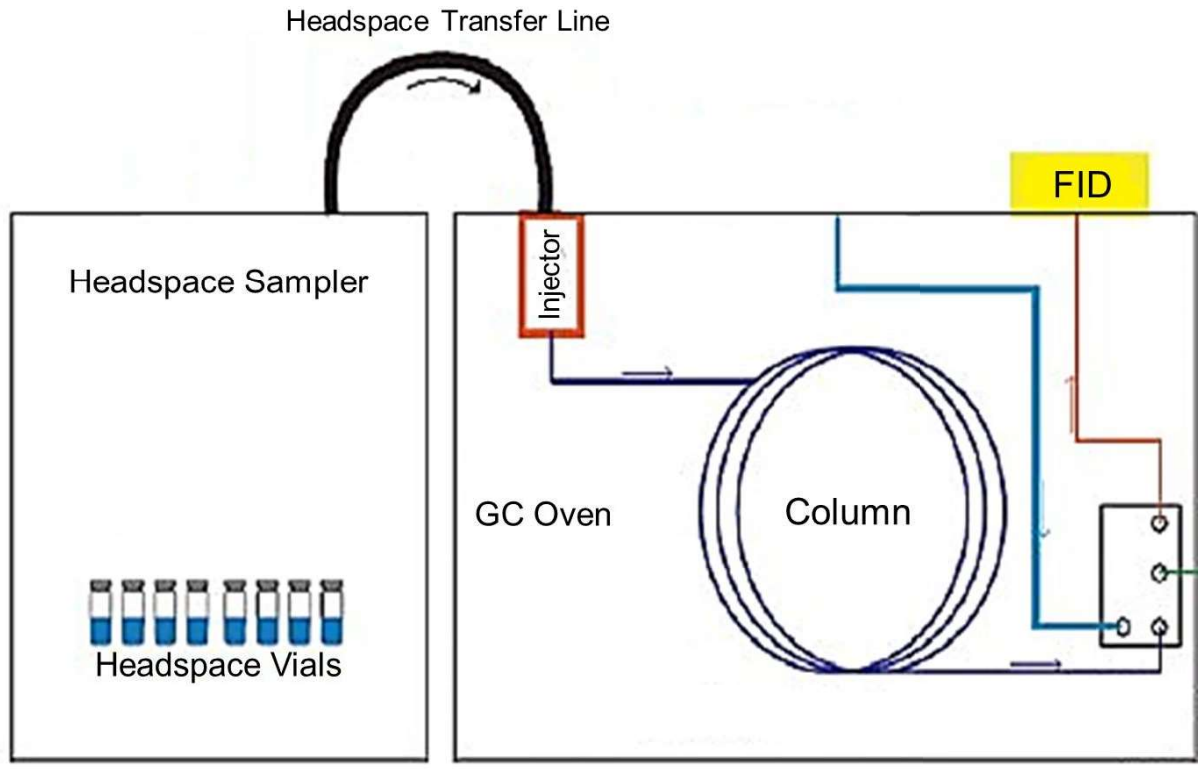


Figure 2.3: HSGC-FID instrument configuration. Figure adapted from (Jacq *et al.*, 2008)

2.2.2. Limitations of post-mortem ethanol testing

The method described above is the gold standard to identify and quantify ante mortem ethanol intoxication in decedents and is used routinely during medico legal investigations (Jones, 2019b). This analytical method is a simple procedure that is accurate, precise and specific however, this method is limited by the behaviour of ethanol in a decedent prior to sampling (Kugelberg and Jones, 2007). This complicates the interpretation and evaluation of the BEC results obtained (Kugelberg and Jones, 2007).

2.2.2.1. Natural decomposition processes

The major limitations associated with post mortem BEC testing are associated with the natural decomposition processes of a dead body (Jones, 2019b). Post mortem redistribution and putrefaction are factors that alter the concentration of ethanol in post mortem blood (Jones, 2019b). Post mortem redistribution is the diffusion of ethanol and other drugs through the stomach wall and into surrounding tissues after death (Kugelberg and Jones, 2007). This may deceptively raise the concentration of ethanol or other drugs not only in these tissues, but also in the central and peripheral blood network (Kugelberg and Jones, 2007). Post mortem redistribution is amplified in traumatic deaths where the internal organs of the decedent have ruptured (Kugelberg and Jones, 2007). In these post mortem cases, it is imperative to sample

blood from the femoral veins which are less susceptible to post mortem redistribution than the chest or pleural cavity however some contamination can still occur at these peripheral sites (Kugelberg and Jones, 2007).

Putrefaction is the breakdown or decomposition of tissues and organs by microbial activity (Tsokos, 2007). After death, exogenous microorganisms (originating from the environment) and endogenous microorganisms (originating from the intestines) invade the tissues and bodily fluids of the decedent and generate ethanol through the fermentation of carbohydrates (Ziavrou *et al.*, 2005, Kugelberg and Jones, 2007). Glucose is the main substrate for this neoformation of ethanol since the amount of glucose in the blood increases after death (Kugelberg and Jones, 2007). Ethanol neoformation increases the post-mortem BEC by approximately 0.7 g/100mL however some cases, concerning decomposed bodies, have reported BEC increases of up to 2.2 g/100mL due to post-mortem neoformation (Ziavrou *et al.*, 2005, Jones, 2019b).

2.2.2.2. Circumstances of death

The circumstances of death can complicate the interpretation of post mortem BEC results (Kugelberg and Jones, 2007). An increase or a decrease in BEC can occur in cases of drowning or when the body of the deceased is submerged in water (Kugelberg and Jones, 2007). A decrease is due to the dilution of body fluids with water whilst an increase is due to microbial activity (Kugelberg and Jones, 2007). Further complications transpire in cases where the decedent experienced severe traumatic events, such as burning or exsanguinations, prior to death (Kugelberg and Jones, 2007). In these cases there may be an insufficient volume of blood present in the decedent's body to adequately sample (Kugelberg and Jones, 2007).

2.2.2.3. Contamination

For post mortem BEC interpretation, it is important that blood samples are not contaminated before or after death (Kugelberg and Jones, 2007). Hospital procedures, such as the administration of intravenous solutions or medications, can alter the blood composition and give inaccurate post mortem BEC results (Kugelberg and Jones, 2007). Intravenous solutions may either dilute the blood which decreases the BEC or promote post mortem ethanol formation (Kugelberg and Jones, 2007). An example is the intravenous solution called mannitol, used to relieve swelling in the brain, which is a substrate for post mortem ethanol fermentation by microbial activity (Kugelberg and Jones, 2007). Generally, no blood is sampled for post-mortem BEC analysis if the decedent received emergency medical care or was admitted to hospital prior to death (Du Plessis *et al.*, 2016).

2.2.2.4. Short half-life of ethanol

The pharmacokinetics of ethanol prevents BEC analysis from determining ethanol intoxication more than 12 hours prior to death (Dahl *et al.*, 2011). As discussed previously, ethanol is rapidly eliminated from the body by metabolic and excretory processes (Cabezas *et al.*, 2016). Since ethanol is excreted from the body at a rate of 0.01-0.02 g/100mL per hour, it can only be detected in blood up to 12 hours after consumption, depending on the concentration and volume consumed (Wigmore, 2014, Dahl *et al.*, 2011). Ethanol metabolites can be analysed in order to determine ethanol intoxication more than 12 hours prior to death (Cabezas *et al.*, 2016).

2.3. ALTERNATIVE MATRICES FOR POST-MORTEM ETHANOL TESTING

Research has suggested that the limitations of post-mortem ethanol testing can be overcome by analysing ethanol in alternate biological matrices such as urine and vitreous humour (Hunsaker *et al.*, 2015, Kugelberg and Jones, 2007, Krabseth *et al.*, 2014). The analysis of ethanol in urine and vitreous humour offers several advantages over analysing post-mortem blood (Refer to Table 2.4). (Kugelberg and Jones, 2007, Dinis-Oliveira *et al.*, 2010, Hunsaker *et al.*, 2015).

Femoral blood, urine and vitreous humour are the recommended samples for ethanol testing in post mortem investigations (Kugelberg and Jones, 2007). However, studies have suggested the use of alternate matrices, including bone marrow, cerebrospinal fluid (CSF), synovial fluid and bile, in extreme post-mortem cases where these matrices are unattainable, e.g., severe trauma (Kugelberg and Jones, 2007, Hunsaker *et al.*, 2015). Post-mortem ethanol testing has also been conducted on tissue samples including brain, liver and skeletal as well as hair samples (Hunsaker *et al.*, 2015, Cabarcos *et al.*, 2015).

Table 2.4: A comparison of the advantages and disadvantages of using post-mortem blood, urine and vitreous humour for ethanol testing. Information obtained and summarised from (Dinis-Oliveira *et al.*, 2010, Hunsaker *et al.*, 2015, Kugelberg and Jones, 2007).

Matrix	Sampling site	Storage conditions	Advantages	Disadvantages
Blood	Femoral veins	• 4°C • In tubes containing sodium fluoride	1. Published reference values related to physiological symptoms 2. Simple sample preparation 3. Generally, large volumes are available for sampling	1. Clotting can cause difficulties during sampling 2. Highly susceptible to post-mortem redistribution and putrefaction 3. Severe trauma can result in insufficient sample volumes 4. Hospital procedures can alter composition
Urine	Intact bladder	• -20 °C • In cups containing sodium fluoride	1. Less susceptible to post-mortem redistribution and putrefaction than blood 2. Simple sample preparation 3. Easy sampling procedure	1. At autopsy, there is generally little or no urine in the decedent's bladder 2. Urine is pooled in the bladder over time therefore ethanol concentrations are volume and time dependent 3. Ethanol neoformation can occur in decedents suffering from diabetes or glycosuria
Vitreous humour	Globe of the eye	• 4°C or -20 °C • In 5 mL tubes containing sodium fluoride	1. Least susceptible matrix to post-mortem redistribution and putrefaction 2. Easy sampling procedure 3. Simple sample preparation	1. Only 5 mL of sample can be obtained from each decedent 2. Poor sample integrity due to various ante mortem health factors 3. Unavailable when decedent has suffered severe head trauma

2.3.1. Vitreous Humour

As highlighted in Table 2.4, vitreous humour (VH) offers several advantages over post-mortem blood and urine when it comes to ethanol testing. VH is a clear, gelatinous substance found in the posterior chamber of the eye between the lens and the retina (Bévalot *et al.*, 2016). Each eye contains approximately 4 mL of VH which functions to maintain the shape of the eye and keep the retina in place (Bévalot *et al.*, 2016). VH consists mainly of water (98-99.7%) but also contains collagen fibres, carbohydrates (glucose), hyaluronic acid, electrolytes (sodium, potassium and lactate) and other proteins such as opticin (Dinis-Oliveira *et al.*, 2010, Jones, 2019a, Kugelberg and Jones, 2007, Bévalot *et al.*, 2016)..

2.3.1.1. Movement of analytes into the VH

Before analytes enter the VH, they need to pass through the retina (Cunha-Vaz, 2004). The retina has a selective barrier, called the blood-retinal barrier (BRB), which functions to regulate the environment of the VH (Cunha-Vaz, 2004, Bévalot *et al.*, 2016). Analytes, unbound to plasma proteins, move from the blood through the BRB by passive or active transport and into the VH (Bévalot *et al.*, 2016). Small analytes move in and out of the VH via diffusion while analytes that are hydrophilic or have a large molecular weight require active transport to cross the BRB and enter the VH (Łukasik *et al.*, 2018, Bévalot *et al.*, 2016). Ethanol and most drugs are small and move through the BRB via diffusion (Łukasik *et al.*, 2018).

2.3.1.2. VH sampling

VH is sampled from a decedent by puncturing the globe of the eye, using a 20-gauge needle, slightly above the junction between the upper and lower eyelids (Refer to Figure 2.4) (Dinis-Oliveira *et al.*, 2010). A maximum of 2 mL of VH is slowly aspirated, using a syringe, from the middle of the globe of each eye (Bévalot *et al.*, 2016). The specific volume and slow aspiration technique is used in order to avoid epithelial cells, from the retina or iris, entering the sample (Bévalot *et al.*, 2016). Water or a saline solution can be used to replace the withdrawn VH in order to reproduce the natural integrity of the eye (Bévalot *et al.*, 2016, Dinis-Oliveira *et al.*, 2010). VH samples are collected in 5 mL tubes to limit the evaporation of volatile substances such as ethanol (Bévalot *et al.*, 2016). Studies recommend adding sodium fluoride to the sample to prevent the neoformation of ethanol, especially in samples from diabetic decedents (Dinis-Oliveira *et al.*, 2010). Samples should be stored at either 4 °C or -20 °C for a maximum of three months prior to analysis (Dinis-Oliveira *et al.*, 2010, Bévalot *et al.*, 2016).



Figure 2.4: The sampling of vitreous humour from the eye. Permission obtained from (Dinis-Oliveira *et al.*, 2010).

2.3.1.3. Advantages of VH analysis

VH is rarely contaminated by post mortem redistribution and is the most well preserved biological matrix (Dinis-Oliveira *et al.*, 2010). Due to the anatomical location of the eye, it is isolated from the stomach and other abdominal tissues that are involved in post mortem redistribution (Dinis-Oliveira *et al.*, 2010). The globe of the eye is also deficient of blood vessels which prevents analytes from entering the vitreous humour after death (Hunsaker *et al.*, 2015).

The VH is less susceptible to putrefaction than other biological matrices including blood and urine (Harper, 1989, Honey *et al.*, 2005). The avascularity and anatomical location of the eye limits the invasion of the VH by microorganisms (Dinis-Oliveira *et al.*, 2010). This subsequently prevents the neoformation of ethanol post-mortem in the VH and allows VH to be used to distinguish ante mortem ethanol ingestion from post-mortem neoformation (Dinis-Oliveira *et al.*, 2010, Cowan *et al.*, 2016).

VH is easily sampled during post mortem investigations and does not require complex analytical procedures (Levine and Jufer, 2008). The sampling technique, as described previously, is straightforward and can be done without dissecting the decedent (Levine and Jufer, 2008). Since VH is a simple matrix that consists mainly of water, samples do not require complex sample preparation and analysis procedures (Bévalot *et al.*, 2016, Dinis-Oliveira *et al.*, 2010). VH samples therefore require little or no sample preparation prior to analysis (Bévalot *et al.*, 2016, Levine and Jufer, 2008).

VH can, in several post mortem cases, be the only adequate biological matrix available for sampling and analysis (Harper, 1989). Due to its anatomical location, VH is protected from decomposition processes, trauma and charring (Levine and Jufer, 2008). VH is the only matrix that can be analysed in decomposition cases while in cases where the decedent has suffered severe trauma, such as exsanguination, VH may be the only available matrix to sample and analyse (Levine and Jufer, 2008, Kugelberg and Jones, 2007, Teixeira *et al.*, 2004).

2.3.1.4. Limitations of VH analysis

The main limitation of analysing VH samples is the sample volume (Levine and Jufer, 2008). A maximum of 5 mL of VH can be sampled from each decedent however to preserve sample integrity, between 2 and 3 mL is generally sampled (Levine and Jufer, 2008). This small sample volume limits the sensitivity of toxicological analyses and may prevent the analysis of certain analytes of interest (Levine and Jufer, 2008).

The integrity of VH samples may vary depending on ante mortem health factors (Levine and Jufer, 2008, Kugelberg and Jones, 2007). VH is unsuitable for toxicological analysis in decedents who have received ophthalmic surgeries because these surgeries alter the composition of the VH and contaminate it with various fluids and analytes (Kugelberg and Jones, 2007, Parsons *et al.*, 2003). The interpretation of VH ethanol concentrations (VEC) requires careful consideration in decedents with diabetes (Kugelberg and Jones, 2007). The VH of these decedents may contain excess glucose which can result in post mortem ethanol neoformation (Kugelberg and Jones, 2007).

The circumstances of death can play a role in the sampling of VH (Levine and Jufer, 2008, Hunsaker *et al.*, 2015). In cases of severe trauma to the head or eyes, VH is unavailable for sampling and analysis (Hunsaker *et al.*, 2015). The sampling of VH is also difficult in decedents with advanced stages of decomposition (Hunsaker *et al.*, 2015). During advanced decomposition, VH dehydrates and dries up thus complicating the sampling process (Hunsaker *et al.*, 2015).

2.3.1.5. Applications of VH analysis

In post mortem cases, VH is sampled and analysed to determine the degree of ethanol intoxication (Tormey, 2016). The VH ethanol concentration (VEC) is used to interpret the degree of inebriation in post-mortem cases where blood is not available for collection; the body has signs of decomposition or the decedent has been embalmed (Kugelberg and Jones, 2007, Dinis-Oliveira *et al.*, 2010, Ioan *et al.*, 2015). The average ratio of VEC:BEC is 1.15-1.20:1 however, a conservative approach is required when estimating BEC from VEC because this ratio has large interindividual variations (Hunsaker *et al.*, 2015, Kugelberg and Jones, 2007). This conservative approach requires the VEC to be divided by two to estimate the BEC (Hunsaker *et al.*, 2015). This estimated BEC value is lower than the true BEC value but has a high degree of confidence (Hunsaker *et al.*, 2015).

VEC is analysed in conjunction with BEC to determine the pharmacokinetic phase of the consumed ethanol (Refer to Table 2.5) (Ioan *et al.*, 2015, Honey *et al.*, 2005). Pathologists can use this pharmacology to either confirm or reject the circumstances of death of a decedent by interpreting the time interval between the ante mortem consumption of ethanol and death

(Refer to Table 2.5) (Hunsaker *et al.*, 2015, Honey *et al.*, 2005). The VEC to BEC ratio can also aid pathologists in identifying post mortem ethanol neoformation and blood sample contamination, due to trauma or sample collection (Honey *et al.*, 2005).

In addition to being used to determine ethanol intoxication, the analysis of VH can be used to determine chronic ethanol use and alcoholism (Kugelberg and Jones, 2007, Tormey, 2016). The detection and quantification of ethanol metabolites in VH can provide extended detection windows for ethanol intoxication ranging from 48 hours to up to 4 weeks (Dinis-Oliveira, 2016, Oppolzer *et al.*, 2016).

Table 2.5: The interpretation of BAC to VAC ratios from decedents (Honey *et al.*, 2005)

VEC to BEC ratio	Ethanol pharmacokinetic phase	Pathologist interpretation
VEC > BEC	Elimination	Death occurred a few hours after the consumption of ethanol
VEC ≈ BEC (1.15-1.20: 1)	Distribution (Equilibrium)	Death occurred shortly after or during ethanol consumption
VEC < BEC	Absorption	• Death occurred rapidly after ethanol consumption • Post-mortem ethanol formation • Blood sample contaminated

2.4. ALTERNATIVE ANALYTES FOR ETHANOL ANALYSIS

The evaluation of ethanol intoxication is of great importance in both ante mortem and post mortem toxicology (Oppolzer *et al.*, 2016). In addition to analysing alternate matrices, the limitations of analysing ethanol in blood can be negated by detecting and quantifying ethanol biomarkers in various biological samples (Kugelberg and Jones, 2007, Oppolzer *et al.*, 2016) A biomarker is a substance, structure or process that reflects an interaction between a biological system and a potential hazard (chemical, physical or biological) (Strimbu and Tavel, 2010). In the case of ethanol intoxication, ethanol is the potential hazard and the biomarkers are substances such as metabolites, cells and enzymes (Oppolzer *et al.*, 2016, Cabarcos *et al.*, 2015). This section will highlight the different ethanol biomarkers and discuss the advantages and limitations of each biomarker.

2.4.1. Ethanol biomarkers

There are two types of ethanol biomarkers, indirect and direct (Cabarcos *et al.*, 2015). Indirect ethanol biomarkers are cells or enzymes that undergo changes in response to ethanol

consumption and include carbohydrate-deficient-transferrin (CDT), gamma glutamyl transferase (GGT), aspartate amino transferase (AAT), alanine amino transferase (ALT) and the mean corpuscular volume of the erythrocytes (MCV) (Andresen-Streichert *et al.*, 2018, Hastedt *et al.*, 2013). The use of these biomarkers to determine the degree of ethanol intoxication is limited by the lack of specificity and sensitivity since increased concentrations of these biomarkers are detected in people with liver disorders, unrelated to ethanol abuse, and also after the administration of certain medications such as barbiturates (Cabarcos *et al.*, 2015, Helander and Beck, 2008).

Direct ethanol biomarkers are formed through the non-oxidative metabolism of ethanol and contain the two original carbon atoms from the ethanol molecule (Cabarcos *et al.*, 2015, Andresen-Streichert *et al.*, 2018). The direct ethanol biomarkers, summarized in Table 2.6, are fatty acid ethyl esters (FAEE), phosphatidylethanol (PEth), ethyl glucuronide (EtG) and ethyl sulphate (EtS) (Cabarcos *et al.*, 2015). These biomarkers have large detection windows which allows for the determination of ethanol intoxication more than 12 hours after consumption (Maenhout *et al.*, 2013, Cabarcos *et al.*, 2015).

Table 2.6: Summary of the direct ethanol biomarkers including enzymatic formation and the analysis advantages and disadvantages. (Information obtained and summarised from (Dinis-Oliveira, 2016, Heier *et al.*, 2016, Maenhout *et al.*, 2013, Oppolzer *et al.*, 2016, Nanau and Neuman, 2015).

Ethanol biomarker	Overview	Enzymatic formation		Detection window	Analysis		
		Location	Process		Matrices	Advantages	Disadvantages
FAEE	A group of 20 lipophilic analytes	Most tissues	FAEE synthase esterifies ethanol with a fatty acid chain	Up to 99 hours	Blood Hair Meconium Liver tissue	High ethanol specificity	Cannot be detected in hydrophilic matrices such as urine
	Consists of an ethyl group linked to a fatty acid chain						Complicated analysis
							Unstable in biological samples if inadequately stored
PEth	Type of phospholipid consisting of 48 different homologues[1]	Cell membrane	Phospholipase D catalyses the reaction of a phospholipid with ethanol	Up to 4 weeks	Blood Hair Breath	Highly sensitive and specific to ethanol consumption	Cannot be detected in hydrophilic matrices such as urine
	Consists of a phosphoethanol head and two long chain carboxylic acid residues					Analysis is easy and cost effective	Can only detect chronic ethanol use
							Precise sample storage conditions required (either 4°C or -80°C)
EtG	Highly polar, non-volatile, and acidic	Liver	UGT[2] catalyses the glucuronidation of ethanol with glucuronic acid	Urine- Up to 80 hours	Urine Blood VH[4] Hair Tissue Meconium	Most sensitive and specific ethanol biomarker	Stability in biological samples
	0.5-1.6% of consumed ethanol is metabolised to form EtG			Blood- Up to 36 hours		Detectable in various matrices within an hour of ethanol consumption	Susceptible to false positive results
EtS	Highly polar, non-volatile, and acidic	Liver	SULT[3] catalyses the sulphonation of ethanol with sulphuric acid	Urine- Up to 80 hours	Urine Blood VH[4] Hair Tissue Meconium	Sensitive and specific ethanol biomarker	Very sensitive analytical technique required due to the low concentrations in biological matrices
	0.1% of consumed ethanol is metabolised to form EtS			Blood- Up to 36 hours		Detectable in various matrices within an hour of consumption	
						Most stable ethanol biomarker	

[1]Homologues: structurally similar compounds that differ from each other by a repeating unit such as a methyl group (Tewari and Vishnoi, 1976), [2]UGT: UDP-glucuronosyltransferase (UDP- uridine 5'-diphospho), [3]SULT: Sulphotransferase, [4]VH: Vitreous humour

2.4.2. EtG and EtS

EtG and EtS are proven to be the most reliable and practical biomarkers to analyse as alternative analytes for ethanol testing (Elrasheed *et al.*, 2017). The reliability of these biomarkers is attributed to their sensitivity and specificity to ethanol consumption as well as the fact that they are detectable in various biological matrices within an hour of ethanol consumption (Oppolzer *et al.*, 2016, Dinis-Oliveira, 2016). EtG and EtS are minor non-oxidative ethanol metabolites that are highly polar, non-volatile and of acidic nature (Oppolzer *et al.*, 2016). Both analytes are formed by enzymatic reactions in the liver and can be detected in biological samples and tissues (Nanau and Neuman, 2015, Baranowski *et al.*, 2008).
Approximately 0.5-1.6% of consumed ethanol is metabolised to form EtG while only 0.1% forms EtS (Refer to Figure 2.2) (Dinis-Oliveira, 2016). In the liver, UGT catalyses the enzymatic transfer of a glucuronyl functional group ($C_6H_9O_6$) from UDP-glucuronic acid to ethanol to from EtG (Heier *et al.*, 2016). EtS is formed by the enzymatic transfer, catalysed by SULT, of a sulphonate functional group (SO_3^-) from 3'-phosphoadenosine-5'-phospho-sulphate to ethanol (Heier *et al.*, 2016).
EtG and EtS are excreted mainly in the urine but can be detected in various biological samples and tissues (Heier *et al.*, 2016). EtG appears in biological samples within an hour of ethanol consumption and peak concentrations occur approximately four hours after consumption (Dinis-Oliveira, 2016). EtS has a similar pharmacokinetic time-course to EtG but EtS concentrations are relatively lower in biological matrices (Helander and Beck, 2004)

2.4.2.1. Applications

There are a large variety of practical applications for EtG and EtS analysis (Helander and Beck, 2008). The fact that these biomarkers are extremely sensitive and specific for ethanol consumption and can be analysed in various biological samples makes them of practical use in both ante mortem and post mortem settings (Helander and Beck, 2008).

- Ante mortem

Several studies have highlighted the ante mortem applications of EtG and EtS in both the clinical and forensic field (Dahl *et al.*, 2011, Erim *et al.*, 2007, Nanau and Neuman, 2015, Himes *et al.*, 2014, Hegstad *et al.*, 2013). The most common clinical application is the use of these biomarkers to confirm abstinence or detect relapse in patients undergoing ethanol withdrawal treatment (Dahl *et al.*, 2011). These biomarkers are also used to determine ethanol consumption in liver transplant patients especially patients with alcoholic liver disease (Erim *et al.*, 2007). Prior to transplantation surgery, EtG and EtS testing is required to monitor

ethanol abstinence and identify ethanol abuse in patients applying for new organs (Erim *et al.*, 2007). Patients with high EtG and EtS levels may not be considered for transplantation due to ongoing ethanol usage (Erim *et al.*, 2007).

The analysis of these biomarkers can also be used to detect ethanol abstinence in the workplace (Skipper *et al.*, 2004). This workplace testing is of particular importance in safety sensitive jobs such as in the mining and the healthcare professions (Skipper *et al.*, 2004). In Europe, EtG and EtS analysis is performed for driving licence regranting legislation (Kummer *et al.*, 2016). A person can have their driving licence confiscated if they are deemed unfit to drive, due to various driving infringements i.e. driving under the influence of ethanol (Kummer *et al.*, 2016). Legislation requires a six month period of proven ethanol abstinence in order for a person's licence to be reinstated which is proven using EtG and EtS analysis (Kummer *et al.*, 2016).

In sexual assault cases, EtG and EtS analysis in blood is used to contribute objective evidence on the victim's degree of intoxication (Hegstad *et al.*, 2013). EtG and EtS analysis is used in anti-doping cases to determine the degree of ethanol intoxication because the concentration of testosterone in blood and urine is influenced by the presence of ethanol (Lostia *et al.*, 2013, Sarkola and Eriksson, 2003). EtG and EtS may also be analysed in maternal hair or meconium to detect long-term utero ethanol exposure which can cause foetal alcohol spectrum disorder (FASD), cognitive and growth disorders of the foetus (Himes *et al.*, 2014, Nanau and Neuman, 2015).

- Post-mortem

The primary post-mortem application of EtG and EtS analysis is to differentiate between the ante mortem consumption of ethanol and the post-mortem formation of ethanol (Høiseth *et al.*, 2010, Krabseth *et al.*, 2014). Additionally, in certain cases and situations, the analysis of these biomarkers can aid pathologists in clarifying the circumstances of death (Sundström *et al.*, 2014).

Since EtG and EtS are reliable biomarkers for ante mortem ethanol consumption, the analysis of these biomarkers is used to rule out the post mortem formation of ethanol, discussed in section 2.2.2.1 (Høiseth *et al.*, 2007). The absence of EtG and EtS associated with the presence of ethanol in biological samples gives conclusive evidence of post mortem ethanol formation (Høiseth *et al.*, 2010). Contrarily, the presence of EtG and EtS associated with the absence of ethanol provides evidence of ethanol consumption 12-24 hours prior to death (Høiseth *et al.*, 2010, Dahl *et al.*, 2011). It is therefore highly recommended to analyse these biomarkers in post mortem biological samples especially in cases whereby BEC results are negative (Krabseth *et al.*, 2014).

EtG and EtS analysis can assist pathologists in cases relating to public transport, safety-sensitive occupations, infants and alcoholic ketoacidosis (Krabseth *et al.*, 2014). Pathologists require EtG and EtS analysis to refute the post mortem formation of ethanol and prove that ethanol concentrations are due to ante mortem ethanol ingestion which is of particular importance in deaths related to safety-sensitive occupations and public transport (Sundström *et al.*, 2014). Literature recommends the use of EtG and EtS analysis in suspected ethanol poisoning cases of infants to provide conclusive evidence as to the origin (ante mortem ingestion or post-mortem formation) of the ethanol detected in the infant (Sundström *et al.*, 2014, Appenzeller *et al.*, 2008). EtG and EtS analysis provides confirmation of ante mortem ethanol consumption in cases where the cause of death is attributed to alcoholic ketoacidosis (Sundström *et al.*, 2014). The longer detection window of these biomarkers allows for the confirmation of ante mortem ethanol consumption despite ethanol concentrations often being negative (Sundström *et al.*, 2014).

2.5. ANALYSIS OF ETG AND ETS

Research into the detection and quantification of EtG and EtS began with the development of methods to analyse EtG using GC coupled with mass spectrometry (GC-MS) (Maenhout *et al.*, 2013, Oppolzer *et al.*, 2016, Schmitt *et al.*, 1995). Further research has developed methods to detect and quantify EtG using various techniques including liquid chromatography coupled with mass spectrometry (LC-MS), immunoassays and capillary electrophoresis (CE) (Křivánková *et al.*, 2005, Zimmer *et al.*, 2002, Nishikawa *et al.*, 1999). Generally, EtG is analysed using either GC-MS or LC-MS due to the high sensitivity and specificity of these techniques in comparison to immunoassays and CE (Oppolzer *et al.*, 2016, Maenhout *et al.*, 2013). EtS analysis was initially accomplished by extending the analytical run of an established LC-MS method for EtG analysis (Helander and Beck, 2004). CE methods have also been developed to analyse EtS however these methods suffer from low sensitivities (Caslavska *et al.*, 2011).

2.5.1. Advantages to EtG analysis

The major advantage of EtG analysis is the specificity and sensitivity of EtG to detect ethanol consumption which exceeds that of other direct ethanol biomarkers (Helander and Dahl, 2005, Oppolzer *et al.*, 2016). Studies have reported sensitivities and specificities close to 100% when analysing EtG to determine ethanol consumption in post-mortem blood and hair samples (Boscolo-Berto *et al.*, 2014, Høiseth *et al.*, 2007, Oppolzer *et al.*, 2016).

In contrast to other ethanol biomarkers, the high polarity of EtG allows for its detection in various biological matrices including blood, urine, vitreous humour, sweat, meconium, tissue and hair (Himes *et al.*, 2014, Heier *et al.*, 2016, Dinis-Oliveira, 2016). This allows for EtG to

be detected in cases where recommended biological samples, such as blood and urine, are insufficient for analysis (Heier *et al.*, 2016). EtG is also more stable in comparison to other ethanol biomarkers (Maenhout *et al.*, 2013). The concentration of EtG remains stable for up to five weeks if stored at 4 °C (Maenhout *et al.*, 2013).

EtG has a longer half-life than ethanol, approximately three hours, and is therefore still present in biological samples after ethanol has been eliminated (Dinis-Oliveira, 2016, Høiseth *et al.*, 2009). Furthermore, EtG has a large detection window of up to 80 hours in urine and 36 hours in blood (Hegstad *et al.*, 2017, Heier *et al.*, 2016, Nanau and Neuman, 2015, Oppolzer *et al.*, 2016). Due to the long half-life and large detection window, EtG is known as a short-to-medium term ethanol biomarker and can be used to determine ethanol consumption as well as to monitor ethanol abstinence (Wurst *et al.*, 2006, Dinis-Oliveira, 2016).

2.5.2. Limitations to EtG analysis

The stability of EtG is a major limiting factor associated with EtG analysis (Maenhout *et al.*, 2013). During sample storage, EtG can be formed or degraded in biological samples (Baranowski *et al.*, 2008). EtG formation can occur if the biological sample is infected with *Escherichia coli* in the presence of ethanol (Helander *et al.*, 2007). This EtG formation is rapid and is not prevented by the addition of sodium fluoride or the storage of the sample at 4 °C (Helander *et al.*, 2007). Alternatively, EtG can be degraded in samples infected by bacteria with β-glucuronidase activity (Helander and Dahl, 2005). These bacteria degrade the EtG through hydrolysis which may result in false-negative or deceptively low EtG results (Helander and Dahl, 2005).

The sensitivity of EtG to the presence of ethanol can be a limitation of EtG analysis, despite its advantages (Nanau and Neuman, 2015). This sensitivity can prevent EtG analysis from differentiating between dermal ethanol exposure and ethanol consumption which can lead to false positive results (Nanau and Neuman, 2015). In the abstinence of ethanol consumption, the use of alcohol-based hand sanitizers can result in EtG levels between 300 and 500 ppb (Nanau and Neuman, 2015). Additional false positives can arise from the use of mouthwashes, the consumption of sauerkraut and mature bananas as well as the exposure to medications that contain ethanol (Maenhout *et al.*, 2013, Jatlow and O'malley, 2010).

Several other factors can significantly affect EtG concentrations in biological matrices including age, gender, marijuana use and kidney disease (Wurst *et al.*, 2004). Age and gender affect EtG concentrations due to renal functioning and the expression of the UGT enzyme (Wurst *et al.*, 2004). Kidney disease can decrease EtG concentrations due to the overexcretion of the analyte while marijuana use can increase EtG levels by increasing the glucuronidation of ethanol (Wurst *et al.*, 2004, Nanau and Neuman, 2015).

2.5.3. Advantages to EtS analysis

The benefits of analysing EtS to determine ethanol intoxication are similar to those of EtG with regards to sensitivity, specificity, the long detection window and the various biological matrices (Maenhout *et al.*, 2013). In addition, EtS is more stable than EtG in biological samples because degradation or formation of EtS only occurs when biological samples have a high bacterial density over an extended period of time (Baranowski *et al.*, 2008, Maenhout *et al.*, 2013, Dinis-Oliveira, 2016, Halter *et al.*, 2009). High bacterial density conditions only occur in post mortem samples where the body of the decedent has reached advanced stages of decomposition (Halter *et al.*, 2009). A further advantage is that EtS levels in biological samples are not affected by dermal ethanol exposure (Nanau and Neuman, 2015, Reisfield *et al.*, 2011). EtS can therefore distinguish ethanol consumption from ethanol exposure from alcohol-based hand sanitizers (Reisfield *et al.*, 2011). .

