Submitted to the Division of Postgraduate Research of Kingston University, London

Development of a headspace GC-FID method for the detection of alcohol in samples of UK motorist's blood and urine.

This thesis is submitted in fulfilment of the requirements of Kingston University for the degree of:

Masters by research in Chemistry

By: Luke Taylor

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Contents

Abstract

Ethanol is one of the most well studied and researched drugs available to man and the effects of ethanol on the human body are of particular interest with the effects on decision making and reaction times in relation to driving being a key area of research, with legislation relating to levels of alcohol within the human body which can be considered safe for vehicle operation being written as a direct result of these studies. Analysis of blood and urine alcohol content is one of the most common forensic toxicological analyses carried out and methods developed to quantify and detect alcohol in biological samples such as blood and urine are vital in areas such as road traffic toxicology where an accurate and trustworthy quantitation is required to determine if a suspected drunk driver is in fact under the influence of alcohol whilst in charge of a vehicle. The method development and validation process are designed to demonstrate the capability of an analytical instrument to achieve this quantitation. A method was developed using headspace gas chromatography with flame ionisation detection (HS- GC-FID) using an HTA 200 H headspace auto sampler, BAC plus 1 and plus 2 columns and dual FID 2014 detectors and tertiary butanol as an internal standard. The accuracy, precision and parameters such as linearity were tested and validated according to a pre- determined validation plan. It was determined that a separation of ethanol and acetone was achieved on one column and the accuracy and precision is within the set parameters of the validation plan. The method was successfully validated, and the next step of the experimentation was the testing of storage stability which involved testing the stability of blood, urine and QC samples at room temperature for up to one month and the testing of freeze thaw stability for up to two-cycles. It was determined that most samples were stable throughout the stability testing period and there was largely no significant effect on alcohol concentration throughout the testing phases.

Introduction

The History, chemical structure and pharmacology of alcohol

Ethanol, also known as ethyl alcohol or grain alcohol has a long history of consumption and abuse within human society throughout the world. It has been shown to have medical applications and was viewed as a treatment for ailments with alcohol being termed “Aqua vitae” or water of life by monks and physicians of the middle ages (Seward, 1979). It has also been consumed as an alternative to contaminated water (Crews, 2018) or to protect from diseases such as the bubonic plague (Hanson, 1995). It is difficult to pinpoint when exactly humans began distilling alcohol for consumption, the earliest evidence of alcohol brewing was found as ethanol residues in pottery from china dating around 7000 to 6600 BC and an alcoholic drink from distilled rice was identified in India known as Sura from between 3000 and 2000 BC (Mandelbaum, 1965) with evidence of alcohol brewing by the Sumerians in Mesopotamia. At around the same time vineyards in Ancient Greece became one of the earliest recorded producers of wine and providing wine to guests as an act of hospitality became commonplace (Scott, 2015). Many of these Greek traditions of wine production and festivities related to alcohol consumption translated over into Roman cultures with wine playing a big role in their society, it was also used in medical applications within the Roman Empire. Wine also played a role in religion within the Roman Empire with a cult to the Roman and Greek god Dionysus/Bacchus being formed and spreading throughout the society of the empire (Scott, 2015). By the 1400s, spirits were beginning to be distilled with whiskey, gin and rum being referenced around this period and by 1494 whiskey within Scotland appeared to be well established with records of consumption being recorded in the UK Exchequer accounts (Bower, 2016).

Ethanol is a polar primary alcohol with the formula C_2H_6O (Figure 1). It is a clear colourless liquid with a distinctive odour and taste with a melting point of -173.4^{o}F (-114.1 ^{o}C) and a boiling point of 173.3 ° F (78.5 ^{o}C) (Silberberg, 2015). It is produced as a product of plant fermentation of sugars or starch and can also be produced via the hydration of ethylene (Chang, 2016). Ethanol has many useful applications including use in cosmetic products, dyes, explosives and synthetic drugs (Chang, 2016). In addition, ethanol is often mixed into beverages for recreational consumption, and ethanol can induce significant effects on the central nervous system (CNS) dependent on the dose. Once ingested the pathway of detoxification and removal from the body takes place in four major steps according to the principle of pharmacokinetics which are defined under the acronym ADME; (absorption, distribution, metabolism and elimination). The rate which this process takes place is dependent on a host of factors including age, gender, metabolic rate and nutrition.

