CHAPTER 1

1.0 Introduction

All deaths that occurs outside a hospital compound (e.g.: sudden, unexplained, natural and accidental) will be sent to Forensic Medicine Department or unit by police officer for post-mortem examination. This practice is accordance with section 330 and 331 of Criminal Procedure Code Act 593 which requires any death investigating officer to request for post-mortem examination to be conducted by a registered medical officer in any government hospital (Forensic Medicine Dept. /Unit).

A considerable percentage of this death is alcohol related in some way. Malaysian has been named by the World Health Organization (WHO) as the world's 10th largest consumer of alcohol despite its small population size. Statistics by the international body in 2011 also showed that Malaysians spent over US$500mil (RM1.5bil) on alcohol with a per capita consumption of seven liters (Tan, 2011).

According to a survey done by Institute of Public Health in 2006, the prevalence of current alcohol consumer in the country was about 7.4% and where else in Kuala Lumpur city alone the prevalence was 12.1% (IPH, 2008). It is admitted by all society that consumption of alcohol not only causing a lot of health issues but there is significant evidence that it also contribute to rise in accident numbers and violent crime.

Based on the research report "Alcohol and Drug Use among Fatally Injured Drivers in Urban Area of Kuala Lumpur" fatal crashes involving drivers under the influences of alcohol and drug are alarming (Norlen *et al*.2012). This study indicates that in the year 2012 about 23.3% of total fatal accident involved drivers are positive for alcohol. If the data is seen combined for substance abuse then the figure rises to 36.6% of total fatal

fatalities reported. This indicates that almost one third of fatal accidents than occur in Kuala Lumpur are due to under influence of substances either alcohol, drug or both. Data for the national level is not available due to lack of reporting. The only comprehensive report available is the above quoted report.

WHO has reported that driving under the influence of alcohol are among the well documented risk factors for road traffic accident. As per other few selected countries according to WHO Health Report, although the number of alcohol related traffic fatalities decreased over a decade, but the alcohol use remains a significant traffic safety matter. (Refer table 1)

Table 1: Road traffic fatalities involving alcohol (% of all road traffic fatalities)

Country	**Year / per 100,000 population**		
	1990	2000	2010
United State of America	50.6	41.4	38.9(2009)
Japan	-	16.3	6.7 (2009)
Germany	-	13.6	11.7(2008)
Republic of Korea	3.1	11.9	14.3 (2005)
New Zealand	46.0	-	30.0

The numbers showing it's a serious matter that need to be solved with prompt action. The reason is not only the driver endangering himself but also put others especially the passenger's and road users as well at risk. If we are looking at the law perspective in

Malaysia, based on the section 45G, Road Traffic Act, the legal blood alcohol concentration/limit is set at 0.08g/100ml of blood. Any level higher than that for any driver is considered as an offence (RTA, 1987).

There are few studies over the years has been conducted to evaluate the minimum level of blood alcohol concentration (BAC) that will give significant effect on the general population and according to Compton a BAC level of 0.04 g/dl or 0.04g/100ml will increase significant risk of involving in a crash (Compton *et al*.2002). There is also sufficient scientific evidence showing meaningful decrements in driving-related performance at BAC level of 0.05 g/dl with the younger and inexperienced drinkers who are drivers appear to be at greatest risk (Howard P, 1991).

The incidence of drinking and driving may differ amongst nations, but many findings have shown that drink drivers have substantially higher risk of being involved in a road crash than drivers who have not consumed alcohol (Norlen *et al*.2012). There is no disputing the scientific evidence which proves that even at low levels, alcohol impairs drivers. Motor vehicle accident are multifactorial and alcohol is one of the major risk factor which is if eliminated will reduce the number of accident and fatalities. Many studies has given evidence that even at a lower BAC level impairment of critical driving functions does take place (Ferrara et al.1994,Compton et al.2002).

The relative risk of being involved in a fatal crash as a driver is up to 10 times greater for drivers with BACs between 0.05 and 0.07g/dl, compared with drivers with 0.00g/dl (Zador, Krawchuk, & Voas, 2000). Whereas Compton and colleague reported that drivers have a considerable higher risk of being involved in a collision (18%) at 0.04g/dL (Compton et al. 2002).

This research has become the pushing factor in the policy change in few countries. When the reduced level of BAC is introduced positive reductions in collision and motor vehicle accident fatalities is noted (Refer Table 2).

Table 2: Reduced BAC level and decline in road traffic collision.

Country	BAC Level	Reduction in Collision/ Percentage (%)
Netherlands	0.05g/dL or lower	12 (1974)
France		36 (1996)
Austria		9 (1988)
New South Wales		13 (1994)
South Australia		14 (1991)
Sweden		20.7 (1997)
Japan	0.03 g/dL	11 (2007)

Source: (Fell & Voas , 2009)

One of the major hurdle faced by the authority is whether the postmortem blood alcohol concentration reliable. This is because the postmortem blood alcohol concentration tends to be less reliable and stable than the BAC freshly obtaining from driving drinkers. A number of reasons have been highlighted by researchers, among others are the postmortem blood is not sterile. The postmortem blood is fairly exposed and contaminated by bacteria and fungi that convert available glucose in the blood to alcohol (Wigmore, 2013).

It is also stated that the postmortem blood will have higher concentration of glucose. The putrefaction of glucose will produce fair amount of acetaldehyde then converted to ethanol which diffuses out mainly for the guts to peripheral blood vessel and elevated the blood alcohol concentration. This gives us insight that the ethanol concentration quantified from postmortem blood may not be the true concentration level at the point of the fatal event. It may be produced later from the putrefaction process that onset about 2 hour upon death. Corry (1978) have highlighted that microbes are capable of producing significant quantity of ethanol in blood and urine particularly from postmortem samples.

The production of ethanol can take place both in corpse and also bodily fluid even after the sample is taken from the dead body. The main factors deciding the production depends on the numbers and nature of the organism present in the sample including the type and quantities of substrates available in the sample. Substrate such as glucose, glycerol, fatty acid and ribose are reported to be high in concentration in postmortem body fluid (Rawat, 2008).

Determination of ethanol in postmortem body fluids of those who have suffered from unnatural deaths such as suicide, homicide and accidental death, and sudden natural death has become very important for finding the ante mortem intake because of the medico legal requirement. Accurate interpretation of blood ethanol concentration at the time of death presents a daunting task given that the presence of detected ethanol in postmortem cases whether corpses or sample after sample collection may vary. Furthermore the concentration of ethanol detected in post mortem blood need to be interpreted whether the person had consumed alcohol at time of death or it's a post mortem artifacts. As mention earlier many country including Malaysia have a very strict law that need to abide, so there

will be some consequences when accident and insurance claims will be invalid if the died person had the concentration of alcohol above the permitted level (Kugelberg & Jones, 2006).

Many factor need to be considered when such interpretation is required because the result that is given might not serve justice to family member who just lost a family member and maybe the sole breadwinner for the family. To avoid such situation it should be a standard procedure to conduct the analysis as soon as possible once the blood sample is obtained from the body during the post mortem examination. The scenario in Malaysia is the entire medico legal sample collected in any police investigation need to analyze by government laboratory facility. The current practice are all sample collected during the postmortem will be handed over to the investigation police officer which will be sent to the Chemistry Department of Malaysia for the analysis.

The duration a sample reaching the Chemistry Department and the result is received on average will take 1 to 2 months based on the data available at the department of Forensic Medicine, Sungai Buloh Hospital. It is believed that same timeline is applicable to the other Hospital in Malaysia too. The delay could happen during the transit of the sample from the Hospital to Chemistry department and at the time, the sample stability and integrity will be affected. The delay and the lack of cold chain through the transit itself might have introduced certain amount of ethanol due to the decomposition of glucose or any microbial activity.

1.1 Purpose of the Study

Qualitative and quantitative determination of ethanol in body fluid by using gas chromatography with a flame ionization detector is a well-established and popular method in every part of the world. Sample injection could be manual and as current trend more laboratory are using headspace sampling technique which is easier and accurate (Kugelberg & Jones, 2006). The recent advancement toward headspace sampling preferences is for determination of volatile substance because of the fact that its offer better chromatographic column protection from being contaminated by non-volatile substance from matrix when compared to direct injection.

Therefore Head Space-Gas Chromatography (HS-GC) has become the choice of method for detecting ethanol in blood or other specimens due to the accuracy and sensitivity that it provides. As to date there are lack of studies conducted using Headspace Gas Chromatography coupled with mass spectrometry to determine the concentration of ethanol in post mortem body fluid in Malaysia. Forensic Medicine Laboratory in Hospital Sungai Buloh has developed a method for automated headspace sampling coupling with gas chromatography mass spectrometer to detect, determine and quantify the ethanol concentration in post mortem blood sample collected during postmortem examination conducted in the department. The aim of the study is to validate and analyze the sample collected in order to provide fast and accurate result to be used by the pathologist and police officer in concluding any medico legal investigation.

1.2 Objectives of Study

1. To validate method of Headspace Sampling Gas Chromatography – Mass Spectrometry (HS-GCMS) for ethanol quantitative analysis using isopropanol as internal standard.
2. To determine the ethanol concentration in post-mortem blood sample collected during the post mortem examination.
3. To evaluate the duration taken from sample collection and result released.

Chapter 2

2.0 Literature Review

2.1 Blood and its constituents

2.1.1. Introduction

Blood is a vital component of life. The blood is a highly specialized tissue comprised of more than 4,000 distinct kinds of components. Each of these components performs well defined and unique functions physiologically (O'Neil, 2013) .The major advantages of blood is that the components can be isolated separately and tested in a laboratory, thereby providing vital information about a person's health status including foreign substance that remain in the blood. Therefore plasma serve as carriers for other molecules (Rogers, 2011).

2.1.2. Human Blood

Human body contains many different type of tissues. These tissues are defined by four categories i.e. epithelium tissue, connective tissue, nerve tissue and muscle tissue. Human blood is a tissue that falls in the category of connective tissue. Blood can be characterized as a fluid mixture, consisting of cellular components and plasma that circulates throughout the body via the arterial, venous, and capillary systems. On the average total volume of blood that circulate in adult human body will be in the range of 5 to 7 litres. Blood contributes approximately 8% of the total human body weight (Rogers, 2011). Blood is mostly red liquid with abundant of cells and proteins suspended in it, making blood more viscous than water (Heart Health Centre, 2008).

2.1.3 Function of Blood

The main function of blood is to serve as the body's transport system. Literally it means the blood transports almost all the essential substance and nutrient such as glucose, oxygen and hormones throughout the body to each every cell. It's also responsible in removing the waste product that release by the cells in the body and flush it out from the body in the form of urine, sweat, faeces or through lung as carbon dioxide gas (Nordqvist, 2010). Beside that another important role played by the blood (white blood cell) is the body's defense mechanism against infection. Till date there is no alternative to replace blood.

2.1.4. Components of Blood

Four of the most important component are red blood cells, white blood cells, platelets, and plasma *(refer figure 1).*

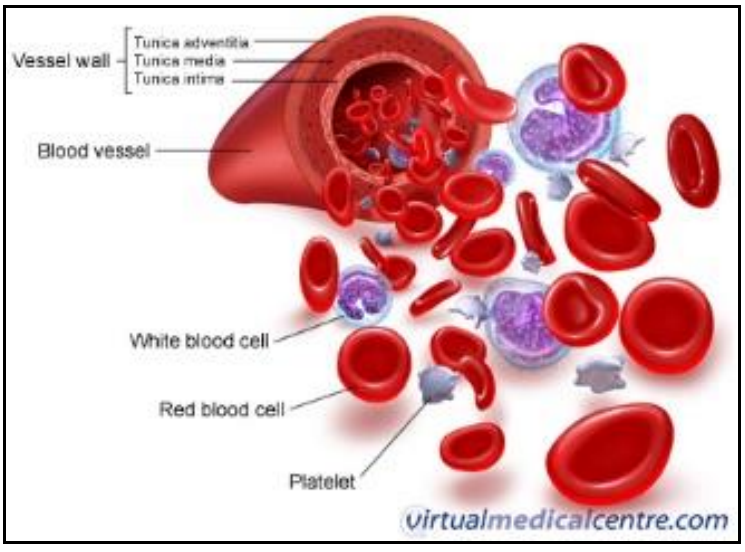

Figure 1: Blood components (source: virtualmedicalcentre.com)

2.1.4.1 Red Blood Cell

Red blood cells (RBCs) also known as erythrocytes, comprises of haemoglobin (iron containing protein molecule) which are responsible for transporting oxygen from the lungs throughout the body through the arterial system and sends carbon dioxide to the lungs for expiration through the venous system. RBCs normally make up 40-50% of the total blood volume. The red cells are formed constantly in our bone marrow from stem cells at a rate of about 2-3 million cells per second (O'Neil, 2013). This is due to fact each RBCs has a very short life span approximately 120 days before it will be removed from circulation through liver.

Blood shows a bright red colour due to the oxyhemoglobin complexing in oxygen rich blood within the arteries where else the blood within the venous system will be darker in colour since it is oxygen deficient. Oxygen can also be released from the haemoglobin by exposure to the atmosphere and becomes progressively darker as it loses its oxygen to the surrounding environment. It is a normal phenomenon encounter when whole blood is drawn during post mortem examination (James & Eckert, 1999).

2.1.4.2 White Blood Cell and Platelet

White blood cells (WBCs) also referred to as leukocytes (Nordqvist, 2010). It only make up to 1% of the total blood volume but perform vital functions related to the immunological response of the body to foreign substances and infection. They can be divided into granulocytes (contain colour granules) and agranulocytes. Granulocytes comprise of neutrophils, eosinophils and basophils. In contrast, agranulocytes do not contain granules and divided to lymphocytes and monocytes. Leukocytes are not restricted to blood instead it can be anywhere in the body while notably in the spleen, liver, and

lymph glands. WBCs contains nucleus therefore they serve as important forensic sample in DNA profiling (O'Neil, 2013).

Platelets serve as an essential part of the clotting mechanism of the blood. It also referred as thrombocytes. They are the smallest cell in the blood. Platelet response to any bleeding by forming blood clot (thrombus) and ceased the blood loss. It plays important role in maintaining blood remain in circulation within the blood vessel. The action will ensure the pressure of blood is maintained all time. Reduce in blood pressure and severe loss of blood can lead to death of a person (Rogers, 2011).