2.5.4. Limitations to EtS analysis

The main limitation associated with EtS analysis is the small percentage of EtS (0.1%) that is formed subsequent to ethanol consumption (Maenhout et al., 2013). Consequently, the EtS concentrations in biological samples are relatively low (Maenhout *et al.*, 2013, Dinis-Oliveira, 2016). Very sensitive analytical techniques and instrumentation are required in order to quantify these low concentrations which is extremely costly and requires highly skilled analysts (Maenhout et al., 2013).

Like EtG, the sensitivity of EtS to the presence of ethanol is a disadvantage because it can produce false positive results (Thierauf *et al.*, 2011, Maenhout *et al.*, 2013). However, of the factors that cause false positive EtG results, only mouthwashes can produce false positive EtS results in the absence of ethanol consumption (Maenhout *et al.*, 2013).

2.5.5. Simultaneous analysis

Several studies have recommended the simultaneous analysis of EtG and EtS, in blood and urine, to determine ethanol intoxication (Helander and Dahl, 2005, Jatlow and O'malley, 2010, Lostia *et al.*, 2013, Wurst *et al.*, 2006). There is a high and significant correlation between EtS and EtG concentrations in biological samples which is attributed to the similar detection windows and pharmacokinetic profiles of these biomarkers (Maenhout *et al.*, 2013, Wurst *et al.*, 2006, Helander and Dahl, 2005). In addition, the analytical technique required to simultaneously analyse EtG and EtS is not more complex or time-consuming than analysing each biomarker independently (Maenhout *et al.*, 2013).

Since EtG and EtS are formed via different metabolic pathways, their simultaneous analysis increases the specificity and sensitivity of the method to determine ethanol intoxication (Wurst *et al.*, 2006, Oppolzer *et al.*, 2016, Elrasheed *et al.*, 2017). Furthermore, a simultaneous

detection method can rule out the formation or degradation of EtG, prior to analysis, from bacteria as well as decrease the probability of false positive results from dermal ethanol exposure (Helander *et al.*, 2007, Lostia *et al.*, 2013, Elrasheed *et al.*, 2017).

2.5.6. LC tandem MS method

For EtG and EtS analysis, LC provides an advantage over GC because GC methods require a laborious, time-consuming derivatization step during sample preparation (Oppolzer *et al.*, 2016). This derivatization step alters the chemical properties of the analytes, through acetylation, which increases their volatility allowing for their detection by GC (Levine and Jufer, 2008, Nishikawa *et al.*, 1999). Research therefore suggested that LC coupled with tandem mass spectrometry provides a rapid, sensitivity analytical method to detect and quantify both EtG and EtS in various biological samples (Nishikawa *et al.*, 1999, Kugelberg and Jones, 2007).

2.5.6.1. Principle

LC tandem MS is a highly sensitive, selective and accurate analytical technique that can detect and quantify a wide range of analytes in a single analytical run (Pitt, 2009, Ramachandram and Dinesh, 2016). This is a hyphenated technique that combines the physicochemical separation abilities of liquid chromatography with mass spectrometry capabilities (Chromacademy, 2016b, Ramachandram and Dinesh, 2016).

- Liquid chromatography

LC is an analytical technique that uses the basic principle of chromatography to separate analytes of interest in a liquid sample (Kumar *et al.*, 2018). In LC, the mobile phase is a solvent that is pumped at high pressure through a packed column (Ramachandram and Dinesh, 2016). The packed column is a stainless-steel tube (30 - 300 mm long) packed with small chemically modified silica particles (3-10 µm in diameter), known as the stationary phase (Bird, 1989, Kumar *et al.*, 2018).

Analytes within a sample partition between the stationary and mobile phase based on physicochemical properties, such as polarity (Hussain and Maqbool, 2014, Bird, 1989). The partitioning of these analytes is determined by the chemical nature of the stationary phase as well as the polarity of the mobile phase (Bird, 1989, Ramachandram and Dinesh, 2016). Based on these aspects, there are two classes of LC namely reverse phase and normal phase (Ramachandram and Dinesh, 2016). In reverse phase LC, the stationary phase is non-polar (chemically modified with hydrocarbon chains e.g. C_6 or C_{18}) and the mobile phase is polar (e.g. water-acetonitrile) (Ramachandram and Dinesh, 2016). Normal phase LC consists of a polar stationary phase (chemically modified with polar functional group e.g. an amino or a

cyano) and non-polar mobile phase (nonpolar solvents e.g. hexane) (Ramachandram and Dinesh, 2016).

During analysis, the sample is carried, at high pressure, by the mobile phase across the bed of the stationary phase (Ramachandram and Dinesh, 2016). Analytes with similar physicochemical properties to the stationary phase take longer to move through the column while analytes with similar properties to the mobile phase move through the column faster (Bird, 1989). A detector, attached to the column outlet, identifies each peak as it leaves the column on the basis of its RT, and quantifies each peak as a function of the detector output (signal) (Bird, 1989). There are a number of different types of detectors such as ultraviolet (UV), diode array, fluorescence and MS (Kumar *et al.*, 2018)

- Tandem mass spectrometry (MS)

Mass spectrometry (MS) is an analytical technique, based on the principle of ionization of analytes, that identifies and quantifies based on mass-to-charge ratios (Ramachandram and Dinesh, 2016). MS can be used to quantify analytes within a sample, identify unknown compounds and determine the elemental composition and chemical structure of a compound (Ramachandram and Dinesh, 2016, Kang, 2012). A MS instrument consists of three components; an ion source, mass analyser and a detector (Kang, 2012).

The ion source is the interface between the LC and the MS and is responsible for the ionization of the analytes forming ions (Ramachandram and Dinesh, 2016). LC tandem MS generally uses electrospray ionization (ESI) which converts analytes dissolved in the LC mobile phase into ions suspended in a gas phase (Ramachandram and Dinesh, 2016). The solvent eluted from the LC column is pumped through a metal capillary, with a voltage of 3-5 kV, that nebulises the solvent to form a fine spray of charged droplets (Pitt, 2009). These droplets are rapidly evaporated by heat and dry nitrogen in a process referred to as ion evaporation (Pitt, 2009, Korfmacher, 2005). This process causes the droplets to decrease in size resulting in individually charged ions of the analyte which are directed into the mass analyser by apertures and focusing voltages (Pitt, 2009, Korfmacher, 2005).

The mass analyser is maintained under vacuum; this is the MS component that separates the ionized analytes according to their mass-to-charge (m/z) ratio in an electric and/or magnetic field (Ramachandram and Dinesh, 2016, Kang, 2012). There are many different types of mass analysers that can be used in conjunction with LC, e.g. single quadrupole, triple quadrupole, ion trap and time-of-flight analysers (Pitt, 2009). A triple quadrupole analyser, also known as tandem MS is most frequently used for targeted bioanalytical analyses (Korfmacher, 2005).

A quadrupole consists of a set of four parallel metal rods; each rod has a combination of a constant and varying voltage applied to it (Pitt, 2009). At a specific time point, two opposite rods will have an applied negative varying voltage while the other two rods will have a positive

varying voltage (Pitt, 2009). An oscillating electric field is created by the varying voltage oscillating from one pair of rods to the other which allows ions of a specific m/z ratio to pass along the axis of the rods (Kang, 2012). The voltages applied to the rods can be changed with time to allow for different ions with a range of m/z ratios to pass through the rods which allows for a quadrupole to act as a mass-selective filter (Kang, 2012).

Tandem MS consists of three of these quadrupoles connected in series (Kang, 2012). The first quadrupole (Q1) scans across a specific m/z ratio range and selects a specific ion/s, precursor ion (Kang, 2012). The second quadrupole (Q2) does not function as a mass-selective filter but rather acts as a collision cell (Kang, 2012). Ions from Q1 enter the collision cell and are bombarded by an inert gas, such as argon (Pitt, 2009). This gas collides with the ions which causes them to fragment (Pitt, 2009). The fragment ions produced are then transmitted to the third quadrupole (Q3) (Pitt, 2009). Q3 separates and analyses the fragment ions, product ions, produced in the collision cell (Kang, 2012).

The detector is the final component of a MS instrument (Kang, 2012). The detector quantifies the ions that exit the mass analyser by measuring the charge induced or current produced when an ion passes or hits the surface of the detector (Kang, 2012). This charge induced or current produced is converted into an electric signal which is recorded on a computer system and produces a mass spectrum (Kang, 2012).

2.5.6.2. Method

A number of research papers have reported on the development of LC tandem MS methods to analyse EtG and EtS in various biological samples (Thierauf *et al.*, 2011, Al-Asmari *et al.*, 2010, Himes *et al.*, 2014, Albermann *et al.*, 2012, Hegstad *et al.*, 2013, Bicker *et al.*, 2006, Morini *et al.*, 2007, Lostia *et al.*, 2013, Høiseth *et al.*, 2010, Favretto *et al.*, 2010). These developed methods differ with regards to sample preparation, LC and tandem MS conditions (Refer to Table 2.7).

The most frequent sample preparation technique used was protein precipitation (PP) with methanol (MeOH) or acetonitrile (ACN) (Oppolzer *et al.*, 2016). After the addition of the precipitation solvent, the solutions were centrifuged and the supernatant was injected onto the HPLC instrument (Thierauf *et al.*, 2011, Lostia *et al.*, 2013). Across all publications, reverse phase chromatography with either an isocratic or a gradient elution profile was used (Oppolzer *et al.*, 2016). These articles primarily analysed these biomarkers using a negative ESI (electrospray ionization) ion source in MRM (Multiple reaction monitoring) mode (Thierauf *et al.*, 2011, Favretto *et al.*, 2010, Himes *et al.*, 2014, Lostia *et al.*, 2013, Albermann *et al.*, 2012, Al-Asmari *et al.*, 2010). In four of the methods, the ionization efficiency of the analytes was

increased with the post column addition, using a T-union, of 2-propanol or acetonitrile (Thierauf *et al.*, 2011, Favretto *et al.*, 2010).

Table 2.7: Summary of published methods referring to the analysis of EtG and EtS in various biological samples including sample preparation, LC and MS conditions

Reference	Matrix	Sample preparation		LC conditions				Tandem MS conditions			Ion transitions m/z (Quantifier; Qualifier)			
		Method	Reagents	Phase	Stationary phase	Mobile phase	Elution	Mode	Ion source	Ion spray voltage (eV)	EtG	EtG-d5	EtS	EtS-d5
(Albermann *et al.*, 2012)[1]	Urine	PP	Methanol (MeOH)	Reverse	Polar-RP[4]	A- 0.1% formic acid B- 100% ACN	Gradient	MRM	ESI negative	-4500	221/75 221/85	226/85 226/75	125/97 125/80	130/98 130/80
(Bicker *et al.*, 2006)	Urine	Dilute and shoot	1:20 with mobile phase	Reverse	Mixed modal RP[4]/ weak anion exchange	Acetic acid in ACN/water	Isocratic	MRM	ESI negative	-4500	221/75 221/85	226/75 226/85	125/97 125/80	130/98 130/80
(Lostia *et al.*, 2013)	Urine	Dilute and shoot	1:10 with mobile phase A	Reverse	Polar-RP[4]	A- 0.1% formic acid B- 100% ACN	Gradient	SRM[5]	ESI negative	-4500	221/85 221/75	N/A	125/80 125/97	N/A
(Lostia *et al.*, 2013)	Blood (Serum)	PP	Acetonitrile (ACN)	Reverse	Polar-RP[4]	A- 0.1% formic acid B- 100% ACN	Gradient	SRM	ESI negative	-4500	221/85 221/76	N/A	125/80 125/98	N/A
(Thierauf *et al.*, 2011)[2]	Vitreous humour Urine	PP	MeOH	Reverse	Polar-RP[4]	0.1% formic acid in ACN	Isocratic	MRM	ESI negative	-4500	221/75 221/85	226/75	125/97 125/80	130/98
(Thierauf *et al.*, 2011)[2]	Blood (Serum)	PP	ACN	Reverse	Polar-RP[4]	0.1% formic acid in ACN	Isocratic	MRM	ESI negative	-4500	221/75 221/85	226/75	125/97 125/80	130/98
(Himes *et al.*, 2014)	Meconium	SPE[3]	Cartridge: Anion-exchange Wash: ACN and MeOH Elute: Hydrochloric acid in ACN	Reverse	RP[4]-C18	A- 0.1% formic acid in water B- 0.1% formic acid in MeOH	Gradient	MRM	ESI negative	-3500	221/75 221/85	226/75 226/85	125/97 125/80	130/98 130/80
(Favretto *et al.*, 2010)[1]	Urine	SPE[3]	Cartridge: Aminopropyl weak anion exchange Wash: Pentane Elute: 0.1% formic acid, format and ACN solution	Reverse	Hypercarb Graphitic Carbon	A- Water, 0.1% formic acid, ammonium formate B- ACN, 0.1% formic acid, ammonium formate	Gradient	SRM	APCI[6]	10	221/75 221/85	226/75 226/85	125/97 125/80	130/98 130/80

[1]Ionization efficiency enhanced by the addition of acetonitrile post-column via a T-union, [2]Ionization efficiency enhanced by the addition of 2-propanol post-column via a T-union, [3]SPE- Solid Phase Extraction, [4]RP- Reverse Phase, SRM[5] – Single Reaction Monitoring, APCI[6] - Atmospheric Pressure Chemical Ionization

2.5.7. Reference values and Interpretation

Currently, there is no scientific correlation between EtG/ EtS concentrations in biological matrices and BEC (Elrasheed *et al.*, 2017, Rainio *et al.*, 2013). This is because EtG and EtS concentrations are dependent on several factors such as genetics, sex, the volume and concentration of ethanol consumed and the time since the ethanol was consumed (Elrasheed *et al.*, 2017). Additionally, there are no published articles that relate the physiological symptoms of ethanol intoxication to the concentration of EtG or EtS in biological matrices (Elrasheed *et al.*, 2017). Studies have however established EtG and EtS concentration cutoffs, a concentration that distinguishes between positive and negative test results, in specific biological matrices (Refer to Table 2.8) (Elrasheed *et al.*, 2017, Reisfield *et al.*, 2011, Jatlow *et al.*, 2014). These concentration cutoffs are used to interpret ethanol intoxication of patients or decedents over a certain time period (Elrasheed *et al.*, 2017, Jatlow *et al.*, 2014).

At present, solely using EtG or EtS results for forensic purposes is unsupported and inadmissible in court (Elrasheed *et al.*, 2017). However, studies have suggested using EtG and EtS concentrations in conjunction with BEC to interpret ethanol intoxication (Armer *et al.*, 2016, Lostia *et al.*, 2013, Høiseth *et al.*, 2007, Wurst *et al.*, 2006). The simultaneous interpretation of ethanol, EtG and EtS results allows a trained professional to determine ethanol intoxication, distinguish between ethanol consumption and post-mortem ethanol formation as well as to determine false positive results (Refer to Figure 2.5) (Høiseth *et al.*, 2007, Armer *et al.*, 2016, Lostia *et al.*, 2013, Rainio *et al.*, 2013).

Table 2.8: Published EtG and EtS concentration cutoffs in various biological matrices

Reference	Analyte	Biological matrix	Concentration cutoff	Detection window
(Elrasheed *et al.*, 2017)	EtG	Hair	7 pg/mg	Up to 3 months
(Kharbouche *et al.*, 2012)	EtS	Hair	9 pg/mg	Up to 3 months
(Elrasheed *et al.*, 2017)	EtG	Urine	500 ppb	80-96 hours
	EtS	Urine	500 ppb	80-96 hours
(Høiseth *et al.*, 2010)	EtG	Blood	100 ppb	24 hours
(Dasgupta, 2015)	EtS	Blood	25 ppb	24 hours

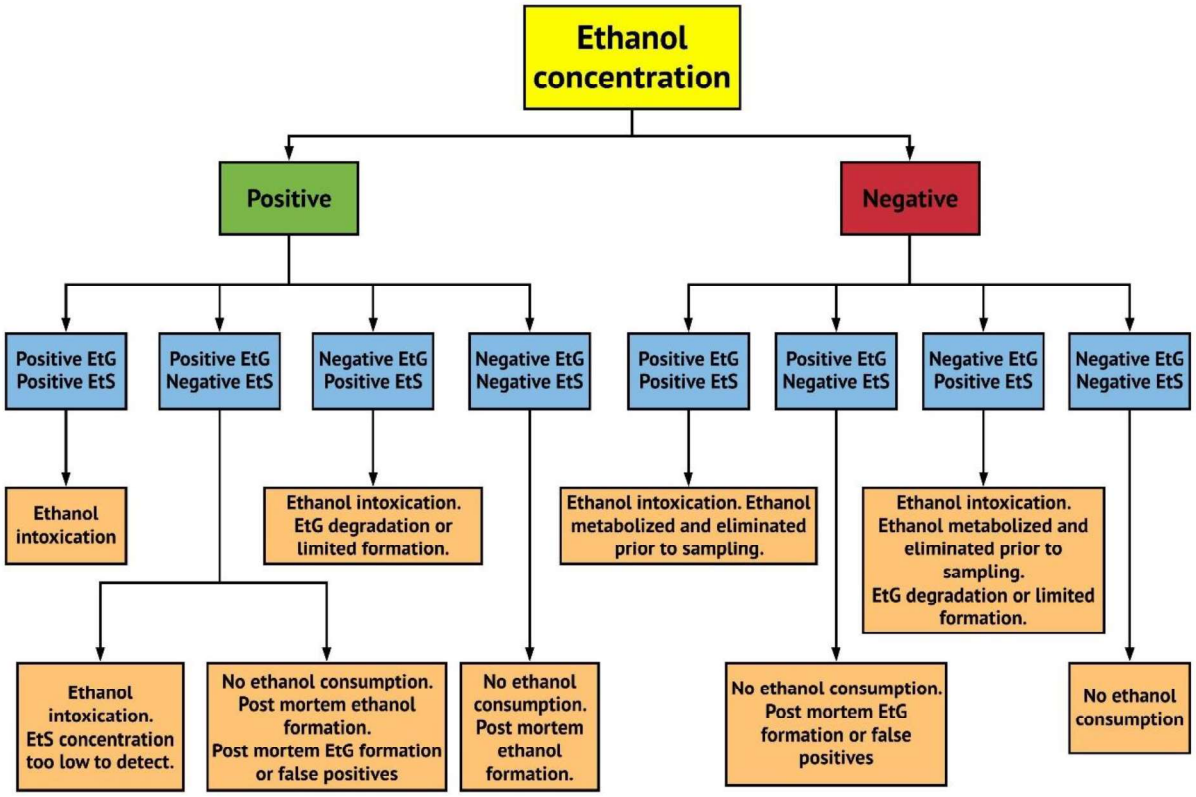


Figure 2.5: A summary of the interpretation of ethanol intoxication using BEC, EtG and EtS results. Information obtained and summarised from (Høiseth *et al.*, 2007, Armer *et al.*, 2016, Lostia *et al.*, 2013, Rainio *et al.*, 2013).

2.6. SUMMARY AND STUDY RATIONALE

In ante mortem and post-mortem toxicology, it is essential to accurately determine the degree of ethanol intoxication in order to interpret and deduce the physiological impairments associated with the intoxication (Kelly and Mozayani, 2012, Thierauf *et al.*, 2011). Ethanol is not only a leading risk factor for mortality worldwide but also contributes to morbidity and disability (World Health Organization, 2018). The analysis of BEC using HSGC-FID is the gold standard method to determine post-mortem ethanol intoxication (Kugelberg and Jones, 2007). However, BEC results can be misinterpreted using this method due to the behaviour of ethanol in a decedent prior to sampling (Kugelberg and Jones, 2007).

Research on more stable ethanol analytes as well as alternative biological samples has been carried out to address the limitations of post-mortem BEC analysis (Kugelberg and Jones, 2007). The simultaneous analysis of the EtG and EtS provides a sensitive and specific method to determine recent ethanol consumption (Oppolzer *et al.*, 2016). Urine and VH are the most suitable biological samples for the analysis of these biomarkers (Rainio *et al.*, 2013). VH was however the sample of choice for this study due to its advantages in post-mortem analysis and the further limitations associated with the post-mortem sampling of urine.

The outcomes of this study will result in: (I)-the technical development and validation of methods to detect and quantify ethanol and its biomarkers EtG and EtS in post-mortem VH samples and (II) the application of these validated methods to a pilot study from the JHB FPS MLL (Johannesburg Forensic Pathology Services Medicolegal Laboratory) post-mortem population.

The development of these methods will potentially assist forensic pathologists in death investigations as well as facilitate the police service and insurance companies. These methods will provide useful information of the quantity and origin of ethanol within post-mortem biological samples as well as provide information regarding the circumstances of death of a decedent.

CHAPTER 3: AIM AND OBJECTIVES

The aim of this study was to develop and validate, according to European Medicines Agency (EMA) guidelines, a method to simultaneously analyse EtG and EtS in VH using LC-MS/MS. In addition, a method to quantitatively determine ethanol in VH using HSGC-FID was developed and validated, according to EMA guidelines. These methods were applied to the VH samples of a sample of decedents from the Johannesburg Forensic Pathology Service Medico Legal Laboratory.

The objectives of this study were:

1. To develop and validate a LC-MS/MS method for the determination of EtG and EtS in post-mortem VH.
2. To develop and validate a HS-GC/FID method to quantitatively determine the concentration of ethanol in post-mortem VH samples.
3. To apply these developed and validated methods to describe the presence of ethanol, EtG and EtS levels in the VH of a post-mortem population from the JHB FPS MLL.

CHAPTER 4: MATERIALS AND METHODS

This chapter will outline the methods used to simultaneously analyse EtG and EtS as well as analyse ethanol in VH samples. The procedure followed for the method validation component of this study is also described.

This study consisted of three processes as summarized in Figure 4.1. The first process involved the development of methods to analyse ethanol as well as EtG and EtS in VH. These methods were subsequently validated before analysing a pilot study of post-mortem VH samples.

The method used to analyse ethanol in VH was optimized based on an existing validated method used, by the National Institute for Occupational Health (NIOH), to analyse ethanol in blood using HSGC-FID (NIOH0410) (Southon, 2019). The method to simultaneous analyse EtG and EtS, using UPLC-MS/MS, was developed and optimised based on a method found in literature (Ohouo *et al.*, 2018, Thierauf *et al.*, 2011, Albermann *et al.*, 2012).

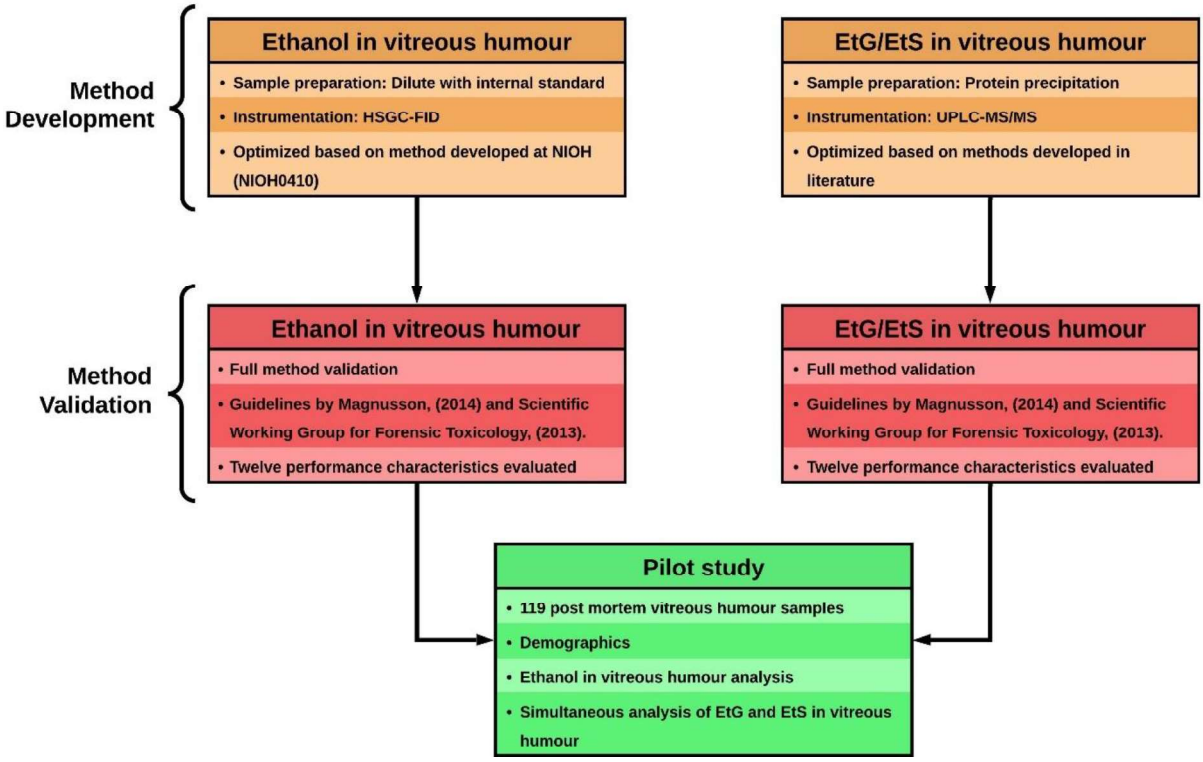


Figure 4.1: A flow diagram illustrating the experimental process followed for this study.

4.1. MATERIALS

BD Vacutainer Eclipse blood collection needles (21G x 0.8 mm x 32 mm), BD Vacutainer one-use holders and BD Vacutainer 4 mL tubes with no additives were purchased from Becton Dickinson (Pty) Ltd. Clear, flat bottom headspace vials (10 mL), grey butyl headspace septa and 20 mm unlined aluminium headspace crimp caps were purchased from Separations (Pty) Ltd. A SUPELCOWAX® 10 capillary GC column (30 m length, 0.25 mm internal diameter and 0.50 µm film thickness) was purchased from Merck (Pty) Ltd. Clear, graduated microcentrifuge tubes with attached cap (1 500 µL) were purchased from Lasec SA (Pty) Ltd. A Waters® Acquity UPLC CSH Phenyl-Hexyl column (150 mm length, 2.1 mm inner diameter and 1.7 µm particle size) and Waters® clear glass screw neck Total Recovery vials with caps (12 X 32 mm, 1 mL volume and preslit PTFE/ Silicone Septum) were purchased from Microsep (Pty) Ltd.

4.2. REAGENTS

Hycolin™ Hospital disinfectant was purchased from Coventry Chemicals Ltd. Sodium fluoride (ACS reagent, ≥99%), tertiary butanol (99.7% purity), 2-propanol (99.9% purity), acetaldehyde (99.5% purity), acetone (99.9% purity), acetonitrile (99.8% purity), methanol (liquid chromatography grade), formic acid (98% purity), hyaluronic acid sodium salt (from *Streptococcus equi.*) and agar were purchased from Sigma-Aldrich. ROMIL LC Wash solution AMPW1 (acetonitrile/methanol/2-propanol/water 25:25:25:25) was purchased from Microsep (Pty) Ltd. The following concentrations of aqueous ethanol certified reference materials (CRM's) were purchased from the National Metrology Institute of South Africa (NMISA): 0.01 g/100 mL, 0.015 g/100 mL, 0.02 g/100 mL (two CRM's with different batch numbers), 0.05 g/100 mL (three CRM's with different batch numbers), 0.1 g/100 mL, 0.2 g/100 mL (three CRM's with different batch numbers) and 0.5 g/100 mL. The following CRM's were purchased from Leco Africa (Pty) Ltd: ethyl-β-D-glucuronide (EtG, 99%, 1 000 µg/mL in methanol), ethyl-β-D-glucuronide-d5 (EtG-d5, 99%, 1 000 µg/mL in methanol), ethyl sulphate sodium salt (EtS, 99%, 1 000 µg/mL in methanol) and ethyl sulphate-d5 sodium salt (EtS-d5, 99%, 1 000 µg/mL in methanol). Ultrapure water (18 MΩ) was obtained from a Milli-Q® water EVOQUA® Ultra Clear purification system.

4.3. EQUIPMENT

The following equipment was used in this study to collect vitreous humour samples: forceps, laboratory squeeze wash bottle (500 mL) and a glass beaker (500 mL).

A Hamilton Microlab® 600 series dilutor (Separations, South Africa), an Agilent® G1888 headspace sampler coupled to an Agilent 6890 N gas chromatography system that is fitted with a flame ionization detector (HSGC-FID) (Chemetrix (Pty) Ltd., South Africa), a Vortex®

Genie 2 mixer (Scientific Industries Inc, United States of America) and A-Grade volumetric flasks (100 mL) were used for the analysis of ethanol in vitreous humour.

The analysis of EtG and EtS in vitreous humour used the following equipment: amber stained Duran® glass bottles with a standard screw caps (500 mL), Precisa 290SCS series balance (Precisa, Switzerland), Vortex® Genie 2 mixer (Scientific Industries Inc, United States of America), A-Grade volumetric flasks (100 mL), glass beakers (500 mL), Pierce Reacti-Therm III heating/stirring module (Separations, South Africa), Ortoalresa Biocen 22 R microcentrifuge (Ortoalresa, Spain), Waters® Acquity Ultra-Performance Liquid Chromatography system coupled with a Waters® Quattro Premier XE tandem mass spectrometer (UPLC-MS/MS) and a Waters® 510 HPLC pump (Microsep, South Africa).

4.4. SAMPLE COLLECTION

Vitreous humour samples were collected at the JHB FPS MLL situated at 25A Hospital Street, Braamfontein, 2001. The JHB FPS MLL services the Johannesburg metropolitan area which includes Alexandra, Booysens, Bramley, Brixton, Cleveland, Diepsloot, Douglasdale, Fairlands, Hillbrow, Honeydew, Jeppe, Johannesburg Central, Langlaagte, Linden, Midrand, Moffatview, Mondeor, Norwood, Parkview, Randburg, Rosebank, Sandringham, Sandton, Sophiatown, and Yeoville.

4.4.1. Ethical clearance

Unconditional ethical clearance was granted from the Wits Human Research Ethics Committee (Clearance certificate number: M171152) (See Appendix 1). Permission to attend post-mortem autopsies and access the case files of the decedents was obtained from the manager of the JFP MLL and the Head of the Division of Forensic Medicine and Pathology at the University of the Witwatersrand.

For confidentiality, this write-up does not contain the names or body numbers of the decedents but rather assigned research numbers.

4.4.2. Population

Vitreous humour was randomly sampled from 119 decedents received at the Johannesburg (JHB) Forensic Pathology Service (FPS) Medico Legal Laboratory (MLL) located at 25A Hospital Street, Braamfontein. According to the National Health Act (Act no. 61, 2003), the primary role for the JHB FPS MLL is to conduct medico-legal investigation on all unascertained or unnatural deaths in the southern Gauteng region. This includes the examination of the body with the intentions of determining the cause of death and factors linked with the death.

The researcher attended autopsy sessions three times a week between June and December 2019 and obtained samples from decedents that adhered to the set inclusion and exclusion criteria. Additional information was gathered from the case files of the decedents including the

demographics, BAC reported in the Forensic Chemistry Laboratory (FCL) reports, cause and circumstances of death (Refer to the data collection sheet in Appendix 2).

4.4.2.1. Inclusion criteria

The following criteria were used to include decedents into this study:

- Both male and female decedents who were 18 years or older
- Decedents in which adequate vitreous humour samples were available (1 - 1.5 mL)

4.4.2.2. Exclusion criteria

The following criteria were used to exclude specific decedents from this study:

- Decedents with advanced stages of decomposition or skeletonization. These stages of decomposition were defined and identified as per the Total Body Score method (Megyesi *et al.*, 2005).
- Decedents with damaged or missing eyes
- Decedents younger than 18 years of age
- Decedents who had received hospital treatments prior to death

4.4.3. VH sample collection procedure

The procedure used, for this study, to sample post-mortem vitreous humour was adapted from a procedure described by Dinis-Oliveira et al., (2010). The sample collection procedure used is illustrated in Figure 4.2.

The clear cap was removed from the BD Vacutainer Eclipse blood collection needle. The BD Vacutainer one-use holder was screwed onto the Eclipse needle until it was tightly secured. Forceps were used to part the upper and lower eyelids of the decedent to exposure the globe of the eye. The green cap of the Eclipse needle was removed, and the needle was inserted into the outer corner of the eye, slightly above the junction between the upper and lower eyelids. A labelled BD Vacutainer 4 mL tube with no additives was inserted into the one-use holder and 1 - 1.5 mL of vitreous humour was slowly aspirated into the tube. The needle was removed from the eye and was covered with the pink safety cover. The tube was removed from the holder and both the needle and holder were discarded into a biohazardous sharps container. The forceps were disinfected with 2% Hycolin solution and saline solution was injected into the eye of the decedent to replicate the natural integrity of the eye.

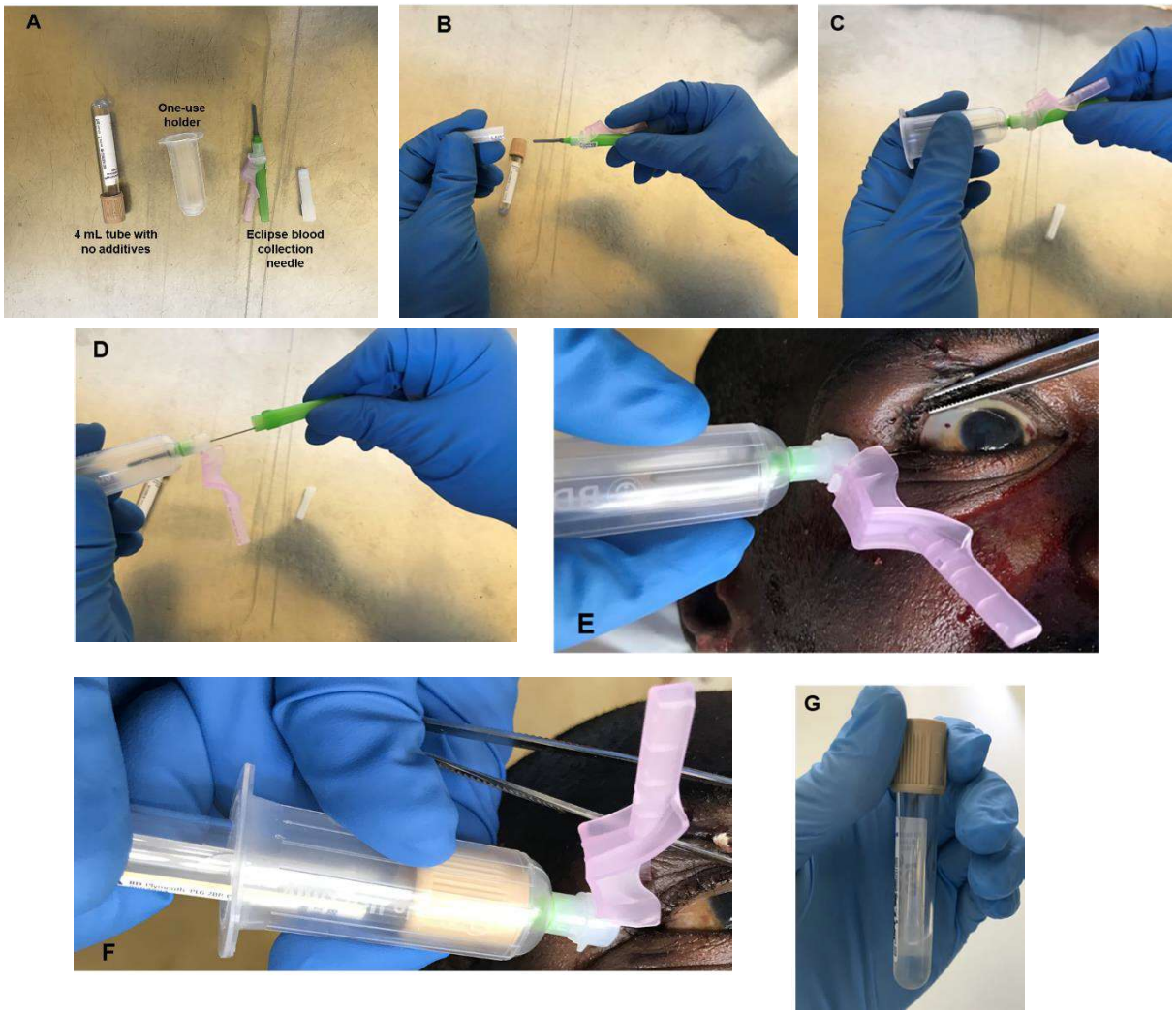


Figure 4.2: The procedure followed to collect vitreous humour samples from the sample of decedents. A) A BD Vacutainer Eclipse blood collection needle, a BD Vacutainer one-use holder and a BD Vacutainer 4 mL tube with no additives. B) The removal of the clear cap from the Eclipse needle. C) The one-use holder was screwed onto the Eclipse needle. D) The green cap was removed from the Eclipse needle. E) Forceps parted the upper and lower eyelids and the needle was inserted into the outer corner of the eye. F) The tube was inserted into the one-use holder and the vitreous humour was aspirated. G) A tube containing the collected vitreous humour sample.