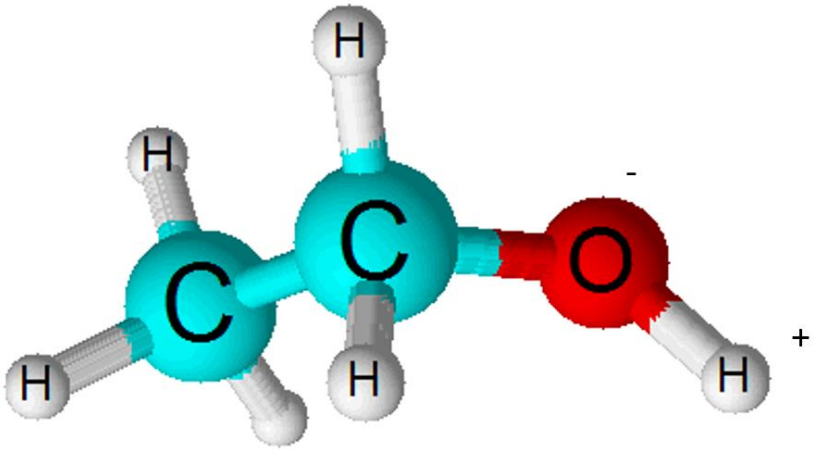


Figure 1: A 3-D representation of a molecule of ethanol, the OH group represents the polar section of the molecule, with the CH_4 section representing the non-polar section.

Absorption takes place primarily in the gastrointestinal tract, alcohol is absorbed slowly through the stomach and rapidly through the small intestine, and this is due to the larger surface area of the intestinal tract when compared to that of the stomach. The rate at which ethanol is absorbed depends on two major factors. The gastric emptying rate, which is the rate at which the stomach empties its contents, if the stomach is empty prior to consuming alcohol, such as following a period of fasting for example, the gastric emptying rate will be higher thus ethanol absorption will occur more rapidly (Gentry, 2000). Nutrients such as carbohydrates, fats and proteins will slow the rate of gastric emptying increasing the time taken for ethanol to transfer from the stomach to the duodenum. (Gentry, 2000). The second major factor is the concentration of ethanol ingested (Dubowski, 1985). Upon absorption ethanol enters the portal vein and is transported to the liver, where it undergoes a phenomenon known as the first pass effect or first pass metabolism, which is the uptake and metabolism of a substance within the liver before entering (the) systemic circulation (Gentry, 2000). There is a connection between gender and the rate of first pass metabolism, it is believed that males have a higher first pass metabolism rate (although the gender difference in practice is minor) (Ammon *et al.,* 1996) when compared to females which is caused by a lower gastric alcohol dehydrogenase (ADH) (Seitz *et al.*, 1993). This corresponds to a slightly higher tolerance to alcohol in men. When absorption of ethanol into a cell occurs it is facilitated by diffusion (Lodish, 2013), a passive process meaning it does not require any transport molecules to facilitate the action, which is defined as the movement of a substance from an area of high concentration to an area of low concentration according to Fick's Law (Lodish, 2013).

After absorption from the gastrointestinal tract and first pass metabolism, ethanol enters systemic circulation and is transported to the right side of the heart, following on to the lungs and then following the circulation back to the heart where it then travels throughout the circulatory system organs with a higher blood flow rate and water content such as the brain will receive ethanol from the blood at a higher rate than tissues and organs that have a lower blood flow such as skeletal muscle (Jones,2011). Ethanol also distributes into the total body water which for an average male of 70 Kg is said to be 42 litres (42 Kg) of water distributed throughout the body, approximately 60% of the body mass is attributed to water with up to a third of the total body water being found in the extra cellular

fluid component (Rosenfield, 1996). This high-water content is responsible for ethanol's widespread distribution in the body.

Ethanol like most drugs is primarily metabolised by the liver. The metabolic pathway responsible for ethanol metabolism has two major branches within humans, the first involves the enzymatic breakdown of ethanol into acetaldehyde by the enzyme alcohol dehydrogenase (figure 2). This uses NAD+ as a cofactor which is reduced into NADH in the process, Zinc also plays a role in this function, it acts as a guide to position the ethanol within the active site (Goodsell, 2001). This process is detailed in figure 3. The primary enzyme implicated in ethanol metabolism is ADHI (Alcohol dehydrogenase class I), this has the highest affinity for ethanol when compared to the class II and III isoforms of ADH, although these will bind more readily to ethanol in higher BACs (Blood alcohol concentrations) (Ramchandani, 2013). Acetaldehyde is a toxic metabolite which is a possible cause of hangover symptoms (Swift, 1998), although other possible causes such as a disturbance in electrolyte balance or other biologically active compounds or congeners such as methanol have been suggested. It is also possible that a hypoglycaemic episode may be responsible for the headache and disorientation symptoms exhibited during a hangover, although the exact cause is unknown it is possibly a mixture of one or more of these factors (Swift, 1998). Alcohol is also a diuretic and suppresses the activity of anti-diuretic hormone within the kidney resulting in an increased urinary output, in events of extended drinking with no proportional water consumption this can lead to dehydration which can contribute to the headaches experienced with a hangover (Rang *et al.,* 2016). It is important for acetaldehyde to be metabolised quickly to avoid toxicity, acetaldehyde is processed into acetate by the enzyme ALDH (aldehyde dehydrogenase). It is shown that individuals of Asian descent can possess variants of the ADH and ALDH genes which makes the metabolism of acetaldehyde less efficient resulting in a build-up of the metabolite causing a phenomenon known as alcohol flush response (Jones, 2011). The symptoms of alcohol flush response include redness of the skin around the face, neck and occasionally the entire body; this is a result of vasodilation caused by the acetaldehyde build-up. The resulting metabolic process within the liver also disrupts the NAD+ and NADH balances within the liver with an increase being observed in both cytosolic and mitochondrial fractions resulting in a disturbance in other metabolic processes and functions, such as the metabolism of certain drugs. (Rang *et al.,* 2016). Acetate may be processed within the liver or enter the bloodstream and be processed within peripheral tissues. The eventual end point of metabolism in this pathway takes place in the muscle tissue and the end products are carbon dioxide and water, methanol follows the same metabolic pathway and is metabolised into formaldehyde and then to formic acid (Zimmerman, 1999).