2.1.4.3 Blood Plasma

Plasma contributes almost 55% of the blood volume in human body. It's fairly clear and pale yellow liquid mainly compose of water (95% of total plasma volume). Plasma also contain fibrinogen (soluble) which play crucial role in blood clotting mechanism. If the fibrinogen is absent due to formation of blood clot, then the remaining part (liquid) will no longer identified as plasma instead referred as serum (O'Neil, 2013). The plasma also help to preserve the blood slightly basic at a stable pH and regulate the distribution of water between the blood and tissue fluid by producing what is known as a colloid osmotic pressure (VMC, 2010).

Plasma and serum have no cellular component but mainly contain comparatively greater content of water than does whole blood. This is noteworthy since ethanol disperses into the various body compartments in proportion to their water content. Therefore the plasma and serum with high water content (95% to 97%), relatively retain more ethanol than the whole blood (85% water content) (Mahaney & Kalin, 2009). Nowadays, plasma is

considered ideal for many laboratory investigations because the constituents in plasma reflect better the pathological situation of a patient than in serum (HSeT, 2012).

2.2 Alcohol and its effects

2.2.1 Chemistry of Ethanol

Ethanol (ethyl alcohol) is a clear, colourless liquid with a pleasant characteristic odour and burning taste. Ethanol, CH_3CH_2OH or C_2H_5OH (refer figure 2) is an alcohol, a group of chemical compounds with a functional group of hydroxyl group, –OH, which is covalently bonded to a carbon (Shakhashiri, 2009). Ethanol melts at –114.1°C, boils at 78.5°C, and has a density of 0.789 g/mL at 20°C (NPI, 2013). Ethanol is a rather small molecule that is completely miscible (soluble) in water but it is lighter than water due to the specific gravity of 0.789 and 1.000 respectively (Mahaney & Kalin, 2009).

The word alcohol derives from Arabic term '*al-kuhul'*, which means a fine powder of antimony used as an eye makeup. Alcohol initially suggested as any fine powder, but medieval alchemists later applied the term as "to the refined products of distillation", and this led to the current usage (Harper, 2014).

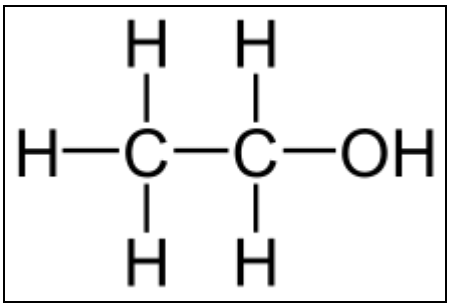

Figure 2 : Ethyl Alcohol @ ethanol structural formula

Alcohol has various daily uses varying from as solvents, cleaning products, fuel for table top cookery, industrial applications and even medical uses. Despite that, ethanol in chemically known as the psychoactive drug in alcoholic drinks such as beers, wines and spirits (Harrison, 2014). With the exception of alcoholic beverages, nearly all the ethanol used industrially is a mixture of 95% ethanol and 5% water, which is known simply as 95% alcohol (Leary, 2000). Beverages alcohol is commonly referred as ethanol or ethyl alcohol or just as alcohol. Ethanol is one member in family of alcohol which contain hydroxyl as functional group. When the term alcohol used it mainly referring to ethanol.

2.2.2 Production of Ethanol

Centuries have past but alcohol beverages are still made by fermentation of sugars using enzyme (*zymase*). Alcohol fermentation was discovered by French chemist and naturalist, Louis Pasteur in mid nineteen century. The fermentation process occur when yeast performs cellular respiration to produce energy in the absent of oxygen gas. In this process simple sugars will be converted to ethanol and carbon dioxide as represented by simple equation:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

Industrial ethanol can be made in several way including the fermentation reaction. This type of alcohol is commonly manufactured using synthetic route which is by reacting ethene with steam (refer reaction equation below) in the presence of phosphoric acid (catalyst) and suitable condition (temperature: 300^{o} C, pressure: 60 -70 atm) (Clark, 2013)

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$$CH_2{=}CH_{2(g)} + H_2O_{(g)} \rightleftharpoons CH_3CH_2OH_{(g)} \quad \Delta H = -45\ kJ\ mol^{-1}$$

To avoid misuse of industrial ethanol it is denatured to prevent its use as a beverage. This denatured ethanol contains small amounts (1-2%) of several different unpleasant or poisonous substances. The removal of all these substances would involve a series of treatments which is very expensive, therefore not cost effective (Harrison, 2014). Thus preventing the usage of industrial ethanol as beverages.

2.2.3. Alcohol consumption and prevalence

Malaysia considered as a small nation (28 million people), is the tenth largest consumer of alcohol in the world. Every year Malaysians spend over US$500 million on alcohol. Alcohol consumption in Malaysia at 11 litres per capita is equivalent to that of European countries known for their high consumption. The easy accessibility to alcoholic drinks in coffee shops, supermarkets, sundry shops and plantations together with insistent advertising and promotions are the main reasons that influence Malaysians to consume alcohol. The average age for alcohol addiction is 22 years (Asuntha et al.2001)

Earlier studies on the prevalence of alcohol consumption were mainly done in hospital setting. Amongst the pioneer research is done by Azman, (Azman et al.1989)

where 883 patients blood was sent for blood alcohol analysis in General Hospital Kuala Lumpur and reported that 30 % of them had actually had concentration of alcohol of higher than 50mg/100ml which mainly involving male patient (91%) with age ranging from 20 to 50 years old. A couple of year later Saroja (Saroja & Kyaw, 1993) recruited all patients from the age of 16 and above whom are admitted to orthopaedic, surgical and medical ward in General Hospital of Kuala Lumpur and concluded that 48% of her study sample are either alcohol consumer or user with 12% from that are dependent or abuser. She further added that 90 % of the alcohol consumer are all male. A year later Maniam, (Maniam T. , 1994) conducted studies on patients visiting the general hospital and reported that 70% of Chinese, 11 % of Malays and 42% of Indians have used or are currently using alcohol and a vast majority of them was social drinkers.

Studies also conducted on post-mortem examination cases, out of 155 post-mortem cases at General Hospital of Kuala Lumpur done in 1988 and 1989, 59(38%) of death involving road traffic injuries. Thirteen (22%) of the case had blood alcohol levels of more than 50mg/100m (Sharom, 1991). The most recent studies is done in 2012 at Forensic Medicine Department Hospital Kuala Lumpur, involving analysis of blood from post mortem examination. According to Norlen (Norlen, et al., 2012) 23.3% of fatal drivers were positive for alcohol, 11% positive for drug and 2.3% were positive for both drug and alcohol. She also highlighted that, 17% of fatally injured drivers were under the influence of alcohol below the blood alcohol concentration (BAC) legal limit (0.08g/100ml) stated in the Road Traffic Act 1987.

Major obstacle faced during conducting of the studies are due to the fact that toxicology result is not obtained from the testing laboratory. Norlen stated that out of 670 cases of road traffic accident selected for the studies only 76 % (509) of cases where the

toxicology report was made available which is useful for the study. Therefore 24 percent selected case was not dropped from the studies because of the result still not received by the requesting department. Though the studies was conducted in 2012 but the result for the sample collected from 2006 till 2009 is still not obtained. There is a need for more rapid and simple analysis required in order to fulfil the requirement of post mortem reporting.

2.2.4 Alcohol and its effect in human body

Alcohol associated problems has become subject of concern in many nations. An alcohol dependent individual will tend to develop psychiatric and physical complications that leads to social problems. Alcohol has also been identified as one of the contributing factor in motor vehicle accidents that causes injuries and even death. Alcohol has been classified as Central Nervous System (CNS) depressant which main mechanism is slowing down the brain action that's affect the body response and reflexes. Blood is often chosen a representative sample over brain tissue in chemical test for impairment studies due to fact that blood is the medium to deliver ethanol to and from the brain and furthermore blood can be obtained rather through simple procedure compared to brain tissues (Mahaney & Kalin, 2009).

Ethanol is a small molecule therefore it is easy for it to diffuse through the wall of stomach and delivered to the brain within a short period and if consumed in empty stomach the person easily becomes intoxicated (Murty, 2004). Murty further elaborated that drinking pattern of a person also will determine the level of intoxication because if the person consume alcohol slowly, the alcohol will be collected at liver and get processed without much effect on the other part of body but the opposite will take place if consume

in rapid manner because the distribution of alcohol will bypass the liver and flow the other part of body especially the brain.

Dr. Kurt M. Dubowski form Department of Medicine, University of Oklahoma has develops a chart that shows the clinical signs and symptom's caused by consumption of alcohol which is based on the blood alcohol concentration measured in grams in 100ml.

Table 3: Stages of Alcohol Intoxication

Concentration BAC (g/100ml)	State/Condition	Clinical sign & Symptoms
0.01-0.05	Subclinical	• Influence/effects usually not apparent or obvious • Behaviour nearly normal by ordinary observation • Impairment detectable by special tests
0.03-0.12	Euphoria	• Mild euphoria, sociability, talkativeness • Increased self-confidence; decreased inhibitions • Diminished attention, judgment and control • Some sensory-motor impairment • Slowed information processing • Loss of efficiency in critical performance tests
0.09-0.25	Excitement	• Emotional instability; loss of critical judgment • Impairment of perception, memory and comprehension • Decreased sensory response; increased reaction time • Reduced visual acuity & peripheral vision; and slow glare recovery • Sensory-motor in-coordination; impaired balance; slurred speech; vomiting; drowsiness
0.18-0.30	Confusion	• Disorientation, mental confusion; vertigo; dysphoria • Exaggerated emotional states (fear, rage, grief, etc) • Disturbances of vision (diplopia, etc.) and perception of colour, form, motion, dimensions • Increased pain threshold • Increased muscular incoordination; staggering gait; ataxia • Apathy, lethargy

0.25-0.40	Stupor	• General inertia; approaching loss of motor functions • Markedly decreased response to stimuli • Marked muscular incoordination; inability to stand or walk • Vomiting; incontinence of urine and faeces • Impaired consciousness; sleep or stupor
0.35-0.50	Coma	• Complete unconsciousness; coma; anaesthesia (Miller, 2005) • Depressed or abolished reflexes • Subnormal temperature • Impairment of circulation and respiration • Possible death
0.45	Death	• Probable death from respiratory arrest

(Mahaney & Kalin, 2009)

2.2.5 Pharmacokinetic of ethanol in human body

Ethanol will undergo four distinguish pharmacokinetic processes in body which include absorption, distribution, metabolism and excretion (Benjamin, 1996). These four processes occur at one and the same until all the alcohol is take up from the gastro-intestinal (GI) tract which means all the alcohol has been metabolized and no longer detected in the blood. Pharmacokinetics is the study of the rates and factors involved in the absorption of drugs into the body, their distribution throughout, and ultimately their elimination from the body (Miller, 2005).

Firstly absorption is the process by which alcohol is made available to the fluids of distribution of the body (e.g., blood, plasma, serum, aqueous humour, lymph, etc.). Nearly 80% of orally consumed ethanol is absorbed from the small intestines, and the balance is absorbed from the stomach. In the fasting state, a bulk of alcohol will be absorbed within 15 minutes and reaches maximum blood level approximately in 20 minutes, with 80-90% complete absorption reached within 30-60 minutes (Benjamin, 1996). Ethanol is amphipathic (contain both lipophilic and hydrophilic regions), therefore it can be easily absorbed by passive diffusion across the cell membranes without the need for conversion and fully soluble in water thus has an analogous volume of distribution to total body water (Kent, 2012).

Once the absorption process occur the ethanol that absorbed from the stomach and/or intestines (GI Tract) enter into the blood and circulated to some level to all parts of the body as long as blood flow is there and this process is identified as distribution. Distribution of ethanol follows simple diffusion process which is diffused from areas of high alcohol concentration to areas of low concentration until a concentration equilibrium

is established. According to Jones (Jones, 2007) body size and composition of a person will also have a substantial impact on the volume of distribution because alcohol is better distributed in tissues with higher water contents and with good blood supply. Female will have lower volume of distribution and higher BAC as compared to males when same amount of alcohol in consumed due to the fact female have smaller lean body mass and blood volume (Kent, 2012).

Alcohol in the body must be deactivated and eliminated from the body. This process is set off by modifying the chemical structure of the ethanol in such a way as to stimulate its excretion. The alteration of the alcohol molecule into a chemically linked substance that is more straightforwardly excreted from the body is called metabolism (detoxification) (Benjamin, 1996). Metabolism of alcohol is a complex process due the fact that the individuals varied in relations to absorption, distribution and elimination of ethanol in the body (Caballeria, 2003). According to Caballeria, "…multiple factors which influence these variations, genetic factors especially those related to the different alleles of ADH2 and ALDH2, beside nutrition and gastric metabolism." Ethanol is metabolized in the liver by the enzyme alcohol dehydrogenase, to acetaldehyde and is consequently break down by the enzyme aldehyde dehydrogenase to acetate (refer figure 3), a substance very similar to acetic acid or vinegar which also serve as an indicator for "chronic drinking" (Zakhari, 2006).

Excretion is the process by which a drug is removed from the body. The kidney and lungs eliminate only 5-10% of an absorbed dose of ethanol in unchanged form. The remainder must be break down before it undergoes excretion.