4.5. SAMPLE STORAGE

The collected vitreous humour samples were preserved by adding 320 µg of sodium fluoride to each BD Vacutainer tube. The tubes were subsequently stored at 4 °C until analysis. For

ethanol analysis, the tubes were stored under these conditions for a maximum of three months while for EtG and EtS analysis, the tubes were stored for a maximum of six months.

4.6. SAMPLE PREPARATION

This section describes the sample preparation procedures as well as the procedures followed to prepare the calibration standards and QC's, for each method. The analysis of ethanol in vitreous humour used a simple preparation procedure that involved diluting the samples with an internal standard. The simultaneous analysis of EtG and EtS utilized a protein precipitation procedure.

4.6.1. Ethanol

4.6.1.1. Dilutor verification

The calibration standards, QC's and samples were prepared using a Hamilton Microlab® 600 series dilutor. Prior to its use, the dilutor was verified according to the Hamilton dilutor verification procedure (NIOH0410) of the NIOH.

In this procedure, five empty headspace vials were weighed and the values recorded on the Hamilton dilutor verification form (refer to Appendix 3). A volume of 1 mL of ultrapure water was added to each headspace vial, using the dilutor. The headspace vials were reweighed and the values recorded. The temperature of the ultrapure water was recorded and the relative density of the water was obtained from Tanaka et al., (2001). Finally, the percent volume of the dispensed water was calculated using the procedure in the verification form. The dilutor was verified fit for purpose if the volume percentage was between 95% and 105%

4.6.1.2. Preparation of calibration standards

Fourteen calibration standards and a blank were prepared, using the dilutor, for this method to have a working range of 0.01 to 0.5 g/100 mL (Refer to Table 4.1). Each blank and calibration standard was prepared, in a 10 mL headspace vial, using various ethanol CRMs (0.01 g/100 mL, 0.02 g/100 mL, 0.05 g/100 mL, 0.1 g/100 mL, 0.2 g/100 mL and 0.5 g/100 mL), ultrapure water and 0.1% tertiary butanol as an internal standard (IS). The vials were sealed with headspace septa, capped using aluminium crimp caps and briefly mixed on a vortex.

Table 4.1: The preparation of the ethanol calibration standards including the concentration of each standard, the CRM used to prepare each standard, the volume of the CRM used, the volume of ultrapure water and the volume of internal standard (0.1% tertiary butanol).

Standard	Concentration (g/100 mL)	CRM used (g/100 mL)	CRM volume (µL)	H_2O volume (µL)	IS volume (µL)
Blank	0	N/A	0	250	250
1	0.01	0.01	250	0	250
2	0.02	0.02	250	0	250
3	0.03	0.05	150	100	250
4	0.04	0.05	200	50	250
5	0.05	0.05	250	0	250
6	0.06	0.10	150	100	250
7	0.07	0.10	175	75	250
8	0.08	0.10	200	50	250
9	0.09	0.10	225	25	250
10	0.10	0.10	250	0	250
11	0.20	0.20	250	0	250
12	0.30	0.50	150	100	250
13	0.40	0.50	200	50	250
14	0.50	0.50	250	0	250

4.6.1.3. Preparation of quality controls (QCs)

For the validation of this method, the three ethanol CRM's used as external QCs had concentrations of 0.015 g/100 mL, 0.05 g/100 mL and 0.2 g/100 ml. These three CRM's had different batch numbers to the CRM's used to prepare the calibration standards. For the application of the method to post-mortem vitreous humour samples, the following CRM's were used to prepare the three QCs: 0.02 g/100 mL, 0.05 g/100 mL and 0.2 g/100 mL.

Each QC sample was prepared using the dilutor. The dilutor added 250 µL of ethanol CRM and 250 µL of the IS to a 10 mL headspace vial. The headspace vial was sealed with a septum, capped using an aluminium crimp cap and briefly mixed on a vortex. Each QC was prepared and analysed in duplicate.

4.6.1.4. Preparation of samples

Using the dilutor, each sample was prepared by adding 250 µL of sample and 250 µL of the IS to a 10 mL headspace vial. The headspace vials were sealed with a septum, capped using an aluminium crimp cap and briefly mixed on a vortex. Each sample was prepared and analysed in duplicate.

4.6.2. EtG, EtS

4.6.2.1. EtG working solutions

Three EtG working solutions (WS) were prepared in 1 500 µL microcentrifuge tubes, using serial dilutions of the EtG stock standard (99%, 1 000 000 ppb). EtG WS1 (10 000 ppb) was prepared up by adding 15 µL of EtG stock standard to 1485 µL of methanol. EtG WS2 (1 000 ppb) was prepared by adding 150 µL of EtG WS1 to 1 350 µL of methanol. EtG WS3 (500 ppb) was prepared up by adding 75 µL of EtG WS1 to 1425 µL of methanol. These solutions were stored at 4 °C and reprepared after three months.

4.6.2.2. EtS working solutions

Four EtS WS were prepared in 1 500 µL microcentrifuge tubes, using serial dilutions of the EtS stock standard (99%, 1 000 000 ppb). EtS WS1 (10 000 ppb) was prepared up by adding 15 µL of EtS stock standard to 1485 µL of methanol. EtS WS2 (1 000 ppb) was prepared by adding 150 µL of EtS WS1 to 1 350 µL of methanol. EtS WS3 (100 ppb) was prepared up by adding 15 µL of EtS WS1 to 1485 µL of methanol. EtS WS4 (10 ppb) was prepared up by adding 15 µL of EtS WS2 to 1485 µL of methanol. These solutions were stored at 4 °C and reprepared after three months.

4.6.2.3. Internal standard

The internal standard was prepared, in a 1 500 µL microcentrifuge tube, by adding 75 µL of EtG-d5 stock (99%, 1 000 000 ppb) and 9 µL of EtS-d5 stock (99%, 1 000 000 ppb) to 1416 µL of methanol. The internal standard had an EtG-d5 concentration of 50 000 ppb and an EtS-d5 concentration of 6 000 ppb. This solution was stored at 4 °C and reprepared after three months.

4.6.2.4. Synthetic vitreous humour

Due to the lack of a blank human vitreous humour matrix, vitreous humour was synthesized, according to a method by Kummer et al., (2007), from hyaluronic acid, agar and ultrapure water. Ultrapure water (500 mL) was heated in a 500 mL beaker at 100 °C for 15 minutes using the Reacti-Therm. Fifty milligrams (50 mg) Hyaluronic acid and 150 mg of agar were weighed out and added to a 100 mL volumetric flask and made up to 100 mL using the heated ultrapure water. The solution was stirred and heated at 90 °C for 15 minutes, using the Reacti-Therm. The solution was then left to cool and gel for 24 hours at room temperature.

4.6.2.5. Preparation of calibration standards

Ten calibration standards and a blank were prepared according to Table 4.2. Each standard and the blank were prepared, in a 1 500 µL microcentrifuge tube, using synthetic vitreous humour and different volumes of various EtG and EtS working solutions. Once prepared the tubes were briefly mixed.

Table 4.2: The preparation of the blank and ten calibration standards. The Table indicates the volume of synthetic vitreous humour (VH), the volume and concentration of EtG working solution (WS) and the volume and concentration of EtS working solution (WS).

Standard	Synthetic VH volume (µL)	EtG WS used (ppb)	EtG WS volume (uL)	[EtG] (ppb)	EtS WS used (ppb)	EtS WS volume (uL)	[EtS] (ppb)
Blank	100	N/A	0	0	N/A	0	0
1	40	500	10	50	10	50	5
2	70	500	20	100	100	10	10
3	55	500	30	150	100	15	15
4	40	500	40	200	100	20	20
5	25	500	50	250	100	25	25
6	0	1 000	50	500	100	50	50
7	15	1 000	75	750	1 000	10	100
8	70	10 000	10	1 000	1 000	20	200
9	35	10 000	15	1 500	1 000	50	500
10	65	10 000	25	2 500	10 000	10	1 000

4.6.2.6. Preparation of QCs

Three QCs were prepared in-house using synthetic vitreous humour and various EtG and EtS WS's. Each QC had a different EtG and EtS concentration and was prepared in a 1 500 µL microcentrifuge tube, according to Table 4.3.

Table 4.3: The preparation of the three in-house prepared QC samples. The table indicates the volume of synthetic vitreous humour (VH), the volume and concentration of EtG working solution (WS) and the volume and concentration of EtS working solution (WS).

QC	Synthetic VH volume (µL)	EtG WS used (ppb)	EtG WS volume (uL)	[EtG] (ppb)	EtS WS used (ppb)	EtS WS volume (uL)	[EtS] (ppb)
1	57	500	25	125	100	18	18
2	27	1 000	60	600	1 000	13	130
3	20	10 000	20	2 000	1 000	60	600

4.6.2.7. Sample, standard and QC preparation procedure

Initially, 100 µL of sample, QC or calibration standard was pipetted into a 1 500 µL microcentrifuge tube. IS (10 µL) was added to each tube and the tubes were briefly mixed using a vortex. For PP, 330 µL of 0.1% formic acid in methanol was added to each tube and the tubes were centrifuged at 10 000 x g for 10 minutes. After centrifugation, 300 µL of the supernatant was removed from each tube and added to a Waters® Total recovery vial. The solution was dried down by incubating the vial, without a cap, at 60 °C in a fume hood for approximately 60 minutes. Thereafter, the vials were removed from the Reacti-Therm and left to cool for 20 minutes in the fume hood. Finally, the dried extracts were reconstituted with 50 µL 0.1% aqueous formic acid and mixed briefly.

4.7. SAMPLE ANALYSIS

Each collected vitreous humour sample was analysed for ethanol as well as EtG and EtS. The method to analyse vitreous humour for ethanol was performed using a headspace sampler coupled with a gas chromatography system fitted with a flame ionization detector (HSGC-FID) at the NIOH situated at 25 Hospital Street, Braamfontein, 2001. EtG and EtS analysis of the vitreous humour samples occurred at Microsep (Pty) Ltd. situated at 2 Saturn Crescent, Linbro Business Park, 2096 using an Ultra-Performance Liquid Chromatography system coupled with a tandem mass spectrometer (UPLC-MS/MS). Prior to accepting method validation and pilot study results, certain criteria had to be met.

4.7.1. Criteria for accepting results

The vitreous humour samples were prepared and analysed in duplicate, for both methods, and the mean of the two results was reported. The repeatability of a set of results was evaluated by calculating the percent RSD using Eq. (4.1). Results with a percent RSD above 10% were considered unacceptable and were reprepared and reanalyse. Results less than the LOQ of the method were reported as <LOQ and results greater than the upper limit of the working range were diluted with ultrapure water and reprepared and reanalysed.

$$\%RSD = \left[\frac{Standard\ devaition\ of\ samples}{Mean\ of\ samples}\right] \times 100 \qquad \textbf{(4.1)}$$

Before the analysis of the vitreous humour samples, calibration standards were analysed to establish a linear calibration curve. The calibration curve was accepted if the correlation coefficient was greater than 0.999 (Scientific Working Group for Forensic Toxicology, 2013). Duplicate sets of QC samples were analysed after the establishment of an acceptable calibration curve as well as after every ten analytical runs. The results of each QC sample were plotted on a Levey-Jennings chart and were accepted or rejected according to the modified Westgard rules as described by the QC procedure of the NIOH (NIOH0167) (Appendix 4).

4.7.2. Ethanol in VH using HSGC-FID

4.7.2.1. Instrument parameters

The instrument parameters for the analysis of ethanol in vitreous humour, on the Agilent® G1888 Headspace sampler and the Agilent 6890 N gas chromatography system fitted with a flame ionization detector, are described in Table 4.4.

Table 4.4: The HSGC-FID conditions used for the analysis of ethanol in vitreous humour

Headspace:			
Oven temperature	70 °C	Pressurisation time	0.20 min
Loop temperature	80 °C	Loop fill time	0.20 min
Transfer line temperature	90 °C	Loop equilibration time	0.05 min
GC cycle time	3.4 min	Injection time	0.50 min
Vial equilibration time	10.0 min		
Front inlet:			
Mode	Split	Initial temperature	150 °C
Gas type	Helium	Pressure	160 kPa
Split ratio	52:1	Total flow	119.4 mL/min
Split flow	114.4 mL/min	Injection volume	2 µL
SUPELCOWAX® 10 capillary GC column:			
Flow	2.2 mL/min	Pressure	160 kPa
FID detector:			
Temperature	260 °C	Injection volume	2 µL
Mode	Constant column + makeup flow	Combined flow	25.0 mL/min
Hydrogen flow	40.0 mL/min	Make-up gas type	Nitrogen
Medical air flow	400.0 mL/min	Lit offset	2.0

4.7.2.2. Data processing

Data processing and instrument control was done using Agilent® Open Lab CDS ChemStation® for GC Systems software. This software was used to establish a calibration curve with the y-axis illustrating signal response and the x-axis illustrating ethanol concentration. The signal response was the ratio of the integrated peak area of ethanol to that of the internal standard. The software calculated the ethanol concentration of the QC and

vitreous humour samples by plotting the signal response of these samples on the established calibration curve and subsequently obtaining the corresponding ethanol concentration.

4.7.3. EtG, EtS

4.7.3.1. Instrument parameters

The instrument parameters of the Waters® Acquity Ultra-Performance Liquid Chromatography system coupled with the Waters® Quattro Premier XE tandem mass spectrometer (UPLC-MS/MS) were optimized, based on a method by Ohouo et al., (2018).

- UPLC conditions

The optimal UPLC conditions for this method are described in Table 4.5.

Table 4.5: UPLC conditions for the analysis of EtG and EtS in vitreous humour

<table>
<tr><td>Column</td><td colspan="4">Waters® Acquity UPLC CSH Phenyl-Hexyl column (150 mm length, 2.1 mm inner diameter and 1.7 µm particle size)</td></tr>
<tr><td>Run time</td><td colspan="4">6 min</td></tr>
<tr><td>Mobile phase A</td><td colspan="4">0.1% Aqueous formic acid</td></tr>
<tr><td>Mobile phase B</td><td colspan="4">0.1% Formic acid in acetonitrile</td></tr>
<tr><td rowspan="7">Gradient elution profile</td><td>Time (min)</td><td>Mobile phase A (%)</td><td>Mobile phase B (%)</td><td>Slope</td></tr>
<tr><td>Initial</td><td>90</td><td>10</td><td>0</td></tr>
<tr><td>0.10</td><td>90</td><td>10</td><td>6</td></tr>
<tr><td>3.00</td><td>40</td><td>60</td><td>6</td></tr>
<tr><td>3.10</td><td>5</td><td>95</td><td>1</td></tr>
<tr><td>4.10</td><td>90</td><td>10</td><td>1</td></tr>
<tr><td>6.00</td><td>90</td><td>10</td><td>1</td></tr>
<tr><td>Flow rate</td><td colspan="4">0.3 mL/min</td></tr>
<tr><td>Seal wash solvent</td><td colspan="4">ROMIL LC Wash solution AMPW1 UpS- acetonitrile/ methanol/ 2-propanol/ water 25:25:25:25</td></tr>
<tr><td>Strong wash solvent</td><td colspan="4">ROMIL LC Wash solution AMPW1 UpS- acetonitrile/ methanol/ 2-propanol/ water 25:25:25:25</td></tr>
<tr><td>Strong wash volume</td><td colspan="4">800 µL</td></tr>
<tr><td>Weak wash solvent</td><td colspan="4">2% Methanol solution</td></tr>
<tr><td>Weak wash volume</td><td colspan="4">1000 µL</td></tr>
<tr><td>Column temperature</td><td colspan="4">45 °C</td></tr>
<tr><td>Sample temperature</td><td colspan="4">4 °C</td></tr>
<tr><td>Injection volume</td><td colspan="4">10 µL</td></tr>
</table>

- MS/MS conditions

The conditions used for the Waters® Quattro Premier XE tandem mass spectrometer are described in Table 4.6. A Waters® 510 HPLC pump was used for the post-column addition of acetonitrile, at a flow rate of 0.6 mL/min, through a T-union before UPLC flow was directed into the MS/MS. Multiple-reaction monitoring (MRM) was used for the quantification of the analytes of interest. The MRM parameters used for this method are described in Table 4.7.

Table 4.6: The MS/MS conditions used to analyse EtG and EtS

Ionization mode	ESI negative
Capillary voltage	2.80 kV
Extractor voltage	3 V
Source temperature	120 °C
Desolvation temperature	350 °C
Desolvation gas flow	800 L/hr
Cone gas flow	20 L/hr
Collision gas flow	0.60 µL/min
Multiplier	650 V

Table 4.7: The MRM parameters used to quantify EtG, EtS, EtG-d5 and EtS-d5

Analyte	EtG		EtG-d5		EtS		EtS-d5
Inter Scan delay (secs)	0.050		0.050		0.050		0.050
Inter Channel delay (secs)	0.050		0.050		0.050		N/A
Span (Da)	0.100		0.100		0.000		0.000
Time segment (minutes)	0-3		0-3		3-6		3-6
Precursor ion (Da)	221.90		226.90		125.00		130.00
Product ion (Da)	74.90	85.90	74.90	85.90	79.86	96.84	98.00
Dwell (secs)	0.080	0.080	0.080	0.080	0.100	0.100	0.100
Cone voltage (V)	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Collision energy (eV)	17.0	22.0	20.0	20.0	22.0	22.0	22.0

4.7.3.2. Data processing

Instrument control and data processing was done using Waters® MassLynx™ Software. The sample results were acquired, processed and reported using the TargetLynx™ Application Manager. This applications manager used automatic algorithms to integrate analyte peaks with relevant retention times and m/z ratios (Refer to Table 4.7). Calibration curves were established with the y-axis illustrating signal response (the analyte to internal standard response ratio) and the x-axis illustrating EtG or EtS concentration. The software calculated the EtG and EtS concentrations of the QC and vitreous humour samples by plotting the signal response of these samples on the established calibration curve and subsequently obtaining the corresponding concentrations.

4.8. METHOD VALIDATION

Method validation is the process of performing a detailed investigation and providing evidence that an analytical method is fit for purpose and effective in producing results with a high degree of accuracy (Araujo, 2009). Analytical methods must be validated when it is necessary to verify that the performance parameters of the method are fit for a particular application (Scientific Working Group for Forensic Toxicology, 2013). Method validation is required in the following scenarios (Kalra, 2011, Magnusson, 2014):

- Newly developed analytical methods
- Modifications or amplifications of an established analytical method
- Analytical methods used outside their intended scope i.e. different biological sample
- Established method used in a different laboratory or with different equipment

Before the analysis of vitreous humour samples, both methods in this study required full method validations. These method validations were performed based on the guidelines highlighted by Magnusson, (2014) and the Scientific Working Group for Forensic Toxicology, (2013). Sample blanks, QCs, calibration standards and spiked samples were used for these method validations. Table 4.8 highlights the 12 performance characteristics used to validate these methods and briefly summarizes the procedures followed and relevant acceptance criteria used.

Table 4.8: The method validation performance characteristics assessed in this study including the procedures followed and acceptance criteria.

Characteristic	Procedure followed	Acceptance criteria
LOD	Ten blank samples analysed and Eq. (4.2) was used to calculate the LOD	N/A
LOQ	Ten blank samples analysed and Eq. (4.3) was used to calculate the LOQ Six replicates of the lowest non-zero calibrator analysed. The precision and accuracy were calculated using Eq. (4.1 and 4.4).	N/A % Accuracy ±10 %, %RSD ≤ 10%
Working range	The working range was the range from the LOQ to the highest calibration standard	N/A
Linearity	Calibration models established by analysing a blank and multiple calibration standards over a period of three analytical runs. Calibration models were evaluated using the correlation coefficient (r) and residual plots.	r ≥ 0.999. Randomly distributed residual plots
Selectivity	<u>Ethanol:</u> Suspected Interferences- Samples spiked with ethanol, tertiary butanol and suspected interferences (acetaldehyde, acetone and 2-propanol) were analysed in duplicate. The peak resolutions of ethanol and tertiary butanol were evaluated. Matrix effects- Calibration standard 12 analysed five times (Sample type 1). Spiked vitreous humour samples, with an ethanol concentration of 0.3 g/100 mL, analysed five times (Sample type 2). Eq. (4.5) used to calculate percent matrix effects.	 The peak resolution of ethanol and tertiary butanol was not affected % Matrix effects 85-115%.
	<u>EtG, EtS:</u> Matrix interferences- Ten blank matrix samples without internal standard analysed and S/N ratios of the analytes were evaluated. Internal standard interferences- Ten blank samples with internal standard analysed and EtG and EtS concentrations evaluated. Matrix effects- Water samples, spiked with EtG concentration of 1000 ppb and EtS concentration of 200 ppb, analysed five times (Sample type 1). Calibration standard 8 analysed five times (Sample type 2). Eq. (4.5) used to calculate percent matrix effects.	 S/N ratio ≤ 3 Concentrations ≤ LOQ % Matrix effects 85-115%.
Accuracy	QC samples analysed in triplicate over five different analytical runs. Eq. (4.4) was used to calculate percent accuracy	Percent accuracy = ±10 %
Precision	Within-run- QC samples analysed six times within the same analytical run. Eq. (4.1) was used to calculate percent RSD Intermediate- QC samples analysed in triplicate over three different analytical runs. Percent RSD calculated using Eq. (4.1)	%RSD ≤ 10%

UOM	Uncertainty calculated using a precision study, a bias study and included the influence of other factors.		N/A
Ruggedness	QC samples analysed in triplicate over three different analytical runs, with varying conditions. Eq. (4.1) used to calculate percent RSD		%RSD ≤ 10%
Carryover	The highest QC sample analysed followed by three blank samples.		Blank concentrations ≤ 20% of LOQ
Stability	<u>Processed sample stability</u>		Reanalysed sample results within a range of ± 10 % of the mean of the time zero results.
	Ethanol- QC3 samples analysed in duplicate to obtain time zero results. Samples reanalysed after being stored in the autosampler tray of the headspace for 48 hours.	EtG/EtS- QC3 samples analysed in duplicate to obtain time zero results. Samples reanalysed after being stored in the autosampler tray of the UPLC for 72 hours.	
	<u>Long-term stability</u>		
	Ethanol- Samples spiked to 0.15 g/ 100 mL ethanol analysed in duplicate to obtain time zero results. Samples reprepared and reanalysed after storage at 4 °C for 3 months	EtG/EtS- QC2 samples analysed in duplicate to obtain time zero results. Samples reprepared and reanalysed after being stored at 4 °C for 6 months	

4.8.1. Limit of Detection

The limit of detection (LOD), also known as detection limit, is the "lowest concentration of an analyte that can be detected by the method at a specified level of confidence, generally 95%" (Barwick, 2016). At this concentration the analyte can be detected but not quantified (Shrivastava and Gupta, 2011). The LOD, of both methods, was estimated by analysing ten blank samples and subsequently calculating the LOD using Eq. (4.2) (Magnusson, 2014).

$$LOD = 3 \times SD \quad \textbf{(4.2)}$$

Where SD is the standard deviation of the concentration of ten blank samples (Magnusson, 2014).

4.8.2. Limit of Quantitation

The limit of quantitation (LOQ), also known as limit of quantification, is the "lowest concentration of an analyte that can be determined with an acceptable level of uncertainty" (Barwick, 2016). The LOQ, of both methods, was estimated by analysing ten blank samples and calculating the LOQ using Eq. (4.3) (Magnusson, 2014).

$$LOQ = 10 \times SD \quad \textbf{(4.3)}$$

Where SD is the standard deviation of the concentration of ten blank samples (Magnusson, 2014).

For both these methods, the lowest non-zero calibrator was used as the LOQ instead of the calculated LOQ's. The accuracy and precision of each LOQ was evaluated by preparing and analysing six replicates of the lowest non-zero calibrator. The accuracy of the replicates was calculated using Eq. (4.4) and the precision of the replicates was evaluated by calculating the percent relative standard deviation (%RSD) using Eq. (4.1). The acceptance criteria for percent accuracy was ±10 % and the %RSD acceptance criteria was ≤ 10% (Scientific Working Group for Forensic Toxicology, 2013).

$$\%\, Accuracy \; = \left[\frac{(Mean\ of\ test\ results - Reference\ value)}{Reference\ value}\right] \times 100 \quad \textbf{(4.4)}$$

4.8.3. Working range

The working range is the concentration interval for which a method provides results with an acceptable uncertainty (Barwick, 2016). For these methods, the lower limit of the working range was the LOQ and the upper limit was the highest calibration standard (Magnusson, 2014).

4.8.4. Linearity

Linearity is the ability of an analytical method to obtain a response that is directly proportional to the concentration of the analyte standard (David, 2006). The linearity of the methods was assessed by establishing and analysing calibration models (Scientific Working Group for Forensic Toxicology, 2013). The calibration models were established by analysing a blank and multiple calibration standards (14 for ethanol and 10 for the EtG, EtS method) over a period of 3 analytical runs. These calibration models were constructed by plotting the detector response (y-axis) of each standard against the standard concentration (x-axis) (Magnusson, 2014). Regression statistics, including correlation coefficient and residual plots, were used to evaluate the linearity of the calibration model and therefore the linearity of the method (Magnusson, 2014, Scientific Working Group for Forensic Toxicology, 2013). The acceptance criteria for the correlation coefficients were ≥0.999 while the residual plots required a random distribution about zero with no tendencies and outliers (Boqué *et al.*, 2002, Unodc, 2009).

4.8.5. Selectivity

Selectivity, also known as specificity or interferences, is “the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components with similar characteristics” (Barwick, 2016). Interferences are non-targeted analytes which may affect the ability of the method to detect and quantify the analytes of interest (Scientific Working Group for Forensic Toxicology, 2013). In method validation, selectivity requires the evaluation of interferences from matrix components, internal standards, suspected interferences and matrix effects (Scientific Working Group for Forensic Toxicology, 2013, Peris-Vicente *et al.*, 2015).

4.8.1.1. Ethanol

The selectivity of the method to analyse ethanol in vitreous humour was assessed by evaluating suspected interferences and matrix effects. The effect of suspected interferences, including acetaldehyde, acetone and 2-propanol, on the analysis of ethanol and tertiary butanol was evaluated by spiking samples containing ethanol and tertiary butanol with these suspected interferences (Magnusson, 2014, Scientific Working Group for Forensic Toxicology,

2013). These samples were prepared and analysed in duplicate and the effect of these interferences on the peak resolution of ethanol and tertiary butanol was evaluated.

Matrix effects reduces the accuracy and reproducibility of an analytical method (Peters *et al.*, 2007, Matuszewski *et al.*, 2003). To assess these matrix effects, two different types of samples were prepared and analysed fivefold (Matuszewski *et al.*, 2003). Sample type one consisted of calibration standard 12 and type two consisted of spiked vitreous humour samples with an ethanol concentration of 0.3 g/100 mL. The average response of each sample type was used to calculate percent matrix effects using Eq. (4.5). The acceptance criteria for percent matrix effects was 85-115% (Matuszewski *et al.*, 2003).

$$\%\ Matrix\ effects = \left[\frac{Response_{Sample\ type\ 2}}{Response_{Sample\ type\ 1}}\right] \times 100 \qquad \textbf{(4.5)}$$

4.8.1.2. EtG and EtS

The selectivity of the method to simultaneously analyse EtG and EtS was assessed by evaluating two interference studies, interferences from the matrix and the internal standards, as well as matrix effects. Matrix interferences were evaluated by analysed ten blank matrix samples without the addition of the internal standard (Scientific Working Group for Forensic Toxicology, 2013). The signal-to-noise (S/N) ratios of each analyte (EtG, EtS, EtG-d5 and EtS-d5) were automatically calculated using the MassLynx™ Software. The acceptance criterium was a S/N ratio less than or equal to three which indicated the absence of matrix interferences.

In mass spectrometry methods, the use of stable-isotope labelled internal standards can interfere and affect the detection and quantification of the analyte of interest (Scientific Working Group for Forensic Toxicology, 2013). The stable-isotope internal standard may either contain the analyte of interest as an impurity or may contain fragment ions with the same mass-to-charge ratio as the significant fragment ions of the analyte of interest (Scientific Working Group for Forensic Toxicology, 2013). The interferences of the internal standards were evaluated by preparing and analysing ten blank samples with internal standard. Internal standard interferences were absent if the concentrations of EtG and EtS in the blank samples were less than the LOQ's of the method.

In LC-MS/MS, matrix effects cause either enhancement or suppression of the ionization of the analytes of interest (Peters *et al.*, 2007). This suppression or enhancement reduces the accuracy and reproducibility of the analytical method (Peters *et al.*, 2007, Matuszewski *et al.*, 2003). To assess these matrix effects, two different types of samples were prepared and analysed fivefold (Matuszewski *et al.*, 2003). Sample type one consisted of pure water samples spiked with an EtG concentration of 1000 ppb and an EtS concentration of 200 ppb.

Sample type two consisted of calibration standard 8. The average response of each sample type was used to calculate percent matrix effects using Eq. (4.5). The acceptance criteria for percent matrix effects was 85-115% (Matuszewski *et al.*, 2003). A percent matrix effects of 100% indicated that there were no matrix effects. A percent matrix effects greater than 100% indicated an ionization enhancement while less than 100% indicated an ionization suppression (Matuszewski *et al.*, 2003).

4.8.6. Accuracy

The accuracy, also known as bias and trueness, of an analytical method is the difference between the obtained test results and an accepted reference value (Peters *et al.*, 2007). The accuracy of these methods was evaluated by preparing and analysing each QC sample in triplicate over five different analytical runs (Scientific Working Group for Forensic Toxicology, 2013). The percent accuracy of each QC sample was calculated using Equation (4.4) and an acceptance criteria of ±10% for each QC was used to evaluate the accuracy of the methods (Scientific Working Group for Forensic Toxicology, 2013).

4.8.7. Precision

Precision is a "measure of how close results are to one another" (Magnusson, 2014). Precision evaluates the distribution of replicate QC results analysed under specific conditions (Magnusson, 2014, Peters *et al.*, 2007). There are three types of precision: within-run precision, intermediate precision and reproducibility (Magnusson, 2014). Within-run precision, also known as repeatability, represents the smallest variation in results. It is defined as the measure of the variation in results when the analysis is performed by the same analyst using the same method and equipment on the same day (Barwick, 2016, Magnusson, 2014). Intermediate precision, also known as between-run precision, is the measure of variability in results when the analysis is performed using the same method but under variable conditions including different analyst, different day/s, or different equipment (Barwick, 2016). Reproducibility represents the largest variation in results and is defined as the measure of the variability in results analysed in different laboratories or using different methods (Magnusson, 2014).

Only within-run and intermediate precision were evaluated in these method validations. For each method, within-run precision was evaluated by preparing and analysing each QC sample six times within the same analytical run (Scientific Working Group for Forensic Toxicology, 2013). The mean and standard deviation results of each QC sample was used to calculate the %RSD using Eq. (4.1).

Intermediate precision was evaluated by preparing and analysing each QC sample in triplicate over three different analytical runs (Scientific Working Group for Forensic Toxicology, 2013).

The means and standard deviations of the results of each QC were used to calculate the %RSD using Eq. (4.1) (Scientific Working Group for Forensic Toxicology, 2013). The acceptance criteria for both within-run and intermediate precision was a %RSD ≤ 10% (Scientific Working Group for Forensic Toxicology, 2013).

4.8.8. Measurement uncertainty

Measurement uncertainty, also known as uncertainty, is a numerical figure (standard deviation or confidence interval range) associated with the result of an analytical measurement (Ellison *et al.*, 2012). This figure "describes the dispersion of values that could reasonably be attributed to the analytical result" and is a statement of the uncertainty of the results which arises from the various errors during sampling and analysis (Ellison *et al.*, 2012).
For these method validations, measurement uncertainty was calculated based on the procedure by Ellison et al., (2012). This is a stepwise process that involved the following steps: a precision study, a bias study, the calculation of the influence of other factors on uncertainty and the calculation of combined and expanded uncertainty (Ellison *et al.*, 2012).

4.8.8.1. Precision study

The precision study evaluates the uncertainty ($\mathcal{U}$) of the overall precision (run to run variation) of the analytical method (Ellison *et al.*, 2012). Equation (4.6) was used to calculate the uncertainty of the precision of each analytical method (Ellison *et al.*, 2012). For the ethanol method, the results of each QC sample analysed 21 times was used to calculate this uncertainty while for the EtG/EtS method, the results of each QC sample analysed 18 times was used.

$$\%\mathcal{U}_{Precision} = \sqrt[2]{\frac{\left[\left(CV_{QC1}\right)^2 \times \left(n_{QC1}-1\right)\right] + \left[\left(CV_{QC2}\right)^2 \times \left(n_{QC2}-1\right)\right] + \left[\left(CV_{QC3}\right)^2 \times \left(n_{QC3}-1\right)\right]}{\left(n_{QC1}+n_{QC2}+n_{QC3}\right)-2}} \quad \textbf{(4.6)}$$

Where $\mathcal{U}_{Precision}$ is the percentage of uncertainty of the precision of the method, CV is the coefficient of variation (also known as RSD) of the results for each QC sample and n is the number of repeated runs of each QC sample (Ellison *et al.*, 2012).

4.8.8.2. Bias study

The bias study evaluates the bias of the analytical method and determines if the bias significantly contributes to the uncertainty of measurement (Ellison *et al.*, 2012). For these method validations, the bias study consisted of two parts.