The second pathway which plays a lesser role in the metabolism of ethanol, and usually only contributes during higher blood alcohol concentrations or chronic episodes of alcohol consumption are encountered is the microsomal oxidative pathway. This pathway uses the cytochrome P450 enzyme CYP2E1 which is located in the smooth endoplasmic reticulum. This pathway leads to the production of Reactive Oxygen Species (ROS) and plays a role in metabolism of ethanol into acetaldehyde.

Only a small proportion of ethanol is excreted unchanged and the process of excretion may happen through three routes, urine, sweat and exhalation. Jones *et al.*, 2009 estimates that only around 2% of ethanol is excreted in the urine unchanged (Jones *et al.*, 2009). Ethanol elimination is believed to follow zero-order kinetics, which means it is dose independent (Baselt, 1995), the elimination rate of ethanol has been estimated to be between 10-15ml of absolute alcohol per hour for a non-tolerant

person (Leonard, 2005) with 18 mg being shown as a lower elimination rate for a person considered to be tolerant (Wilkens *et al.,* 1998). A small proportion of ethanol is conjugated into ethyl glucuronide, ethyl sulphate or ethyl esters. Ethyl glucuronide is formed as a minor metabolite of ethanol by the enzyme UDP-glucuronosyltransferase whilst ethyl sulphate is formed by sulfotransferase and although this and sweat does account for a portion of the elimination of ethanol overall this only accounts for a small fraction of elimination. Around 90% of ethanol ingested is eliminated via the oxidative metabolic pathways and under 10% of the total elimination occurs via sweat, urine or breath (Cederbaum, 2013).

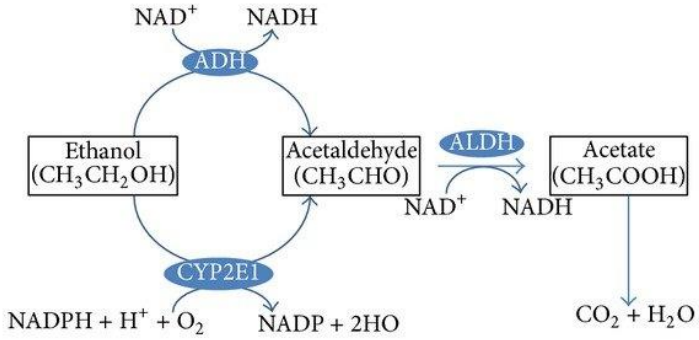


Figure 2: The oxidative reaction pathway for the elimination of ethanol showing the oxidation of ethanol by ADH into acetaldehyde and the second oxidation stage which is the oxidation of acetaldehyde into acetate (Kawaratani, 2013).