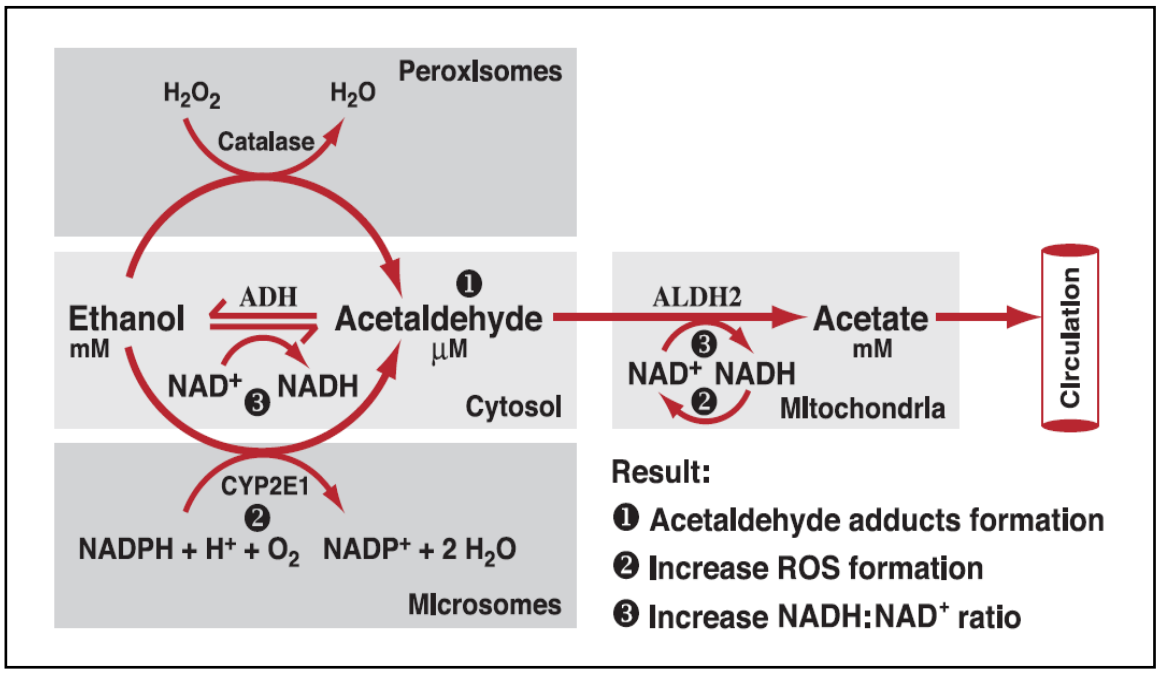


Figure 3: Oxidative pathways of alcohol metabolism (Zakhari, 2006)

The liver is responsible for the removal (undergo metabolism) of 95% of ingested alcohol from the body. The remainder of the alcohol is eliminated through excretion of alcohol in breath, urine, sweat, faeces, milk and saliva. The body uses several different metabolic pathways as in oxidation of alcohol to acetaldehyde to acetic acid to carbon dioxide and water as shown in figure 3. Normal person metabolize alcohol at consistent rate, as rule of thumb, a person will able to remove drinks of 15 ml alcohol per hour. This rate usually influence by a couple of factors such as the eliminations tend to be higher when the blood alcohol concentration in the body is very high. As for the chronic drinkers the metabolism of alcohol take places significantly higher rate than the average. Ultimately one cannot rule out that the body's ability to metabolize alcohol swiftly tend to reduces with aging (Kent, 2012).

2.2.6 Post-mortem blood alcohol concentration

A few studies has been conducted to understand the condition and the process that involved in formation of alcohol in the progression of putrefaction. According to Bogusz , (Bogusz, Guminska, & Markiewicz, 1970) it is noted that when blood decompose in vitro condition, rapid breakdown glucose start to occur after three days and only then alcohol concentration appeared to be present in blood, subsequently reaching the highest concentration on 10^{th} to 12^{th} day of putrefaction. The ethanol formation could occur in two different putrefying blood sample which is either while in cadaver or in vitro blood (test tube).

Moriya and Ishizu (Moriya & Ishizu, 1994) disclosed that in antemortem condition, unusual endogenous ethanol production was confirmed where concentration of ethanol detected in intra-abdominal is 2.45mg/g(body fluid) which is higher compared to blood taken from the heart which was only 1mg/g detected. It is noted that significant quantity of ethanol was produced in the bodily fluid due to the microbiological organisms. There are numerous species of bacteria, yeast, or even fungi that have the ability to produce ethanol and other volatile organic compounds in postmortem specimens especially blood.

According to O Neil and Poklis, *Esccherichia coli* and *Candida albicans* are the primary causes of post-mortem ethanol synthesis, whereas there are about 58 other species of bacteria and 17 species of yeasts that can also produce ethanol during purification of human body (blood) (O Neil & Poklis, 1996). This same group of bacteria that responsible for ethanol production also produced other volatile compounds i.e. methyl alcohol, formaldehyde, n-propyl alcohol, propionic acid, acetone, acetic acid, acetaldehyde, n-butyric acid and iso-butyric acid (Bogusz, Guminska, & Markiewicz, 1970).

To establish whether the ethanol concentration determined from post mortem blood sample is post mortem production or due to ingestion prior to death , further corroboration with vitreous humour and urine sample is suggested. This two sample are well preserved from the decontamination of post-mortem blood decomposition.

2.2.6 Measurement of blood alcohol for forensic purposes

Detection volatile compound especially alcohol in forensic sample from post-mortem examination has become one of the routine techniques in the field of forensic toxicology. Forensic analysis is often conducted is to identify the source of post-mortem ethanol, to determine probable cause of natural death, to ascertain the cause of intoxications, to corroborate for pathological diagnosis or even identifying the location of clandestine burials and human remains (Boumba, Kourkoumelis, Ziavrou, Fragkouli, & Vougioklakis, 2012).

Many species of bacteria, yeast, and fungi have the ability to produce ethanol and other volatile organic compounds in post-mortem specimens. The ethanol formation cause difficulties in interpreting positive ethanol results from accident victims. Therefore, the inhibition of ethanol formation at all steps starting from specimen collection is a priority. Preservative that is usually used is sodium fluoride for post-mortem specimens. Studies have been published detailing the effectiveness of sodium fluoride for the prevention of ethanol formation in blood and urine specimens. The post-mortem blood specimens collected in Forensic Medicine department sometimes may have been exposed to numerous microbial species capable of ethanol production. Studies conducted by Lewis and his co researcher found that without preservative, specimens stored at 4 ^{o}C for 96 hour

showed an increase in ethanol concentration ranging from 22 to 75mg/100ml whereas at 25 ^{o} C, these same specimens showed an increase ranging from 19 to 84mg/100ml. Addition of 1% sodium fluoride was found to be no significant increase in ethanol concentration at either temperature. (Lewis, Johnson, Angier, & Vu, 2004)

Blood alcohol content (BAC) analysis is one of the most common test for forensic purposes. It is generally performed using headspace gas chromatography (GC) with a flame ionization detector (FID). Recently with the introduction of Headspace technology and mass spectrometry the analysis of alcohol in blood has become simple, rapid and accurate. For purposes of law enforcement, blood alcohol content is used to define the level of intoxication. Blood alcohol level can also provide a rough measure of impairment. Most countries forbid operation of motor vehicles and heavy machinery by anyone who has levels of blood alcohol content above a legal limit. The content of alcohol in blood is most commonly expressed as grams per deciliter (g/dL) (Caruso & Santoro, 2013).

In forensic alcohol analysis bodily fluid will be collected, either from the corpse or living person. The only different is in the technical part of measuring the ethanol production in the body fluid. The latter sample does not much contribute much problem to the analysis. However the sample obtained from the autopsy are documented to provide in the some problems in the interpretation of the data.

2.3 Head Space Analysis

2.3.1 Development of Headspace

Headspace gas chromatography (HS-GC) for determination of ethanol content of blood is commonly used as a preferred method by most of the forensic laboratories (Pfannkoch & Whitecavage, 2007). Most of the sample type that is sent in for forensic analysis are non-volatile samples i.e. body fluid such as blood, urine, and saliva, vitreous humour and etc. The main concern is the constituent that is analyzed from this sample matrix are volatile in nature. Introducing liquid state sample into gas chromatography will cause the volatile component of the sample to vaporized and carried to the column by carrier gas (mobile phase) and the non-volatile (liquid) component will remain at the injection port. This will eventually has higher chances to contaminate the gas chromatography system especially the column (Ettre, 2002).

The condition is even challenging if the sample matrix in solid state because the difficulty in extracting the volatile component without undergoing a series of extraction on the analyte of interest. This also include sample that has higher boiling point which make the extraction process tedious and time consuming that is considered as pitfall in forensic analysis. The problem that arise has been practically solved with the invention of Headspace sampling. The headspace injection sampling technique has evolved to a great benefit forensic toxicology field by offering clean injection, lower consumptions of gas chromatograph consumables (carrier gas), nor to forget its simplicity and further reduces the likelihood of artefacts during the analysis that lessens the possibility of contamination and accurately quantifies analytes of interest (Bernal, 2012).

Headspace sampling become very versatile method for sample injection especially involving volatile samples because it can be easily integrated with gas chromatography and furthermore eliminate the need for sample preparation using solvent extraction technique.

There are several technique that has been developed for headspace based on the needs of different industry and the nature of the sample that is analyzed. Among them are *static headspace analysis*, *purge and trap headspace technique* or *dynamic headspace analysis*, and finally the latest introduction to the headspace series is *solid phase micro extraction (SPME) headspace analysis*.

Static headspace technique allows the analyst to take an aliquot of gas vapour in equilibrium within the contact with liquid or solid state. Basically once equilibrium is achieved the analyte of interest will be distributed between the two phase depending on their partition coefficients. The dynamic headspace technique on the other hand, the liquid sample is purged continuously with inert gas until all the volatile component is flash out through a trap either using by lower temperature or absorbent. Then the trap will be rapidly heated and the volatile analyte is released into the system where the quantification will be done. Finally, for the SPME technique a fused-silica fibre coated on its surface with a stationary phase and fixed in a modified syringe is exposed to the headspace above the sample. The fibre is detached upon equilibrium, and the analytes accumulated in the stationary phase coating are desorbed thermally in the injector of the gas chromatograph and transferred by the carrier gas into the column (Ettre, 2002).

The technique employment is determined based on some considerations, for example type of sample tested, quantitative or qualitative analysis is favoured, sensitivity, automation and budget (Bernal, 2012). For the determination of ethanol

in blood sample the static headspace analysis is more appropriate because due to the fact that ethanol is highly volatile and easily vaporized when heated, therefore most of the laboratory analysts prefer this method which is cost effective compared to other two technique which incur extra costs.

2.3.2 Principle of Static Headspace

Static headspace sampling techniques can be used for both qualitative and quantitative analysis of volatile component in the forensic samples. Collected sample is placed in a closed vial and heated, this is to promote the volatile analytes of interest to diffuse into the headspace of the vial (refer figure 4), once equilibrium is reached between the analyte concentration in the headspace and the analyte concentration in the sample, a portion of the headspace is taken and injected into the gas chromatograph. This can be done manually or with an automated sampler. All the process will be normally performed at a pressure and temperature higher than ambient conditions (Smith, 2003). In headspace analysis there are two basic principle that need be determined before analysis being done and they are partition coefficient and phase ratio.

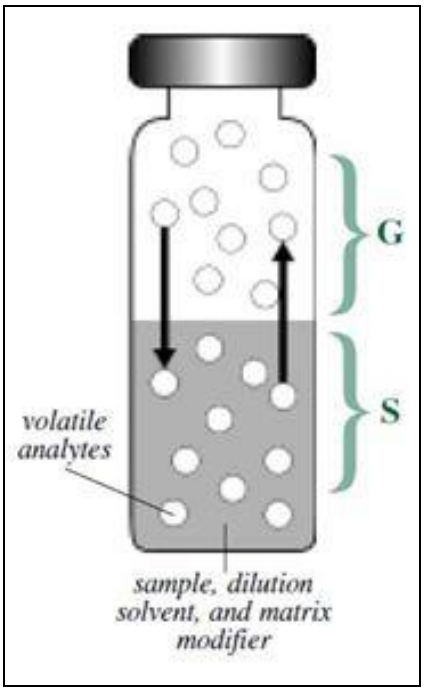


Figure 4 : Schematic representation of the phases of the headspace in the vial.

G: Gas phase (headspace), S: Sample Phase

Source: (Restek, A Technical Guide for Static Headspace Analysis Using GC, 2000)

2.3.1.1 Partition Coefficient

Samples preparation is very crucial, the analyst must ensure the concentration of the volatile components in the headspace is obtained at maximum level, while minimizing undesirable component or contamination from compounds in the sample matrix. Concentration of analyte of interest in the headspace can be determined once the partition coefficient (K) is calculated (refer Equation 1). C_s is refer to the concentration of analyte in sample phase and C_g is the concentration of analyte in gas phase. A low K values of analyte of interest indicate tendency to partition more readily into

the gas phase, and having reasonably high responses and low limits of detection. The K value can be bring down by altering the temperature at which the vial is equilibrated or by changing the composition of the sample matrix (Zhao & Barron, 2012).

$$K = C_s/C_g \qquad \text{Equation 1}$$

2.3.1 Phase Ratio

The phase ratio (β) is termed as the relative volume of the headspace compared to volume of the sample in the sample vial (refer equation 2). The V_s refers to volume of sample phase and V_g is to volume of gas phase. Lower values of β, will produce higher responses for volatile compounds. Nonetheless, diminishing the β value will not always increase in response needed to improve sensitivity. According to Zhao and Barron when β is decreased by increasing the sample size, compounds with high K values partition less into the headspace compared to compounds with low K values, and yield correspondingly smaller changes in C_g. Samples that contain compounds with high K values need to be enhanced to deliver the lowest K value before changes are made in the phase ratio (Zhao & Barron, 2012).

$$B = V_g/V_s \qquad \text{Equation 2}$$

2.3.3 Advantages of Headspace Sampling

The headspace injection technique can be used to analyse a very low to trace levels of volatile component whether it's from liquid or solid samples. There are varies advantages that offer by this technique and among others such as (Leap, 2010):

- easy sample preparation
- minimize time of analysis
- eliminates Gas Chromatography contamination
- improves detection limits
- reduction in method development times
- reduction of matrix interference from sample matrix
- extended life of the GC column
- reduction of instrument down time.

The technique are simple and can provide higher sensitivity for the post mortem blood alcohol determination.

2.3.4 Blood Alcohol Determination using Headspace Auto sampler

It is well documented that Headspace Gas Chromatography (HS-GC) has revolutionize the determination of ethanol content for blood or any other volatile compound. G. Machata (1960) from University Forensic Institute in Vienna was the person that published first use of HS-GC for quantitative analysis for blood alcohol content determination using device called precolumn. Machata developed a method by injecting blood sample into this small column (precolumn), which

contained glass wool or some inert packing material. After injection, the precolumn was heated rapidly and flushed with the carrier gas, which transported the evaporated volatile compounds into the chromatographic column while the non-volatile part of the sample remained adsorbed to the packing of the precolumn" (Ettre, 2002).

Even though Machata discoveries have become popular in Europe and made commercially available but it only served as routine analysis in few countries. Nonetheless the manual sample preparation and injection limits the number of samples that can be analyzed at any point of time. Therefore he continue to explore new possibilities of automating the whole manual sample preparation and injection. In 1967 the collaboration Machata and Bodenseewerk Perkin-Elmer & Co introduced the first automated headspace gas chromatography system (Ettre, 2002).