The first part of the bias study involved the calculation of the bias (Ellison *et al.*, 2012) using Eq. (4.7 to 4.9). For the ethanol method, the bias was calculated using 21 analytical results of QC1 while for the EtG/EtS method, the bias was calculated using 18 analytical results of QC3.

a. Equation (2.7) was used to calculate the percentage of measurement bias.

$$\%Bias = \left[\frac{\bar{X} - X_0}{X_0}\right] \times 100 \quad \textbf{(4.7)}$$

Where, $\bar{X}$ refers to the mean of the specific QC results and X_0 is the reference value of the QC sample.

b. Equation (2.8) was used to calculate the uncertainty of the mean of the QC sample.

$$\mathcal{U}_{\bar{X}} = \frac{SD}{\sqrt[2]{n}} \quad \textbf{(4.8)}$$

Where, $\mathcal{U}_{\bar{X}}$ is the uncertainty of the mean of the specific QC sample, SD is the standard deviation of the specific QC and n is the number of repeated results of the specific QC.

c. Equation (2.9) was used to calculate the percentage of uncertainty of the bias.

$$\%\mathcal{U}_{Bias} = \sqrt[2]{\%\ Bias^2 + \mathcal{U}_{\bar{X}}{}^2} \quad \textbf{(4.9)}$$

Where, $\%\mathcal{U}_{Bias}$ is the percentage of the uncertainty of the bias

The second part of the bias study assessed the significance of the bias and determined if the uncertainty of the bias was required in the overall uncertainty calculation (Ellison *et al.*, 2012). The significance of the bias was assessed using a t test (Ellison *et al.*, 2012). The calculated t-value was calculated using Eq. (4.10) and compared to the critical t-value at a 95% confidence interval for a specific number of degrees of freedom (Ellison *et al.*, 2012). If the calculated t-value was greater than the critical t-value then the bias was significant and was included in the estimation of measurement uncertainty. If the calculated t-value was less than the critical t-value then the bias was insignificant and was ignored when estimated measurement uncertainty (Ellison *et al.*, 2012).

$$t_{calc} = \frac{|\%\ Bias|}{\%\mathcal{U}_{Bias}} \quad \textbf{(4.10)}$$

Where t_{calc} is the calculated t-value

4.8.8.3. Influence of other factors on uncertainty

The significance of the influence of other factors on measurement uncertainty was evaluated independently and combined, using Eq. (4.11), to obtained a single uncertainty percentage (Ellison *et al.*, 2012).

For the ethanol method, the calculation of the uncertainty of other factors only included the uncertainties of the CRM's used as the QC samples (Ellison *et al.*, 2012). For the EtG/EtS method, the calculation of the uncertainty of other factors included the independent uncertainties of the EtG and EtS stock standard purities and uncertainties of the pipettes used (Ellison *et al.*, 2012).

$$\%\mathcal{U}_{Other} = \sqrt{\left(\%\mathcal{U}_{Stock\ purity}\right)^2 + \left(\%\mathcal{U}_{Volumetric\ flask}\right)^2 + \left(\%\mathcal{U}_{Pipette}\right)^2 + \left(\%\mathcal{U}_{Balance}\right)^2} \quad \textbf{(4.11)}$$

4.8.8.4. Calculating the combined and expanded uncertainty

For each method, the calculated uncertainties were added together to acquire the combined uncertainty ($\%\mathcal{U}_c$) of the analytical method using Eq. (4.12) (Ellison *et al.*, 2012).

$$\%\mathcal{U}_c = \sqrt[2]{(\%\mathcal{U}_{Bias})^2 + (\%\mathcal{U}_{Precision})^2 + (\%\mathcal{U}_{Other})^2} \quad \textbf{(4.12)}$$

The expanded uncertainty was used when reporting results since it gives a larger interval of values that could reasonably be attribute to the reported result (Menditto *et al.*, 2007). The expanded uncertainty was calculated using Eq. (4.13) where k represented a coverage factor of 2.

$$\%\mathcal{U}_{exp} = k \times \%\mathcal{U}_c \quad \textbf{(4.13)}$$

4.8.9. Ruggedness

Ruggedness, also known as robustness, is the measure of an analytical methods ability to remain unaffected by small variations in the method parameters which might occur during routine sample analysis (Magnusson, 2014, Peters *et al.*, 2007). These small variations include changes to pH, temperature, mobile phase composition or analyst (Peters *et al.*, 2007).

For both methods, ruggedness was evaluated by preparing and analysing each QC sample in triplicate over three different analytical runs. Each analytical run had small variations in the method parameters including different analysts and variations in the sample preparation temperatures. The means and standard deviations of the results of each QC were used to calculate the %RSD using Eq. (4.1) (Scientific Working Group for Forensic Toxicology, 2013). The acceptance criteria for the %RSD was ≤ 10% (Scientific Working Group for Forensic Toxicology, 2013).

4.8.10. Carryover

Carryover is the contamination of a sample by a previously injected high concentration sample (Wille *et al.*, 2017). Carryover is due to the analyte of interest adsorbing onto surfaces of the analytical instrument and leads to inaccurate analytical results (Wille *et al.*, 2017, Scientific Working Group for Forensic Toxicology, 2013). Carryover was evaluated by analysing the highest QC sample followed by three blank samples (European Medicines Agency, 2011, Scientific Working Group for Forensic Toxicology, 2013). Carryover was acceptable when the concentration of the analyte of interest in the blank samples was less than 20% of the LOQ of the method (European Medicines Agency, 2011).

4.8.11. Stability

Stability in method validation refers to the "chemical stability of an analyte in a sample matrix under specific conditions for given time intervals" (Peters *et al.*, 2007). The stability of the analyte during the analytical procedure, from sample collection to analytical results, is significant for accurate quantification (Peters *et al.*, 2007). During method validations, stability experiments for the various stages of analysis are required (Peters *et al.*, 2007).
For these method validations, two types of stability studies were performed: processed sample stability and long-term stability. Processed sample stability refers to the stability of the analyte in a sample after the sample preparation step and before the sample is analysed on the analytical instrument (Peters *et al.*, 2007). Long-term stability refers to the stability of the analyte in the sample matrix prior to sample preparation and analysis (Peters *et al.*, 2007).

4.8.11.1. Processed sample stability

This stability study was evaluated by preparing and analysing QC3 samples in duplicate to obtain time zero results. Subsequently, these samples were subjected to a specific stability study design and reanalysed. For the ethanol method, the samples were reanalysed after being stored in the autosampler tray of the headspace for 48 hours. For the EtG/EtS method, the samples were stored in the autosampler tray of the UPLC for 72 hours. The samples were

considered stable under these specific conditions if the reanalysed sample results fell within a range of ± 10 % of the mean of the time zero results.

4.8.11.2. Long-term stability

This stability study was evaluated by preparing and analysing spiked samples in duplicate to obtain time zero results. For the ethanol method, blank vitreous humour samples were spiked to an ethanol concentration of 0.15 g/100 mL while the EtG/EtS method used QC2 samples. These samples were subsequently stored at 4 °C in 4 mL tubes containing sodium fluoride. The samples were reprepared and reanalysed after three months for the ethanol method and six months for the EtG/EtS method. The samples were considered stable under these specific storage conditions if the reanalysed sample results fell within a range of ± 10 % of the mean of the time zero results.

4.9. DATA ANALYSIS

All data generated for method development, method validation and the pilot study was analysed using Microsoft® Excel®. TIBCO Statistica™ was used to generate relevant tables and figures as well as to calculate statistical parameters. The decedents from the pilot study were categorized into one of eight categories according to the ethanol, EtG and EtS results of their VH samples (refer to Figure 4.3). Ethanol, EtG and EtS results were considered positive if they were greater than or equal to the method LOQ (ethanol- 0.01 g/100mL, EtG- 50 ppb and EtS- 5 ppb) while results less than the LOQ were considered negative. Subsequent to the categorization of each decedent, Figure 4.4 was used to determine ethanol intoxication 24 hours prior to death, to distinguish between ethanol consumption and post-mortem ethanol formation as well as to determine false positive and false negative ethanol, EtG and EtS results

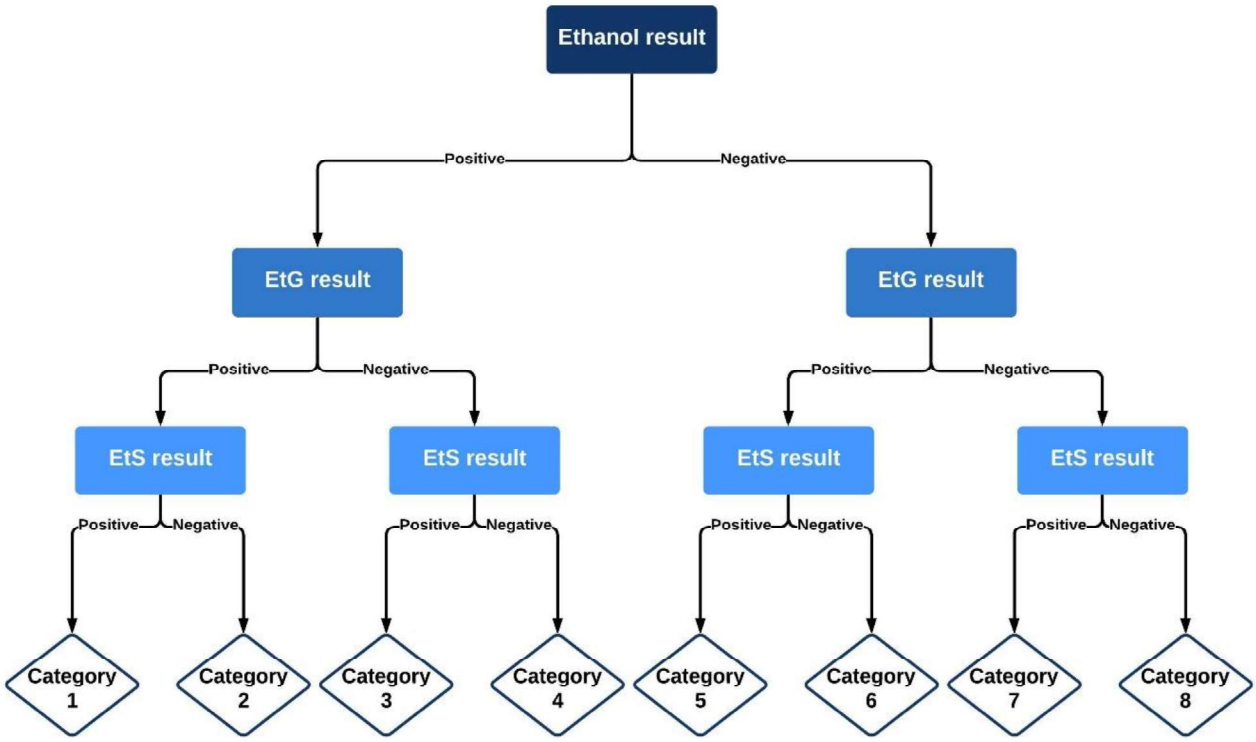


Figure 4.3: The process used to categorize the decedents according to the ethanol, EtG and EtS results of their VH samples

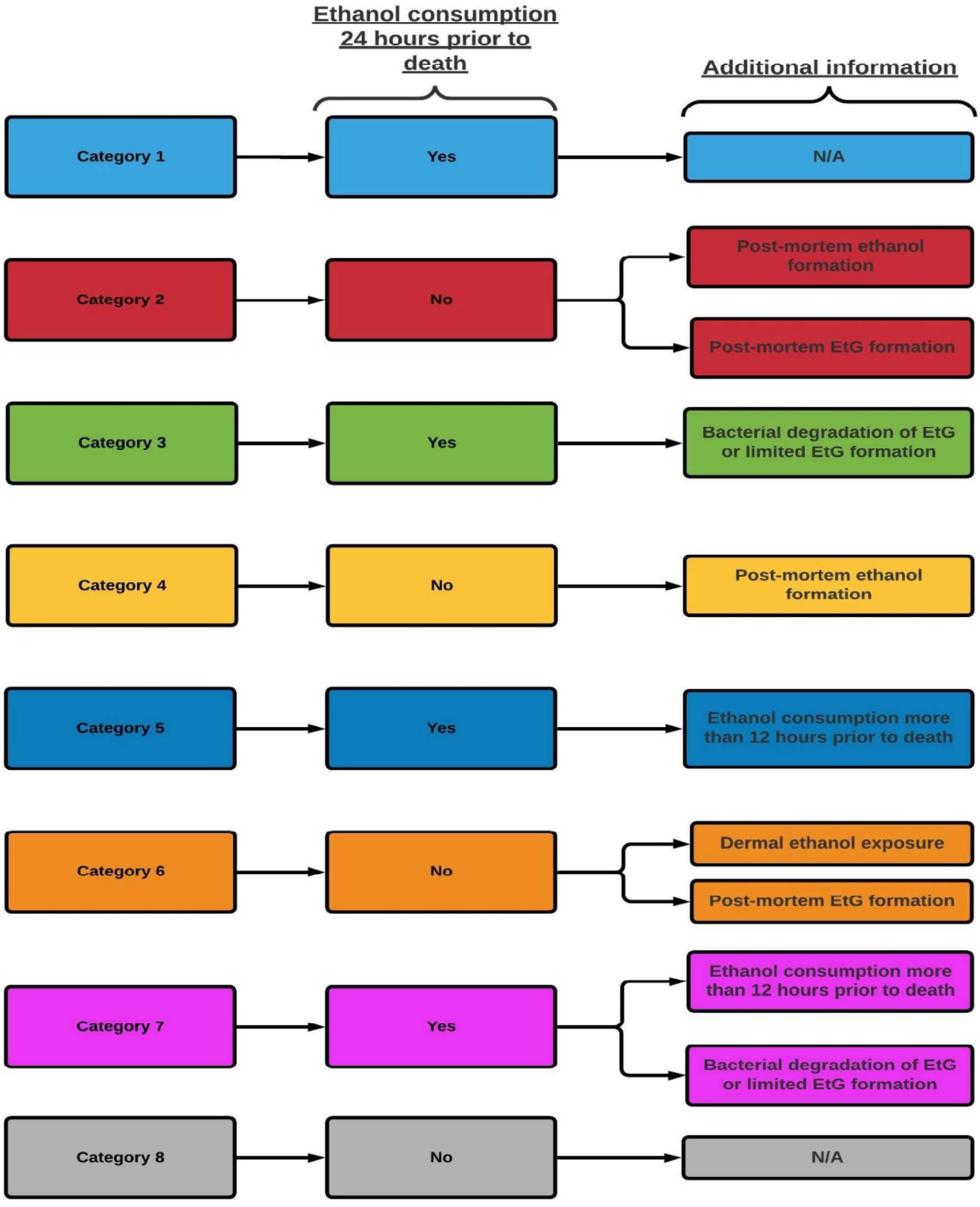


Figure 4.4: A flow diagram describing the process of determining ethanol intoxication 24 hours prior to death, distinguishing between ethanol consumption and post-mortem ethanol formation as well as to determine false positive and false negative results

CHAPTER 5: RESULTS

5.1. ETHANOL IN VH: METHOD VALIDATION

The analytical method used in this study to quantify ethanol in vitreous humour was modified from a validated method to quantify ethanol in blood. A full method validation was required because of the modifications made to the method. The method was validated using the performance characteristics and acceptance criteria highlighted in Table 4.8.

5.1.1. Limit of Detection

The calculated LOD of the method was 0.00198 g/100mL, using a $r^2 \geq 0.999$ (Refer to Table 5.1).

Table 5.1: The concentrations of the ten blank samples (ultrapure water) measured in g/ 100 mL using HSGC-FID. The mean, standard deviation and the calculated LOD of the method is also illustrated.

Sample name	Analyst	Result (g/100ml)
Blank 1	Gareth Riley	0,00221934
Blank 2	Gareth Riley	0,00037539
Blank 3	Gareth Riley	0,00007959
Blank 4	Gareth Riley	0,00000000
Blank 5	Gareth Riley	0,00000000
Blank 6	Gareth Riley	0,00000000
Blank 7	Gareth Riley	0,00000000
Blank 8	Gareth Riley	0,00000000
Blank 9	Gareth Riley	0,00000000
Blank 10	Gareth Riley	0,00000000
Mean		0,00026743
Standard deviation (SD)		0,00066012
LOD (3 X SD)		0,00198035

5.1.2. Limit of Quantitation

The calculated LOQ for this method was 0,00660 g/100 mL, using a $r^2 \geq 0.999$ (Refer to Table 5.2). However, as stated in Section 4.8.3, the LOQ used for this method was the lowest non-zero calibrator with a value of 0.01 g/100 mL. The percent accuracy of the lowest non-zero calibrator over six analytical runs was -5.92% while the percent RSD was 9,55% (Refer to Table 5.3).

Table 5.2: The concentrations of the ten blank samples (ultrapure water) measured in g/ 100 mL using HSGC-FID. The mean, standard deviation and the calculated LOQ of the method is also illustrated.

Sample name	Analyst	Result (g/100ml)
Blank 1	Gareth Riley	0,00221934
Blank 2	Gareth Riley	0,00037539
Blank 3	Gareth Riley	0,00007959
Blank 4	Gareth Riley	0,00000000
Blank 5	Gareth Riley	0,00000000
Blank 6	Gareth Riley	0,00000000
Blank 7	Gareth Riley	0,00000000
Blank 8	Gareth Riley	0,00000000
Blank 9	Gareth Riley	0,00000000
Blank 10	Gareth Riley	0,00000000
Mean		0,00026743
Standard deviation		0,00066012
LOQ (10 X SD)		0,00660115

Table 5.3: The six replicate results (g/ 100 mL) of the lowest non-zero ethanol calibrator for the HSGCFID method including the mean, standard deviation, reference value, percent accuracy and percent RSD.

Analytical run	Analyst	Result (g/100ml)
1	Gareth Riley	0.00947308
2	Bianca Southon	0.00954177
3	Gareth Riley	0.00809204
4	Gareth Riley	0.00844514
5	Gareth Riley	0.01048170
6	Gareth Riley	0.0104146
Mean		0.00941
Standard Deviation		0.00090
Reference value*		0.01
%RSD		9.55352
% Accuracy		-5.91945

*Reference value refers to the concentration of the CRM stated by the manufacturer

5.1.3. Working Range

The linear working range for the method to quantify ethanol in VH using HSGC-FID was 0.01 g/ 100 mL to 0.5 g/ 100 mL.

5.1.4. Linearity

The linearity of the method, between 0.01 g/ 100 mL and 0.5 g/ 100 mL, was evaluated by establishing a linear calibration model using three different analytical runs. According to the acceptance criteria, each analytical run had a $r^2 \geq 0.999$. The established calibration model had a correlation coefficient of 0.9996 (Refer to Figure 5.1). The residual plot of the established calibration model had a random distribution about zero with no tendencies and outliers.

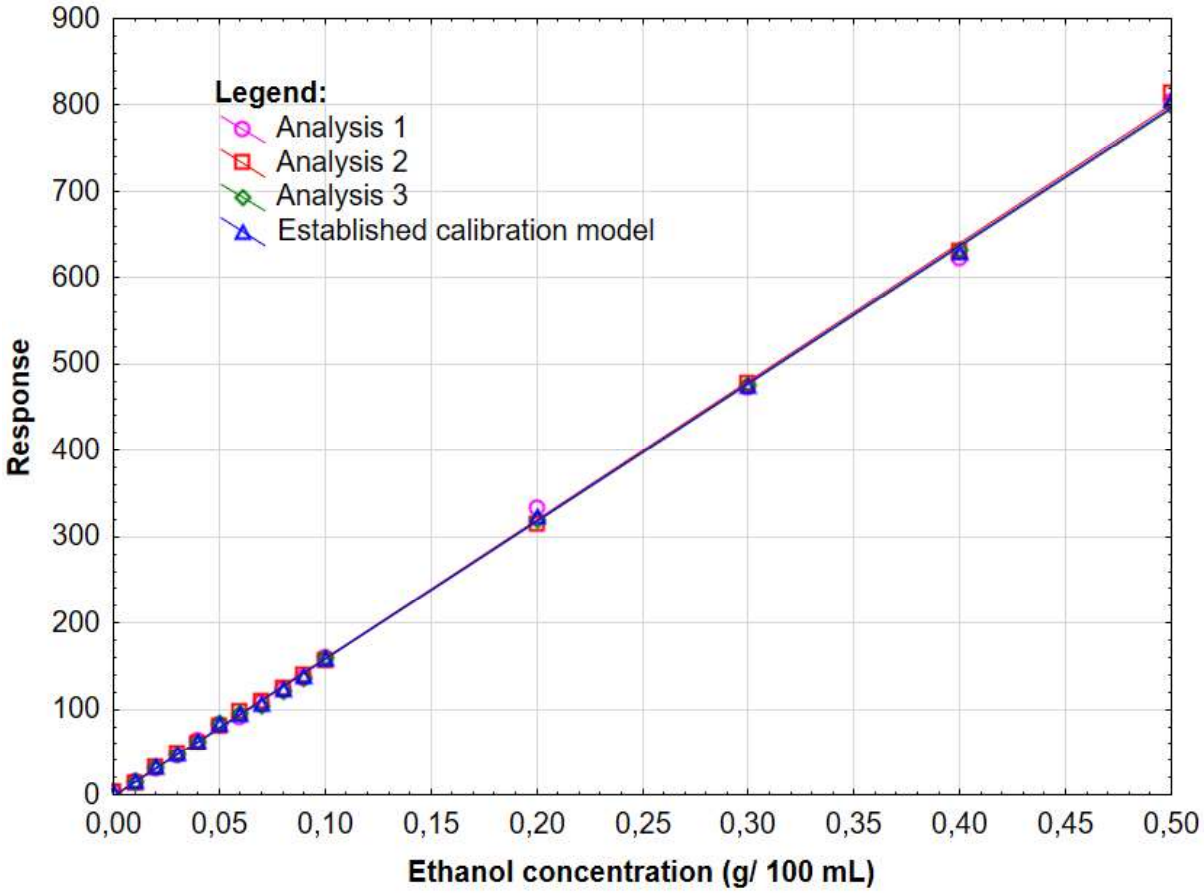


Figure 5.1: A scatterplot illustrating the calibration curves of the three different analytical runs as well as the established calibration model (r = 0.9996, $y = 1597.9817x - 1.3815$).

5.1.5. Selectivity

The selectivity of the method was assessed by evaluating the effect of suspected interferences and the effect of the matrix on ethanol and tertiary butanol results.

5.1.1.1. Suspected interferences

The suspected interferences evaluated, which are common by-products found in post-mortem samples, were acetaldehyde, acetone and 2-propanol. The retention times of the interferences notably differ from the retention times of ethanol and tertiary butanol (Refer to Table 5.4) and the chromatogram illustrates good peak resolution with no co-eluting peaks (Refer to Figure 5.2).

Table 5.4: The chromatographic retention times of acetaldehyde, acetone, 2-propanol, ethanol and tertiary butanol.

Analyte	Retention time (±SD)
Acetaldehyde	1,072 (±0,001)
Acetone	1,205 (±0,002)
Tertiary Butanol	1,355 (±0,001)
2-Propanol	1,433 (±0,000)
Ethanol	1,462 (±0,001)

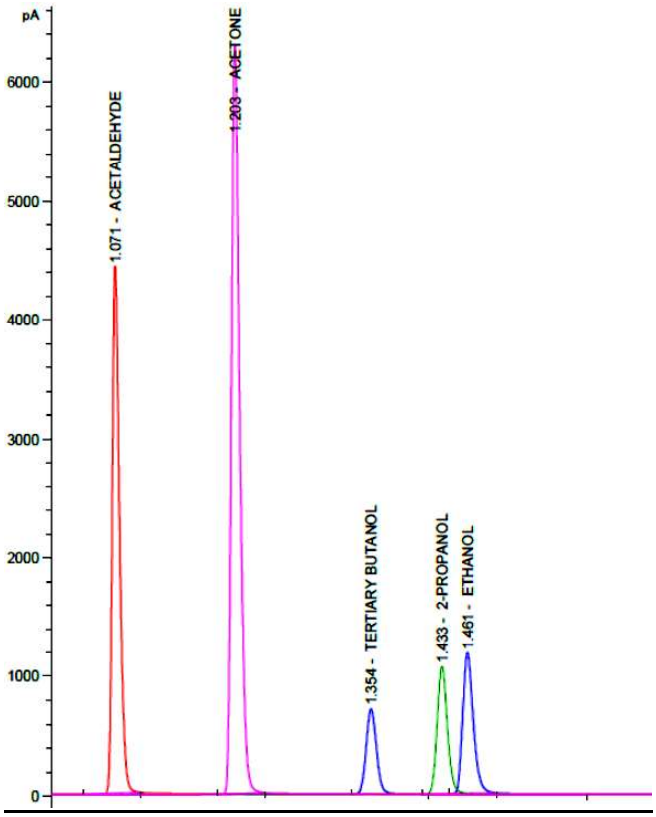


Figure 5.2: A GC-FID chromatogram showing the retention times of acetaldehyde, acetone, tertiary butanol, 2-propanol and ethanol.

5.1.1.2. Matrix effect

The percentage matrix effect was 99.36% (Refer to Table 5.5).

Table 5.5: Matrix effects results showing the five analytical results obtained for sample type 1 (calibration standard 12) and sample type 2 (blank vitreous humour samples spiked to an ethanol concentration of 0.3 g/100mL) as well as the calculated percent matrix effect.

Analysis number	Sample type 1 concentration (g / 100 mL)	Sample type 2 concentration (g / 100 mL)
1	0,286649	0,286235
2	0,287579	0,292618
3	0,297303	0,287703
4	0,284651	0,281319
5	0,284579	0,283651
Mean	0,288152	0,286305
% Matrix effects		99,35901930

5.1.6. Accuracy

The percent accuracies for QC1 (0.015 g/100mL), QC2 (0.05 g/100mL) and QC3 (0.2 g/100mL) were -0.75%, -0.60% and -0.17%, respectively (Refer to Table 5.6).

Table 5.6: The results of each QC analysed in triplicate over five different analytical runs. The table includes the reference values of each QC as well as the mean, standard deviation and percent accuracy calculations.

Analytical run	Number	Analyst	QC1 (g/100ml)	QC2 (g/100ml)	QC3 (g/100ml)
1	1	Gareth Riley	0,015341	0,050653	0,19891
	2		0,015566	0,050428	0,19791
	3		0,015192	0,049866	0,20010
2	4	Bianca Southon	0,015048	0,050710	0,19926
	5		0,015771	0,050294	0,20204
	6		0,015575	0,050333	0,19987
3	7	Gareth Riley	0,015544	0,050333	0,19978
	8		0,014626	0,050351	0,20074
	9		0,014640	0,050834	0,19872
4	10	Gareth Riley	0,014575	0,051091	0,20039
	11		0,015003	0,050932	0,20036
	12		0,014685	0,050901	0,19980
5	13	Bianca Southon	0,014765	0,050640	0,19984
	14		0,014821	0,049178	0,19762
	15		0,014848	0,048320	0,19946
Mean			0,015067	0,050324	0,199653
Standard deviation			0,00039087	0,0007052	0,00106558
Reference value			0,01518	0,05063	0,2
% Accuracy			-0,74707949	-0,60373955	-0,17356667

5.1.7. Precision

The precision of this method was assessed by evaluating the percent RSD of within-run and intermediate precision.

5.1.7.1. Within-run precision

The percent RSD for QC1 (0.015 g/100mL), QC2 (0.05 g/100mL) and QC3 (0.2 g/100mL) were 1.48%, 0.49% and 0.46%, respectively (Refer to Table 5.7).

Table 5.7: The results of each QC analysed six times over one analytical run. The table includes the calculated means, standard deviations and percent RSD.

Analytical run	Number	Analyst	QC1 (g/100ml)	QC2 (g/100ml)	QC3 (g/100ml)
1	1	Gareth Riley	0,015341	0,050653	0,19891
	2		0,015566	0,050428	0,19791
	3		0,015192	0,049866	0,20010
	4		0,014980	0,050215	0,19723
	5		0,014953	0,050485	0,19855
	6		0,014995	0,050354	0,19921
Mean			0,015171	0,050333	0,198652
Standard deviation			0,00022387	0,00024738	0,00091812
% RSD			1,47564261	0,49147423	0,46217663

5.1.7.2. Intermediate precision

The percent RSD for QC1 (0.015 g/100mL), QC2 (0.05 g/100mL) and QC3 (0.2 g/100mL) were 2.03%, 0.51% and 0.31% respectively (Refer to Table 5.8).

Table 5.8: The results of each quality control analysed in triplicate over three analytical runs. The table includes the calculated means, standard deviations and percent RSD.

Analytical run	Number	Analyst	QC1 (g/100ml)	QC2 (g/100ml)	QC3 (g/100ml)
3	10	Gareth Riley	0,015544	0,050333	0,19978
	11		0,014626	0,050351	0,20074
	12		0,014640	0,050834	0,19872
4	13	Gareth Riley	0,014575	0,051091	0,20039
	14		0,015003	0,050932	0,20036
	15		0,014685	0,050901	0,19980
6	19	Gareth Riley	0,014595	0,050824	0,20007
	20		0,014735	0,050942	0,19924
	21		0,015043	0,051005	0,20060
Mean			0,014827	0,050801	0,199966
Standard deviation			0,00030063	0,00025732	0,00062259
% RSD			2,02757396	0,50651296	0,31134916

5.1.8. Measurement uncertainty

The combined uncertainty of measurement calculated based on the procedure by Ellison et al., (2012) was 1.55% whereas the expanded uncertainty was 3.11%.

5.1.9. Ruggedness

The percent RSD for QC1 (0.015 g/100mL), QC2 (0.05 g/100mL) and QC3 (0.2 g/100mL) were 2.76%, 1.55% and 0.58%, respectively (Refer to Table 5.9).

Table 5.9: The results of each QC analysed in triplicate over three analytical runs. Each analytical run had small variations in the method parameters including different sample preparation temperatures and different analysts. The table includes the calculated means, standard deviations and percent RSD.

Analytical run	Number	Sample preparation temperature (°C)	Analyst	QC1 (g/100ml)	QC2 (g/100ml)	QC3 (g/100ml)
2	7	20,4	Bianca Southon	0,015048	0,050710	0,19926
	8			0,015771	0,050294	0,20204
	9			0,015575	0,050333	0,19987
3	10	21,4	Gareth Riley	0,015544	0,050333	0,19978
	11			0,014626	0,050351	0,20074
	12			0,014640	0,050834	0,19872
5	16	21,2	Bianca Southon	0,014765	0,050640	0,19984
	17			0,014821	0,049178	0,19762
	18			0,014848	0,048320	0,19946
Mean				0,015071	0,050110	0,199703
Standard Deviation				0,00041622	0,0007767	0,00116219
%RSD				2,76178322	1,54997272	0,5819566

5.1.10. Carryover

The three blank samples (ultrapure water), analysed after the highest QC sample (QC3- 0.2 g/100mL), had ethanol concentrations of:

- 0.00103 g/ 100 mL
- 0.00107 g/ 100 mL
- 0.00108 g/ 100 mL

Since all these concentrations were less than 20% of the LOQ (0.002 g/100mL), the acceptance criteria for carryover was accepted.

5.1.11. Stability

The stability of the method was assessed by performing a processed sample stability study and a long-term stability study.

5.1.11.1. Processed sample stability study

The individual results of the time zero samples were 0.2476 g/100 mL and 0.2539 g/ 100 mL (Mean: 0.2507 g/ 100 mL). The acceptable range for the reanalysed samples was calculated as 0.2257 g/ 100 mL to 0.2758 g/ 100 mL (mean ± 10%).
The results of the reanalysed samples were 0.2551 g/ 100 mL and 0.2591 g/ 100 mL, respectively.

5.1.11.2. Long-term stability study

The individual results of the time zero samples were 0.1520 g/ 100 mL and 0.1560 g/ 100 mL (Mean: 0.1540 g/ 100 mL). The acceptable range for the reanalysed samples was calculated as 0.1390 g/ 100 mL to 0.1690 g/ 100 mL (mean ± 10%).
The results of the reanalysed samples were 0.1489 g/ 100 mL and 0.1511 g/ 100 mL, respectively.

5.1.12. Conclusion

The HSGC-FID method used to analyse ethanol in VH met all the acceptance criteria for method validation. This method can therefore be deemed fit for purpose across the defined linear working range, 0.01 g/100mL to 0.5 g/100mL.

5.2. ETG/ ETS IN VH: METHOD DEVELOPMENT

The method to simultaneously analyse EtG and EtS in VH was developed and optimized based on various methods obtained from literature (Ohouo *et al.*, 2018, Thierauf *et al.*, 2011, Albermann *et al.*, 2012). The development of this method involved a stepwise process that consisted of the following procedures:

- The optimization of MS/MS conditions
- The optimization of chromatographic separation
- The optimization of the sample preparation procedure
- The optimization of MS response and sensitivity
- The verification of synthetic vitreous humour as a surrogate matrix

5.2.1. MS/MS conditions

The optimal MS/MS conditions for this method are described in Tables 4.6 and 4.7 above. The ionization mode, quantification method and MS/MS transitions were obtained from literature while the remaining conditions were optimized, using the autotune function in the MassLynx software, by the direct pump infusion of neat standards (EtG, EtG-d5, EtS, EtS-d5) into the MS/MS.

5.2.2. Chromatographic separation

The optimal UPLC conditions are summarized in Table 4.5. A gradient elution profile using 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile as mobile phases provided the optimal resolution for the analytes of interest (Refer to Figure 5.3).

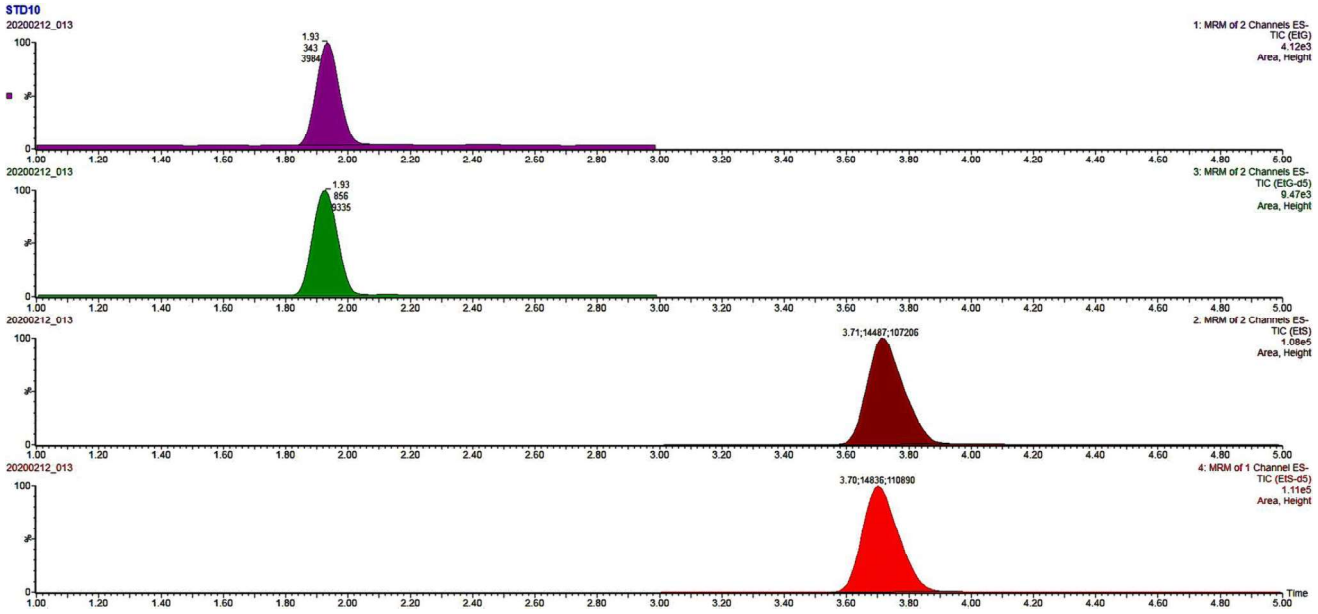


Figure 5.3: A chromatogram indicating the retention times of neat EtG, EtS, EtG-d5 and EtS-d5 standards at a concentration of 5000 ppb.