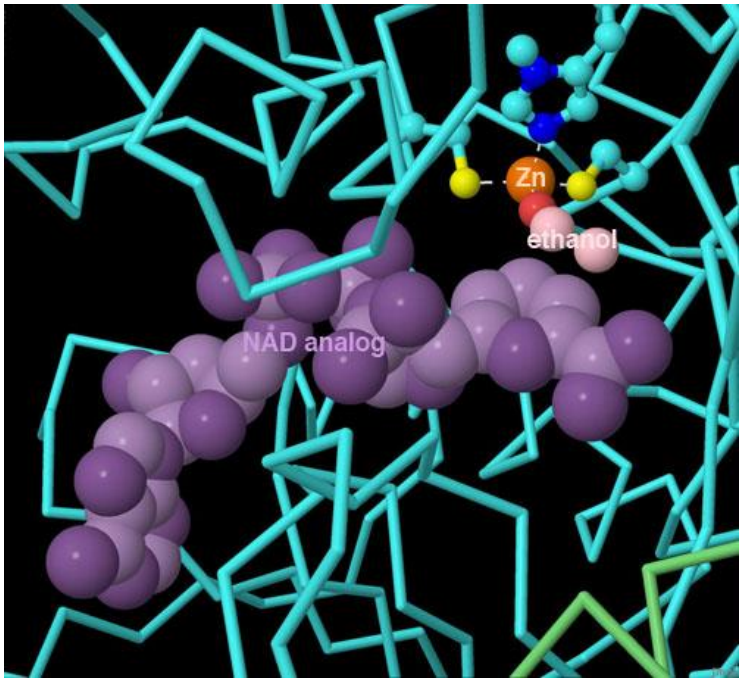


Figure 3: The molecular representation showing the role zinc plays in anchoring ethanol in the active site of ADH during the enzymatic metabolism of ethanol into acetaldehyde (Goodsell, 2001). As shown, this process utilises NAD+ and the molecule used in this metabolism is no longer available for other metabolic processes such as metabolism of medicinal drugs demonstrating the disruptive effect of ethanol on liver metabolic activity.

Alcohol on the CNS

The effects of alcohol concentration on an individual were examined in a seminal study by Kurt Dubowski in 1977. This study documented the stages of alcohol intoxication, the clinical effects observed and the BAC range at which the specific states are experienced. It has been noted that consistent intake of small volumes of alcohol, one or two units a day rapidly leads to a tolerance build –up, however this tolerance is rapidly lost within only a few days of abstinence (Leonard, 2005). Table 1 demonstrates these stages with conversions of the original BAC values which were given as a percentage into mg/dl, the unit currently used by the UK. The blood alcohol content at which inhibition of response times and fine performance tasks occurs, happens at relatively low levels of ethanol starting at as low as 30 mg/dl and it has been shown that these effects are more prominent as alcohol levels are increasing as opposed to when they are decreasing. This effect is known as the Mellanby effect and is suggested to be a result of a phenomenon known as acute tolerance (Ginsburg *et al.,* 2008). This develops rapidly after the consumption of alcohol, and the secondary effect is the sensation associated with intoxication and this decreases as the ethanol is eliminated. Studies in rats have verified this effect and a conclusion was drawn that proprioceptive response (the awareness of one's own body parts) was less accurate with the rising of BAC than when BAC was declining (Wang *et al.,* 1993). This could infer that a motorist that has undertaken driving while in the BAC absorption phase and prior to peak absorption may still have a degree of impairment even if they were under the prescribed 80 mg/dl legal limit.

The development of alcohol addiction is attributed to a major system of the brain associated with reward and addiction known as the limbic system. This is the collection of areas of the brain associated with the release of reward related neurotransmitters such as dopamine, Gamma Amino Butyric Acid (GABA) and glutamate. This system is formed of several regions of the brain including the hippocampus which is associated with memory processing, the thalamus and hypothalamus which are linked to control of the endocrine system as well as body temperature regulation and feeding, while the amygdala and septum are the emotional centres (Leonard, 2005). This interconnected signalling nexus provides the necessary combination of reward related neurotransmitters, memory centres to reinforce the feelings and sights of the exposure to alcohol and the emotional centres to experience the mood-altering effects of ethanol. The reinforcement of behaviours such as consumption of alcohol in this case can be a major factor in a transition from casual or social ethanol consumption into full alcohol seeking behaviour and alcoholism. There are two types of reinforcing behaviour, a positive reinforcement which can be described as a rewarding stimulus such as the euphoria of ethanol consumption which can lead to alcohol seeking behaviours and a negative reinforcement behaviour which may be an aversive response in the case of alcohol this may be symptoms associated with withdrawal such as anxiety, aggression or dysphoria (Gilpin and Koob, 2008). Essentially an individual may exhibit alcohol seeking behaviours to avoid these unpleasant symptoms.

Two key neurotransmitter receptors within the central nervous system (CNS) are affected by ethanol consumption and particularly by heavy and chronic drinking episodes. Alcohol is a known depressant and has an effect on the inhibitory neurotransmitter GABA. By binding to the $GABA_A$ receptor, it acts as an agonist for activity allowing an increased binding of GABA to the endogenous receptor. It also acts as an antagonist at NMDA (N methyl -D- Aspartate) type glutamate receptors which results in a

decreased responsiveness to released glutamate (Julien, 2011). The effect of the inhibited glutamate response alongside the enhanced GABA neurotransmission can lead to an adaptation response where there is an upregulation of NMDA receptors which results in excess glutamate release upon the removal of ethanol's effects on GABA thus leading to withdrawal symptoms (Julien, 2011). The influence of ethanol on $GABA_A$ receptors also triggers a dysregulation of GABAergic transmission and this in turn stimulates an increase in activation of opioid receptors that result in an activation of dopaminergic reward neurons within the ventral tegmental area (VTA) (Julien, 2011), (Gilpin and Koob, 2008). This agonistic effect on GABAergic receptors is also believed to be linked to a positive reinforcement effect of ethanol (Julien, 2011).