The availability of an automated instrument for headspace GC transform blood alcohol determination. Subsequently Machata's method has been customized by many nations in Europe and North America as the standard method for Blood Alcohol Concentration (BAC) measurements. Machata methods also introduced the usage of internal standard compound together with the sample matrix. There are many internal standard available commercially or can also be prepared in laboratory. Among the internal standard that is used in alcohol content determination are *n-propanol, t-butanol, acetonitrile,methanol, acetaldehyde and iso-propanol.* The selection on internal standard to be use depends on the type of column utilized in the GC instrument (Restek , 2000).

The internal standard technique uses one or more designated compounds (almost similar to the analyte of interest) at known concentrations spiked into the sample. The response of the compounds of interest are then compared to the results of the internal standard. Therefore the interference by the matrix of the sample can be minimized and accurate result able to be obtained (Restek, 2007).

Chapter 3

3.0 Methodology

The methodology is divided into two part:

Part A – Validation of determining the concentration of ethanol in post mortem blood using isopropanol as internal standard and

Part B – Determining concentration of ethanol in postmortem sample collected during post mortem examination.

Part A

3.1 Instrumentation

Gas chromatography Trace GC Ultra coupled with mass spectrometer DSQ II (brand: Thermo Fisher Scientific) was used for validation and determination of ethanol using 0.1% iso-propanol as the internal standard. Headspace sampling was done using HS Triplus Autosampler from Thermo Scientific. The chromatogram and spectrum were analyzed using Xcalibur software develop by Thermo Scientific to calculated the response detected by the detector to quantify the amount of ethanol presence in a sample. The column was a TR-WAXMS (0.25 mm i.d. x 0.25 μm x 30 m). Parameter on Gas Chromatography for the analysis was set as shown in table below.

Table 4: GC Instrumentation Parameter

Parameter	GC condition
Oven Temperature	40 ^{o}C
Injector Port Temperature	180^{o}C
Transfer Line Temperature	220 ^{o}C
Ion Source Temperature	220 ^{o}C
Mode	Split
Carrier Gas	Helium
Flow rate	20 ml/min
Scan Mode	Full Scan (range :28 – 150)
Scan Rate	500.0 amu/s
Scan per sec	3.7594

3.2 Procedure for Method Validation (ICH, 1996)

The analytical method validation was carried out as stated in ICH guidelines. The validation parameters addressed were selectivity, specificity, precision, linearity, and limit of detection, limit of quantitation and robustness (ruggedness) (Peters, 2007).

3.2.1 Retention Time and Analyte Resolution:

The chromatograms generated in the precision test were used to analyze the variation in analyte retention time (tR) and resolution (RS).

3.2.2 Precision and Accuracy

Precision was determined using the repeatability between 10 replicate samples of blood spiked with three different concentration (40mg/dl,100mg/dl,300mgdl) of the calibration curve and reported as a coefficient of variation/Relative Standard Deviation (percentage).

The accuracy was calculated as the percent recovery from 8 replicates of blood samples spiked with six different concentration of 25mg/dl, 40mg/dl, 80mg/dl, 100mg/dl, 200mg/dl and 300mg/dl.

3.2.3 Limits of Detection and Quantification:

The limit of detection (LOD) for each analyte in blood was defined as the lowest detectable concentration yielding an S/N of 3 and the limit of quantification (LOQ) was defined as the lowest concentration yielding an S/N of 5. To determine the LOD and LOQ, ten individual samples were prepared by spiking with the lowest acceptable concentration of ethanol, 25mg/dl @ 0.025 %.

3.2.4 Linearity: Linear least squares regression calibration curves were constructed by plotting the peak area ratios of the analyte to the IS versus the expected concentrations of the standard solution.

3.3 Calibration Standards:

The headspace gas chromatography is calibrated each time before an analysis is done. For this purpose blood was used as standard matrix (expired blood form blood bank) and ethanol standard that is used was ranging from 10mg/dl (0.01%) to 300mg/dl (0.30%). The standard was purchased from manufacturer, Cerriliant Corporation from Texas, USA. The standard is used as calibrator and controls because of different concentration and lot numbers. Each of the standards with concentration of 40mg/dl, 50mg/dl, 80mg/dl, 100mg/dl, 200mg/dl and 300 mg/dl of ethanol were spiked (0.1 mL) with a fresh blood (0.5 mL) into six headspace vials. A 0.1% working internal standard of Isopropanol solution was made by serial dilution. Once the sample and internal standard pipetted into the 20 ml vial it was sealed with crimp cap incorporated with silicon septa using manual crimper.

3.4 Internal standard

The internal standard 0.1% is prepared as follows:

1 mL of Isopropanol is pipetted and transfer to 10mL volumetric flusk then filled with de-ionized water till the mark. The solution is mix thoroughly. The Stock Internal Standard is then diluted x 100 to make the working Internal standard which is sealed and stored in refrigerator.

3.5 External standard

A series of concentrations of ethanol is prepared as external standard during the analysis. Control blood obtained from blood bank were use in preparing seven headspace vials and then spiked with internal standard (isopropanol). Each of the vials was labelled according and spiked with 40 mg/dl, 50 mg/dl, 80 mg/dl, 100 mg/dl, 200mg/dl and 300mg/dl (w/v) of ethanol standards from Cerriliant®. These standards were used to determine the amount of analytes based on the calibration curve plotted.

Part B

3.6 Sample collection

All postmortem blood sample is treated as potentially infectious therefore it was always handled with caution and safer laboratory practice is adhered all the time. All procedure was performed using protective glove. Blood sample were taken during postmortem examination which is conducted in mortuary at Forensic Medicine, HSgB. The collected blood is placed in a grey coloured vacutainer tube which is pre-added with preservatives [one percent of sodium oxalate]. Collected sample were labeled with all the relevant particular as stated in departmental specimen collection guidelines attached with completed request form for toxicological to Forensic Toxicology Laboratory within the Forensic Medicine Department. All sample received will be analyzed on the same day and if not then then sample will be kept in sample refrigerator in evidence room. The temperature of refrigerator is always maintained in the range of 2 to

8^{O}C.Precaution was taken to limit the exposure to blood and aerosols. Prior to aliquoting, the blood sample is inspected, to ensure that blood is not clotted.

3.7 Internal Standard Preparation

Internal Standard is prepared freshly in laboratory using commercially available iso-propanol. 1 ml of iso-propanol is pipetted and transferred to volumetric flask 10ml labeled 10 %. It further diluted second time (1%) and finally to obtain 0.1% of concentration. The prepared 0.1% iso-propanol is transferred to small glass container with screw cap. Date of preparation is labeled on the container and are kept under refrigeration.

3.8 Sample Preparation

20 mL headspace vials is labeled for each standard, control and blood sample to be analyzed. Standards and controls are analyzed for single time and the unknown sample in duplicate.100 µL of blood sample is pipetted in labeled headspace vial accordingly. 20 µL of internal standard is added and stopper is inserted. Blank sample is prepared with 100 µL of standard blood which is obtain from Blood Bank of the hospital and 20 µL of internal standard added before the vial is crimped with the cap. For each batch on analysis a control sample is prepared which contain 20 µL of internal standard and 100 µL of ethanol standard. This is to monitor whether the analysis is running properly as required. The vials was gently swirl until homogeneous and crimp seal it tightly. The sample headspace vial is ready for the analysis. Work list for the samples, standard and controls is prepared

accordingly and the data is key in to software for sequencing and parameter setting.

3.9 Experimental Procedure

3.9.1 Sample vials are arranged accordingly on the headspace auto-sampler rack. The samples and standards information prepared were keyed into software spreadsheet (Xcalibur). Headspace sampling was done using HS Triplus Autosampler. During this stage the sample is preheated at 80^{o}C for duration of four minutes. 1.0ml of headspace is removed from the vial with a gas-tight syringe and injected into injection port upon the four minute incubation.

3.9.2 Gas Chromatography Condition

The gas chromatography injector is set at 180^{o}C and the detector temperature is at 220^{o}C. The oven was held isothermally at 40^{o}C for the duration of six minutes run. Helium was use as the gas chromatography carrier gas at a flow rate of 20 ml/min. The identification and integration of all the peak reported by the MS spectrum was done using Xcalibur software. The software integrated all peaks in a chromatogram with a signal-to-noise ratio of 5 or greater. Peak area was analyzed and calculated for the purpose of quantification of the analytes based on the chromatogram produce. The retention times for ethanol and isopropanol were also documented.

CHAPTER 4

4. Results & Discussion

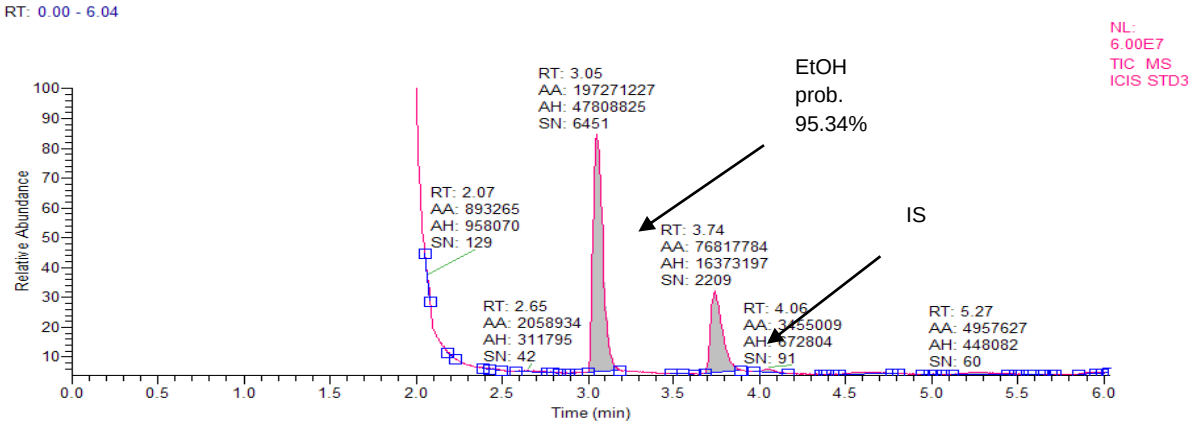
Part A: Method Validation

4.1. Calibration curve

In obtaining a reliable quantification, an appropriate calibration model need to be chosen. Beside this it is necessary to ensure that the relationship between the concentration of analyte in the sample and the response produced which is based on the area ratio of analyte against the internal standard. The calibrators used must be matric based and should cover the whole range of calibration (Peters, Drummer, & Musshoff, 2006). At least six point different concentration level of calibrator must be used to establish the calibration model (Chan, 2008).

The calibration curve is established by the analysis of six calibration levels and each points corresponding to standard solution with known concentration respectively i.e. 0.04%, 0.05%, 0.08%, 0.10%, 0.20% and 0.30%. The calibration curve is best fitted to a linear curve. The examples of chromatograms and spectrums obtained are shown in **Figure 5** and **Figure 6** whereas the calibration curves are presented in **Figure 7**.

A)



B)

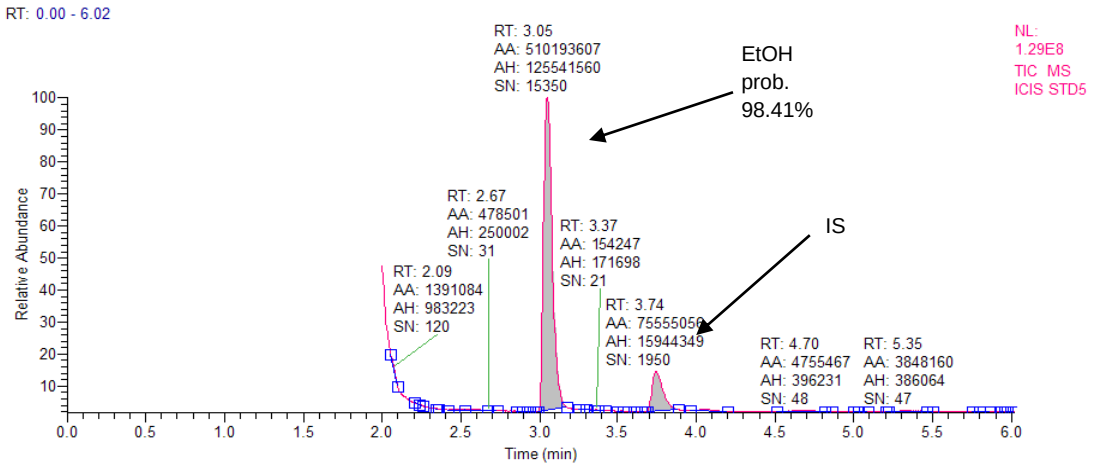
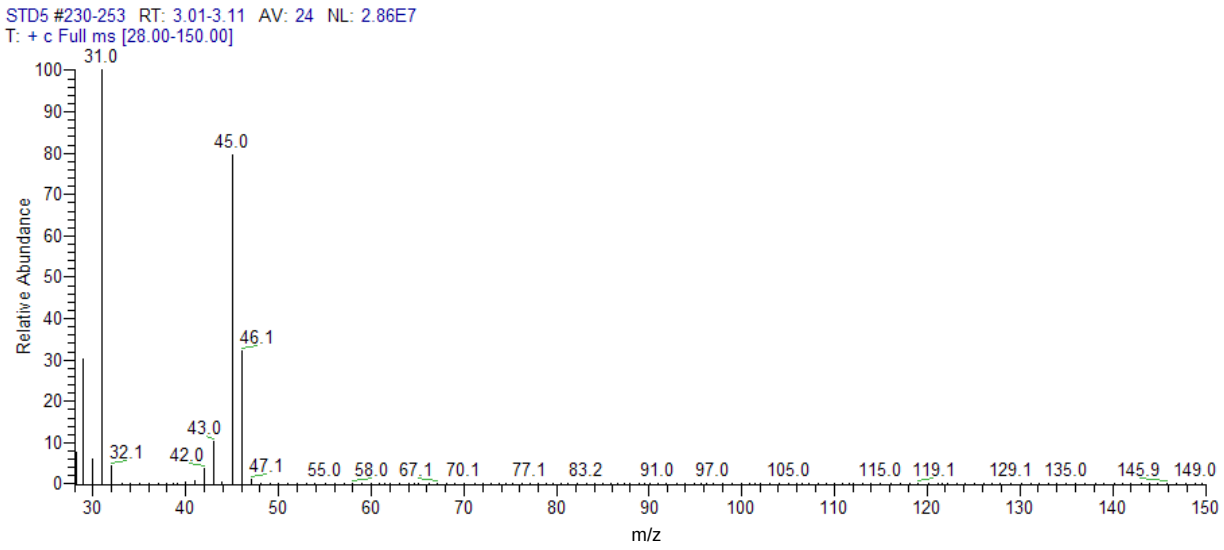


Figure 5: Chromatograms for ethanol for blood sample spiked with (A) 0.08% (or 80 mg/dl); and (B) 0.20% (or 200 mg/dl).