5.2.3. Sample preparation procedure

Seven different sample preparation procedures were trialled during this method development (Refer to Table 5.10). Sample preparation procedure two was used for this method because this procedure produced the most sensitive MS/MS responses, based on peak area, for EtG and EtS (Refer to Figure 5.4).

Table 5.10: A summary of the procedures followed and solvents used for each of the seven sample preparation procedures

Procedure:	Reference:	Procedure followed:	Solvents used:
1	(Thierauf *et al.*, 2011)	PP[2]	MeOH[5]
		Supernatant evaporated and reconstituted	Mobile phase A
2	N/A[1]	PP[2]	MeOH[5] plus 0.1% FA[6]
		Supernatant evaporated and reconstituted	Mobile phase A
3	(Thierauf *et al.*, 2011)	PP[2]	ACN[7]
		Supernatant evaporated and reconstituted	Mobile phase A
4	N/A[1]	Cation exchange SPE[3]	Load: 4% Phosphoric acid
			Wash: FA[6] and MeOH[5]
			Elute: 5% Ammonium hydroxide
5	(Himes *et al.*, 2014, Favretto *et al.*, 2010)	Anion exchange SPE[3]	Load: 5% Ammonium hydroxide
			Wash: MeOH[5] and ACN[7]
			Elute: 2% FA[6]
6	N/A[1]	HLB[4] SPE[3]	Load: 2% HCl[8]
			Wash: ACN[7]
			Elute: 5% Ammonium hydroxide
7	N/A[1]	Pass-through using HLB[4] SPE[3]	Load: 2% HCl[8]

N/A[1]- Method not published, PP[2]- Protein precipitation, SPE[3]- Solid Phase Extraction, HLB[4]- Hydrophilic-Lipophilic Balance, MeOH[5]- Methanol, FA[6]- Formic Acid, ACN[7]- Acetonitrile, HCl[8]- Hydrochloric acid

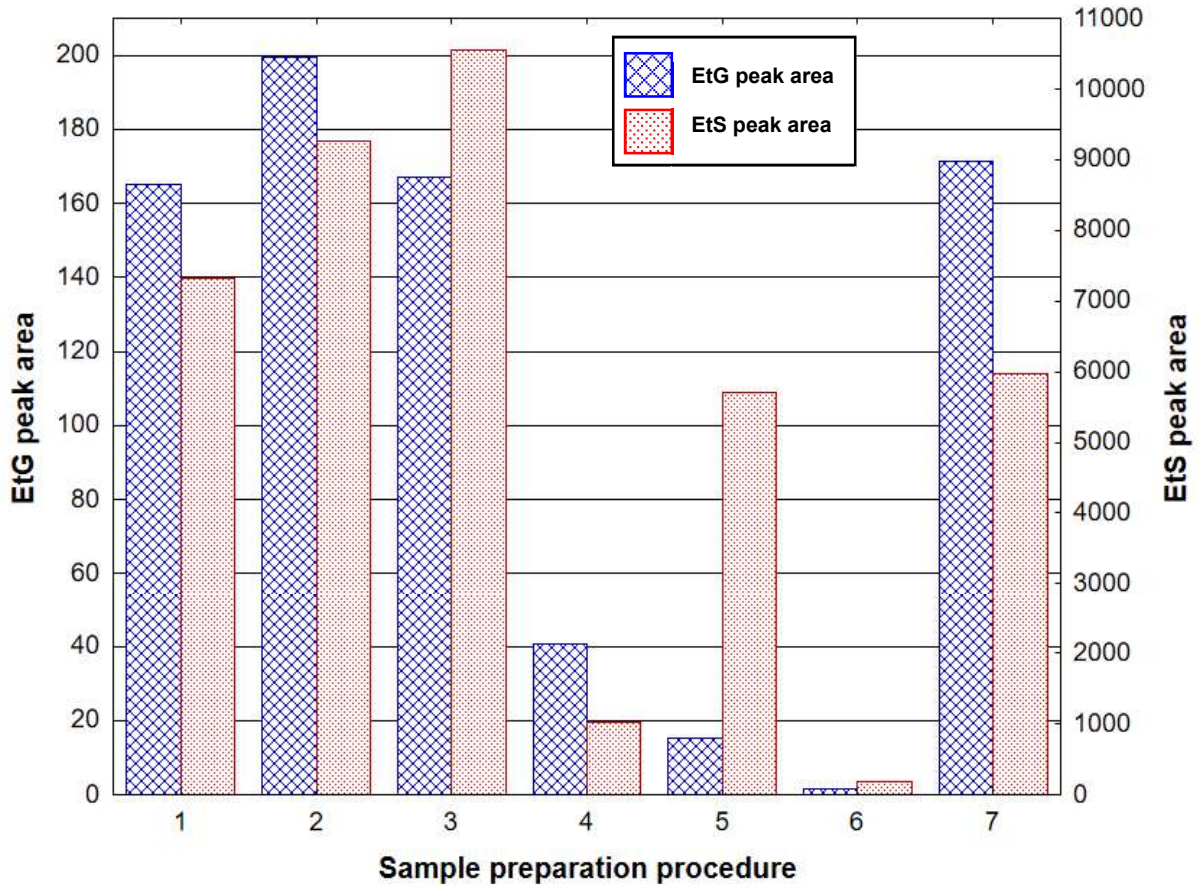


Figure 5.4: The peak area of EtG (left y-axis) and EtS (right y-axis) obtained after performing the various sample preparation procedures on blank vitreous humour samples spiked to a EtG and EtS concentration of 1000 ppb (n=3).

5.2.4. MS response and sensitivity

The addition of acetonitrile post-column, at a flow rate of 0.6 mL/min, increased the ionization efficiency of both EtG and EtS. As a result of this increased ionization, the LOQ of EtG decreased approximately 9 times while the LOQ of EtS decreased 2.5 times (Refer to Table 5.11). The MS sensitivity was further increased by dividing the chromatogram into two time-segments.

Table 5.11: The calculated LOD and LOQ of EtG and EtS with and without the post-column injection of acetonitrile.

	EtG concentration (ppb)		EtS concentration (ppb)	
Sample	**Without post-column injection**	**With post-column injection**	**Without post-column injection**	**With post-column injection**
Blank 1	72,343	20,892	0,650	2,890
Blank 2	42,490	24,699	1,126	2,949
Blank 3	65,451	21,929	0,576	3,165
Blank 4	73,959	22,336	0,922	3,156
Blank 5	27,432	22,378	0,927	3,053
Blank 6	26,917	24,872	0,793	2,948
Blank 7	25,521	21,881	0,661	3,029
Blank 8	24,786	23,471	0,986	2,978
Blank 9	24,646	29,171	0,539	2,954
Blank 10	26,792	25,040	1,192	2,943
Mean	41,034	23,667	0,837	3,007
SD	20,057	2,282	0,217	0,088
LOD	60,172	6,846	0,652	0,265
LOQ	200,574	22,821	2,172	0,883

5.2.5. Verification of Synthetic Vitreous Humour as a surrogate matrix

This method required synthetic vitreous humour, as a surrogate matrix, to prepare calibration standards, blanks and quality controls. Before the use of this synthetic vitreous humour, it was important to verify its fitness for use as a surrogate matrix. Two linear calibration curves were established for each analyte: one in authentic matrix and the other in surrogate matrix (Refer to Figures 5.5 and 5.6). Each calibration curve was established by analysing a blank and five calibration standards (EtG- 150, 300, 500, 1000 and 7500 ppb; EtS- 5, 20, 100, 500 and 2500 ppb) over three different analytical runs.

There was no significant difference between the slopes of the EtG calibration curves in the two different matrices, $t(4) = -0.685$, $p=0.531$. There was also no significant difference between the slopes of the EtS calibration curves in the two different matrices, $t(4) =0.0695$, $p=0.948$.

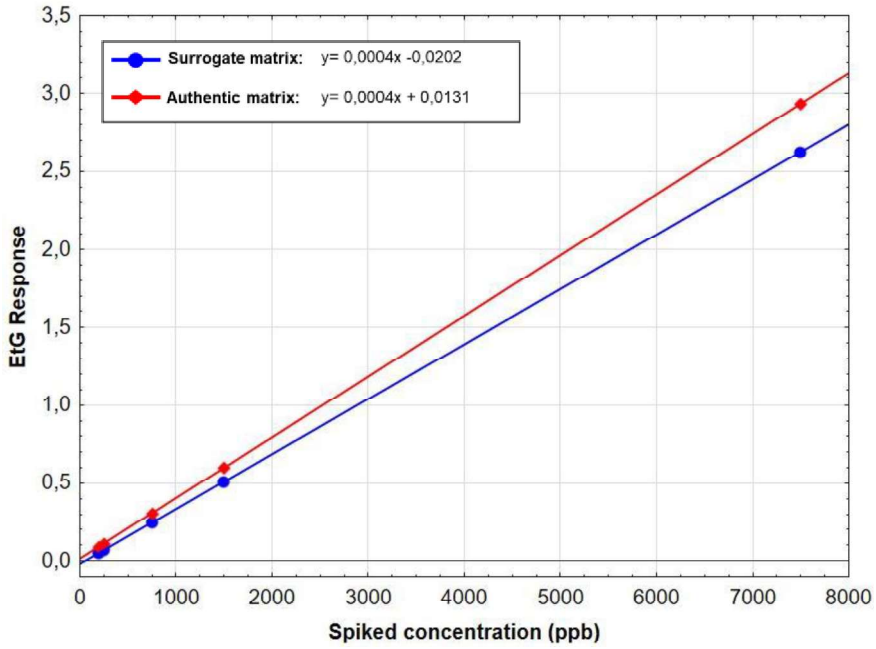


Figure 5.5: The established calibration curves of EtG in surrogate matrix (blue) and authentic matrix (red).

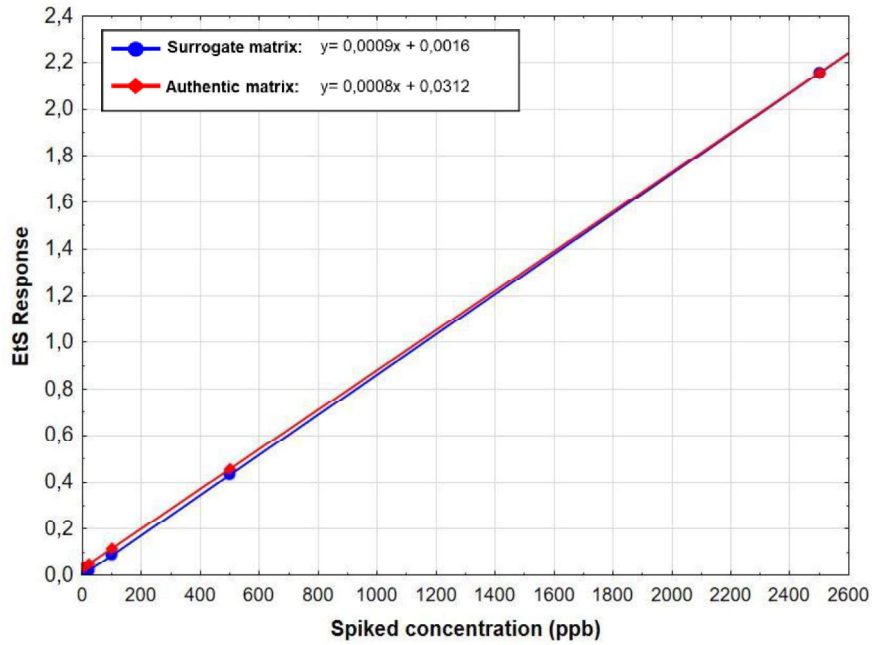


Figure 5.6: The established calibration curves of EtS in surrogate matrix (blue) and authentic matrix (red).

5.3. ETG/ ETS IN VH: METHOD VALIDATION

The newly developed analytical method to simultaneously analyse EtG and EtS in vitreous humour required a full method validation. The method was validated using the performance characteristics and acceptance criteria highlighted in Table 4.8.

5.3.1. Limit of Detection

The calculated LOD's of the method were 6.85 ppb for EtG and 0.27 ppb for EtS (Refer to Table 5.12).

Table 5.12: The EtG and EtS results for the ten blank samples (synthetic vitreous humour without analytes of interest) including the calculation of the mean, standard deviation and the LOD's of the method

Sample name	Analyst	EtG concentration (ppb)	EtS concentration (ppb)
Blank 1	Gareth Riley	20,892	2,890
Blank 2	Gareth Riley	24,699	2,949
Blank 3	Gareth Riley	21,929	3,165
Blank 4	Gareth Riley	22,336	3,156
Blank 5	Gareth Riley	22,378	3,053
Blank 6	Gareth Riley	24,872	2,948
Blank 7	Gareth Riley	21,881	3,029
Blank 8	Gareth Riley	23,471	2,978
Blank 9	Gareth Riley	29,171	2,954
Blank 10	Gareth Riley	25,040	2,943
Mean		23,667	3,007
Standard deviation (SD)		2,282	0,088
LOD (3 X SD)		6,846	0,265

5.3.2. Limit of Quantitation

The calculated LOQ's for this method were 22.82 ppb for EtG and 0.88 ppb for EtS (refer to Table 5.13). However, as stated in Section 4.3, the LOQ used for this method was the lowest non-zero calibrator which was 50 ppb for EtG and 5 ppb for EtS. The percent accuracies of the lowest non-zero calibrator over six analytical runs were 3.23% for EtG and 5.26% for EtS while the percent RSD's were 3.01% for EtG and 5.48% for EtS (Refer to Table 5.14).

Table 5.13: The EtG and EtS results for the ten blank samples (synthetic vitreous humour without analytes of interest) including the calculation of the mean, standard deviation and the LOQ's of the method

Sample name	Analyst	EtG concentration (ppb)	EtS concentration (ppb)
Blank 1	Gareth Riley	20,892	2,890
Blank 2	Gareth Riley	24,699	2,949
Blank 3	Gareth Riley	21,929	3,165
Blank 4	Gareth Riley	22,336	3,156
Blank 5	Gareth Riley	22,378	3,053
Blank 6	Gareth Riley	24,872	2,948
Blank 7	Gareth Riley	21,881	3,029
Blank 8	Gareth Riley	23,471	2,978
Blank 9	Gareth Riley	29,171	2,954
Blank 10	Gareth Riley	25,040	2,943
Mean		23,667	3,007
Standard deviation (SD)		2,282	0,088
LOQ (10 X SD)		22,821	0,883

Table 5.14: The EtG and EtS concentrations (ppb) for the analysis of six replicates of the lowest non-zero calibrator including the mean, standard deviation, reference value, percent accuracy and percent RSD.

Analytical run	Analyst	EtG concentration (ppb)	EtS concentration (ppb)
1	Gareth Riley	50,436	5,013
2	Bianca Southon	51,317	4,997
3	Gareth Riley	50,377	4,936
4	Gareth Riley	54,551	5,507
5	Bianca Southon	52,688	5,660
6	Gareth Riley	50,315	5,465
Mean		51,614	5,263
Standard Deviation		1,553	0,288
Reference value		50,000	5,000
%RSD		3,009	5,475
% Accuracy		3,228	5,260

5.3.3. Working Range

For this analytical method, EtG and EtS were analysed simultaneously therefore the working range was established by analysing a blank and 10 calibration standards containing various concentrations of both EtG and EtS (Refer to Table 4.2). The linear working ranges for this analytical method were:

- 50 ppb to 2 500 ppb for EtG
- 5 ppb to 1 000 ppb for EtS

5.3.4. Linearity

The linearity of this method was evaluated by establishing a calibration model for each analyte.

5.3.4.1. EtG

The established calibration model, to assess the linearity of EtG between 50 ppb and 2500 ppb for EtG, had a correlation coefficient of 0.9998 (Refer to Figure 5.7). The residual plot of the established calibration model shows residuals randomly distributed about zero with no tendencies or outliers.

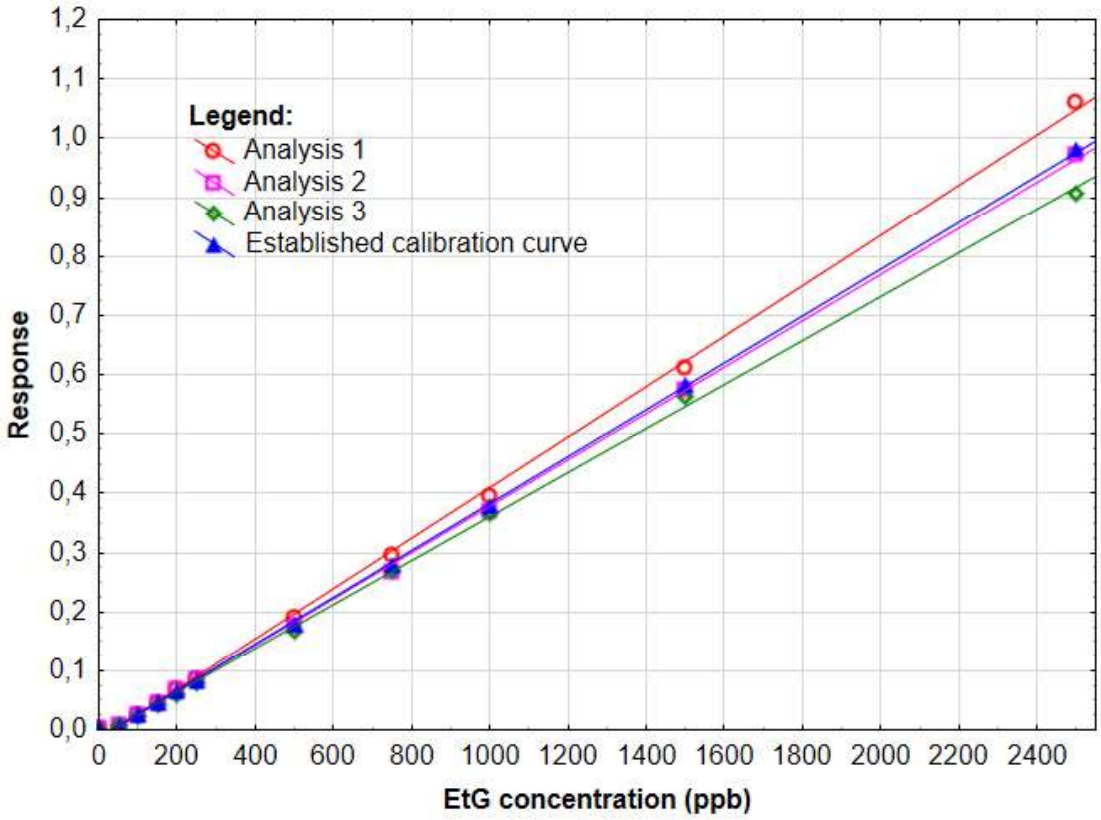


Figure 5.7: A scatterplot illustrating the calibration curves of three different analytical runs as well as the established calibration model for EtG (r = 0.9998, $y = 0.004x - 0.0128$) (n=3).

5.3.4.2. EtS

The established calibration model, to assess the linearity of EtS between 5 ppb and 1000 ppb, had a correlation coefficient of 0.9999 (Refer to Figure 5.8). The residual plot of the established calibration model shows residuals randomly distributed about zero with no tendencies or outliers.

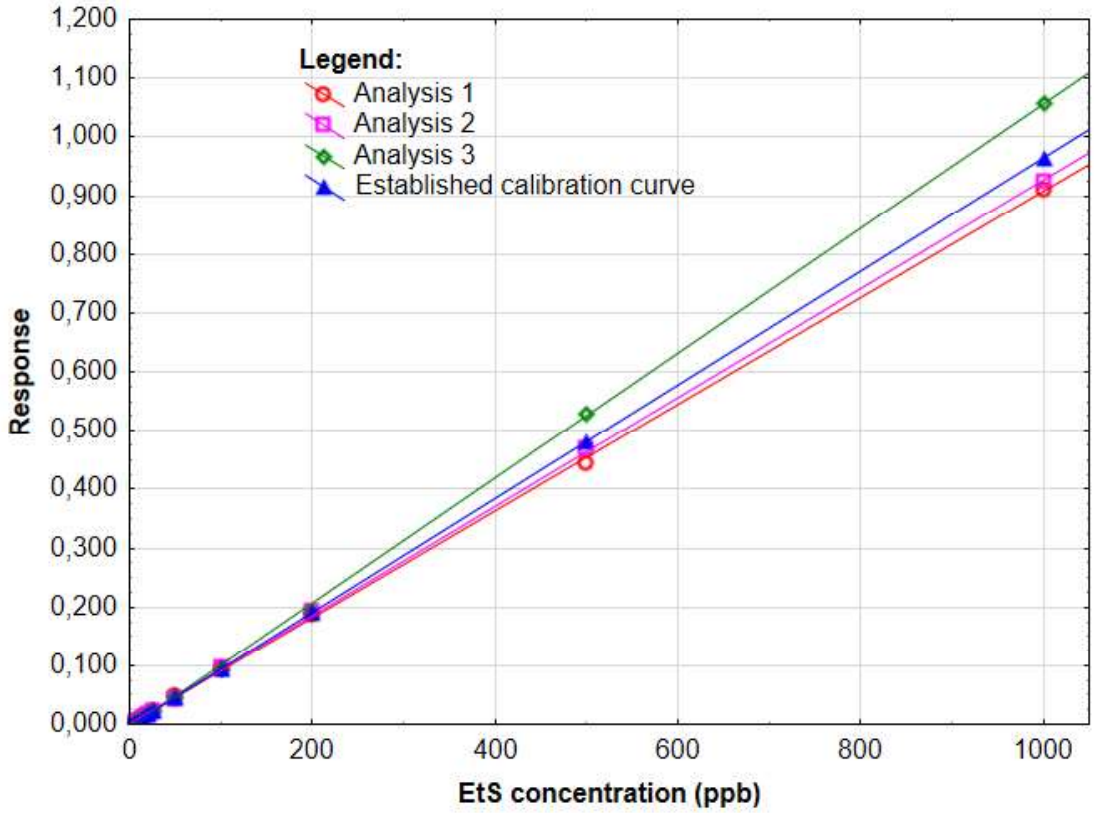


Figure 5.8: A scatterplot illustrating the calibration curves of three different analytical runs as well as the established calibration model for EtS (r = 0.9999, $y = 0.001x - 0.0017$) (n=3).

5.3.5. Selectivity

The selectivity of this method was assessed by evaluating two interference studies as well as the effects of the matrix. The two interferences studies evaluated were matrix interferences and interferences from the internal standards.

5.3.5.1. Matrix interferences

Each analyte (EtG, EtG-d5, EtS and EtS-d5) was analysed in ten blank samples (synthetic vitreous humour without analytes of interest or internal standards). There were no matrix interferences as the S/N ratios of each analyte in these samples was less than 3 (Refer to Table 5.15).

Table 5.15: The S/N ratios of EtG, EtG-d5, EtS and EtS-d5 in the ten blank samples containing no internal standard (IS)

Sample name	EtG S/N ratio	EtG-d5 S/N ratio	EtS S/N ratio	EtS-d5 S/N ratio
Blank with no IS 1	1,444	2,299	1,481	1,507
Blank with no IS 2	1,523	1,261	2,341	0,888
Blank with no IS 3	0,316	0,848	2,740	2,302
Blank with no IS 4	1,335	2,427	2,503	1,831
Blank with no IS 5	1,837	0,994	1,866	2,090
Blank with no IS 6	0,955	1,563	2,926	2,584
Blank with no IS 7	1,074	1,305	1,337	1,225
Blank with no IS 8	1,188	2,682	1,943	0,464
Blank with no IS 9	0,315	0,626	2,587	2,517
Blank with no IS 10	0,488	0,946	2,401	2,193

5.3.5.2. Interferences from the internal standards

The EtG and EtS concentrations of ten blank samples (synthetic vitreous humour without analytes of interest) were less than the LOQ's of the method (Refer to Table 5.16). Therefore, the stable isotope labelled internal standards did not interfere with the analysis of the analytes of interest.

Table 5.16: The EtG and EtS concentrations (ppb) of the ten blank samples containing the internal standards. This table also includes the LOQ's for EtG and EtS.

Sample name	Analyst	EtG concentration (ppb)	EtS concentration (ppb)
Blank 1	Gareth Riley	20,892	2,890
Blank 2	Gareth Riley	24,699	2,949
Blank 3	Gareth Riley	21,929	3,165
Blank 4	Gareth Riley	22,336	3,156
Blank 5	Gareth Riley	22,378	3,053
Blank 6	Gareth Riley	24,872	2,948
Blank 7	Gareth Riley	21,881	3,029
Blank 8	Gareth Riley	23,471	2,978
Blank 9	Gareth Riley	29,171	2,954
Blank 10	Gareth Riley	25,040	2,943
LOQ		50,000	5,000

5.3.5.3. Matrix effects

The percentage matrix effects were 97.43% for EtG and 101.15% for EtS (Refer to Table 5.17).

Table 5.17: The EtG and EtS concentrations (ppb) obtained for the five analytical runs of sample type 1 (pure water spiked with an EtG concentration of 1000 ppb and an EtS concentration of 200 ppb) and sample type 2 (calibration standard 8) as well as the calculated percent matrix effects.

EtG- Matrix Effects		
Analysis number	**Sample type 1 concentration (ppb)**	**Sample type 2 concentration (ppb)**
1	1105,220	1085,732
2	944,302	1026,652
3	1017,013	933,690
4	985,890	939,095
5	1055,171	990,949
Mean	1021,519	995,224
% Matrix effects		97,426

EtS- Matrix Effects		
Analysis number	**Sample type 1 concentration (ppb)**	**Sample type 2 concentration (ppb)**
1	231,206	221,800
2	200,272	205,069
3	192,302	197,376
4	199,265	211,521
5	201,126	200,173
Mean	204,834	207,188
% Matrix effects		101,149

5.3.6. Accuracy

The calculated percent accuracies for EtG QC1 (125 ppb), QC2 (600 ppb) and QC3 (2000 ppb) were -1.31%, -0.06% and 1.372%, respectively while the calculated percent accuracies for EtS QC1 (18 ppb), QC2 (130 ppb) and QC3 (600 ppb) were 0.68%, -0.19% and 1.45%, respectively (Refer to Table 5.18).

Table 5.18: The EtG and EtS results of QC1, QC2 and QC3 analysed in triplicate over five different analytical runs. The table includes the reference values of each quality control as well as the calculated mean, standard deviation and percent accuracy.

Analytical run	Number	Analyst	EtG concentration (ppb)			EtS concentration (ppb)		
			QC1	QC2	QC3	QC1	QC2	QC3
1	1	Gareth Riley	127,636	605,189	1867,866	17,793	129,723	600,688
	2		124,251	577,722	2034,538	18,321	128,553	605,289
	3		114,234	585,115	1965,552	18,687	127,291	587,922
2	4	Bianca Southon	123,169	584,520	2011,280	18,827	132,402	593,195
	5		123,510	584,716	2045,894	18,982	128,627	602,279
	6		125,619	602,950	2019,032	18,850	130,602	623,429
3	7	Gareth Riley	125,360	629,893	2087,071	17,619	135,865	624,823
	8		132,438	586,148	1977,094	17,919	130,177	641,533
	9		122,860	570,105	2038,497	18,635	126,068	581,887
4	10	Gareth Riley	120,478	623,597	2077,937	17,927	129,373	613,388
	11		124,421	620,683	2073,846	18,346	132,864	617,262
	12		118,111	599,288	1982,230	17,453	129,789	613,349
5	13	Bianca Southon	121,373	624,665	2093,022	17,331	128,908	612,448
	14		124,283	617,472	2062,401	18,060	127,495	627,871
	15		122,731	582,479	2075,246	17,091	128,547	585,238
Mean			123,365	599,636	2027,434	18,123	129,752	608,707
Standard deviation			3,970	18,991	58,187	0,578	2,376	16,557
Reference value (ppb)			125,000	600,000	2000,000	18,000	130,000	600,000
% Accuracy			-1,308	-0,061	1,372	0,682	-0,191	1,451

5.3.7. Precision

The precision of this method was assessed by evaluating the percent RSD of within run and intermediate precision.

5.3.7.1. Within-run precision

The percent RSD's for EtG QC1 (125 ppb), QC2 (600 ppb) and QC3 (2000 ppb) were 2.33%, 2.39% and 2.24%, respectively while the percent RSD's for EtS QC1 (18 ppb), QC2 (130 ppb) and QC3 (600 ppb) were 2.58%, 1.55% and 1.53%, respectively (Refer to Table 5.19).

Table 5.19: The EtG and EtS concentrations (ppb) of QC1, QC2 and QC3 analysed six times during a single analytical run. The table includes the calculated means, standard deviations and percent RSD's.

Analytical run	Number	Analyst	EtG concentration (ppb)			EtS concentration (ppb)		
			QC1	QC2	QC3	QC1	QC2	QC3
4	1	Gareth Riley	120,478	623,597	2077,937	17,927	129,373	613,388
	2		124,421	620,683	2073,846	18,346	132,864	617,262
	3		118,111	599,288	1982,230	17,453	129,789	613,349
	4		126,090	589,872	2090,397	18,687	134,695	600,716
	5		121,191	632,454	2019,072	17,531	134,101	590,099
	6		119,073	618,587	1979,243	17,548	133,361	605,319
Mean			121,561	614,080	2037,121	17,915	132,364	606,689
Standard deviation			2,831	14,702	45,720	0,461	2,052	9,254
% RSD			2,329	2,394	2,244	2,576	1,550	1,525

5.3.7.2. Intermediate precision

The percent RSD's for EtG QC1 (125 ppb), QC2 (600 ppb) and QC3 (2000 ppb) were 4.06%, 3.39% and 3.32%, respectively while the percent RSD's for EtS QC1 (18 ppb), QC2 (130 ppb) and QC3 (600 ppb) were 2.29%, 2.12% and 2.83%, respectively (Refer to Table 5.20).

Table 5.20: The EtG and EtS concentrations (ppb) of each quality control analysed in triplicate over three analytical runs. The table includes the calculated means, standard deviations and percent RSD's.

Analytical run	Number	Analyst	EtG concentration (ppb)			EtS concentration (ppb)		
			QC1	QC2	QC3	QC1	QC2	QC3
1	1	Gareth Riley	127,636	605,189	1867,866	17,793	129,723	600,688
	2		124,251	577,722	2034,538	18,321	128,553	605,289
	3		114,234	585,115	1965,552	18,687	127,291	587,922
3	7	Gareth Riley	125,360	629,893	2087,071	17,619	135,865	624,823
	8		132,438	586,148	1977,094	17,919	130,177	641,533
	9		122,860	570,105	2038,497	18,635	126,068	581,887
4	10	Gareth Riley	120,478	623,597	2077,937	17,927	129,373	613,388
	11		124,421	620,683	2073,846	18,346	132,864	617,262
	12		118,111	599,288	1982,230	17,453	129,789	613,349
Mean			123,310	599,749	2011,626	18,078	129,967	609,571
Standard deviation			5,011	20,299	66,711	0,414	2,749	17,239
% RSD			4,064	3,385	3,316	2,290	2,115	2,828

5.3.8. Measurement uncertainty

The combined and expanded uncertainty of measurements calculated based on the procedure by Ellison et al., (2012) were:

- EtG: 4.91% and 9.82%
- EtS: 4.68% and 9.36%

5.3.9. Ruggedness

The percent RSD's for EtG QC1 (125 ppb), QC2 (600 ppb) and QC3 (2000 ppb) were 3.18%, 2.60% and 3.04%, respectively while the percent RSD's for EtS QC1 (18 ppb), QC2 (130 ppb) and QC3 (600 ppb) were 2.75%, 1.31% and 1.80%, respectively (Refer to Table 5.21).

Table 5.21: The EtG and EtS concentrations (ppb) of each quality control analysed in triplicate over three analytical runs. Each analytical run had small variations in the method parameters including different sample preparation temperatures and different analysts. The table includes the calculated means, standard deviations and percent RSD's.

Analytical run	Number	Sample preparation temperature (°C)	Analyst	EtG concentration (ppb)			EtS concentration (ppb)		
				QC1	QC2	QC3	QC1	QC2	QC3
1	1	21,9	Gareth Riley	127,636	605,189	1867,866	17,793	129,723	600,688
	2			124,251	577,722	2034,538	18,321	128,553	605,289
	3			114,234	585,115	1965,552	18,687	127,291	587,922
2	4	19,4	Bianca Southon	123,169	584,520	2011,280	18,827	132,402	593,195
	5			123,510	584,716	2045,894	18,982	128,627	602,279
	6			125,619	602,950	2019,032	18,850	130,602	623,429
4	10	22,9	Gareth Riley	120,478	623,597	2077,937	17,927	129,373	613,388
	11			124,421	620,683	2073,846	18,346	132,864	617,262
	12			118,111	599,288	1982,230	17,453	129,789	613,349
Mean				122,381	598,198	2008,686	18,354	129,914	606,311
Standard Deviation				3,886	15,573	61,100	0,504	1,703	10,912
%RSD				3,175	2,603	3,042	2,745	1,311	1,800

5.3.10. Carryover

5.3.10.1. EtG

The three blank samples (synthetic vitreous humour without analytes of interest) analysed after QC3 (2000 ppb), had calculated concentrations of:

- 6,14 ppb
- 8,05 ppb
- 6,41 ppb

These concentrations were all less than 10 ppb which was 20% of the LOQ and carryover was therefore accepted.

5.3.10.2. EtS

The three blank samples (synthetic vitreous humour without analytes of interest) analysed after QC3 (600 ppb), had concentrations of:

- 0,35 ppb
- 0,54 ppb
- 0,41 ppb

These concentrations were all less than 1 ppb which was 20% of the LOQ and carryover was therefore accepted.

5.3.11. Stability

The stability of the method was assessed by performing a processed sample stability study and a long-term stability study for each analyte.

5.3.11.1. EtG processed sample stability study

The individual results of the time zero samples were 1978,71 ppb and 1965,55 ppb (Mean: 1972,13 ppb). The acceptable range for the reanalysed samples was calculated as 1793,64 ppb to 2192,23 ppb (mean ± 10%).
The results of the reanalysed samples were 1992,05 ppb and 1998,71 ppb, respectively.

5.3.11.2. EtG long-term stability study

The individual results of the time zero samples were 577,72 ppb and 585,12 ppb (Mean: 581,42 ppb). The acceptable range for the reanalysed samples was calculated as 530,41 ppb to 648,28 ppb (mean ± 10%).
The results of the reanalysed samples were 635,47 ppb and 617.45 ppb, respectively.

5.3.11.3. EtS processed sample stability study

The individual results of the time zero samples were 591,32 ppb and 593.20 ppb (Mean: 592,26 ppb). The acceptable range for the reanalysed samples was calculated as 535,56 ppb to 654,57 ppb (mean ± 10%).
The results of the reanalysed samples were 629,60 ppb and 616.60 ppb, respectively.

5.3.11.4. EtS long-term stability study

The individual results of the time zero samples were 128,55 ppb and 127,29 ppb (Mean: 127,922 ppb). The acceptable range for the reanalysed samples was calculated as 115,67 ppb to 141,38 ppb (mean ± 10%).
The results of the reanalysed samples were 134,34 ppb and 134,97 ppb, respectively.

5.3.12. Conclusion

The UPLC-MS/MS method used to analyse EtG and EtS in VH met all the acceptance criteria for method validation. This method can therefore be deemed fit for purpose across the defined linear working range, EtG 50 ppb to 2500 ppb and EtS 5 ppb to 1000 ppb.