Table 1: Kurt Dubowski's stages of acute alcoholic influence/intoxication

Blood alcohol concentration (mg/dl)	Stage of alcoholic influence	Clinical signs/ symptoms
10-50	Subclinical	No apparent influence, behaviour will be observably normal. Changes may be detected with specialist tests.
30-120	Euphoria	Mild euphoria observable, talkative and sociable behaviours become notable, loss of inhibition or an increase in self-confidence, reduction in attention, judgement and control. A reduction in efficiency of fine performance tasks.
90-250	Excitement	Emotional instability further decreased inhibitions and critical judgement. Memory impairment may be observed with an increase in reaction time and incoordination will become apparent.
180-300	Confusion	Mental confusion/disorientation and dizziness, exaggerated emotional states such as anger, fear or grief, a decreased pain awareness following a general decrease in sensation slurred speech and impaired balance.
270-400	Stupor	Apathy, sensations of inertia. Approaching paralysis. Greater decrease in sensation to stimuli with a marked increase in muscular incoordination, resulting in an inability to stand or walk. Vomiting and urinary/faecal incontinence. Impaired consciousness resulting in sleep or stupor.
350-500	Coma	Complete loss of consciousness, anaesthesia or coma resulting in depressed or totally inhibited reflexes, subnormal body temperature, and urinary/faecal incontinence. Depressed rates of circulation and respiration resulting in possible death.
450+	Death	Death resulting from respiratory paralysis.

Table 1: Kurt Dubowski's stages of acute alcoholic influence/ intoxication. Original BAC values were given in percent so a conversion into mg/dl was carried out for ease of interpretation of each stage (Dubowski, 1977).

The role of ethanol in forensic toxicology

In England and Wales, the legal blood ethanol limit for driving is currently set at 80mg/dl with a legal urine limit set at 107 mg/dl, in Scotland it is currently 50 mg/dl in blood and 67 mg/dl of urine. The breath limit in the UK is set at 35 µg/dl Drivers found to oversee a vehicle when over these limits are liable to be charged under section 5 of the Road Traffic Act 1988. This act states that a person is guilty of the offence of DUI (Driving whilst under the influence) if a person drives or attempts to drive a motor vehicle on a road or pubic place or is in charge of a motor vehicle on a road or public place after consuming so much alcohol that the blood, urine or breath level exceeds the prescribed limit. Section 7 of the act governs the provision of samples by a police officer and says that in the course of an investigation, a constable may require a specimen of blood urine or breath for a confirmatory test. But this sample may only be taken if the requirement is made at a hospital or police station. The requirement is imposed in circumstances where section 6 applies or the officer is in uniform. Failure to provide a sample is viewed in the same light as being above the *per se* limits. It should be noted that despite the limits in blood urine and breath being set at 80, 107 mg/dl and 35 µg/dl respectively, Gullberg (2012) cited the work of Wallis and Brownlie (1985) showing that an allowance for uncertainty of 6mg% or 6% is subtracted from the analytical value (Gullberg, 2012). Therefore, in practical terms for a blood or urine sample to be over the limit the analytical value must be 87mg% or 113mg%. By contrast the prosecution limit for breath is 40 micrograms per 100 ml of breath.

Two major studies led to the implementation of these limits. The first of these was carried out in 1964 in a town in Illinois United States which later became known as the Grand Rapids by Professor Robert.F. Borkenstein, while the follow up carried out in 1994 in Germany by H.P Krueger *et al*. (Krueger *et al.* 1995) the purpose of both studies was to assess the risks of a driver being involved in an accident when blood alcohol concentration was factored in. The Grand Rapids study analysed and estimated alcohol in breath and blood of drivers involved in accidents. This was then compared to a large group of drivers passing the accident sites at the same time of day or on the same weekday, this group acted as the control group and their alcohol levels in breath and blood was plotted against those involved in accidents as a function of BAC (Lucas, 2000).