(a)



(b)

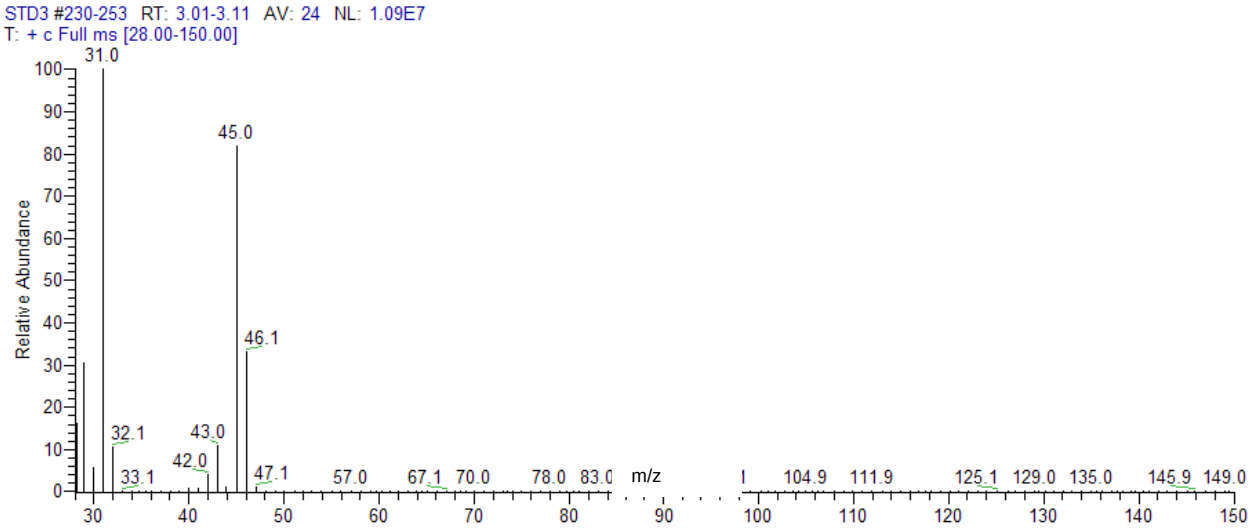


Figure 6: Spectrums for ethanol for blood sample spiked with (a) 0.08% (or 80 mg/dl); and (b) 0.20% (or 200 mg/dl).

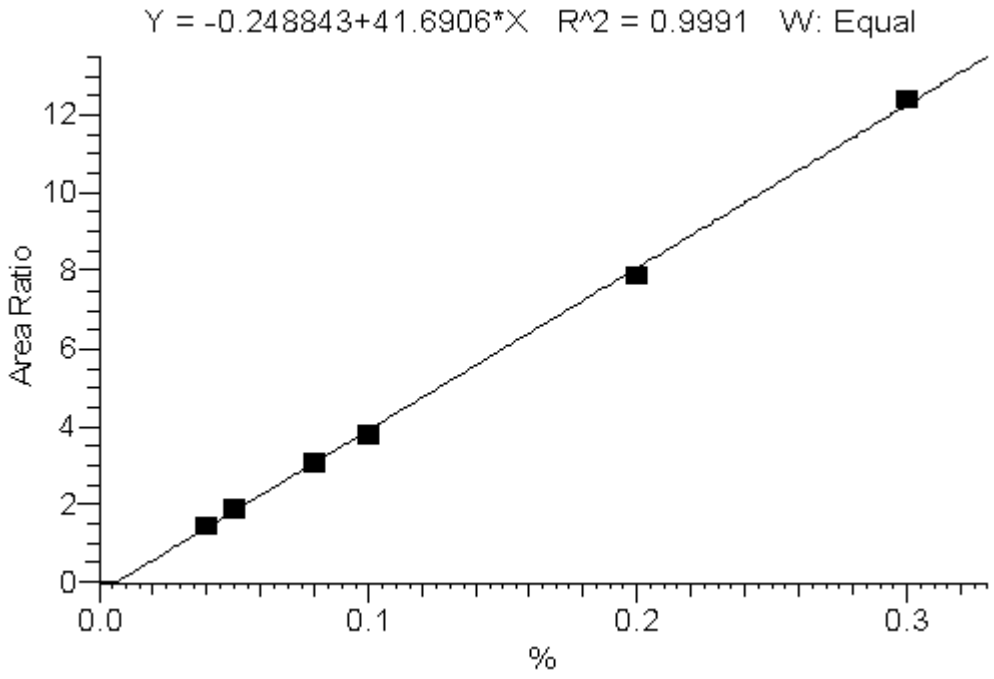


Figure 7: Calibration curve for ethanol with six calibration levels 0.04%, 0.05%, 0.08%, 0.10%, 0.20% and 0.30%.

The retention time for ethanol is at 3.05 in all chromatograms, showing the consistency and stability of the separation and detection, in lower and higher concentrations.

4.2 Linearity Study

To assess the linearity of the method, the calibration curve was established with six points and each point corresponds to a standard solution of known concentration of 0.04%, 0.05%, 0.08%, 0.10%, 0.20% and 0.30%. The characteristic parameters of the curves achieved is presented in table 1 whereas the obtained calibration curve is presented in Figure 3. The regression coefficient (R^2) was measured at 0.9999 and the linear index were calculated to be approximately 1.00. This value is over 0.999 indicating a relationship of direct proportionality between the concentrations of standard solutions and the peak area ratio.

Table 5: Parameters of calibration curve for standard ethanol

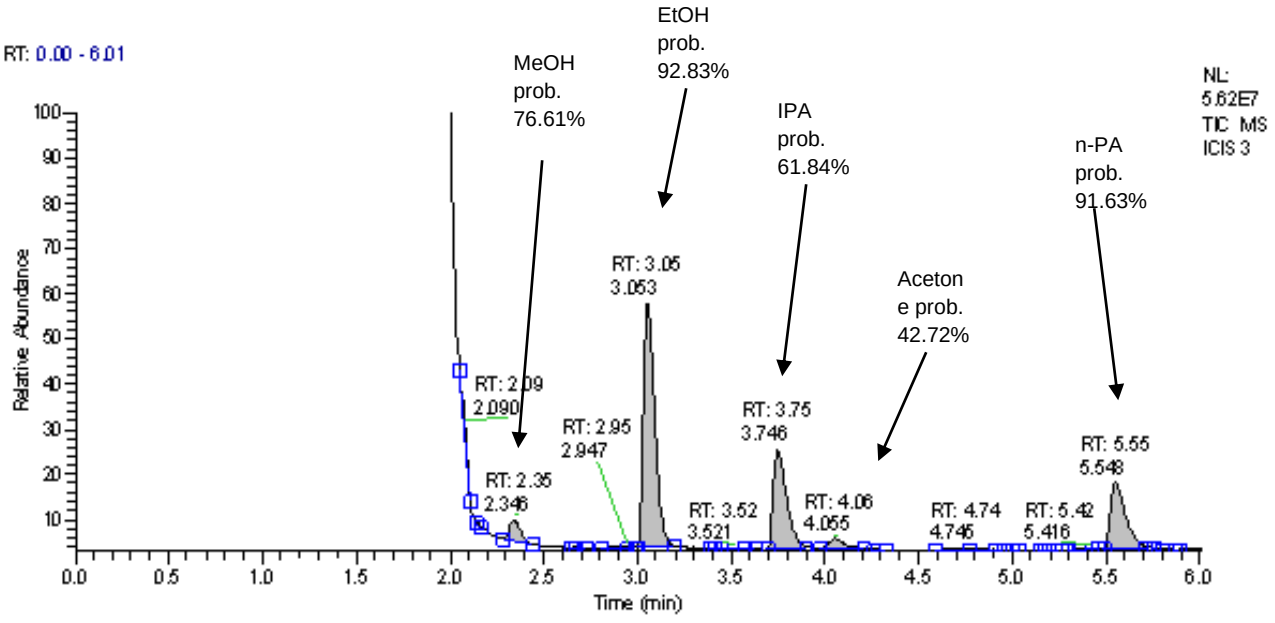
Standard concentration x/%	**Area Ratio y**	**Calculated Concentration "x"/%**	**linear index**
0.04	1.481	0.0415	1.04
0.04	1.575	0.0437	1.10
0.05	1.925	0.0521	1.04
0.05	1.944	0.0526	1.05
0.08	3.100	0.0803	1.00
0.08	3.289	0.0849	1.06
0.10	3.812	0.0974	0.974
0.10	3.835	0.0980	0.980
0.20	7.891	0.1952	0.976
0.20	8.287	0.2047	1.024
0.30	12.400	0.3034	1.011
0.30	12.432	0.3042	1.014

4.3 Selectivity and specificity

Specificity refers to the ability of the method to assess unequivocally an analyte (in this case ethanol) in the presence of components that may be expected to be present in the post-mortem blood. Based on the literature, four other common analyte is chosen to be included in the sample in order to assess the specificity of the method and the column used (Chan, 2008). The term selectivity and specificity are interchangeable but according to International Union of Pure and Applied Chemistry (IUPAC) , its preferred the term selectivity over specificity with reserving the latter for analysis which is completely selective.

Therefore selectivity was analysed using prepared two spiked blood sample with selected common volatile organic compound under alcohol group. They were methanol, ethanol, isopropanol, n-propanol and acetone. The spiked sample were analysed using the prepared analysis protocol. The chromatogram and the spectrum obtained from the analysis were evaluated qualitatively.

(a)



(b)

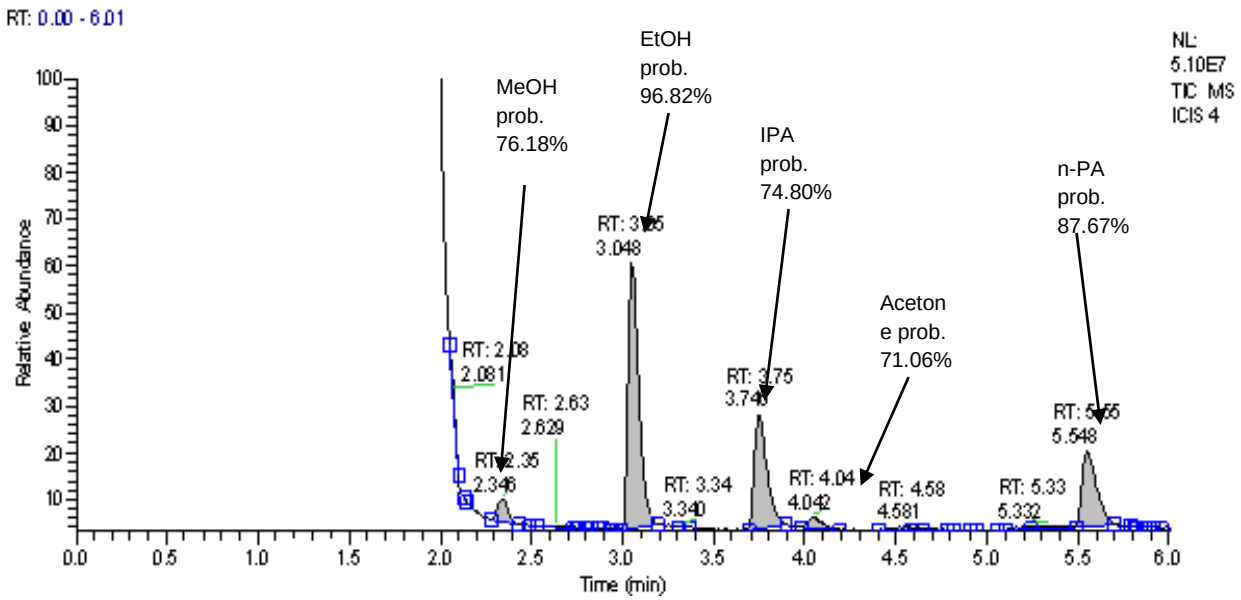


Figure 4 (a) and (b): Chromatograms of five volatile compounds analysed by Gas Chromatography with headspace sampling technique.

Based on the chromatogram obtained it clearly shows that all the volatile compounds spike in the sample (ethanol, methanol, iso-propanol, n-propanol and acetone) were fairly separated in the chromatograms. The peaks of each compound shows variation in retention time for both sample analyzed. The method is specific for ethanol resulting in well-established retention time for both samples. The retention time of ethanol is 3.05 minutes for both sample. For methanol it is at 2.35 minutes for both samples. The retention time for other three compounds are 3.75, 4.04 and 5.55 minutes for iso-propanol, acetone and n-propanol respectively. Therefore, it is noted that the method and the column is highly selective and accurate in determination of the analytes and the quality of the analyses.

4.4 Accuracy

According to International Convention on Harmonization (ICH) accuracy of an analytical procedure as the closeness of agreement between the results of measurement to a true value (Chan, 2008). To determine the accuracy of the method standard solution is run as sample in eight replicate and recovery is calculated. The fresh blood sample is spike with six ethanol level e.g. 25mg/dl, 40mg/dl, 80mg/dl , 100mg/dl , 200mg/dl and 300mg/dl .The measurement is recorded and mean value were also calculated as per Table 2. Recovery is calculated using the formula;

$$\% \text{ Recovery} = \frac{\text{Mean concentration from observation}}{\text{Concentration from CRM}} \times 100$$

Table 6: Result of recovery testing on six standard ethanol solution

Standard Concentration	25mg/dl	40mg/dl	80mg/dl	100mg/dl	200mg/dl	300mg/dl
1	27	39	78	98	203	301
2	26	39	77	98	204	301
3	22	39	76	98	201	300
4	25	38	78	99	200	300
5	26	37	79	99	202	303
6	25	39	77	97	204	298
7	25	38	79	97	201	301
8	25	38	77	100	201	298
Mean	25.12	38.38	77.63	98.25	202.00	300.25
% Recovery	101.24	95.47	95.13	98.25	100.05	101.09

Table 7: Result on the evaluation of accuracy of the method.

Concentration (mg/dl)		**Recovery (%)**
Theoretical	**Determined (Mean)**	
25	25.12	101.24
40	38.38	95.47
80	77.63	95.13
100	98.25	98.25
200	202.00	100.05
300	300.25	101.95
Average		98.68 (RSD 2.94%)

The rate of recovery is fitted between the ranges of 95.13% to 101.95%. The relative standard deviation (RSD) calculates at 2.94 %. This giving confident on the methods and the instrument accuracy in achieving the concentration close to the theoretical values.