5.4. PILOT STUDY

This section of the results chapter describes the ethanol, EtG and EtS concentrations of the post-mortem vitreous humour samples sampled from the 119 decedents received at the JHB FPS MLL. The raw data of the 119 decedents can be seen in Appendix 5 and includes age, sex, post-mortem interval (PMI), circumstance of death, VH ethanol concentration (g/100 mL), VH EtG concentration (ppb) and VH EtS concentration (ppb).

In 7 of these 119 decedents (5.9%), the volumes of vitreous humour sampled were insufficient hence these cases were excluded from this study. Table 5.22 summarizes the demographics of the 112 decedents whose post-mortem vitreous humour samples were sampled.

Table 5.22: Summarized demographics (including age, sex and circumstance of death) of the study participants (N=112).

Category	Sub-category	Number of cases (n=112)	Percentage of cases (%)
Age (years)	18-24	20	17.9
	25-34	32	28.6
	35-44	32	28.6
	45-54	8	7.1
	55+	8	7.1
	[1]N/A	12	10.7
Sex	Male	93	83.0
	Female	19	17.0
Circumstance of death	Assault	9	8.0
	Drowning	2	1.8
	Electrocuted	3	2.7
	Fell from height	3	2.7
	Hanging	6	5.5
	[2]MBA/[3]MVA	6	5.2
	Natural	10	8.9
	Overdose	3	2.7
	Poisoning	11	9.8
	[4]PVA	7	6.2
	Shot	29	25.9
	Stab	17	15.2
	[5]Other	2	1.8
	[1]N/A	4	3.6

[1]N/A- Information unavailable at time of autopsy and during data collection from case file; [2]MBA- Motorbike accident; [3]MVA- Motor vehicle accident; [4]PVA- Pedestrian vehicle accident; [5]Other- Includes n=1 burn victim and n=1 carbon monoxide gassing.

5.4.1. Ethanol, EtG and EtS sample results

Table 5.23 summarizes the ethanol, EtG and EtS concentrations obtained from the 112 vitreous humour samples. The decedents were categorized, using Figure 4.3, into one of eight categories based on the ethanol, EtG and EtS results of their VH samples. For each category, the median and interquartile range (IQR) was quoted as well as the percentage of cases that had an EtG or EtS concentration between the LOQ and the cut-off value, 100 ppb for EtG and 25 ppb for EtS (Dasgupta, 2015, Høiseth *et al.*, 2010). The median and IQR of the results for each analyte within each category were reported because the raw data was nonparametric.

Table 5.23: The categorized ethanol, EtG and EtS concentrations obtained from the 112 vitreous humour samples, including the mean, IQR and percentage of cases within each category with an EtG or EtS result between the LOQ and the cut-off value

Category	Sample result			Number of cases	Percentage of cases (%)	Median [IQR]*			Results between LOQ and cut-off value (%) **	
	Ethanol	EtG	EtS			Ethanol (g/ 100 mL)	EtG (ppb)	EtS (ppb)	EtG	EtS
1	Positive	Positive	Positive	38	33.9	0.171 [0.0639-0.247]	833.70 [246.61-2090.94]	610.30 [332.81-1251.44]	7.9 (n=3)	0.0 (n=0)
2			Negative	0	0.0	-	-	-	-	-
3		Negative	Positive	8	7.1	0.0624 [0.0430-0.142]	-	90.13 [58.034-385.65]	-	0.0 (n=0)
4			Negative	1	0.9	0.0373	-	-	-	-
5	Negative	Positive	Positive	8	7.1	-	130.45 [102.49-213.89]	16.41 [7.011-375.65]	12.5 (n=1)	62.5 (n=5)
6			Negative	14	12.6	-	71.68 [62.09-121.00]	-	64.3 (n=9)	-
7		Negative	Positive	10	8.9	-	-	17.99 [14.32-53.85]	-	80.0 (n=8)
8			Negative	33	29.5	-	-	-	-	-
Total				112	100.0	0,000 [0,000-0,127]	61.68 [0.00-344.80]	16.89 [0.00-516.81]	11.6 (n=13)	11.6 (n=13)

* When no cases were present in a category or the analyte concentration was negative, no median or IQR was stated. No IQR was stated when only one case was present.
** Percentage of cases, within each category, with an analyte concentration between the LOQ and the cut-off value for abstinence, EtG- 100 ppb and EtS- 25 ppb.

5.4.2. Samples with positive results

The ethanol, EtG and EtS results of the 79 vitreous humour samples that were positive for at least one analyte were evaluated using descriptive and inferential statistics.

5.4.3.1. Descriptive statistics

The basic descriptive statistics, of each analyte, for the 79 positive samples are described in Table 5.24. The ethanol results were converted from g/100mL units to ppb.

Table 5.24: The basic descriptive statistics for ethanol, EtG and EtS of the 79 positive samples.

Statistical parameter	[1]Ethanol (ppb)	EtG (ppb)	EtS (ppb)
Distribution	Non-parametric	Non-parametric	Non-parametric
Mean	9.61 x 10^5	735.28	533.47
SD	1.17 x 10^6	1368.38	878.31
Median	4.80 x 10^5	127.85	102.00
Upper quartile	1.75 x 10^6	932.16	705.25
Lower quartile	0.00	54.26	8.44
Maximum	4.52 x 10^6	7596.78	4661.41
Minimum	0.00	0.00	0.00

[1]Ethanol results were converted from g/100mL to ppb using: $ppb = (1 \times 10^7) g/100mL$

5.4.3.2. Inferential statistics

Inferential statistics were used to evaluate the differences and associations between ethanol and EtG/EtS results for the 79 positive samples. A Kruskal-Wallis test was conducted to evaluate the differences between the mean ranks of the three analytes measured in ppb. The test showed that there was a significant difference between the mean ranks of at least one of the analytes, $H(2) = 12.466, p = 0.002$. A Dunn's multiple comparisons of mean ranks *post hoc* test was conducted to determine which mean ranks were significant. The results of this *post hoc* test are illustrated in Table 5.25.

Table 5.25: The results of the Dunn's multiple comparisons of mean ranks test including the mean rank and p-values.

Group 1		Group 2		p-value
Analyte	**Mean rank (ppb)**	**Analyte**	**Mean rank (ppb)**	
Ethanol	140.886	EtG	109.956	0,014
Ethanol	140.886	EtS	106.158	0,0044
EtG	109.956	EtS	106.158	1.00

A Spearman's rank-order correlation test was conducted to determine the strength and direction of the association between ethanol and the two metabolites, EtG and EtS. The results indicate a Spearman's rank-order correlation coefficient of 0.606 (df=77, $p<0.001$) between ethanol and EtG concentrations and 0.800 (df=77, $p<0.001$) between ethanol and EtS concentrations.

5.4.3. Association between EtG and EtS

This section compared and evaluated the EtG and EtS results for the 79 positive samples. A strong, positive relationship ($r = 0,718$; $r^2 = 0,516$; $p = 0.0000$) was observed between the concentrations of EtG and EtS (Refer to Figure 5.9). A Spearman's rank correlation coefficient of 0.664 ($p<0.00001$) was calculated for the two analytes.

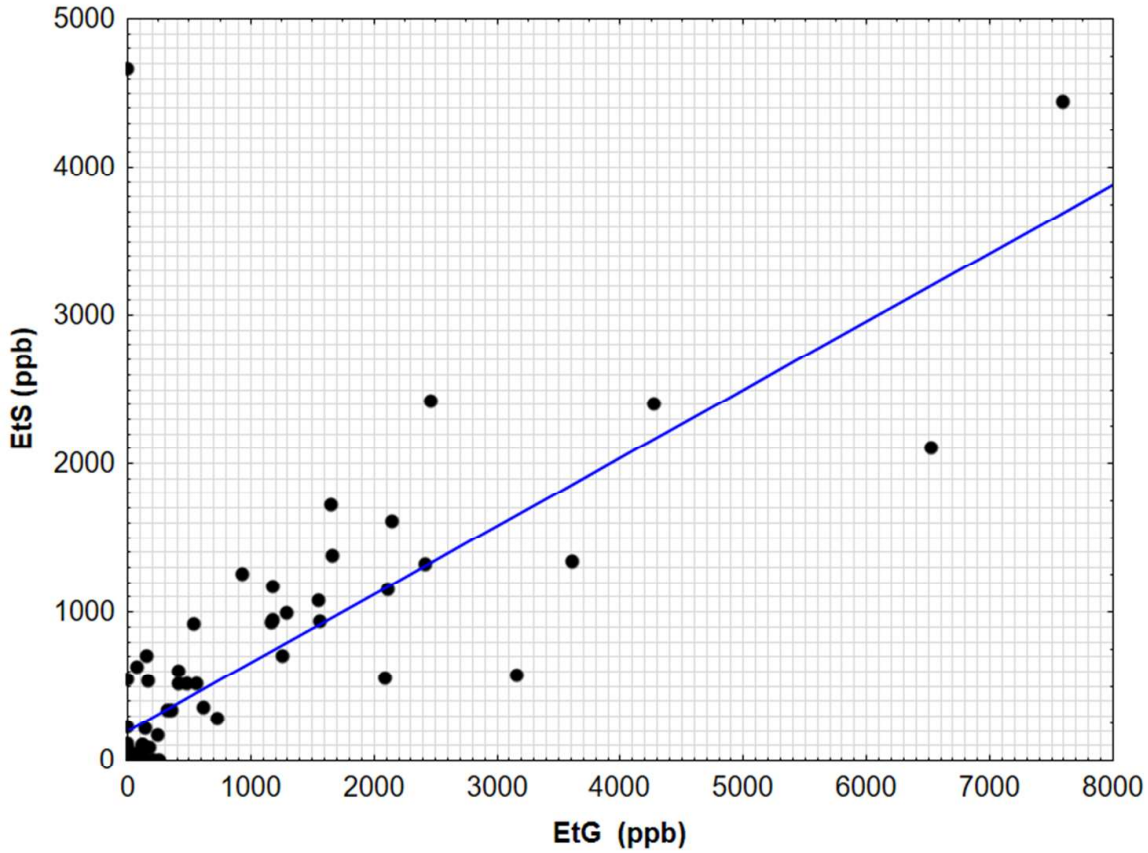


Figure 5.9: A scatterplot of the EtG and EtS concentrations of the 79 positive samples ($y = 0.461x + 194.560; r = 0.718; r^2 = 0.516; p < 0.05$)

5.4.4. Ratio of EtG to EtS

There were 46 vitreous humour samples that were positive for both EtG and EtS. The ratio of EtG to EtS was calculated for these 46 samples (Refer to Figure 5.10). The 46 samples had a mean EtG:EtS ratio of 3.56 (±7.67) with a median of 1.44 (1.013-2.17).

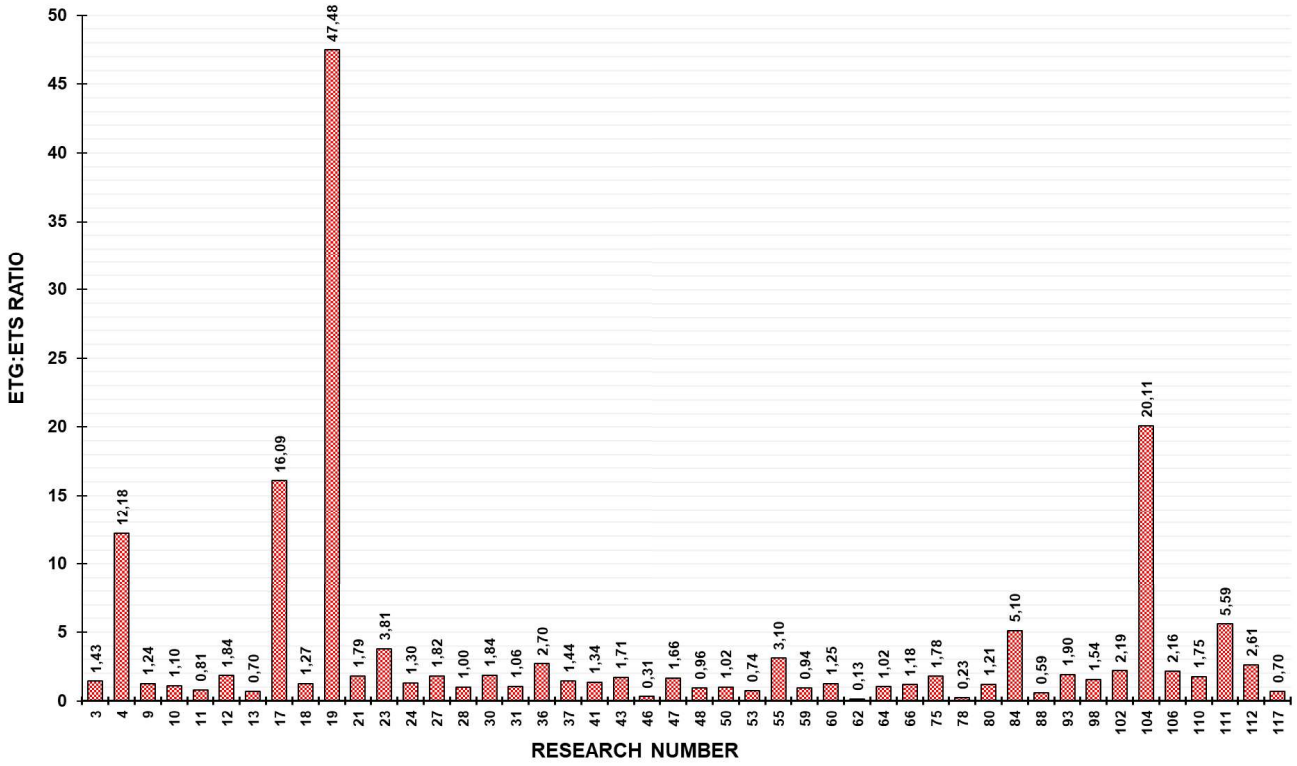


Figure 5.10: A bar graph illustrating the EtG:EtS ratio of the 46 samples positive for both EtG and EtS

CHAPTER 6: DISCUSSION

This chapter discusses the results obtained in Chapter 5 and relates these results to relevant literature. The sections discussed include the method validation results for the ethanol method, the results of the development and validation of the EtG, EtS method and the results obtained from the pilot study.

6.1. ETHANOL IN VH METHOD VALIDATION

The method to analyse ethanol in VH was modified from a previously developed and validated in-house method within the analytical division at the NIOH, NHLS JHB (Southon, 2019). The modifications made to the method were (i) a decreased sample volume and (ii) an alternative GC column.

The rationale to decrease the required sample volume of the method was based on the limited volume of vitreous humour that can be sampled from a decedent, up to 2 mL (Bévalot *et al.*, 2016). For this study, the volume of collected vitreous humour needed to be sufficient for the duplicate analysis of ethanol as well as EtG and EtS using two different sample preparation and analytical techniques. For this reason, a sample volume of 250 µL was used to quantify ethanol in vitreous humour. A SUPELCOWAX® 10 polar GC column was used as an alternative to a HP-INNOWax column. Both GC columns are polar with similar bonded polyethylene glycol stationary phases however, the SUPELCOWAX® 10 column had a highly selectivity for the analysis of alcohols (Merck Kgaa, n.d.).

The alternative sample matrix coupled with the modifications to the method indicated that a full method validation was required (Magnusson, 2014). This method validation was implemented to give evidence that the method was fit for purpose and effective in producing results with a high degree of accuracy (Araujo, 2009, Magnusson, 2014). The following performance characteristics were evaluated for this method validation: selectivity, limit of detection and quantitation, working range and linearity, accuracy, precision, measurement uncertainty, ruggedness, carryover and stability.

6.1.1. Limit of detection and limit of quantitation

The calculated LOD of the method was 0.00198 g/ 100 mL while the calculated LOQ was 0.00660 g/100mL. The LOD and LOQ of the method was calculated by analysing ten blank samples which consisted of sample matrix (vitreous humour) with no analyte of interest (ethanol) (Magnusson, 2014). The standard deviation of the blank samples was used to calculate the LOD and LOQ (Magnusson, 2014).

6.1.2.1. Limit of Detection

The International Union of Pure and Applied Chemistry (IUPAC) strongly recommends the use of a factor of three to calculate LOD using Eq. (4.3) (Analytical Methods Committee, 1987). This factor gives a confidence interval of 95% for false negative and false positive errors (Analytical Methods Committee, 1987). The use of this factor limits the errors regarding the choice and validity of different confidence levels when LOD is being estimated (Analytical Methods Committee, 1987).

6.1.2.2. Limit of Quantitation

The IUPAC applies a default value of ten to calculate the LOQ using Eq. (4.4) (Wenzl *et al.*, 2016, Analytical Methods Committee, 1987). A value of ten corresponds with a RSD of 10% which calculates a LOQ with acceptable precision and bias (Magnusson, 2014).
For this method, the lowest non-zero calibrator (0.01 g/ 100 mL) was used as the LOQ instead of the calculated LOQ because the precision and accuracy of the lowest non-zero calibrator can be evaluated with ease and in some cases is more acceptable than the calculated LOQ (Armbruster and Pry, 2008). The precision of a set of values is determined by the percent RSD (Scientific Working Group for Forensic Toxicology, 2013). Both the percent accuracy (-5,92%) and percent RSD (9,55%) were within the acceptance criteria and therefore the lowest non-zero calibrator was used as the LOQ (Scientific Working Group for Forensic Toxicology, 2013).

6.1.2. Working range

The linear working range for the method to analyse ethanol in VH using HSGC-FID was between 0.01 g/100mL and 0.5g/100 mL. In literature, the general working range is marginally less than the working range of this method (O'neal *et al.*, 1996, Dorubet *et al.*, 2011, Sudhaker and Jain, 2016). However, a method developed by Honey *et al.* (2005) had a working range similar to this method i.e. 0.01 g/ 100 mL to 0.47 g/ 100 mL (Honey *et al.*, 2005).
The working range of this method consisted of 14 calibration standards, with increasing ethanol concentrations. Fourteen calibration standards were included in this method because a report from the National Metrology Institute of South Africa (NMISA) in 2018 stated that a calibration curve consisting of 12 to 14 calibration standards was considered optimal for accurate ethanol analysis (National Metrology Institute of South Africa, 2018).

6.1.3. Linearity

The linearity of this method was assessed by evaluating the correlation coefficient and residual plot of the establishing linear calibration model. The calibration model was established using the calibration curve of three different analytical runs which were run on different days by

different analysts (Magnusson, 2014). The correlation coefficient of the calibration model was 0.99925. The residual plot illustrated residuals randomly distributed about zero with no outliers and no systematic tendencies.

The established calibration model adheres to the acceptance criteria of linearity (Scientific Working Group for Forensic Toxicology, 2013, Magnusson, 2014). Based on the calibration model, this method can be considered linear between ethanol concentrations of 0.01 g/100mL and 0.5 g/ 100 mL.

6.1.4. Selectivity

The selectivity of GC methods is based on the stationary phase of the GC column and the programmed temperature of the oven (Freeman and Kukla, 1986). Hence, the selection of the SUPELCOWAX® 10 GC column was a critical aspect of method development and directly contributed to the selectivity of the method. The selectivity of the method was assessed by evaluating the effect that suspected interferences and the matrix had on the analysis of ethanol and tertiary butanol.

The suspected interferences evaluated were acetaldehyde, acetone and 2-propanol because in post mortem blood, these volatile analytes (excluding ethanol) are the most frequently detected substances (Boumba *et al.*, 2012). These analytes are also the most frequently detected analytes in post mortem vitreous humour because of their high abundance in blood and the transport mechanism of analytes from blood into vitreous humour (Bévalot *et al.*, 2016). The retention times of these three analytes differed from the retention times of ethanol and tertiary butanol (Refer to Table 5.4). As a result, the peak resolutions of ethanol and tertiary butanol were not affected by the presence of these suspected interferences (Refer to Figure 5.2).

The percentage matrix effect was 99.36%, which fell within the acceptance criteria of 85-115%. The headspace sampling technique, employed prior to the GC analysis of the samples, limited the effects of the sample matrix by separating the volatile analytes from the sample matrix (Tian *et al.*, 2012). Only these volatile analytes were then analysed using GC-FID (Kugelberg and Jones, 2007).

The acceptance criteria were met for both the matrix effects and the effects of suspected interferences. This method can therefore be considered selective to analyse ethanol in vitreous humour.

6.1.5. Accuracy

The accuracy of the method was assessed by evaluating the percent accuracy of each QC analysed in triplicate across five analytical runs. The percent accuracy was calculated for each QC using Eq. (4.5) (Scientific Working Group for Forensic Toxicology, 2013). The percent

accuracy for the three QC's ranged from -0.17% to -0.75%; QC1 (0.015 g/100mL) had the largest percent accuracy while QC3 (0.2 g/100mL) had the smallest. The percent accuracy for all three QC's adhered to the acceptance criteria of ±10% and the method was therefore considered accurate (Scientific Working Group for Forensic Toxicology, 2013).

6.1.6. Precision

The precision of the method was assessed by evaluating both within-run and intermediate precision. The third type of precision, known as reproducibility, was not evaluated in this method validation because it requires samples to be analysed in another laboratory or with another method (Scientific Working Group for Forensic Toxicology, 2013). This was unachievable because currently no local laboratories have validated methods to analyse ethanol in vitreous humour.

For the within-run precision study, each QC was analysed six times within a single analytical run and the percent RSD for each QC was calculated using Eq. (4.1). The acceptance criteria for within-run precision (%RSD ≤10%) was met with the percent RSD of the QC's ranging from 1.48% to 0.46%.

The intermediate precision study was executed by analysing each QC in triplicate across three different analytical runs, run on different days by different analysts. The percent RSD for each QC was calculated using Eq. (4.1) and ranged from 0.51% to 2.03%. All three QC's had a percent RSD less than 10% and therefore the acceptance criteria for intermediate precision was met.

The acceptance criteria for within-run and intermediate precision were met for this method validation and therefore the method to analyse ethanol in vitreous humour using HSGC-FID was considered precise.

6.1.7. Measurement uncertainty

The measurement uncertainty of the method was calculated based on the four-step procedure described by Ellison *et al.* (2012). These steps included a precision study, a bias study, the calculation of the influence of other factors on uncertainty and finally, the calculation of the combined and expanded uncertainty (Ellison *et al.*, 2012).

6.1.7.1. Precision study

The precision study was performed to determine the percent precision uncertainty (Ellison *et al.*, 2012). The calculated percent precision uncertainty of this method was 1.55%. This value was calculated using Eq. (4.6) using 21 different analytical results of each QC (Ellison *et al.*, 2012).

6.1.7.2. Bias study

The bias study was conducting using the raw data from 21 analytical runs of QC1 (0.015 g/100mL). QC1 was selected since it had the largest uncertainty in comparison to the other QC's. The lesser uncertainties of the other QC's were therefore incorporated in the uncertainty of QC1.

The bias study consisted of two subsequent steps: (i) the percent bias uncertainty was 1.09%, calculated using Eq. (4.9) which required the calculation of percent bias, Eq. (4.7) and the uncertainty of the mean, Eq. (4.8) and (ii) the significance of the bias was determined using a t-test. The calculated t-value was calculated as 1 using Eq. (4.10) which is less than the critical t-value, t(20) =2.086, at a 95% confidence interval. This deduced that the uncertainty of the bias was insignificant and could be ignored in the calculation of the overall measurement uncertainty of the method.

6.1.7.3. Influence of other factors

The use of CRM's as QCs limited the calculation of the influence of other factors on measurement uncertainty to a single factor, CRM uncertainty (Ellison *et al.*, 2012). The uncertainty of the CRM used for QC1, SD= ±0.00061 g/ 100 mL, was larger than the other two CRM's and was therefore used to calculate this uncertainty. It was essential to convert the standard deviation into a qualified uncertainty which was done by treating it as a rectangular distribution and divided by $\sqrt{3}$ (Ellison *et al.*, 2012). Consequently, the calculated uncertainty of other factors was 0.023%.

6.1.7.4. Combined and expanded uncertainty

The combined uncertainty was calculated as 1.55% using Eq. (4.12). and only the percent precision uncertainty and percent uncertainty of other factors. The expanded uncertainty was calculated as 3.11% using Eq. (4.13). The value of the coverage factor, k, is associated with the level of confidence required for the method (Ellison *et al.*, 2012). For this method a level of confidence of 95% was required therefore a value of two was used as the coverage factor (Ellison *et al.*, 2012).

6.1.8. Ruggedness

The ruggedness of the method was assessed by evaluating the results obtained when small deliberate variations were made to the method parameters or environmental conditions (Peters *et al.*, 2007). The ruggedness of this method was assessed by varying the analyst and the environmental temperature during sample preparation. The environmental temperature ranged from 20.4 °C to 21.4 °C while two different analysts prepared and analysed the

samples. The percent RSD of the results of each QC under the varying conditions were calculated using Eq. (4.1). Since the percent RSD for each QC was below 10%, the method to analyse ethanol in vitreous humour was considered rugged (Scientific Working Group for Forensic Toxicology, 2013).

6.1.9. Carryover

The evaluation of carryover deduced that this method had no sufficient carryover. The analysis of three blank samples after a QC3 sample resulted in ethanol concentrations less than 20% of the LOQ (0.002 g/ 100 mL) of the method.

6.1.10. Stability

The evaluation of stability involved two independent stability studies. These studies included a processed sample stability study and a long-term stability study (Peters *et al.*, 2007).

6.1.10.1. Processed sample stability study

The processed sample stability study was designed based on the maximum time that a sample will remain unanalysed after preparation (Scientific Working Group for Forensic Toxicology, 2013). The conditions of this study design involved a sample being left in the auto sampler tray of the headspace for a period of 48 hours. This specific time period was decided on whilst alluding to different sample analysis conditions including large batches of samples and instrument breakdown. The results of the reanalysed samples were within ±10% of the mean of the time zero results (Scientific Working Group for Forensic Toxicology, 2013). The results of this study design indicated that there were no changes to the concentration of ethanol or tertiary butanol within the samples and therefore the sample is stable for 48 hours after sample preparation.

6.1.10.2. Long-term stability study

The long-term stability study was designed based on the storage conditions of the vitreous humour samples after sample collection (Peters *et al.*, 2007). The samples were stored at 4 °C in 4 mL tubes, containing sodium fluoride (Bévalot *et al.*, 2016, Dinis-Oliveira *et al.*, 2010). According to Dinis-Oliveira et al. (2010), the vitreous humour samples could be stored under these conditions for up to three months. This study was therefore designed by initially analysing samples for time-zero results. The same samples were reanalysed after being stored under the above conditions for three months. The results of the reanalysed samples were within ±10% of the mean of the time zero results (Scientific Working Group for Forensic Toxicology, 2013). As with the processed sample study, the results of this study design indicated that there were no changes to the concentration of ethanol or tertiary butanol within

the samples. The samples are therefore stable for up to three months when stored at these conditions.

6.2. ETG, ETS METHOD DEVELOPMENT

The method to simultaneously analyse EtG and EtS was developed and optimized based on methods published in literature (Ohouo *et al.*, 2018, Thierauf *et al.*, 2011, Albermann *et al.*, 2012). The procedure used to optimize and develop this method was based on the stepwise optimization procedure described by Kostić et al., (2013). This optimization procedure focused on the following steps: MS/MS conditions; UPLC conditions; sample preparation procedures; and MS response and sensitivity (Kostić *et al.*, 2013). In addition to these steps, a verification study was performed to determine the fitness of purpose of the synthetic vitreous humour as a surrogate matrix.

6.2.1. MS/MS conditions

The initial step of this method development was the optimization of the MS/MS conditions.
The following MS conditions were predetermined by literature and was found to not require optimization on the Waters® Quattro Premier XE tandem mass spectrometer (Thierauf *et al.*, 2011):

- The ionization mode (ESI negative)
- The quantification method (MRM mode)
- The MS/MS transitions for each analyte and internal standard (Refer to Table 4.7).

ESI negative was the optimal ionization mode for EtG and EtS because these analytes, due to their acidic nature, form anions in solution (Chromacademy, 2016a, Oppolzer *et al.*, 2016). The use of MRM mode as the quantification method for these analytes allowed for a detection method with a higher sensitivity and selectivity (Woźniak *et al.*, 2017). According to Sargent (2013), the monitoring of multiple MS/MS transitions increases the sensitivity and selectivity of a MS method. This method therefore monitored two MS/MS transitions; the most sensitive transition was used for the quantification of the analytes (EtG- 221.90>74.90; EtS- 125.00>79.86) whereas the second mass ion transition was used for confirmatory purposes (EtG- 221.90>85.90; EtS- 125.00>96.84) (Sargent, 2013).
The remaining MS conditions were optimized by the direct pump infusion of working solution standards of EtG, EtS, EtG-d5 and EtS-d5, at concentrations of 5000 ppb. Subsequently, the auto tune function of the tandem MS determined the MS/MS conditions that produced the optimal sensitivity and selectivity for the analytes (Refer to Table 4.6 and 4.7).

6.2.2. Chromatographic separation

The goal of the chromatographic optimization step was to maximize the response of the analytes and minimize the analytical run time (Kostić *et al.*, 2013). The UPLC conditions used

for this method were based on the conditions described by Ohouo et al., (2018); reverse phase chromatography with a UPLC CSH Phenyl-Hexyl column and a gradient elution profile. The gradient elution profile improved the resolution and sensitivity of the analytes as well as shortened the analytical run time (Lakka and Kuppan, 2019, Kostić *et al.*, 2013).

Two polar mobile phases were used for this gradient elution; 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Initially the mobile phase had a low concentration of organic solvent (acetonitrile) and a high concentration of water which allowed the analytes of interest to be partitioned into the stationary phase (Dong and Boyes, 2018, Lakka and Kuppan, 2019). As the concentration gradient of the mobile phases changed and the concentration of acetonitrile increased, so the analytes of interest partitioned between the mobile and stationary phases and eluted from the column according to their partition coefficients (Lakka and Kuppan, 2019, Dong and Boyes, 2018). EtG is more polar than EtS and therefore eluted from the column first. A pH modifier, 0.1% formic acid, was added to the mobile phases to suppress the ionization of the analytes which improved the chromatographic retention (Dong and Boyes, 2018). The only alteration made to this published method was the optimization of the gradient elution profile to reduce the analytical run time. The elution profile was optimized by altering the ratio of mobile phases of each individual gradient program thus decreasing the retention times (Refer to Table 4.5). This method made use of the MS/MS to not only quantify but to also to differentiate EtG and EtS from their internal standards based on their m/z ratio (Sargent, 2013).

6.2.3. Sample Preparation

In this method development step, seven different sample preparation procedures were trialled based on procedures obtained from literature (Oppolzer *et al.*, 2016). These procedures included three PP procedures and four SPE procedures. The efficiencies of the sample preparation procedures were evaluated using the EtG and EtS responses, based on peak area, generated by the UPLC-MS/MS after the specific sample preparation procedures were applied to 1000 ppb reference standards (Refer to Figure 5.4).

Sample preparation procedures one, two and three used PP to clean-up the samples by denaturing and removing the proteins (Polson *et al.*, 2003). Protein precipitation came about through the addition of organic solvents and acidic additives to the sample, in a 3:1 ratio, which decreased the solubility of the proteins (Polson *et al.*, 2003). The subsequent centrifugation produced a protein pellet and a supernatant containing the analytes of interest (Kole *et al.*, 2011). The evaporation and reconstitution of the supernatant in a low volume of mobile phase A improved the chromatographic separation of the analytes and increased the S/N ratio by concentrating the sample (Li *et al.*, 2013).

Sample preparation procedures four to seven used SPE to remove all contaminants and interfering substances as well as to concentrate the sample (Kole *et al.*, 2011). Procedure four, five and six each used a different microelution SPE sorbent with load, wash and elution steps that were optimized to produce the optimal sensitivity and selectivity for EtG and EtS (Refer to Table 5.10). Procedure seven used a SPE pass-through procedure that involved the loading of the sample, diluted 1:1 with 2% hydrochloric acid, onto the SPE sorbent and collecting the eluates (Lu *et al.*, 2019).

The sample preparation procedures that used PP generated higher responses compared to the SPE methods (Refer to Figure 5.4). The poor responses generated by the SPE procedures were attributed to the contrasting physicochemical behaviours of EtG and EtS which resulted in the analytes not being retained by the SPE sorbents and eluting in the load and wash solutions.

Sample preparation procedure two was the optimal procedure because it generated the highest response for EtG along with a high response for EtS. The responses generated by procedure three were also high however the EtG response for this procedure was lower than for procedure two. Since the ionization efficiency of EtG was poor, the procedure that generated the highest response for EtG was selected as the optimal sample preparation procedure.

6.2.4. MS response and sensitivity

The optimization of the response and sensitivity of the MS involved two aspects: the inclusion of a time-segment approach and the post-column addition of acetonitrile.

6.2.4.1. Time-segment approach

The inclusion of a time-segmented approach divided the total analysis time of the method into several timed-segments with each segment monitoring specific MS transitions (Kostić *et al.*, 2013, Calvo *et al.*, 2011). This allowed the MS/MS to, instead of focusing on all the MS transitions, focus on specific MS transitions within each time-segment which improved the sensitivity and specificity of the method (Kostić *et al.*, 2013, Stahl-Zeng *et al.*, 2007). Two timed-segments were created for this method: one for EtG and EtG-d5 (0 to 3 minutes) and the other for EtS and EtS-d5 (3 to 6 minutes).

6.2.4.2. Post-column addition of organic modifiers

Due to the highly polar nature of EtG and EtS, the chromatographic separation of these analytes on a reverse phase column requires mobile phases with a high-water content (Lamoureux *et al.*, 2009, Oppolzer *et al.*, 2016). These highly aqueous mobile phases are poor

solvents for electrospray ionization, due to water's high surface tension and high heat of evaporation and were associated with the poor ionization efficiencies and reduced responses of EtG and EtS, especially EtG (Lamoureux *et al.*, 2009). Organic modifiers such as acetonitrile and 2-propanol can be added post-column to enhance the ionization efficiencies of EtG and EtS without influencing the chromatographic separation (Janda *et al.*, 2002, Thierauf *et al.*, 2011).

For this method development, two different organic modifiers, at varying flow rates, were added post-column to assess the effects these modifiers had on the ionization efficiencies of EtG and EtS. The post-column addition of 2-propanol, at 0.2, 0.4 and 0.6 mL/min, did not enhance the ionization efficiencies of the analytes. However, the ionization efficiencies of both analytes were enhanced by the post-column addition of acetonitrile at a flow rate of 0.6 mL/min. The enhanced ionization efficiencies lead to an increase in the responses and S/N ratios of the analytes which lowered the LOQ of EtG and EtS. After the post-column addition of acetonitrile, the LOQ of EtG decreased approximately nine times while the LOQ of EtS decreased 2.5 times (Refer to Table 5.11).

6.2.5. Synthetic Vitreous Humour

Blank samples, calibration standards and quality controls used for this method were prepared by spiking a surrogate matrix with the analytes of interest. A surrogate matrix was required due to the limited availability of analyte-free post mortem vitreous humour samples (Hess *et al.*, 2018) and the method validation requirement whereby matrix-matched calibration standards and quality controls are required (Scientific Working Group for Forensic Toxicology, 2013, Elliott *et al.*, 2018).

This method used synthetic vitreous humour as a surrogate matrix. A method by Kummer et al., (2007) was used to synthesize this synthetic vitreous humour using hyaluronic sodium salt, agar and deionized water. Before this surrogate matrix was used, a verification study was performed to demonstrate that the physicochemical properties of the analytes of interest were equivalent in the surrogate and authentic matrix (Hess *et al.*, 2018). In this verification study, two calibration curves for each analyte were established: one in surrogate vitreous humour and the other in authentic vitreous humour (Refer to Figures 5.5 and 5.6). Thereafter, the slopes of the calibration curves for each analyte were compared using a t-test (Hess *et al.*, 2018).