The second study by Krueger *et al*. gave further evidence for a need to reduce the legal limit from 80mg/dl to 50 mg/dl, the design of this study was like that of Borkenstein in that drivers were selected at the roadside by police who were following a random sampling plan (Krueger *et al.* 1995). At a separate checkpoint driver were randomly stopped, interviewed and asked for a breath sample, an accident study was also undertaken, and police were asked to take a breath sample from all accident drivers. This study took samples at a variety of times and days accounting for different times of the day and week where drinking may have been higher (Krueger, 1995). The risk factors of both studies were compiled into a graph by Krueger *et al.* to provide a comparison of the findings from both studies (Figure 4). The Borkenstein study suggested the implementation of a 100mg/dl BAC limit, this was later reduced in areas such as the UK to 80 mg/dl. The Krueger study highlighted that this was still too high however showing that at a BAC of 80mg/dl the risk factor of being involved in an accident was 400 % higher (Lucas, 2000) than that of a driver that had no detectable alcohol in their system and some areas of the world responded to this and further reduced this to a limit of 50 mg/dl.

In the U.K. the road traffic act sets fixed limits on concentrations of intoxicating substances meaning a conviction will be incurred should a person be found to be driving with a higher concentration of a substance outlined within section 5 of this act. Given the effect that such a conviction can have on a person's life it is vital that a method designed to detect these substances is robust, trustworthy and accurate to minimise the possibility of errors that can lead to wrongful convictions or acquittal. It is for this reason that forensic analytical methods are held to such a stringent standard and are so scrupulously examined during each stage of development and validation. With many aspects of the operation being checked, tested and refined including an external test scheme known as proficiency testing (PT) which is run by a wide variety of accredited practitioners and compares inter laboratory results obtained for a specified sample concentration. This highly controlled and regulated method development and validation process results in a high-quality method capable of detecting a wide variety of concentrations of either drugs or alcohol depending on the instrumentation used. For blood alcohol quantification, a method called GC-FID (Gas Chromatography with Flame Ionisation Detection) is frequently used. This is specialised technique for the detection volatile compounds such as alcohols. Given that alcohol is the most commonly used recreational drug worldwide, it is unsurprising that a common analytical test carried out within forensic toxicology is to determine the amount of ethanol in a person's system. The analysis is typically carried out on blood and urine or vitreous humour, this type of analysis is used in variety of case types from drink/drug driving, drug facilitated sexual assault and deliberate and accidental drug overdoses.

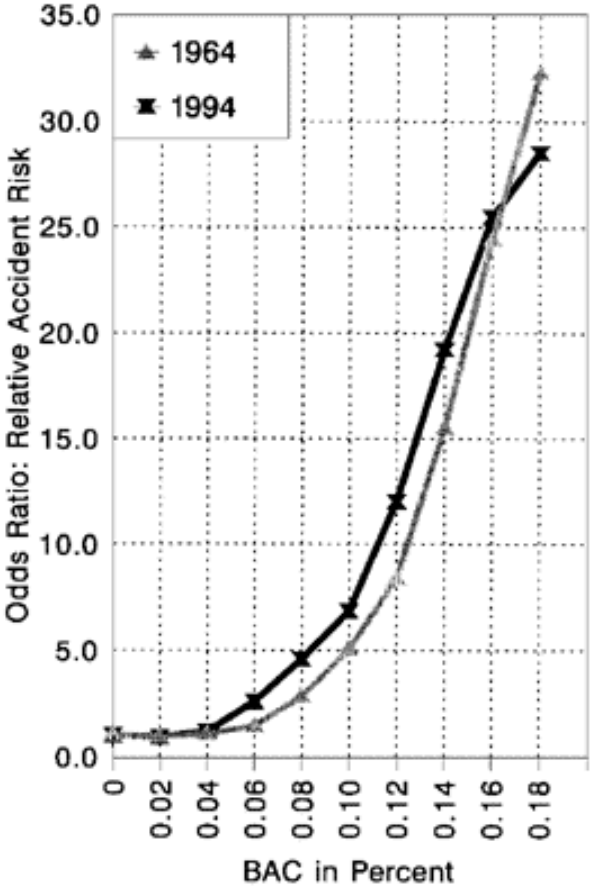


Figure 4: The relative risk odds ratio plotted against the blood alcohol concentration. As demonstrated the risk factors of lower concentrations at 20 and 40 mg/dl (0.02 and 0.04 %) are approximately the same, however from 60 mg and above the risk factors were higher on average in the 1994 study by Krueger (Krueger *et al*., 1995). The grey line represents Borkenstein's original data with the black line showing data from Krueger's 1994 study.

Gas Chromatography with Flame Ionisation Detector

A technique commonly applied within forensic laboratories for the analysis of alcohol in biological samples is gas chromatography (GC). This is often used in conjunction with a headspace auto sampler and flame ionisation detection (FID) (Mermet, 2004) which is referred to as headspace GC-FID. Headspace operates on the principle of Henry's law, which states that the concentration ratio of a volatile substance in the gas phase of a vessel and the concentration in the liquid phase are fixed at a given pressure and temperature (Atkins, 2017). In the case of ethanol this means that the concentration of ethanol in the gas phase to be sampled is directly proportional to the concentration of ethanol found in the sample's liquid phase. Headspace sampling has a major advantage over the alternative sample technique of direct liquid injection in that headspace is an extraction method along with the sampling method. A headspace sampler heats the sample vial to cause evaporation of the volatile components within the liquid phase resulting in the gas phase above the liquid, or the headspace, to reach an equilibrium with the liquid phase (Mermet, 2004). This headspace is then sampled by a syringe found within the auto-sampler, which is then injected into the inlet of the GC, and at this point it passes through the inlet and into the column where a separation of analytes occurs.