4.5. Precision

Precision evaluation is the closeness of agreement between a series of measurements obtained from multiple samples of the same homogenous sample under control condition. It measures the degree of repeatability of an analytical method under normal operation (%RSD). Repeatability is given as the relative standard deviation on the result from two or more analysis conducted on the same sample, by the same technician, using the same instrument in short interval or period of time. ICH suggested that repeatability using a minimum nine determination covering specified range for the procedure. The calculation is conducted via 10 replicates. Each of the three sample were spiked using ethanol standard separately in concentration of 40mg/dl (0.04%), 100mg/dl (0.10%) and 300mg/dl (0.30%).

Table 8: Precision result for low, medium and high ethanol concentration.

Sample	(40mg/dl)		(100 mg/dl)		(300 mg/dl)	
	x_i	$(\overline{x_i} - x)^2$	x_i	$(\overline{x_i} - x)^2$	x_i	$(\overline{x_i} - x)^2$
1	39	0.49	98	0.09	301	0.49
2	39	0.49	98	0.09	301	0.49
3	39	0.49	98	0.09	300	0.09
4	38	0.09	99	0.49	300	0.09
5	37	1.69	99	0.49	303	7.29
6	39	0.49	97	1.69	298	5.29
7	38	0.09	97	1.69	301	0.49
8	38	0.09	100	2.89	298	5.29
9	38	0.09	98	0,09	302	2.89
10	38	0.09	98	0.009	200	0.49

	$\overline{x}$ = 38.3	∑ = 4.1	$\overline{x}$ = 98.3	∑ = 7.52	$\overline{x}$ = 300.3	∑ = 13.7
SD	**0.675**		**0.925**		**1.56**	
RSD	**1.76%**		**0.94 %**		**0.519%**	

Based on the calculation the statistical data obtained for 40mg/dl, average: 38.3, Standard Deviation: 0.675, Relative Standard Deviation: 1.76%; 100mg/dl, average: 98.3, Standard Deviation: 0.925, Relative Standard Deviation: 0.95 %; and 300mg/dl: average: 300.3, Standard Deviation: 1.56, Relative Standard Deviation: 0.519%. In clinical and forensic toxicology analysis the acceptance criteria for precision are below 15 % RSD. The result obtained are much lower indicating the method used shows high in precision.

4.6. Limit of Detection (LOD)

Limit of detection (LOD) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from background noise. LOD is expressed as the analyte concentration corresponds to 0 + 3SD. Ten blanks sample were prepared by spiking the lowest acceptable concentration of ethanol (0.025% @ 25mg/dl. Data obtained is analysed to calculate the LOD which is determined as 3 times the Standard Deviation.

Table 9: Result of ten blank sample for LOD determination.

Sample Blank	Xi (mg/dl)	$(Xi- \bar{X})$
1	27	3.24
2	26	0.64
3	22	10.24
4	25	0.04
5	26	0.64
6	25	0.04
7	25	0.04
8	25	0.04
9	27	3.24
10	24	1.44
Mean	25.2	19.6
SD	-	1.48

LOD Calculation,

LOD = 3SD = 3 x 1.48 = 4.44

Therefore the Limit of Detection (LOD) calculated is approximately **4 mdg/dl.**

4.7 Limit of Quantification

The limit of quantification (LOQ) is the determination of the lowest concentration of analyte that can measure with an acceptable level of repeatability precision and trueness.

It is calculated from the results at the lowest accepted spiked level, as 5 or even 10 times the standard deviation. LOQ is an indicative value and should not be used in decision making.

By referring the Table 9,

LOQ = 5SD = 5x 1.48= 7.4

Therefore, the Limit of Quantification for this method is approximately **7 mg/dl.**

4.8 Robustness

The robustness of the analytical method is the ability of the method to resist the changes or adjustment made to the protocol in order to investigate the effect on precision or accuracy of the method.

This is done by monitoring two different conditions which is, the changes in the sample matrix and the changes in ethanol concentrations.

A. The changes in the sample matrix

i. Blood sample

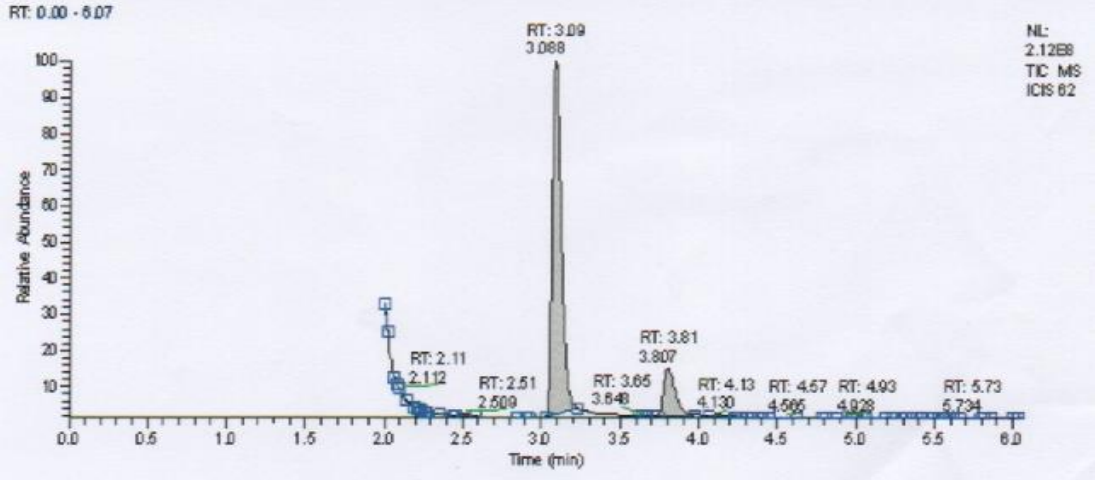


Figure 9: Chromatogram of blood sample

ii. Urine sample

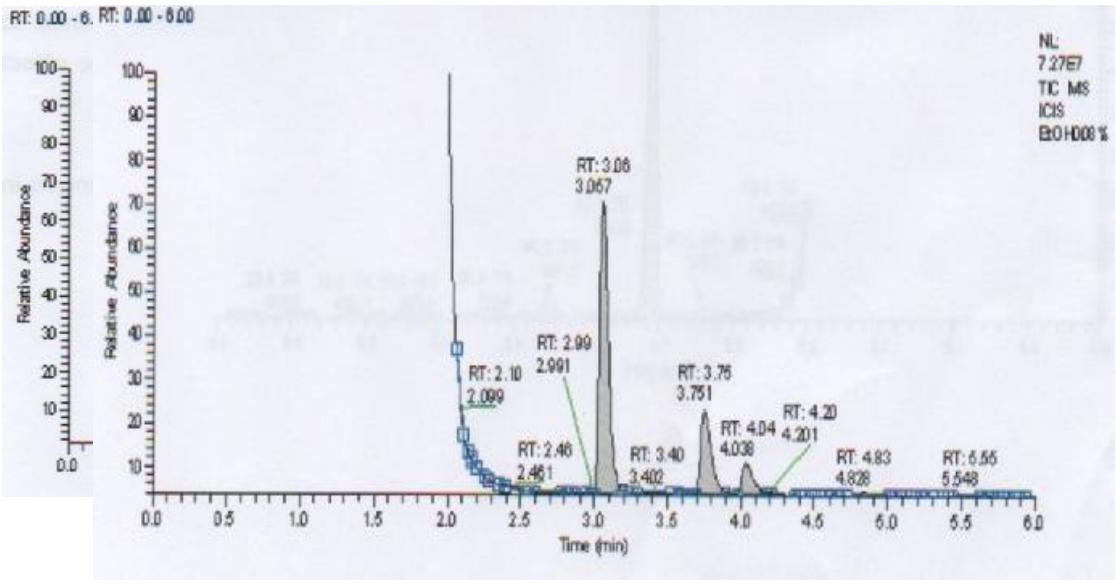


Figure 10: Chromatogram of Urine sample

B. The changes in ethanol concentrations

i. Concentration : 100 µl of Ethanol-40 (40mg/dl) spiked in 100µl blood

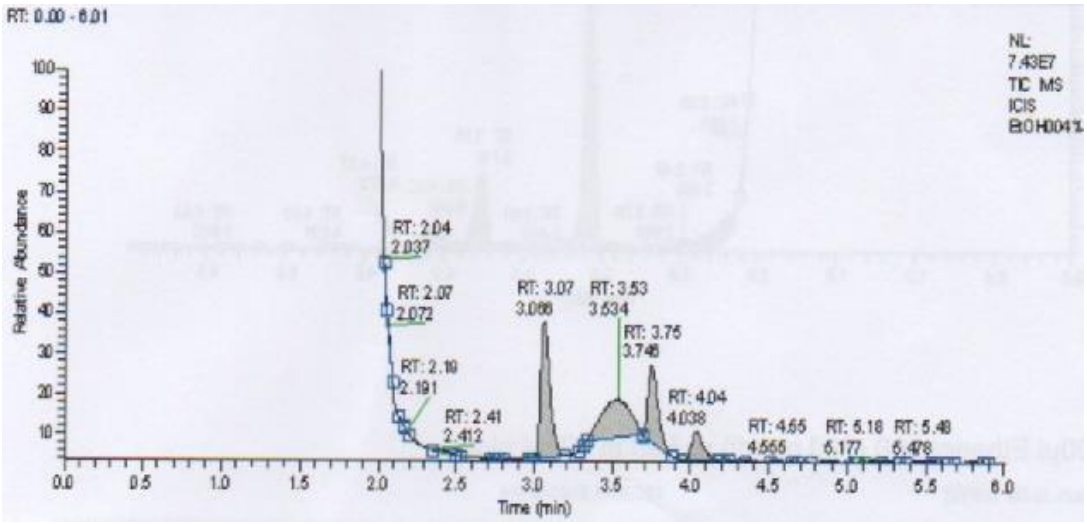


Figure 10: Chromatogram for ethanol-40 (40mg/dl)

ii. Concentration : 100 µl of Ethanol-80 (80mg/dl) spiked in 100 µl blood

Figure 11: Chromatogram for ethanol -80 (80mg/dl)

iii. Concentration: 100µl of Ethanol-100 (100mg/dl) spiked in 100µl blood

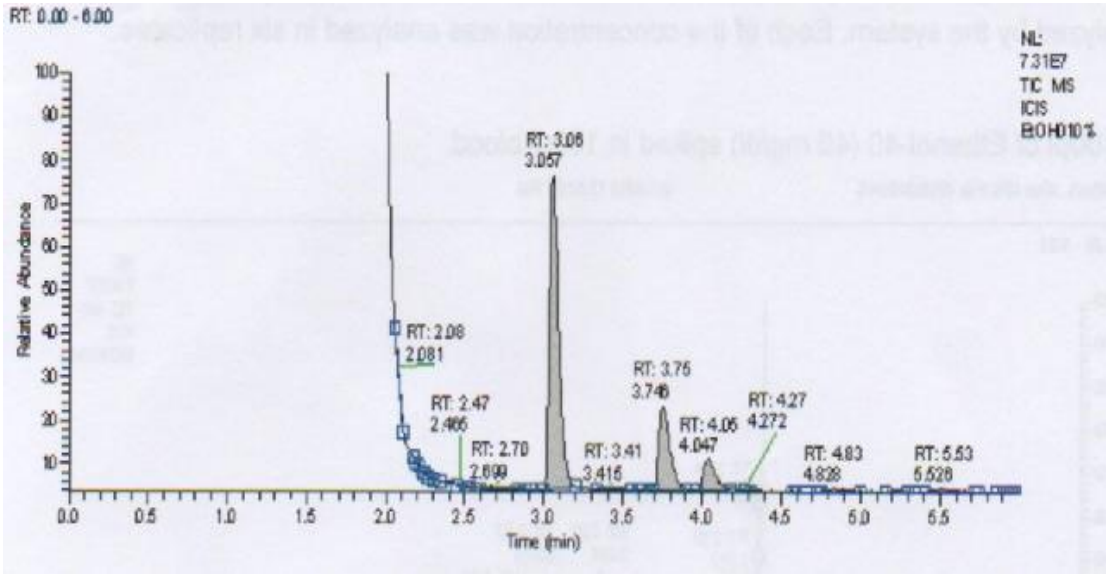


Figure 12: Chromatogram for ethanol -100 (100mg/dl)

iv. Concentration : 100µl of Ethanol-300 (300mg/dl) spiked in 100µl blood

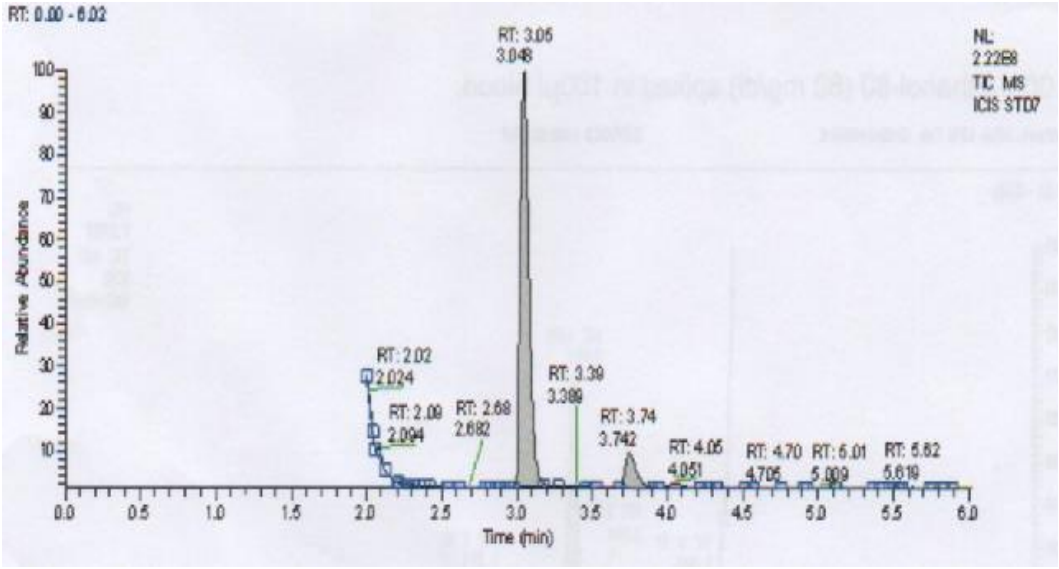


Figure 13: Chromatogram for ethanol -300 (300mg/dl)

Table 10: Result of fortified ethanol concentration from 40 mg/dl to 300mg/dl in six replicates

Concentration (mg/dl)	Mean T_R	Standard Deviation	t_R		Relative Standard Deviation (%)
			Minimum	Maximum	
40 (n= 6)	3.06	0.0063	3.05	3.07	0.21
80 (n= 6)	3.048	0.0041	3.04	3.05	0.13
100 (n= 6)	3.047	0.0052	3.04	3.05	0.17
300 (n= 6)	3.043	0.0052	3.04	3.05	0.17

Although the analysis is conducted in different matrix i.e. blood and urine samples, the peaks are detected with a small differences in retention time. Whereas for the analysis with different concentration the retention time recorded are very much consistence at the range of 3.05 to 3.07 minutes with relative standard deviations from 0.17-0.21%. This give a reasonable accuracy and precision of the method employed.