For both EtG and EtS, the independent samples t-test indicated no significant difference between the slopes of the established calibration curves ($p>0.05$). The slopes of the established calibration curves are therefore equivalent which indicates that the sensitivity of the method is equivalent in both matrices and the physicochemical properties of the analytes of interest were the same in the surrogate and authentic matrix (Hess *et al.*, 2018). The

synthetic vitreous humour was therefore confirmed to be fit for use as a surrogate matrix for this method.

6.3. ETG, ETS METHOD VALIDATION

A full method validation was required for the newly developed method to simultaneously analyse EtG and EtS in vitreous humour (Magnusson, 2014). This method validation study was conducted to provide evidence that the method was fit for purpose and effective in producing results with a high degree of sensitivty and accuracy (Araujo, 2009, Magnusson, 2014). The method validation included the following performance characteristics: limit of detection and quantitation, working range and linearity, selectivity, accuracy, precision, measurement uncertainty, ruggedness, carryover and stability.

6.3.1. Limit of detection and limit of quantitation

The calculated LOD's for this method were 6.85 ppb and 0.27 ppb for EtG and EtS, respectively. The calculated LOQ's for EtG and EtS were 22.82 ppb and 0.883 ppb, respectively. The LOD and LOQ of the method was calculated by analysing ten blank samples (Magnusson, 2014). The blank samples consisted of sample matrix (vitreous humour) with no analytes of interest (EtG and EtS) (Magnusson, 2014).

6.3.2.1. Limit of Detection

The LOD's were calculated using Eq. 2.2. The International Union of Pure and Applied Chemistry (IUPAC) recommends the use of a factor of three to calculate LOD because this factor gives a confidence interval of 95% for false negative and false positive errors and limits the errors regarding the choice and validity of different confidence levels when LOD is being estimated (Analytical Methods Committee, 1987).

6.3.2.2. Limit of Quantitation

The LOQ's were calculated using Eq. 2.4. The IUPAC default value for calculating LOQ is ten because it corresponds with a RSD of 10% (Wenzl *et al.*, 2016, Analytical Methods Committee, 1987). A RSD of 10% calculates a LOQ value that has acceptable precision and bias (Magnusson, 2014). For this method, the lowest non-zero calibrators (EtG- 50 ppb and EtS- 5 ppb) were used as the LOQ's instead of the calculated LOQ's. The precision and accuracy of the lowest non-zero calibrator were evaluated with ease and is more acceptable than the calculated LOQ (Armbruster and Pry, 2008). The relevant acceptance criteria for precision and accuracy were met for the lowest non-zero calibrators of both EtG and EtS. Hence, the concentrations of these calibrators were used as the LOQ's for this method (Scientific Working Group for Forensic Toxicology, 2013). The LOQ's obtained for this method are suitable for the

application of this method in routine post-mortem forensic toxicology because this method can accurately and precisely quantify EtG and EtS concentrations below the concentration cutoff values of 100 ppb and 25 ppb, respectively (Høiseth *et al.*, 2010, Dasgupta, 2015)

6.3.2. Working range

The linear working range for this method was from 50 to 2 500 ppb for EtG and 5 to 1 000 ppb for EtS. This working range was similar to those quoted in literature which ranged from 25 – 2 000 ppb to 50 – 50 000 ppb for both analytes (Albermann *et al.*, 2012, Hegstad *et al.*, 2013, Favretto *et al.*, 2010, Thierauf *et al.*, 2011).

The determination of the upper and lower limits of this working range were based on two method specifications. Firstly, as stated by Albermann et al., (2012), the analysis of EtG and EtS in biological samples (blood and VH) requires a sensitive method that can detect and quantify analyte concentrations less than 100 ppb. This value indicates the abstinence of ethanol consumption over the previous 48 hours and allows for the interpretation of ethanol consumptions during this time period (Albermann *et al.*, 2012, Elrasheed *et al.*, 2017). Additionally, the upper limits of this working range were required to prevent the dilution and reanalysis of samples under circumstances whereby concentrations of EtG and EtS in the sample were greater than this limit.

6.3.3. Linearity

The linearity of this method was assessed by evaluating the correlation coefficient and residual plot of the establishing calibration model. The calibration model was established using the calibration curve of three different analytical runs (Magnusson, 2014). The correlation coefficients of the calibration models were 0.9998 for EtG and 0.9999 for EtS (Refer to Figure 5.7 and 5.8). Both residual plots illustrated residuals randomly distributed about zero with no outliers. There were also no systematic tendencies that were visually observed on the residual plots.

This method can be considered linear within the stipulated linear working ranges since the acceptance criteria for linearity were met for both EtG and EtS.

6.3.4. Selectivity

The selectivity of this method was associated with the use of a MS/MS analyser in MRM mode. MS/MS analysers have an extremely high selectivity in MRM mode because the analyser acts as a double mass filter with the first and third quadrupoles selecting specific precursor and product ions (Wong *et al.*, 2018, Kang, 2012). The high selectivity of the MS/MS analyser prevented the effect of commonly encountered interferences and limited the effect of matrix and internal standard interferences (Nielsen, 2010, Wong *et al.*, 2018). For this method validation, two independently evaluated interference studies were used to evaluate matrix and

internal standard interferences. In addition, a matrix effects study conducted to evaluate the effect (enhancement or suppression) of the matrix on the ionization of EtG and EtS.

6.3.1.1. Matrix interferences

Matrix interferences were assessed by analysing ten blank samples with no internal standard (Scientific Working Group for Forensic Toxicology, 2013). Subsequently, the S/N ratio for each analyte (EtG, EtS, EtG-d5 and EtS-d5) in each sample was calculated (Wille *et al.*, 2017). The S/N ratio of each analyte in each sample was less than three, the estimated LOD (Refer to Table 5.15) (Scientific Working Group for Forensic Toxicology, 2013). S/N ratios ≤ 3 indicate that the presence of interferences in the sample matrix were insignificant and matrix interferences were therefore absent (Wille *et al.*, 2017). The sample preparation procedure coupled with the selectivity of the MS/MS analyser prevented any significant matrix interferences (Wong *et al.*, 2018, Horwitz, 2002).

6.3.1.2. Internal standard interferences

The two internal standards, EtG-d5 and EtS-d5, used for this method were labelled with stable deuterium isotopes. The synthesis of these internal standards involved the substitution of five hydrogen atoms from EtG and EtS standards with five deuterium atoms (H^2) (Yuan *et al.*, 2019). These internal standards performed identically to EtG and EtS during sample preparation, chromatographic separation and MS/MS detection, due to their similar physicochemical properties (Yuan *et al.*, 2019).

The limitation of using stable-isotope labelled internal standard is that they can interfere with the analysis of the analytes of interest (Yuan *et al.*, 2019, Scientific Working Group for Forensic Toxicology, 2013). These internal standards can either contain the analytes of interest as an impurity or can contain fragment ions with the same m/z ratio as the fragment ions of the analyte of interest (Scientific Working Group for Forensic Toxicology, 2013, Yuan *et al.*, 2019). It is therefore imperative to assess the effect of the internal standard interferences on the analysis of the analytes of interest (Yuan *et al.*, 2019).

The internal standard interferences were evaluated by analysing ten blank samples containing both internal standards. Subsequently, the EtG and EtS concentrations of the blank samples were calculated and compared to the LOQ's of the method (Scientific Working Group for Forensic Toxicology, 2013). All ten blank samples had EtG and EtS concentrations below the LOQ's of the method (Refer to Table 5.16). Therefore, the internal standards did not interfere with the analysis of the analytes of interest.

6.3.1.3. Matrix effects

The percent matrix effects for EtG and EtS were calculated from Eq. 2.1 (Matuszewski *et al.*, 2003). Mean EtG and EtS concentrations for each sample type were calculated from the preparation and analysis of each sample five times. Sample type one consisted of a standard prepared in ultrapure water with an EtG and EtS concentration of 1000 ppb and 200 ppb, respectively. Sample type two was prepared by subjecting blank vitreous humour samples to the sample preparation procedure. Subsequently, these samples were spiked with EtG and EtS reference standards to a concentration of 1000 ppb EtG and 200 ppb EtS.

The percentage matrix effects for both EtG (97.43%) and EtS (101.15%) were within the acceptable range of 85-115% however both analytes were influenced by undetected, co-eluting matrix components. The matrix effects percentage of less than 100% indicated that the ionization of EtG was suppressed by these matrix components (Matuszewski *et al.*, 2003). In contrast, the ionization of EtS was enhanced, percent matrix effects greater than 100%, by these matrix components (Matuszewski *et al.*, 2003).

These observed matrix effects were compensated for through the use of stable-isotope labelled internal standards (Matuszewski *et al.*, 2003). The matrix exhibited the same ionization effects on these internal standards as the analytes of interest, due to the similar chromatographic retention times (Matuszewski *et al.*, 2003). These comparable ionization effects coupled with the use of the ratio of analyte-to-internal standard to calculate the analyte concentration allowed for the compensation of these matrix effects. The compensation of these matrix effects improved the accuracy and precision of the method.

The acceptance criteria were met for both interference studies as well as matrix effects. This method can therefore be considered selective for the analysis of EtG and EtS in vitreous humour.

6.3.5. Accuracy

The accuracy of the method was assessed by analysing each QC in triplicate across five analytical runs and calculating the percent accuracy for each QC using Eq. 2.6 (Scientific Working Group for Forensic Toxicology, 2013). The percent accuracies for EtG ranged from -1.31% to 1.37% while for EtS, the percent accuracies ranged from -0.19% to 1.45% (Refer to Table 5.18). This method was considered accurate since all the percent accuracies were less than ±10% (Scientific Working Group for Forensic Toxicology, 2013).

6.3.6. Precision

The precision of the method was assessed by evaluating both within-run and intermediate precision. The third type of precision, known as reproducibility, was not evaluated in this method validation because it requires samples to either be analysed in another laboratory or

with another method (Scientific Working Group for Forensic Toxicology, 2013). This was unachievable because no laboratories in South Africa have validated methods to analyse EtG and EtS in vitreous humour.

Within-run precision was evaluated by analysing each QC six times within a single analytical run. Subsequently, the percent RSD for each QC was calculated using Eq. 2.7 (Magnusson, 2014). For both EtG and EtS, the percent RSD's of each QC were below 10% (Refer to Table 5.19) and therefore the acceptance criteria for within-run precision were met.

The intermediate precision study was performed by analysing each QC sample in triplicate across three different analytical runs and calculating the percent RSD using Eq. 2.7 (Magnusson, 2014). Both analytes met the acceptance criteria for precision since the percent RSD's of all three QC's were less than 10% (Refer to Table 5.20).

The acceptance criteria for within-run and intermediate precision were met for this method validation and therefore the method to analyse EtG and EtS in vitreous humour is precise.

6.3.7. Measurement uncertainty

The measurement uncertainty of each analyte was independently calculated based on the procedure described by Ellison et al., (2012). This procedure is a stepwise process that involved four steps. These steps included a precision study, a bias study, the calculation of the influence of other factors on uncertainty and finally, the calculation of the combined and expanded uncertainty (Ellison *et al.*, 2012).

6.3.7.1. Precision study

The precision study was performed to determine the percent precision uncertainty (Ellison *et al.*, 2012). The calculated percent precision uncertainties were 3.02% and 2.64% for EtG and EtS, respectively. These percentages were calculated using Eq. 2.8 whereby the percent CV of each QC was determined by preparing and analysing each QC 18 times (Ellison *et al.*, 2012).

6.3.7.2. Bias study

The bias study, for each analyte, was conducting using the raw data from 18 analytical runs of QC3. This QC had the largest percent bias uncertainty compared to QC1 and QC2 and was therefore used to conduct this bias study.

The bias study consisted of two subsequent steps. Firstly, the percent bias uncertainty was calculated as 13.35% for EtG and 3.90% for EtS. This value was calculated using Eq. (2.11) which required the calculation of percent bias, Eq. (2.9) and the uncertainty of the mean, Eq. (2.10). Subsequently, the significance of the bias was determined (Ellison *et al.*, 2012). The

significance of the bias was determined using a t-test. A calculated t-value of 0.10 for EtG and 0.30 for EtS was obtained using Eq. (2.12). These calculated t-values was less than the critical t-value, $t(17) = 2.110$, at a 95% confidence interval. This deduced that, for both analytes, the uncertainty of the bias was insignificant and could be ignored in the calculation of the overall measurement uncertainty of the method.

6.3.7.3. Influence of other factors

The use of in-house prepared QC samples for this method validation specified that the other factors that influenced the measurement uncertainty were evaluated independently (Ellison *et al.*, 2012). These other factors included the uncertainty of the stock standard purity and the uncertainty of the pipettes used. The independent uncertainties of each of these factors were calculated and combined to obtain the influence of other factors on uncertainty (Ellison *et al.*, 2012).

The purity of the EtG and EtS stock standards was 99% which indicates an uncertainty of 1%. The conversion of this uncertainty into a qualified uncertainty involved treating this uncertainty as a rectangular distribution and dividing it by $\sqrt{3}$ (Ellison *et al.*, 2012). The qualified uncertainty for the purity of the EtG and EtS stock standards was 0.58%.

Two different pipettes were used to prepare each QC sample. The uncertainties of the pipettes, given as a SD, were obtained from the calibration certificates. The uncertainty of the 10-100µL pipette was 0.6µL and the 100-1000µL pipette had an uncertainty of 6.6µL. Since the calibration of the pipettes was done by an outside supplier, these uncertainty were converted into qualified uncertainties by treating with rectangular distributions and dividing them by $\sqrt{3}$ (Hwang *et al.*, 2017). The addition of the qualified uncertainties of each pipette gave a combined pipette uncertainty of 3.83%.

The combined uncertainty of other factors was calculated, using Eq. (2.13), by the addition of the stock standard uncertainty and the combined pipette uncertainty. The uncertainty of other factors was 3.87% for each analyte.

6.3.7.4. Combined and expanded uncertainty

The combined uncertainty was calculated, using Eq. (2.14), as 4.91% for EtG and 4.68% for EtS. The combined uncertainties were calculated using only the percent precision uncertainty and percent uncertainty of other factors. The expanded uncertainty, calculated using Eq. (2.15), was 9.82% for EtG and 9.36% for EtS. The value of the coverage factor, k, is associated with the level of confidence required for the method (Ellison *et al.*, 2012). For this method a level of confidence of 95% was required therefore a value of two was used as the coverage factor (Ellison *et al.*, 2012).

6.3.8. Ruggedness

The ruggedness of the method was assessed by evaluating the results obtained when small deliberate variations were made to the method parameters or environmental conditions (Peters *et al.*, 2007). The ruggedness of this method was assessed by varying the analyst and the environmental temperature during sample preparation. The environmental temperature ranged from 19.4 °C to 22.9 °C while two different analysts prepared and analysed the samples. The percent RSD of the results of each QC under the varying conditions were calculated using Eq. (2.7). The method to simultaneously analyse EtG and EtS in vitreous humour was considered rugged since the percent RSD for each QC was below 10% (Refer to Table 5.21) (Scientific Working Group for Forensic Toxicology, 2013).

6.3.9. Carryover

The evaluation of carryover deduced that this method had no sufficient carryover. The analysis of three blank samples after a QC3 sample resulted in EtG and EtS concentrations less than 20% of the LOQ's (EtG- 50 ppb and EtS- 5 ppb) of the method. The use of a strong and weak wash solution prevented any sufficient carryover by removing sample residues from the UPLC needle and system (Sargent, 2013).

6.3.10. Stability

The evaluation of stability involved two independent stability studies. These studies included a processed sample stability study and a long-term stability study (Peters *et al.*, 2007).

6.3.10.1. Processed sample stability study

The processed sample stability study was designed based on the maximum time that a sample will remain unanalysed after preparation (Scientific Working Group for Forensic Toxicology, 2013). The conditions of this study design involved a prepared sample being left in the auto sampler tray of the UPLC, at 4°C, for a period of 96 hours. This specific time period was decided on whilst alluding to different sample analysis conditions including large sample batches and instrument breakdown. The results of the reanalysed samples were within ±10% of the mean of the time zero results (Scientific Working Group for Forensic Toxicology, 2013). These results indicated that, under these conditions, the concentrations of EtG, EtS and the internal standards within the sample remained unchanged. The samples are therefore stable for 96 hours after sample preparation

6.3.10.2. Long-term stability study

The long-term stability study was designed based on the storage conditions of the vitreous humour samples after sample collection (Peters *et al.*, 2007). The samples were stored at

4 °C in 4 mL tubes, containing sodium fluoride (Bévalot *et al.*, 2016, Dinis-Oliveira *et al.*, 2010). This study was designed by initially analysing samples for time-zero results. The same samples were reanalysed after being stored under the above conditions for six months. The results of the reanalysed samples were within ±10% of the mean of the time zero results (Scientific Working Group for Forensic Toxicology, 2013). As with the processed sample study, the results of this study design indicated that the concentrations of EtG, EtS and the internal standards remained unchanged. The samples are therefore stable for up to three months when stored under these conditions.

6.4. PILOT STUDY

The two validated methods were applied to a pilot study of vitreous humour samples as a means to evaluate these validated procedures on true post-mortem VH samples and subsequently to critically analysing the obtained results. The vitreous humour samples were collected from 119 decedents received at the JHB FPS MLL but seven decedents were excluded from this study due to insufficient sample volumes to analyse all three analytes. The vitreous humour samples of the remaining 112 decedents were analysed for ethanol, EtG and EtS. This section critically discusses the analytical results of these analyses as well as the demographics of the decedents from which these samples were collected.

6.4.1. Demographics

The demographics of the 112 decedents were summarized according to age, sex and circumstance of death. Table 5.22 indicates that victims were most prevalent in age groups 25-34 and 35-44 years (both 28.6%; n=32). This age distribution deviates from the statistics obtained from Statistics South Africa which stated that, in 2016, 60.3% of unnatural deaths in South Africa occurred in people between the age of 25 and 34 years (Statistics South Africa, 2018). This distribution is, however, similar to the distribution reported in the 2011 NIMSS (National Injury Mortality Surveillance System) annual report on fatal injuries in Gauteng i.e. 27% of victims were in the age group of 25 to 34 years (Medical Research Council, 2013). The 2011 NIMSS report on Gauteng also reported that 79.2% of cases were male decedents which is comparable to this study where 83.0% (n=93) of the decedents were male (Medical Research Council, 2013) (Refer to Table 5.22).

The circumstance of death was categorised into 14 independent categorises as highlighted in Table 5.22. In the NIMSS report on Gauteng in 2011, violent deaths (deaths associated with assault, shootings or stabbings) were the leading circumstance of unnatural deaths and accounted for 30.5% of cases (Medical Research Council, 2013). In comparison, the population of this study had a greater proportion of deaths associated with violence (deaths

associated with assault, shootings or stabbings), 49% (n=55), with the most prevalent circumstance of death being shooting, 25.9% (n=29) of cases.

The case files of all the decedents did not contain information regarding the history of ethanol consumption and whether the decedent suffered from diabetes, eye diseases or other metabolic disorders. In addition, the official BAC results of decedents submitted for testing at the FCL JHB were not available due to administrative delays from the FCL.

6.4.2. Ethanol, EtG and EtS sample results

The eight categories of sample results, highlighted in Table 5.23, were determined based on the positive or negative ethanol, EtG and EtS results. A positive result was defined as a concentration greater than or equal to the LOQ of the method (0.01 g/ 100 mL for ethanol, 50 ppb for EtG and 5 ppb for EtS). In contrast, a negative result was defined as a concentration less than the LOQ of the method. Figure 4.4 was subsequently used to was used to determine ethanol intoxication 24 hours prior to death, to distinguish between ethanol consumption and post-mortem ethanol formation as well as to determine false positive and false negative ethanol, EtG and EtS results.

The last two columns of Table 5.23 indicate the number and percentage of cases, within each category, that had EtG and EtS concentrations between the LOQ of the method and specific cut-off values (EtG- 100 ppb, EtS- 25 ppb). These cut-off values are published EtG and EtS blood concentrations that distinguish between ethanol intoxication and abstinence over the 24 hours prior to death (Elrasheed *et al.*, 2017, Reisfield *et al.*, 2011, Jatlow *et al.*, 2014). There are no published cut-off values for EtG or EtS in VH however these blood cut-off values were used to interpret the results of this pilot study since ethanol, EtG and EtS passively diffuse between the blood and the VH (Łukasik *et al.*, 2018, Bévalot *et al.*, 2016).

No cases from this study population were categorized into category two (positive ethanol results accompanied with positive EtG and negative EtS results). Just less than a third of the study population (29.5%, n=33) did not consume ethanol 24 hours prior to death since the VH samples of these 33 decedents were negative for all three analytes ethanol, EtG and EtS (category eight) (Armer *et al.*, 2016, Høiseth *et al.*, 2007). One can deduce that in these 33 cases, ethanol consumption did not contribute to the circumstances or cause of death (Sundström *et al.*, 2014, Krabseth *et al.*, 2014).

6.4.3.1. Category one



Figure 6.1: The ethanol, EtG and EtS results for category one

More than a third of the samples from the study population (33.9%, n=38) were categorized into category one. These samples were positive for all three analytes which indicates that these decedents consumed ethanol within 24 hours of their death. The median ethanol, EtG and EtS concentrations of these samples were 0.171 (0.0639-0.247) g/ 100 mL, 833.70 (246.61-2090.94) ppb and 610.30 (332.81-1251.44) ppb, respectively. At these concentrations, the decedents would have experienced symptoms such as impaired balance, perception and memory as well as vomiting and drowsiness (Dubowski, 2006).

There were 3 cases (Case 27, 62 and 93) in this category that had EtG concentrations less than the cut-off value for abstinence i.e. 100 ppb (Elrasheed *et al.*, 2017). These 3 cases also all had low ethanol concentrations (<0.05 g/100 mL), with two of these cases having EtS concentrations just above the EtS cut-off value (Case 27- 33.733 ppb and Case 93- 38.395 ppb). From these results, it is suspected that these two decedents either: 1) did not consume an alcoholic beverage prior to death but instead used an ethanol-containing product, e.g. medication (cough syrup) or mouthwash, or 2) consumed a single alcoholic beverage prior to death, e.g. a 350 mL beer with an ethanol concentration of ~5% (Armer *et al.*, 2016, Maenhout *et al.*, 2013, Alcohol Research: Current Reviews Editorial, 2018). Further investigation into these cases indicated that prior to death, medical personal attempted to resuscitate one of the decedents (Case 93) which involved the administration of medication.

Case 62 had low ethanol and EtG concentrations (0.0176 g/ 100 mL and 81.881 ppb) associated with a high EtS concentration of 624.699 ppb. The decedents' cause of death was blunt force head trauma associated with falling while the SAPS 180 form suspected that the decedent consumed a large volume of ethanol prior to death. The low concentration of ethanol in the vitreous humour of the decedent indicated that ethanol consumption occurred more than 12 hours prior to death since most of the ethanol had been metabolised and eliminated from the body (Elrasheed *et al.*, 2017). The high EtS concentration however confirms that ethanol was consumed 24 hours prior to death (Armer *et al.*, 2016). It is suspected that the low EtG concentration was a result of either 1) kidney disease which causes the initial overexcretion of the analyte or 2) Gilbert's syndrome which is a genetic liver disease that limits the expression of the UGT enzyme in the liver and consequentially limited EtG formation (Helander and Dahl, 2005, Wurst *et al.*, 2004). The interpretation of the ethanol, EtG and EtS

results for this decedent deduces that, despite the suspicions made in the SAPS 180 form, the decedent did not consume large volumes of ethanol prior to death and therefore ethanol intoxication did not contribute to the death of the decedent, warranting further evaluation.

6.4.3.2. Category three



Figure 6.2: The ethanol, EtG and EtS results for category three

Eight cases (Case 1, 5, 20, 54, 56, 63, 81 and 115) (7.1%) from the study population were categorized into category three which, according to Figure 4.4, indicates that these decedents had consumed ethanol prior to death. The median concentrations of ethanol and EtS in the vitreous humour samples of these decedents were 0.0624 (0.0430-0.142) g/ 100 mL and 90.13 (58.034-358.65) ppb, respectively. These concentrations indicate that the decedents consumed low volumes of ethanol causing symptoms including reduced attention, judgement and control as well as some sensory-motor impairment (Dubowski, 2006).

The negative EtG results may have been caused by bacterial degradation or the limited formation of the analyte (Helander and Dahl, 2005, Wurst *et al.*, 2004). The infection of the vitreous humour samples, prior to death, by bacteria with β-glucuronidase activity may have degraded the EtG to result in false negative results (Helander and Dahl, 2005). Alternatively, it is feasible to suspect that the decedents suffered from Gilbert's syndrome or kidney disease (Wurst *et al.*, 2004, Krabseth *et al.*, 2014).

In Case 54, the negative EtG result was associated with high ethanol (0.313 g/ 100 mL) and EtS concentrations (4661.407 ppb) while the case file of the decedent suggested that the decedent suffered from liver disease. Unfortunately, there was limited information regarding the type of liver disease however if the decedent suffered from Gilbert's syndrome then the negative EtG result can be justified by the absence of the UGT enzyme (Wurst *et al.*, 2004).

6.4.3.3. Category four

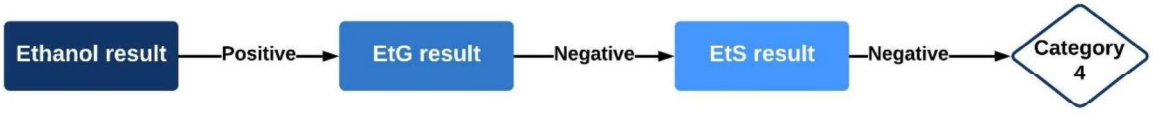


Figure 6.3: The ethanol, EtG and EtS results for category four

One (Case 97) of the 112 cases was categorized into category four because this case had a positive ethanol result (0.0373 g/ 100 mL) associated with negative EtG and EtS results which

indicates that the decedent did not consume ethanol prior to death (Armer *et al.*, 2016). Upon further investigation, it was found that the decedent died on a Friday evening and was only autopsied the three days later on the Monday. Taking this information into account, one can deduce that the false positive ethanol result may have been associated with the post mortem neoformation of ethanol in the vitreous humour sample which, despite its rarity, is plausible if the decedent suffered from diabetes (Kugelberg and Jones, 2007). In diabetic cases, the vitreous humour of the decedent has excess glucose which microorganisms use, as a substrate, for the neoformation of ethanol (Kugelberg and Jones, 2007). This false ethanol result may have had major implications on the interpretation of the cause of death by the forensic pathologist because without the EtG/EtS results, the forensic pathologists may have deduced that ethanol intoxication contributed to the death of the decedent however the decedent did not consume ethanol (Ziavrou *et al.*, 2005, Jones, 2019b).

6.4.3.4. Category five



Figure 6.4: The ethanol, EtG and EtS results for category five

Eight cases (Case 4, 17, 19, 47, 78, 84, 98, 104) (7.1%) from the study population were categorized into category five with negative ethanol results along with positive EtG and EtS results. These results indicate that these decedents consumed ethanol more than 12 hours prior to death (Høiseth *et al.*, 2007, Armer *et al.*, 2016). After 12 hours, ethanol is metabolised and eliminated from blood and vitreous humour therefore ethanol analysis of these samples was negative (Høiseth *et al.*, 2007).

Case 47 had a high EtG (1559.660 ppb) and EtS (938.212 ppb) concentrations which indicated that the decedent was a chronic alcoholic (Høiseth *et al.*, 2009). According to the case history, the decedent died at 07:00 after being stabbed in the abdomen. The ethanol, EtG and EtS results along with the case history of this decedent suggests that, despite the decedent being a chronic alcoholic, he did not consume ethanol 12 hours prior to death and therefore ethanol consumption did not contribute to his death.

There were six cases that had EtG or EtS concentrations less than the cut-off values for abstinence (EtG- Case 98 and EtS- Case 4, 17, 19 and 104). The EtG and EtS results of these six cases indicates that these decedents either: 1) did not consume an alcoholic beverage prior to death but instead used an ethanol-containing product, e.g. medication (cough syrup) or mouthwash, or 2) consumed a single alcoholic beverage prior to death, e.g. a 350 mL beer

with an ethanol concentration of ~5% (Armer *et al.*, 2016, Maenhout *et al.*, 2013, Alcohol Research: Current Reviews Editorial, 2018).

6.4.3.5. Category six

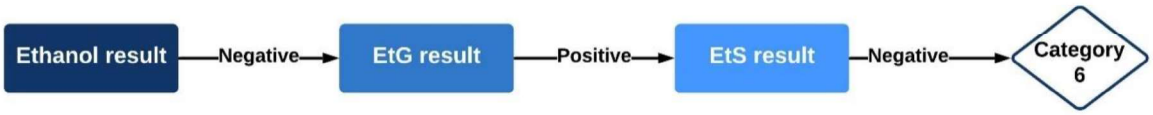


Figure 6.5: The ethanol, EtG and EtS results for category six

Category six comprised of 12.6% (n=14) of the cases (Case 24, 29, 33, 42, 44, 82, 83, 90, 91, 94, 96, 100, 101, 108) which tested negative for ethanol and EtS and positive for EtG. No ethanol was consumed by these decedents prior to death because no EtS was detected in the vitreous humour samples (Armer *et al.*, 2016). The EtG results had a median of 71.68 (62.09-121.00) ppb while 64.3% (n=9) of these cases had an EtG concentration less than the cut-off value.

The positive EtG results can be attributed to either post mortem formation or dermal ethanol exposure (Helander *et al.*, 2007, Nanau and Neuman, 2015). EtG can be formed in post mortem vitreous humour samples if the samples are infected by *Escherichia coli* and ethanol is present however this post mortem formation of EtG was highly unlikely in these cases due to the absence of ethanol (Helander *et al.*, 2007). These positive EtG results can potentially be attributed to dermal ethanol exposure from the use of ethanol-based hand sanitizers (Nanau and Neuman, 2015). The negative EtS results can further argue this hypothesis because EtS concentrations are not affected by dermal ethanol exposure however, more information is required to prove this hypothesis (Reisfield *et al.*, 2011).

6.4.3.6. Category seven

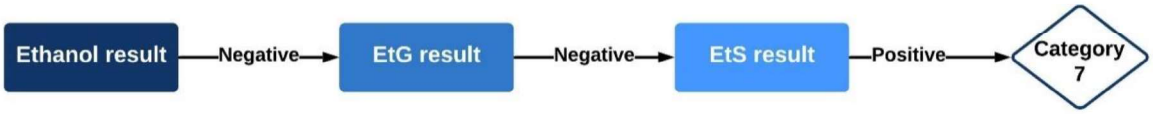


Figure 6.6: The ethanol, EtG and EtS results for category seven

There were ten cases (8.9%) (Case 15, 35, 49, 68, 69, 72, 73, 77, 107, 119) within this study population that were categorized into category seven because these cases tested negative for ethanol and EtG and positive for EtS. The median EtS concentration of these cases was 17.99 (14.32-53.85) ppb with the EtS concentration being less than the cut-off value in 80.0% (n=8) of these cases.

The positive EtS results suggest that these decedents consumed ethanol however the negative ethanol results and low median EtS concentrations suggest this ethanol consumption was more than 12 hours prior to death (Armer *et al.*, 2016, Høiseth *et al.*, 2007). The negative EtG results can be attributed to either the limited formation or degradation of the analyte (Helander and Dahl, 2005, Wurst *et al.*, 2004). It is possible that the decedents in this category suffered from disorders which prevented or limited the formation of EtG (Wurst *et al.*, 2004, Krabseth *et al.*, 2014). Alternatively, prior to death, the eyes of the decedents may have been infected by bacteria with β-glucuronidase activity. The presence of these bacteria in the vitreous humour degraded the EtG by bacterial hydrolysis resulting in negative results (Helander and Dahl, 2005).

6.4.3. Samples with positive results

There were 79 (70.5%) samples from the study population that were positive for at least one analyte. This excluded the 33 samples from category eight that tested negative for all three analytes. The ethanol, EtG and EtS results of these 79 samples were evaluated using descriptive and inferential statistics. Descriptive statistics were evaluated to compare the results of this study with results from literature while inferential statistics were used to compare the results of the three analytes.

6.4.3.1. Descriptive statistics

The following descriptive statistics, as highlight in Table 5.23, were calculated from the concentrations of each analyte in the 79 positive samples: mean, standard deviation, median, upper and lower quartiles and maximum and minimum. The ethanol concentrations were reported as g/100mL however for inferential statistics, these concentrations were converted to ppb in order for all three analytes to have the same units of measurement.

- Ethanol

The 79 vitreous humour samples had a mean ethanol concentration of 0.0961 (±0.117) g/100mL with a median concentration of 0.0480 (0.000-0.175) g/100mL. At this mean ethanol concentration, the decedents would have experienced physiological symptoms ranging from elation and talkativeness to some sensory-motor impairment and reduced attention, judgement and control (Dubowski, 2006). There were three vitreous humour samples that had ethanol concentrations greater than 0.35 g/ 100mL, literature states that these ethanol concentrations cause unconsciousness and possible death (Dubowski, 2006). However, the cause of death in these cases was not stipulated as acute alcohol poisoning which is an indication of ethanol tolerance (Garriott and Aguayo, 2008).

The mean VH ethanol concentration obtained in this study was substantially less than the mean concentrations obtained in literature (Honey *et al.*, 2005, Thierauf *et al.*, 2011). According to a study conducted by Honey *et al.* (2005), the mean ethanol concentration of 209 vitreous humour samples obtained from an American population was 0.230 g/ 100 mL. A similar study conducted by Thierauf *et al.* in 2011, on 26 vitreous humour samples from a German population, had a mean ethanol concentration of 0.201 g/ 100 mL. These studies did however exclude samples with negative ethanol concentrations. Additionally, these studies were conducted on populations with contrasting socioeconomic classes compared to the population of this study. There are however no published studies on ethanol concentrations in vitreous humour from developing populations.

- EtG

The mean and median EtG concentrations of these 79 vitreous humour samples were 735.28 (±1368.38) ppb and 127.85 (54.26-932.16) ppb, respectively. These statistics indicate that, in general, the sample population had EtG concentrations associated with the consumption of ethanol at least 24 hours prior to death (Elrasheed *et al.*, 2017). Five cases (Case 36, 43, 55, 75 and 111) within this study population had EtG concentrations greater than 3000 ppb with a maximum concentration of 7596.776 ppb. These high EtG concentrations suggest that the decedent was either a chronic alcoholic or a heavy drinker (Høiseth *et al.*, 2009).

The median EtG concentration obtained in this study is comparable to the results obtained by Keten *et al.* (2009) and Vezzoli *et al.* (2015). Keten *et al. (2009)* conducted a study on 25 vitreous humour samples, obtained from a Turkish population, that had a median EtG concentration of 90 ppb (Keten *et al.*, 2009). In 2015, Vezzoli *et al.* conducted a similar study that showed that the median EtG concentration of 63 vitreous humour samples from an Italian population was 220 ppb (Vezzoli *et al.*, 2015). Two additional studies from literature presented mean EtG concentrations of 4200 ppb and 2060 ppb however these studies only analysed vitreous humour samples from decedents with a history of ethanol consumption (Rainio *et al.*, 2013, Thierauf *et al.*, 2011).