GC columns come in two primary types, capillary and packed columns, dual capillary columns (utilising different polarities) are more commonly used in forensic blood alcohol analyses in modern analytical laboratories, however packed columns have been utilised in the past although they are rarely utilised today due to the improved overall performance of capillary columns (Holler, 2014), It is important for the dual columns to differ in polarity to ensure the separation properties are different. If both columns had identical separation properties it is a possibility that the presence of acetone for example would make an analysis of a sample very difficult.

Capillary columns have a variety of applications ranging from biological fluid alcohol analysis to the analysis of accelerants in an arson case and some columns are more suited to specific applications than others. For example, the Restek BAC plus 1 and plus 2 columns chosen for this method are especially suited to the separation of alcohols due to the polarity, film thickness and internal diameter allowing for a short run time. The Fused silica used within a capillary column is relatively delicate and so columns are coated in a thin layer of polyimide to provide structural strength to the column, figure 5 shows a cross sectional diagram of a capillary column. The stationary phase of a capillary column is an immobilised liquid which is coated to the walls of the column. This liquid is highly viscous and specifically selected to have a boiling point of 100^{o}C above the selected analysis temperature (Mermet, 2004). This coating can be done in two ways, the first is known as support coated open tubular (SCOT) which is pre-absorbed onto a porous support and the second coating is wall coated open tubular (WCOT) which, as the name suggests involves the liquid phase being thinly coated directly to the capillary wall (Mermet, 2004). In addition to the column, a mobile phase known as a carrier gas is used to carry the analytes along the column. Typically, in gas chromatography one of three gases, either helium, nitrogen or hydrogen is used (Rouessac, 2007). The optimum carrier gas to use for a method can be determined using the Van Deemter plot shown in figure 6, this is a measure of carrier gas efficiency, it should be noted that hydrogen gas is considered the most flexible gas to use as a carrier gas due to the wide range of optimum pressures when compared to helium and nitrogen, however helium is a safer alternative and is commonly utilised (Rouessac, 2007).