Part B

4.9 Analysis of Ethanol in Post-mortem Blood Sample

I. New Column

In the month of December, the Gas Chromatography instrument undergone periodic maintenance and the previous column was replaces with new column. Previously the analysis of ethanol was conducted with the same protocol but using acetonitrile as the internal standard causing the deterioration of the column and furthermore the column was never replaced since its installation in 2009. To validate the column performance, linearity studies was conducted to obtain the calibration curve and regression coefficient for all six level of standard concentration of ethanol. Chromatogram and mass spectrum of each concentration level is shown below.

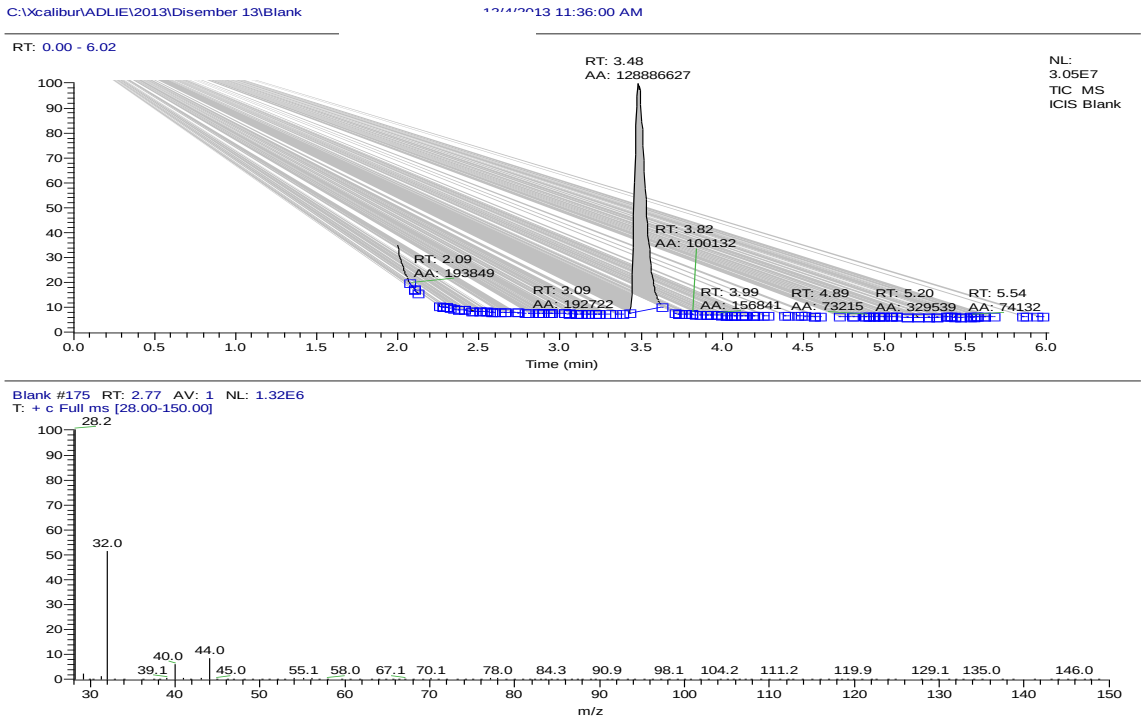


Figure 12 : Chromatogram and Mass Spectra for Blank

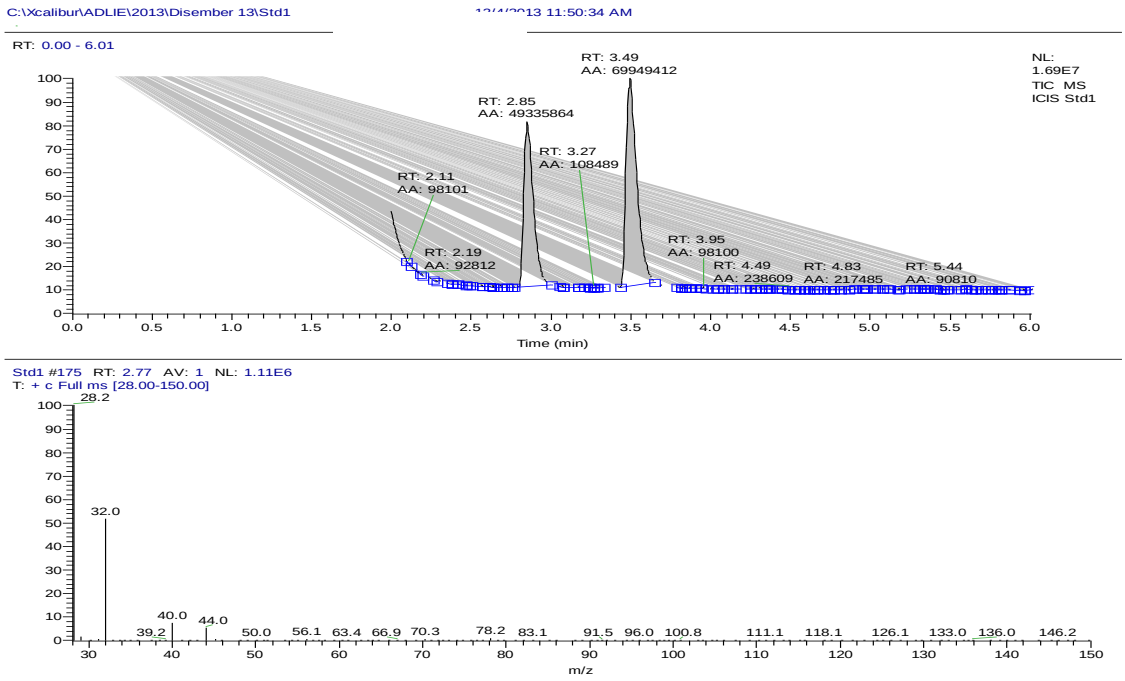


Figure 13 : Chromatogram and Mass Spectra for 0.025% @ 25mg/dl

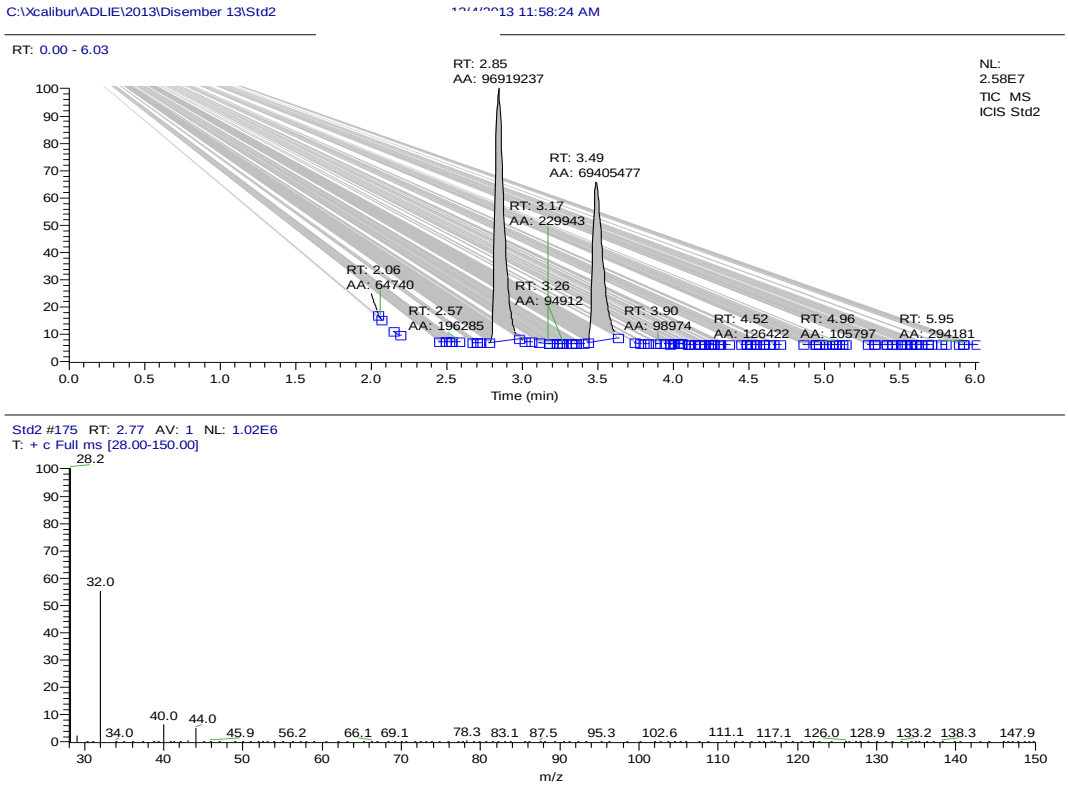


Figure 14 : Chromatogram and Mass Spectra for 0.05% @ 50mg/dl

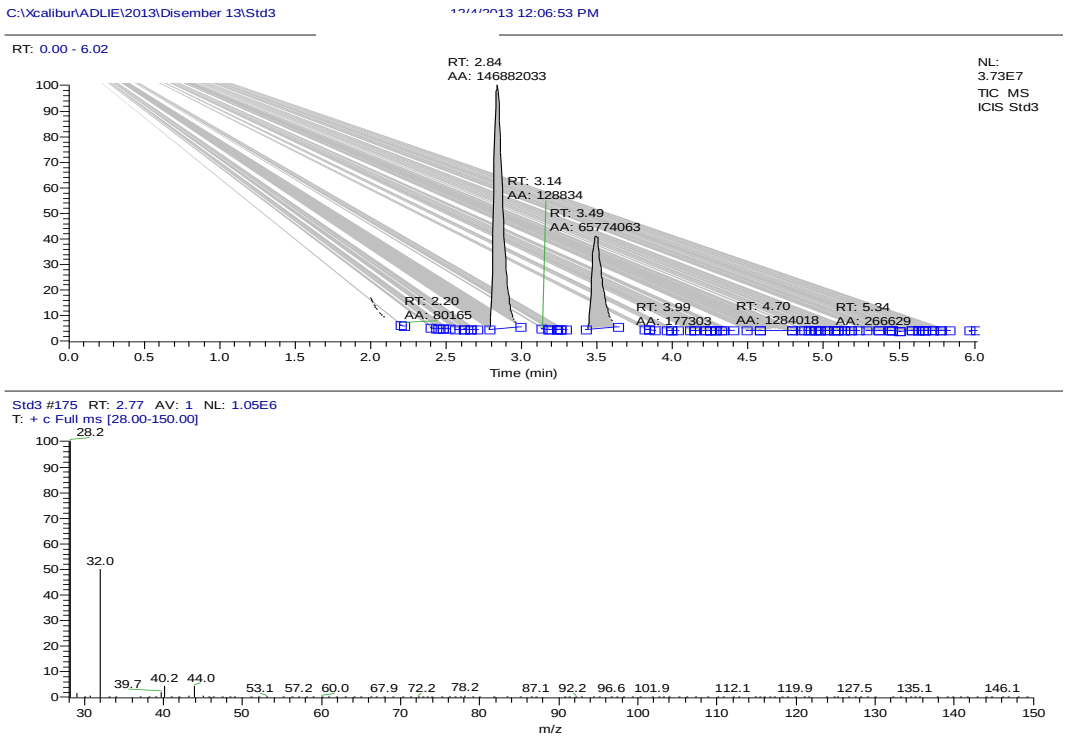


Figure 15 : Chromatogram and Mass Spectra for 0.08% @ 80 mg/dl

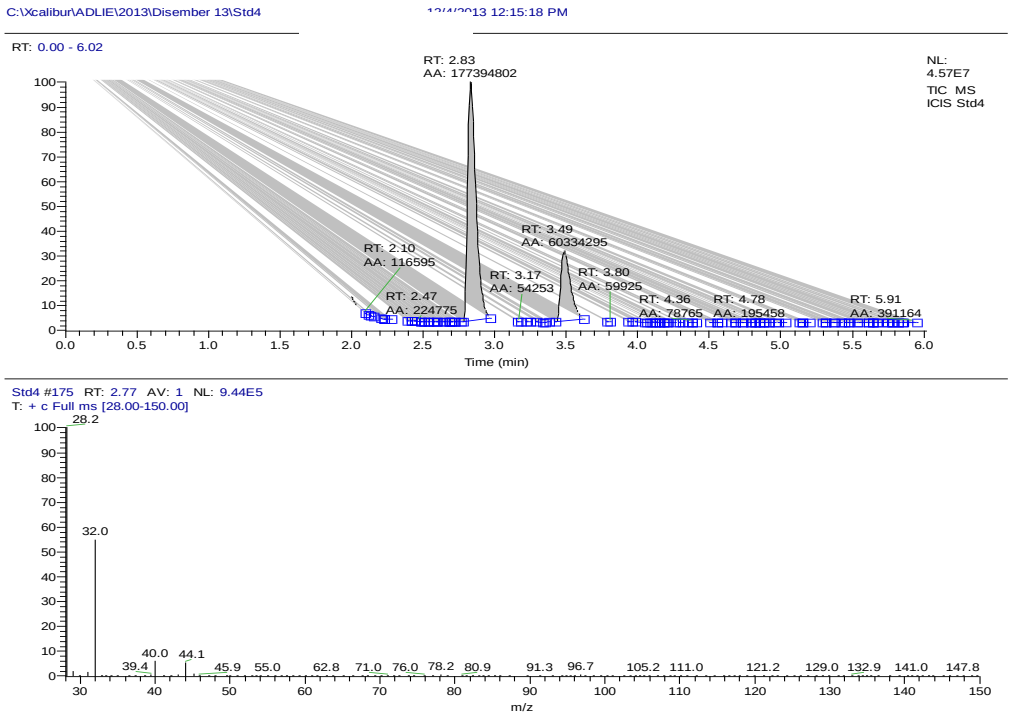


Figure 16 : Chromatogram and Mass Spectra for 0.10% @ 100 mg/dl

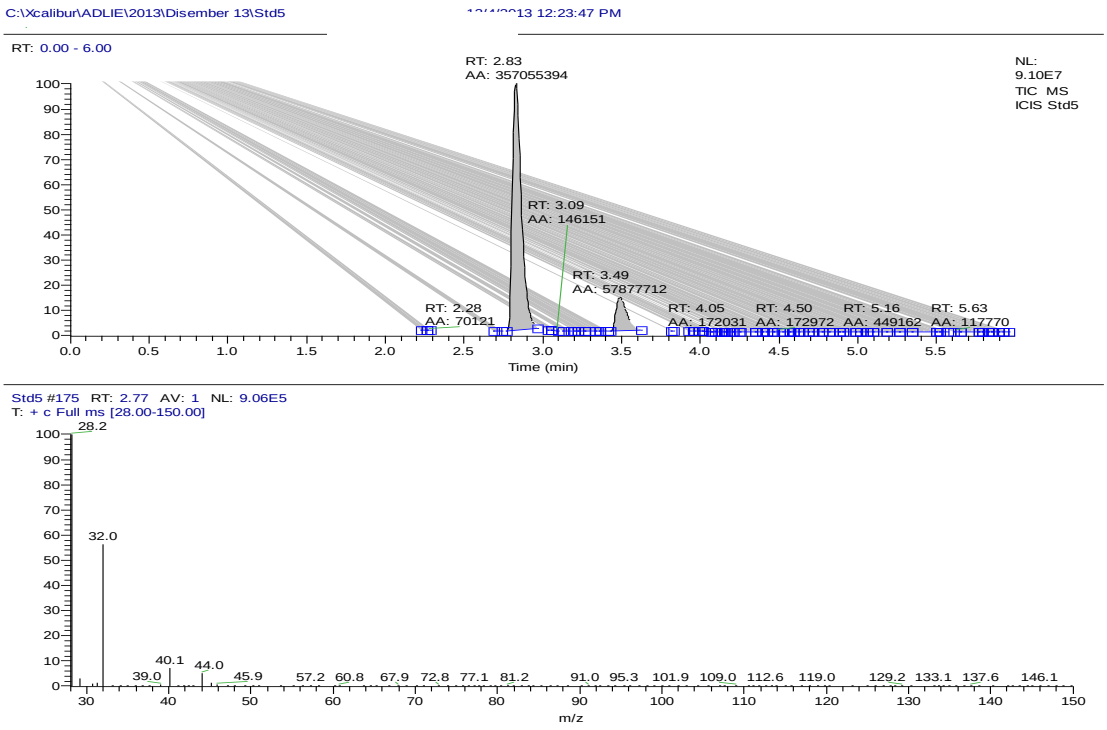


Figure 17 : Chromatogram and Mass Spectra for 0.20% @ 200 mg/dl

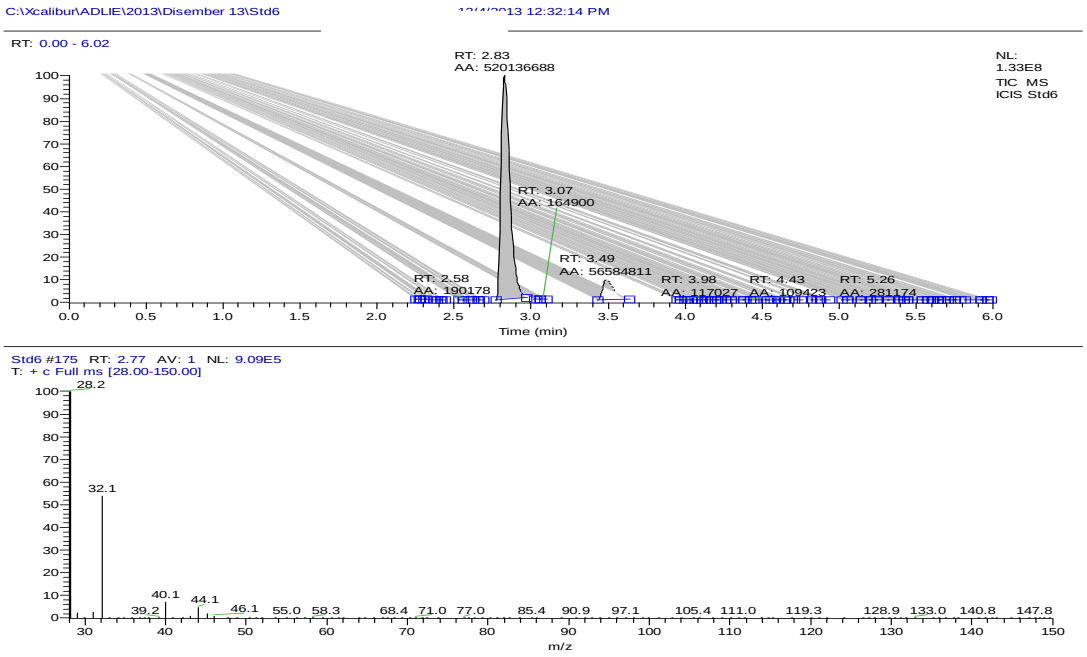


Figure 18 : Chromatogram and Mass Spectra for 0.30% @ 300 mg/dl

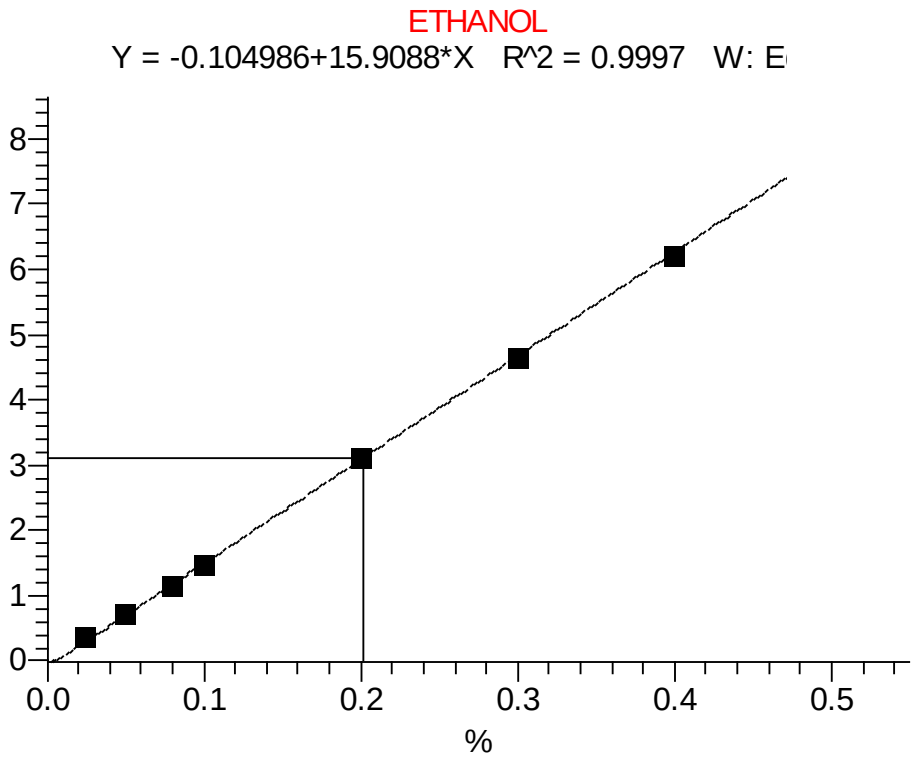


Figure 19 : Calibration Curve for ethanol standards

The retention time for ethanol and isopropanol has been shifted to new value which is for ethanol the retention time is 2.85 and for isopropanol is 3.49 respectively. The calibration curve is linear with regression value of 0.9997. Based on the chromatogram and the spectra of different shows the analysis is valid and revalidation is not conducted. Therefore new value was assigned for the analysis of ethanol in post mortem blood obtain starting from January.

II. Analysis post mortem blood sample

The analysis was conducted on fifty sample collected form postmortem examination conducted in Forensic Medicine Department in Hospital Sungai Buloh various type of case. The summary of all the information obtained from the analysis in the tables below.

Table 11: Summary of result obtained from analysis of 50 sample

	Sample ID	Area	Internal Standard Area	Calculated Amount (mg/dl)	Retention Time	Days of Analysis	Result
1	P225/13	NF	34220603	NF	NF	1	Not Detected
2	P226/13	NF	28321953	NF	NF	1	Not Detected
3	P235/13	NF	30762104	NF	NF	1	Not Detected
4.	P236/13	NF	27699978	NF	NF	1	Not Detected
5	P237/13	NF	30691147	NF	NF	1	Not Detected
6	P239/13	NF	31179860	NF	NF	1	Not Detected
7	P240/13	3174097	30661579	13	2.86	1	Detected
8	P242/13	NF	34605377	NF	NF	1	Not Detected
9	P243/13	NF	33658935	NF	NF	1	Not Detected
10	P246/13	151016132	34872636	279	2.83	1	Detected

NF: Not Found

No	Sample ID	Area	Internal Standard Area	Calculated Amount (mg/dl)	Retention Time	Days of Analysis	Result
11	P249/13	NF	34469154	NF	NF	1	Not Detected
12	P250/13	NF	36044840	NF	NF	2	Not Detected
13	P253/13	28466918	32981236	61	2.85	1	Detected
14.	P257/13	NF	31771375	NF	NF	1	Not Detected
15	P259/13	NF	29474224	NF	NF	2	Not Detected
16	P002/14	121347312	33549427	248	2.83	2	Detected
17	P005/14	NF	28712364	NF	NF	1	Not Detected
18	P010/14	NF	40381918	NF	NF	1	Not Detected
19	P012/14	5875220	32575396	22	2.85	1	Not Detected
20	P013/14	NF	30054838	NF	NF	2	Not Detected
21	P017/14	17776022	28934407	51	2.85	1	Detected
22	P018/14	NF	29533408	NF	NF	2	Not Detected
23	P019/14	NF	34889791	NF	NF	1	Not Detected
24	P021/14	NF	33817422	NF	NF	1	Not Detected
25	P022/14	NF	34207398	NF	NF	1	Not Detected
26	P023/14	NF	33799249	NF	NF	1	Not Detected
27	P025/14	NF	39799290	NF	NF	1	Not Detected