- EtS

The mean EtS concentration of the 79 positive samples was 533.47 (±878.31) ppb while the median concentration of the samples was 102.00 (8.44-705.25) ppb. In general, since these statistical results are greater than 25 ppb, these 79 decedents consumed ethanol 24 hours prior to death (Dasgupta, 2015). There were seven decedents (Case 41, 43, 48, 50, 54, 55, 75) with an EtS concentration greater than 1500 ppb with a maximum concentration of 4661.41

ppb. In these seven cases, the high EtS concentrations suggest that the decedent was either a chronic alcoholic or a heavy drinker (Høiseth *et al.*, 2009).

In literature, there was only one study that analyses EtS in vitreous humour samples. This study was conducted by Thierauf *et al.* in 2011 and analyses 26 vitreous humour samples obtained from a German post-mortem population. The mean EtS concentration of the 26 samples was 920 ppb (Thierauf *et al.*, 2011). This mean EtS concentration obtained by Theirauf *et al.* was greater than the mean EtS concentration obtained in this study. However, the study by Theirauf *et al.* sampled vitreous humour from decedents with a history of ethanol consumption and as a result the mean EtS concentration of the population was inflated.

6.4.3.2. Inferential statistics

The concentrations of all three analytes had non-parametric distributions. Despite attempting various types of data transformations, the raw data could not be manipulated to fit the assumptions of normality. The difference and associations between the concentrations of ethanol and EtG/EtS were therefore assessed using non-parametric tests. For these non-parametric tests, the units of measurement for all three analytes was ppb.

- Differences between ethanol and EtG/EtS

A Kruskal-Wallis test was used to assess the differences between the ethanol, EtG and EtS concentrations of the 79 vitreous humour samples. The null hypothesis for this test was that there was no difference between the mean ranks of the three analytes i.e. the analyte concentrations were the same. The Kruskal-Wallis test results indicated that there was a significant difference between at least one of the mean ranks of the analytes since the p-value was less than 0.05. These results provide strong evidence that there is a difference between the concentrations of at least one of the analytes.

A Dunn's multiple comparisons of mean ranks *post hoc* test was conducted to determine which mean ranks were significant hence determining which analyte/s had different concentrations. The results of the Dunn's test, described in Table 5.24, indicate the p-values and mean rank for each analyte combination e.g., ethanol to EtG, ethanol to EtS and EtG to EtS. The p-values indicate that there was a significant difference between the mean ranks of ethanol and EtG (p-value=0.014) and ethanol and EtS (p-value=0.0044). There was no significant difference between the mean ranks of EtG and EtS (p-value=1.00). These results suggest that the ethanol concentrations of the 79 vitreous humour samples were not the same as either the EtG or EtS concentrations however the concentrations of EtG and EtS in the 79 samples are equivalent. The mean ranks indicate that the ethanol concentration (mean rank: 140.886 ppb)

of the 79 samples was greater than the EtG (mean rank: 109.945 ppb) and EtS concentrations (mean rank: 106.158 ppb).

The results obtained from these non-parametric tests were hypothesised prior to analysis based on the pharmacokinetics of consumed ethanol. The concentrations of EtG and EtS in the vitreous humour were expected to be less than the concentration of ethanol because only a small percentage of consumed ethanol is metabolised to form these metabolites (Dinis-Oliveira, 2016). According to Dinis-Oliveira (2016) and Maenhout *et al.* (2013), between 0.5% and 1.5% of consumed ethanol is metabolised to form EtG while approximately 0.1% is metabolised to form EtS. The high concentration of ethanol in these vitreous humour samples was associated with hydrophilic properties and small molecule weight of ethanol along with the high water content of vitreous humour (National Center for Biotechnology Information, 2019, Dinis-Oliveira *et al.*, 2010). These properties allow ethanol to diffuse, through the BRB, from a lower water content in the blood to a higher water content in the vitreous humour (Łukasik *et al.*, 2018).

- Associations between ethanol and EtG/EtS

In the previous section the differences between the ethanol, EtG and EtS results were established. In this section, the association (also known as relationship) between the three analytes will be established. The association between the ethanol concentrations and the EtG/EtS concentrations of the 79 positive vitreous humour samples was investigated using Spearman's rank-order correlation test because the distribution of the data was non-parametric. The significant Spearman's rank-order correlation coefficients of 0.800 ($df=77$, $p<0.001$) between ethanol and EtS and 0.606 ($df=77$, $p<0.001$) between ethanol and EtG provides evidence that there is a better association between ethanol and EtS than between ethanol and EtG.

The Spearman's rank-order correlation test results suggest that, in vitreous humour, EtS is a more suitable analyte to predict ethanol consumption prior to death than EtG. This further supports the conclusions made by Krabseth *et al.* (2014) and Høiseth *et al.* (2010) which suggests that EtS is the gold standard biomarker to predict ethanol consumption prior to death. However, the low correlation coefficients of 0.800 and 0.606 indicate that various other factors play a role in the relationship between ethanol and these alcohol biomarkers. Literature suggests that there is no association between ethanol and EtG/EtS concentrations because analyte concentrations are influenced by various factors including interindividual variations in medical history, pharmacokinetics, ethanol consumption patterns and post-mortem redistribution and putrefaction (Wurst *et al.*, 2004, Thierauf *et al.*, 2011).

6.4.4. Association between EtG and EtS

Simple linear regression was used to evaluate the association between the EtG and EtS concentrations of the 79 positive vitreous humour samples. The correlation coefficient (r=0.718) of the scatterplot, Figure 5.9, suggests that there is a strong, positive relationship between EtG and EtS. This relationship is attributed to the similar pharmacokinetic processes and detection windows of these biomarkers (Maenhout *et al.*, 2013, Wurst *et al.*, 2006).

The relationship between EtG and EtS was statistically significant ($p<0.001$) and had a r^2 value of 0.516. The r^2 value indicates that half, 52%, the vitreous humour samples had EtG and EtS concentrations that were related by the equation, $EtS = (0.46 \times EtG) + 194.56$, which implies that various other factors influence the relationship between EtG and EtS. According to literature, this relationship is influenced by diseases where the activity of the UGT enzyme is absent or limited, e.g., Gilbert's syndrome (Krabseth *et al.*, 2014, Maenhout *et al.*, 2013). Other factors that influence this relationship include the post-mortem stability of EtG and dermal ethanol exposure (Helander and Dahl, 2005, Nanau and Neuman, 2015).

The non-parametric distributions of the EtG and EtS raw data suggested that the results obtained from the simple linear regression analysis be verified using a Spearman's rank-order correlation test. The non-parametric results of this test indicated a significant Spearman's rank-order correlation coefficient of 0.664 ($df=45$, $p<0.01$) which provided further evidence of the strong, positive relationship between EtG and EtS.

6.4.5. Ratio of EtG to EtS

There were 46 vitreous humour samples that were positive for both EtG and EtS. The ratio of EtG to EtS was calculated for these samples and illustrated in Figure 5.10. The ratios ranged from 0.13 to 47.48 with a mean ratio of 3.56 (±7.67) and a median of 1.44 (1.013-2.17). The distribution of these 46 ratios illustrate that there was no exact ratio between EtG and EtS however 50% of the samples had a ratio between 1.013 and 2.17. These results are in agreement with Reisfield *et al.* (2011) and Thierauf *et al.* (2011) who both concluded that there is no apparent relationship between the concentrations of EtG and EtS in biological samples. Within these 46 ratios, six outliers were identified using the $1.5 \times IQR$ rule which included case 4, 17, 19, 84, 104 and 111. All six of these cases had relatively low EtS concentrations associated with high EtG concentrations. Various assumptions can be made regarding the high EtG concentrations of these samples. Firstly, the vitreous humour samples could have experienced post mortem EtG formation by Escherichia coli (Helander *et al.*, 2007). Alternatively, these high EtG concentrations may be attributed to dermal ethanol exposure through the use of ethanol-based hand sanitizers (Reisfield *et al.*, 2011)

6.4.6. Application in post-mortem forensic toxicology

The results of this pilot study highlighted several important facts related to the analysis of ethanol, EtG and EtS in VH for post-mortem forensic toxicology. These results further justified the conclusion, by Høiseth *et al*. (2010) and Krabseth *et al.* (2014), that the primary post-mortem application of EtG and EtS analysis is to differentiate between ante mortem ethanol consumption and post-mortem formation. There was one decedent from this study that had EtG, EtS and ethanol results that indicated post mortem ethanol formation (category 4) however if ethanol analysis was conducted in blood instead of VH, this phenomenon would be considerably higher because blood is more susceptible to post mortem ethanol formation than VH (Dinis-Oliveira *et al.*, 2010). Unfortunately, it was not possible to obtain BEC results for this study population.

This pilot study provided examples of how the analysis of these analytes can aid pathologists in clarifying the series of events leading up to the death of the decedent (Sundström *et al.*, 2014). An example of this is the decedent (Case 62) who was suspected of consuming large volumes of ethanol prior to falling to their death. The EtG, EtS and ethanol results however indicated that the decedent consumed ethanol more than 12 hours prior to death. This important piece of information may assist the pathologist, police and insurance companies in determining the actual circumstances of death as well as whether the decedent's death was caused by foul play. This study also illustrates how the analysis of these analytes can assist a pathologist in determining whether a decedent suffered from a specific disease or disorder. There were a handful of decedents who, after the analysis of these analytes, were identified as suffering from chronic alcoholism, heavy drinking, diabetes, kidney diseases or liver diseases e.g., Gilbert's syndrome.

This study did however indicate the short comings of solely using EtG and EtS results to determine ethanol intoxication for forensic purposes (Elrasheed *et al.*, 2017). The inferential statistics evaluated indicated that not only did the VH EtG/EtS results significantly differ from the VH ethanol results but that there was also a weak relationship between the ethanol and EtG/EtS results. This further justifies publications that conclude that various factors influence the concentration of EtG and EtS in biological samples and therefore these analytes should not be used independently but in conjunction with BEC to interpret ethanol intoxication (Wurst *et al.*, 2004, Thierauf *et al.*, 2011, Elrasheed *et al.*, 2017).

Finally, the results of this pilot study indicate that EtS is a more suitable analyte to determine ethanol intoxication prior to death than EtG which supports that statements made by Krabseth *et al.* (2014) and Høiseth *et al.* (2010). EtS has a greater specificity to ethanol consumption than EtG because EtS is more stable and can distinguish ethanol consumptions from dermal ethanol exposure (Dinis-Oliveira, 2016, Reisfield *et al.*, 2011).

CHAPTER 7: LIMITATIONS AND RECOMMENDATIONS

This chapter will briefly describe the limitations that were encountered during this study as well as any future recommendations.

7.1. LIMITATIONS

The limitations of this study were primarily attributed to the collection of data. The main limitation was that the requested BEC results for the sample population were not available at the time of data analysis due to administrative delays at the JHB FCL. The lack of these results prevented the researcher from comparing blood ethanol results to VH ethanol, EtG and EtS results which could have provided additional perspectives to this study. The limited information on the history of each decedent (e.g., recent ethanol consumption, circumstances and cause of death) further hindered the interpretative aspect of the ethanol, EtG and EtS results.

7.2. RECOMMENDATIONS

This study revealed several topics in which publications are limited including reference values relating EtG and EtS concentrations to physiological symptoms, pharmacokinetic studies on EtG and EtS in a South African population and comparisons of different ethanol biomarkers across various biological samples. Such research would provide valuable information to the usefulness of EtG and EtS analysis for both ante mortem and post-mortem applications. I highly recommend that any future research studies on this topic be conducted by analysing ethanol, EtG and EtS in both vitreous humour and blood samples as this would provide valuable information for post-mortem forensic toxicology.

As described previously, the analysis of EtG and EtS in post-mortem forensic toxicology has several benefits when it comes to determining ethanol intoxication prior to death. The researcher would recommend that forensic pathologists and toxicologists request the analysis of these analytes in conjunction with BEC analysis in order to differentiate between ante-mortem ethanol consumption and post-mortem formation as well as to providing additional information on the series of events prior to the death of a decedent.

CHAPTER 8: CONCLUSION

The aim and objectives of this study were met by dividing the study into three processes. Initially methods were developed to analyse ethanol as well as EtG and EtS in VH, these methods were subsequently validated before being used to analyse ethanol, EtG and EtS of 112 post-mortem VH samples from a JHB population. The method to analyse ethanol in VH was modified from a validated method used to analyse ethanol in blood using HSGC-FID. The modifications made to the method included a smaller sample volume and an alternative GC column. The method to simultaneously analyse EtG and EtS in VH using UPLC-MS/MS was developed and optimized based on literature. The optimal sample preparation procedure used protein precipitation with 0.1% formic acid in acetonitrile. A Phenyl-Hexyl UPLC column with a gradient elution profile (0.1% formic acid in water and 0.1% formic acid in acetonitrile) provided the optimal chromatographic resolution and the analytes were quantified using ESI negative ionization in MRM mode. The ionization efficiency of the analytes was enhanced by the post-column addition of acetonitrile.

Both methods met all the acceptance criteria for the method validation performance characteristics and were therefore deemed fit for purpose across the following linear ranges: ethanol (0.01-0.5 g/100mL), EtG (50-2500 ppb) and EtS (5-1000 pp). The measurement uncertainties for these methods were 3.11%, 9.82% and 9.36% for ethanol, EtG and EtS, respectively. All results less than the method LOQ's (ethanol- 0.01 g/100mL, EtG- 50 ppb and EtS- 5 ppb) were reported as <LOQ.

The pilot study results highlighted the various benefits, for forensic pathologists and toxicologists, of analysing EtG and EtS in post-mortem VH samples including: 1) to differentiate between ante mortem ethanol consumption and post-mortem formation; 2) to clarify circumstances of death and 3) identify whether a decedent suffered from a specific disease or disorder. Despite the benefits, the study also identified the short comings of solely using EtG and EtS results to determine ethanol intoxication for forensic purposes (Elrasheed et al., 2017). Finally, the pilot study indicated that EtS is a more suitable analyte to determine ethanol intoxication prior to death than EtG because EtS is a more stable analyte in post-mortem samples and can distinguish ethanol consumption from dermal ethanol exposure. This is of particular importance during the current pandemic where the use of ethanol-based hand sanitizers has drastically increased worldwide.

In conclusion, the analysis of EtG and EtS in either post-mortem blood or vitreous humour samples should be considered as a routine test when pathologist request BEC tests. The interpretation of these results can provide extremely useful information that can assist forensic pathologists, police services and insurance companies in death investigations.

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APPENDICES

APPENDIX 1: ETHICS CLEARANCE CERTIFICATE

R14/49 Mr Gareth Riley

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M171152

NAME: (Principal Investigator)	Mr Gareth Riley
DEPARTMENT:	Forensic Medicine and Pathology National Institute of Occupational Health
PROJECT TITLE:	The Determination of Ethyl Glucuronide and Ethyl Sulphate in Vitreous Humour as Biomarkers of Recent Alcohol use in Post Mortem Forensic Cases using Liquid Chromatography Tandem Mass Spectrometry
DATE CONSIDERED:	24/11/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Boitumelo Kgarebe and Ildiko Fenyvesi
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	22/12/2017

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 Research Office Secretary on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised 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 to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review November and will therefore be due in the month of November each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 24/01/2018.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 2: DATA COLLECTION SHEET

Data Collection Sheet

Body number: _____ Pathologist: _____

Research number: _____ Date of autopsy: _____

Demographics:

Age: _____ Sex: Male | Female

Race: White | Black | Indian | Coloured

Death information:

Date of death: _____ Time of death: _____

Circumstances leading up to death (SAPS 180 form): _____

Pathologists cause of death: _____

Reported BAC (g/ 100 mL): _____

Ethanol in vitreous humour:

Date of analysis: _____

Ethanol concentration in VH (g/100ml):

1= _____
2= _____
Average= _____
%CV= _____

EtG and EtS in vitreous humour

Date of analysis: _____

EtG concentration in VH (mg/L):		EtS concentration in VH (mg/L):	
1=		1=	
2=		2=	
Average =		Average =	
%CV=		%CV=	

APPENDIX 3: HAMILTON DILUTOR VERIFICATION FORM

Section A: General Information

Date	
Name of Analyst	

Section B: Balance Information

Make and Model of Balance		
Mass of Calibrated weight used to verify (g)	Reading (g)	Range
1		
2		
10		
20		

Section C: Dilutor Information

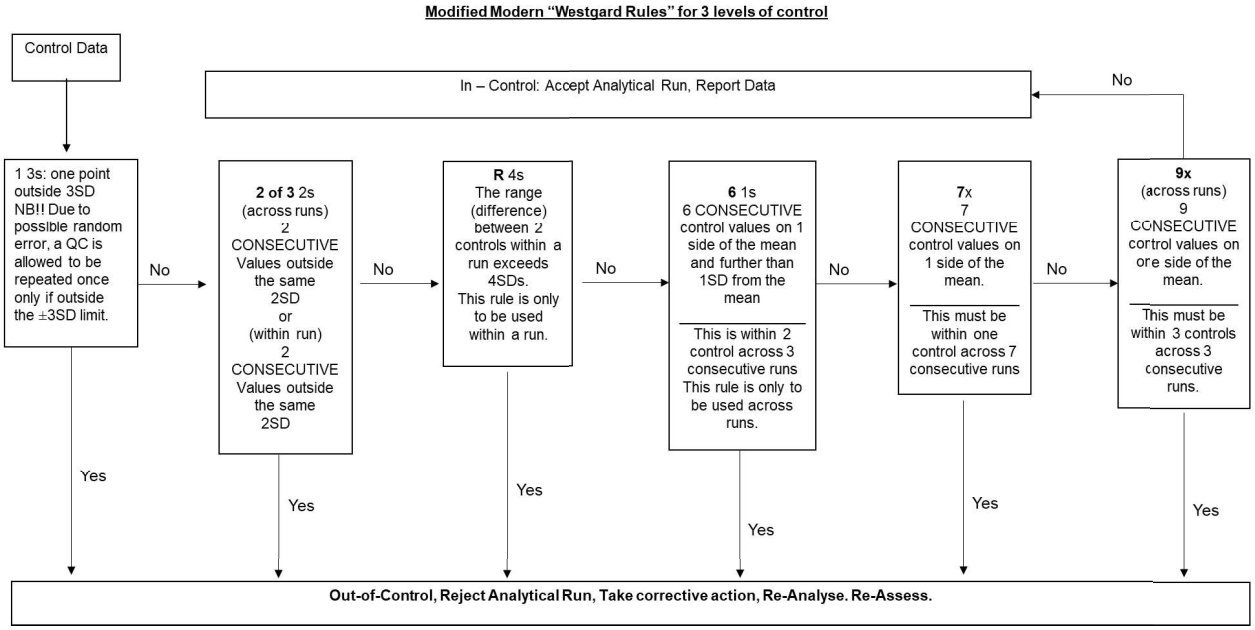
Make and Model of Dilutor			
Unique ID Number			
Temperature of H_2O Being used		Density of H_2O (Tanaka *et al.*, 2001)	

Section D: Verification

Item Weight (g)	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
1. Vial with H_2O dispensed					
2. Empty Vial					
3. Water dispensed (point 1 – point 2)					
4. Average weight (Sum of point 3 ÷ 5)					
5. Calculated average volume (point 4 ÷ density)					
6. % Volume (point 5 ÷ set volume × 100)					

Analyst Signature: ……..

APPENDIX 4: MODIFIED MODERN "WESTGARD RULES"

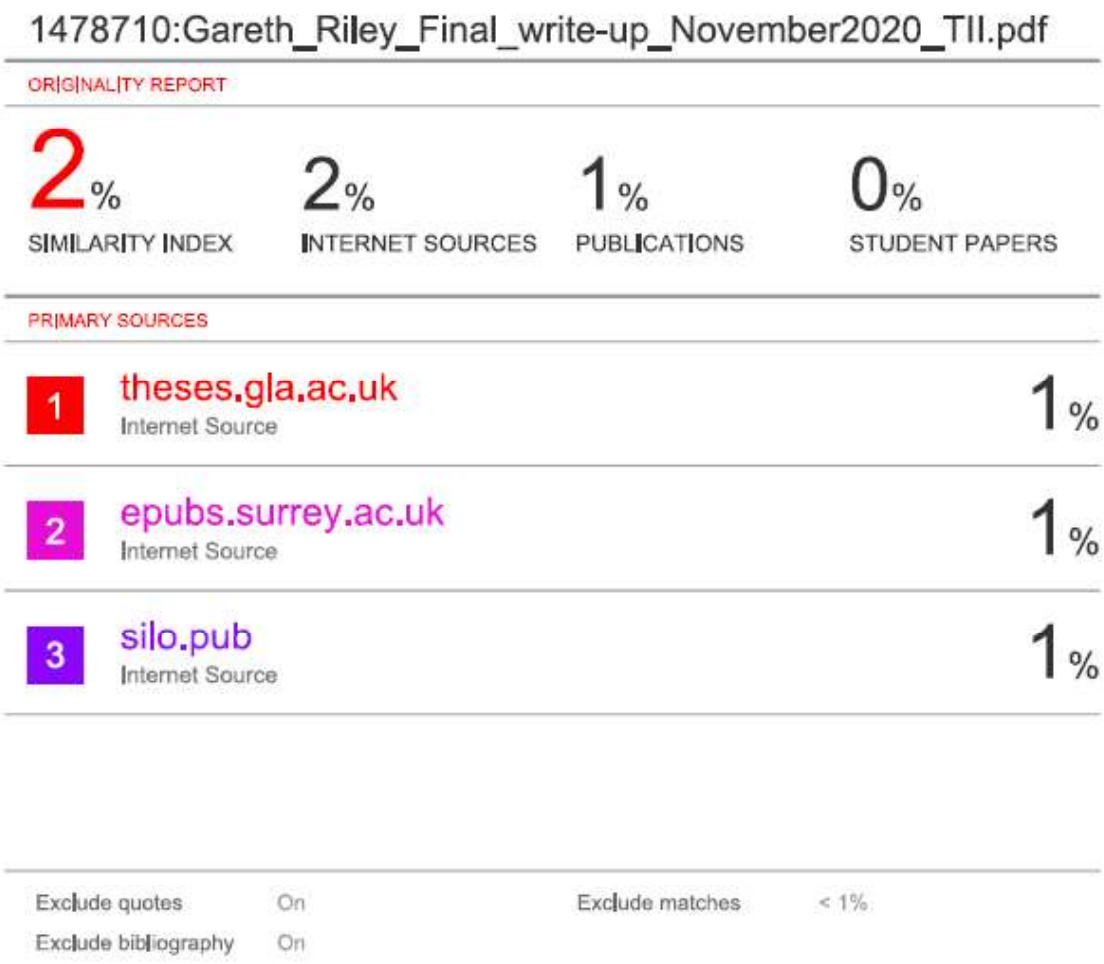


APPENDIX 5: RAW DATA

Case number	Age (years)	Race	Sex	PMI (days)	Category of death	Circumstances of death (according to SAPS 180)	EtOH in VH (g/100ml)	EtG (ppb)	EtS (ppb)
1	35	Black	Male	2	Shot	Deceased shot 5 times	0.048	<LOQ	228.874
2	35	Black	Male	2	PVA	Hit by car (PVA)	Insufficient sample	Insufficent sample	Insufficient sample
3	44	White	Male	1	Overdose	Went to and didn't wake up	0.01955	246.605	171.993
4	35	Black	Male	2	Shot	Deceased shot 7 times	<LOQ	102.791	8.441
5	40	Black	Male	3	Shot	Deceased shot 4 times	0.154	<LOQ	542.423
6	24	Black	Female	2	Overdose	Deceased consumed tablets and died on arrival to hospital	<LOQ	<LOQ	<LOQ
7	19	Black	Male	3	Assault	Found on train tracks. No indications of being hit by train	<LOQ	<LOQ	<LOQ
8	45	Black	Male	3	PVA	Deceased found with multiple injuries (PVA)	0.3825	Insufficent sample	Insufficient sample
9	47	Asian	Male	2	Stab	Multiple stabbings to chest with knife	0.141	126.625	101.999
10	42	Black	Male	2	Assault	Assaulted	0.2845	565.487	512.562
11	35	Black	Male	2	PVA	Deceased hit by car while crossing the road (PVA)	0.237	415.273	514.985
12	UNK	Black	Male	4	Natural	Homeless man went to sleep and didnt wake up	0.1505	2421.882	1317.073
13	28	Black	Male	2	Shot	Deceased shot in chest	0.16	417.382	595.903
14	18	Black	Female	2	Electrocuted	Electrocution	<LOQ	<LOQ	<LOQ
15	26	Black	Male	1	Hanging	Hung himself from a door	<LOQ	<LOQ	4.665
16	29	Black	Male	3	Shot	Multiple GSW	<LOQ	<LOQ	<LOQ
17	41	Black	Male	2	Electrocuted	Electrocution	<LOQ	137.060	8.519
18	44	Black	Male	1	Poisoning	Poisoning	0.159	1169.466	923.629
19	32	Black	Male	3	Poisoning	Poisoning	<LOQ	264.969	5.581
20	33	Black	Male	1	MVA	MVA	0.1305	<LOQ	43.093
21	35	Black	Male	2	PVA	PVA	0.2485	1265.712	705.251
22	UNK	Black	Male	3	Stab	Deceased stabbed in right arm and left shoulder	<LOQ	<LOQ	<LOQ
23	24	White	Female	2	Hanging	Deceased found hanging from waldrobe handle	0.243	2090.943	549.329
24	30	Black	Male	2	Assault	Deceased found on street lying face down with open wounds on head	0.27	1293.403	993.365
25	42	Black	Male	2	Shot	Shot by unknown male	<LOQ	211.523	<LOQ
26	27	Coloured	Male	2	Hanging	Hung himelf with extension cord	<LOQ	<LOQ	<LOQ
27	27	Black	Male	2	Shot	Multiple GSW to head	0.015	61.268	33.733
28	39	Black	Male	3	Stab	Stabbed with a sharp object on left upper chest	0.175	332.081	332.807
29	25	White	Male	3	Overdose	Drug overdose	<LOQ	59.916	<LOQ
30	UNK	Black	Male	2	PVA	PVA	0.452	2116.474	1148.314
31	37	Black	Male	2	PVA	Deceased hit by a motor vehicle	0.2575	357.515	338.657
32	32	Black	Male	3	Stab	Stabbed on right side of the neck	<LOQ	<LOQ	<LOQ
33	58	Black	Male	3	UNK	Deceased found dead in a truck with head inside a bucket of water	<LOQ	88.870	<LOQ
34	85	Asian	Female	1	Drowning	Drowning	<LOQ	<LOQ	<LOQ
35	80	White	Female	2	Shot	Shot and assaulted	<LOQ	<LOQ	18.790
36	40	Asian	Male	4	Poisoning	Suspected posioning	0.0605	3612.097	1339.148
37	44	Black	Male	1	MVA	MVA	0.251	1554.254	1077.632
38	18	Black	Male	3	Hanging	Found hanging by Nylon rope	<LOQ	<LOQ	<LOQ
39	54	Black	Male	2	Natural	Found dead in the street	<LOQ	<LOQ	<LOQ
40	30	Black	Male	2	PVA	Hit by a truck	Insufficient sample	Insufficent sample	Insufficient sample
41	UNK	Black	Male	3	Shot	GSW to back	0.1665	2152.660	1607.652
42	23	Black	Male	2	Poisoning	Suspected food poisoning	<LOQ	70.220	<LOQ
43	34	Black	Male	3	Stab	Stabbed multiple times	0.443	7596.776	4437.283
44	58	Black	Male	2	Assault	Assualted by unknown suspect	<LOQ	195.876	<LOQ
45	27	Black	Male	3	UNK	Deceased found dead in prison	<LOQ	<LOQ	<LOQ
46	30	Black	Male	3	Shot	GSW to upper body, head and leg	0.123	168.843	536.654
47	37	Black	Male	4	Stab	Stabbed in head and chest	<LOQ	1559.660	938.212
48	35	Black	Male	3	PVA	Hit by a car	0.284	1654.696	1717.587
49	30	Black	Male	2	Electrocuted	Electrocution	<LOQ	<LOQ	53.851
50	30	Black	Male	3	Stab	Deceased stabbed in head	0.243	2468.715	2424.829
51	35	Black	Male	2	Shot	Multiple GSW to body	<LOQ	<LOQ	<LOQ
52	44	Black	Female	2	MVA	MVA (Passenger)	<LOQ	<LOQ	<LOQ
53	25	Black	Male	4	Shot	Multiple GSW	0.21	932.156	1251.441
54	37	Black	Male	2	Stab	Stabbed on leg	0.313	<LOQ	4661.407
55	UNK	Black	Male	3	Natural	Body found with signs of vomitting	0.058	6529.386	2103.556
56	37	Black	Male	1	Hanging	Found hanging	0.0711	<LOQ	53.060
57	18	Black	Male	3	Stab	Stabbed on left side of neck	<LOQ	<LOQ	<LOQ
58	61	Black	Female	2	Poisoning	Suspected food poisoning	<LOQ	<LOQ	<LOQ
59	25	Black	Male	1	Shot	Shot	0.236	488.104	518.641
60	35	Black	Male	3	Shot	GSW to chest and back	0.22	1183.390	948.653
61	42	Black	Male	3	PVA	PVA	Insufficient sample	Insufficent sample	Insufficient sample
62	UNK	Black	Male	2	Assault	Suspected of drinking too much alcohol	0.0176	81.881	624.699
63	31	Black	Male	1	MVA	MVA	0.0119	<LOQ	71.028
64	26	Black	Male	2	Shot	GSW to chest	0.05065	1187.308	1164.805
65	42	Asian	Female	3	Drowning	Found dead next to a pool	<LOQ	<LOQ	<LOQ
66	33	Black	Male	2	Stab	Stabbed during a fight at a bar	0.0639	121.198	102.684
67	24	Coloured	Female	2	Hanging	Found hanging in room by a rope	<LOQ	<LOQ	<LOQ
68	53	Black	Male	1	Shot	Multiple GSW by unknown person	<LOQ	<LOQ	14.318
69	22	Black	Male	2	Shot	Shot during a robbery	<LOQ	<LOQ	17.184
70	20	Black	Male	1	PVA	Hit by car (PVA)	<LOQ	<LOQ	<LOQ
71	48	Black	Female	1	Natural	Possible heart attack	<LOQ	<LOQ	<LOQ
72	21	Black	Male	1	UNK	Unknown	<LOQ	<LOQ	16.589
73	46	Black	Male	1	Shot	Shot	<LOQ	<LOQ	81.466
74	41	Black	Male	7	Poisoning	Poisoning	<LOQ	<LOQ	<LOQ
75	42	Black	Male	2	Stab	Multiple stab wounds to body	0.1854	4280.656	2399.596
76	38	Black	Female	3	Hanging	Suspected hanging	Insufficient sample	Insufficent sample	Insufficient sample
77	43	White	Male	2	Shot	Shot	<LOQ	<LOQ	24.560
78	41	Black	Male	3	Natural	Fell and died	<LOQ	162.809	699.807
79	34	Black	Male	3	Stab	Stabbed 5 times	<LOQ	<LOQ	<LOQ
80	43	Black	Male	2	Shot	Multiple gun shot wounds	0.35525	1662.398	1373.377
81	23	Black	Male	2	Poisoning	Suspected cyanide poisoning	0.037935	<LOQ	109.227
82	UNK	Black	Male	3	Natural	Unknown	<LOQ	59.235	<LOQ
83	30	Black	Male	3	Natural	Unknown	<LOQ	121.001	<LOQ
84	27	Black	Female	3	Fell from height	Foam coming out of mouth	<LOQ	123.837	24.302
85	40	Asian	Male	1	Fell from height	Jumped from a building	<LOQ	<LOQ	<LOQ
86	19	Black	Male	2	Assault	Found covered in blood	Insufficient sample	Insufficent sample	Insufficient sample
87	74	Asian	Female	1	Burnt	Deceased was burnt	<LOQ	<LOQ	<LOQ
88	UNK	Black	Male	2	Shot	Multiple gun shot wounds to the head and back	0.057395	541.180	913.380
89	26	Black	Female	2	Poisoning	Suspected poisoning	<LOQ	<LOQ	<LOQ

Case number	Age (years)	Race	Sex	PMI (days)	Category of death	Circumstances of death (according to SAPS 180)	EtOH in VH (g/100ml)	EtG (ppb)	EtS (ppb)
90	23	Black	Male	2	Stab	Stabbed with a knife in the chest	<LOQ	68.208	<LOQ
91	19	Black	Male	4	Shot	Multiple gun shot wounds	<LOQ	73.144	<LOQ
92	35	Black	Male	1	Natural	Sudden death without medical history	<LOQ	<LOQ	<LOQ
93	30	Black	Male	5	Shot	8 gun shot wounds. Attempted resuscitation	0.034645	73.087	38.395
94	32	Black	Male	2	Poisoning	Posioning (Organophosphates)	<LOQ	54.258	<LOQ
95	UNK	Black	Male	1	Natural	Found dead in an open field	<LOQ	<LOQ	<LOQ
96	52	White	Male	1	Poisoning	Suspected anti-freeze poisoning	<LOQ	163.214	<LOQ
97	20	Black	Female	1	Shot	Shot by unknown person	0.03733	<LOQ	<LOQ
98	22	Black	Male	2	Assault	Traumatic head injury due to assault	<LOQ	79.380	51.496
99	UNK	Black	Male	2	Natural	Unknown	<LOQ	<LOQ	<LOQ
100	26	Black	Female	1	Stab	Stabbed in the chest with a knife	<LOQ	62.094	<LOQ
101	18	Black	Male	1	PVA	PVA	<LOQ	114.553	<LOQ
102	41	White	Female	1	Gassing	Gassed herself inside a motor vehicle	0.061765	127.847	58.422
103	65	White	Female	3	Assault	Assaulted and strangled	<LOQ	<LOQ	<LOQ
104	30	Black	Female	3	UNK	Unknown	<LOQ	102.193	5.081
105	UNK	Black	Male	2	Shot	Deceased was shot	<LOQ	<LOQ	<LOQ
106	56	Black	Male	2	Shot	Shot	0.071325	175.285	81.092
107	19	Black	Female	1	Poisoning	Suspected poisoning	<LOQ	<LOQ	6.802
108	54	White	Male	4	MBA	Motorbike driver in fatal accident	<LOQ	66.430	<LOQ
109	25	Black	Male	2	Shot	GSW to left side of chest	<LOQ	<LOQ	<LOQ
110	UNK	Black	Male	4	Stab	Stabbed three times with a sharp object	0.11175	621.972	354.461
111	33	Black	Male	3	Shot	Shot	0.24695	3166.108	566.175
112	31	Black	Male	3	Stab	Stabbed three times	0.13015	735.236	281.504
113	20	Black	Male	2	Assault	Assualted (Mob justice)	<LOQ	<LOQ	<LOQ
114	46	Black	Male	3	Fell from height	Fell from a building	<LOQ	<LOQ	<LOQ
115	31	Black	Male	2	MVA	Deceased was sitting in the back of a bakkie and fell off while the bakkie was driving (Passenger)	0.053635	<LOQ	63.007
116	35	Black	Male	3	Assault	Mob assault by the community	<LOQ	<LOQ	<LOQ
117	18	Black	Male	1	Stab	Stabbed in the chest	0.2406	150.675	215.584
118	25	Black	Male	3	PVA	Hit while crossing the freeway	Insufficient sample	Insufficent sample	Insufficient sample
119	43	Black	Male	1	Shot	Shot and killed	<LOQ	<LOQ	15.604

APPENDIX 6: TURNITIN REPORT



1478710:Gareth_Riley_Final_write-up_November2020_TII.pdf

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