28	P027/14	NF	36178683	NF	NF	1	Not Detected
29	P028/14	NF	33020358	NF	NF	1	Not Detected
30	P031/14	NF	39512483	NF	NF	1	Not Detected

No	Sample ID	Area	Internal Standard Area	Calculated Amount (mg/dl)	Retention Time	Days of Analysis	Result
31	P032/14	NF	38651028	NF	NF	1	Not Detected
32	P033/14	NF	33592602	NF	NF	1	Not Detected
33	P034/14	NF	34470756	NF	NF	1	Not Detected
34	P039/14	NF	34035875	NF	NF	1	Not Detected
35	P040/14	NF	34844568	NF	NF	1	Not Detected
36	P041/14	NF	30707904	NF	NF	1	Not Detected
37	P042/14	NF	34404831	NF	NF	1	Not Detected
38	P047/14	NF	40853951	NF	NF	1	Not Detected
39	P052/14	NF	38029737	NF	NF	1	Not Detected
40	P055/14	NF	35719958	NF	NF	1	Not Detected
41	P056/14	NF	34469154	NF	NF	1	Not Detected
42	P057/14	NF	35404268	NF	NF	2	Not Detected
43	P066/14	NF	38544663	NF	NF	2	Not Detected
44	P076/14	117810241	31337338	257	2.84	2	Detected
45	P078/14	NF	27593738	NF	NF	1	Not Detected
46	P079/14	NF	31941246	NF	NF	1	Not Detected
47	P080/14	NF	27482648	NF	NF	1	Not Detected
48	P081/14	NF	26339613	NF	NF	1	Not Detected
49	P083/14	NF	35014044	NF	NF	1	Not Detected
50	P084/14	NF	34041109	NF	NF	1	Not Detected

Based on the fifty sample analyzed, only for seven sample (P240/13, P246/13, P253/14, P002/14, P012/14, P017/14, P076/14) ethanol concentration has been detected. This is based on the chromatogram and spectra the retention time is between 2.83 to 2.86 which is fairly consistent with the ethanol retention time of 2.85. Both the chromatogram and spectra has been evaluated to indicate all the seven sample are positive for ethanol detection. The ethanol concentration obtained did not surpass the limit of ethanol concentration permitted by the law which 0.08 g/l @ 800mg/dl. The highest concentration detected is for sample P246/13 with the concentration of 279mg/dl. The lowest concentration detected is for sample with the id P240/13 with the concentration of 13mg/dl.

The analysis is conducted within an average of one to two days after the sample is collected during the postmortem examination. The sample were also collected using sample tube with pre added with preservative of sodium fluoride. The addition of the will ceased all the microorganism activities that is related to ethanol production. Considering these two facts, it can be concluded that the concentration of ethanol detected is based on the consumption of alcohol and not from any natural putrefaction or microbial production. The analysis also has been conducted in a shortest time as possible as compared to current practice that take about one to two month for the result to be back.

CHAPTER 5

5.0 Conclusion

The determination of ethanol in post-mortem blood has been successfully conducted using Headspace sampling coupled with Gas Chromatography – Mass Spectrometry technique. The blood alcohol analysis can be conducted easily and rapidly within one day with high accuracy and sensitivity. The limit of detection (LOD) and limit of quantification (LOQ) is 4mg/dl and 7mg/dl respectively indicate that the analysis method are very sensitive and suitable for blood alcohol determination and quantification. Further analysis need to be conducted to show correlation of alcohol consumption and the concentration of ethanol distributed in the blood. This is still a major obstacle because of the sampling of blood need to be conducted via ante mortem and postmortem. Beside that there are many studies shows that postmortem diffusion and redistribution occurs and the concentration of ethanol in postmortem blood is not necessarily the same as the concentration present at the time of death. Therefore further analysis should be conducted not only on the postmortem blood but other bodily fluid such as urine and vitreous humor.

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