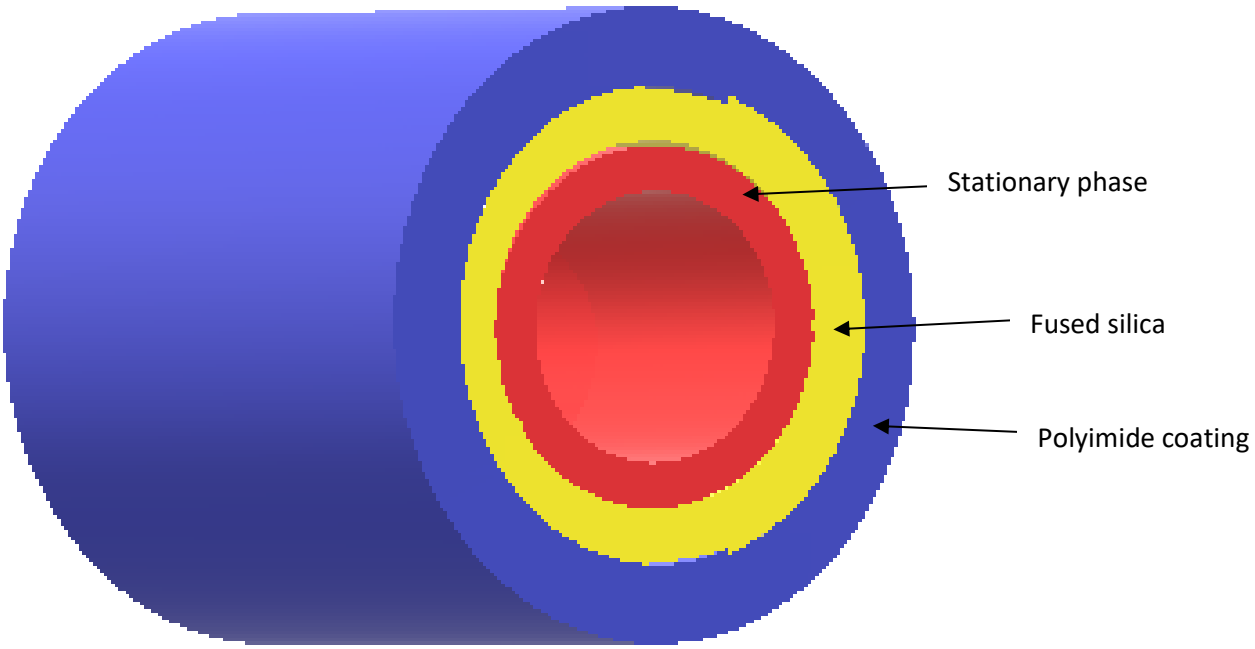


Figure 5: A cross section of a capillary GC column showing the protective outer coating made of polyimide, the fused silica base for the stationary phase and the stationary phase in which seperation occurs, the phase, internal diameter (ID) and film thickness will vary depending on the column type and the purpose of the column. These factors will affect the maximum capacity for a sample a column will have and also how analytes interact and separate on the column.

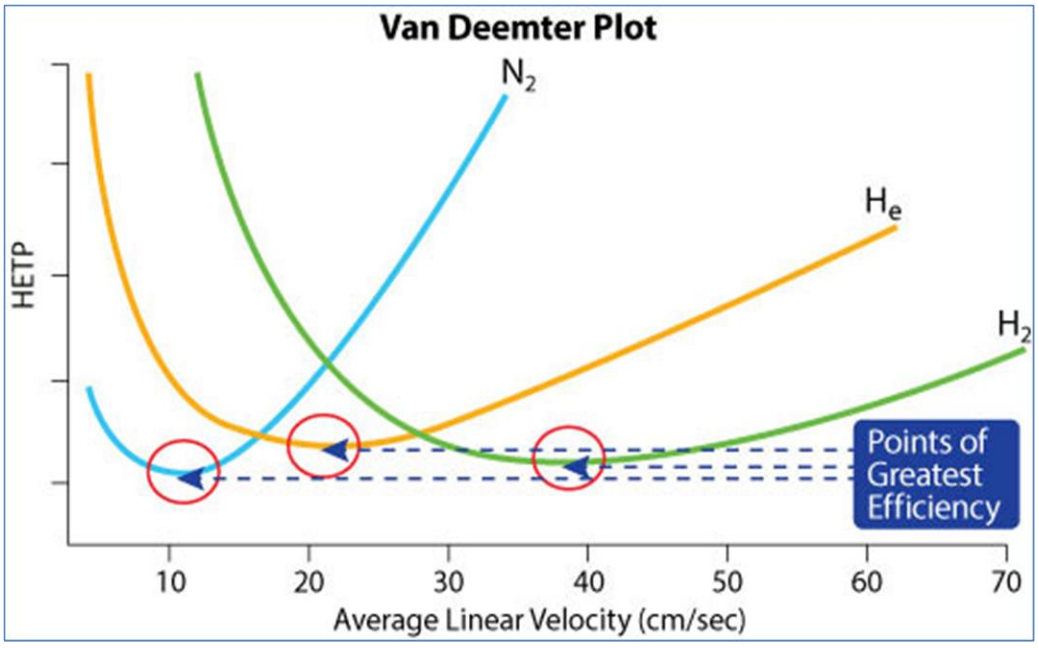


Figure 6: The Van Deemter Plot showing that hydrogen has the widest range of linear velocities at which it will provide optimum efficiency with Nitrogen showing the narrowest range (Oden, 2015).

At the end of the separation process which takes place within the column the analytes must pass through a detector to be identified and the data transformed into a visual display, in the case of gas chromatography this would typically be a peak on a chromatogram. An internal standard is used as a reference and quantitation is achieved based on the ratio of the area of the analyte which has a varying concentration and the internal standard, which is the same concentration across all samples allowing for a calibration curve to be drawn (Rouessac, 2007). The flame ionisation detector is a highly sensitive detector type used in forensic blood alcohol analyses, the detetion limit is reported to be in the order of 2-3 pg/s and a linear range of 10^8 (Holler, 2014). Flame ionisation detectors (FID) are the most widely used type of detector for gas chromatography (Holler, 2014). FIDs operate on the gas flow from the column entering the detector space and being destroyed in a hydrogen flame, the resulting destruction of hydrocarbon compounds creates ions which are charged particles that pass through a weak current of around 10^{-12}A (Amperes) between two electrodes, a ground potential that acts as a polarisation electrode and a collector electrode which surrounds the flame, the signal is amplified to a measureable voltage by an electrometer (Rouessac, 2007). It is believed Carbon atoms produce CH radicals within the flame which produce CHO^+ ions and free electrons. Flame ionisation detectors are sensitive to hydrocarbon compounds such as alcohols, but are insenstitive to compounds such as H_2O, CO_2, SO_2 and NO_X which can be advantageous if samples are diluted into water or contaminated with sulphate or nitrates as they are not detectable and so will not interfere with chromatograms (Holler, 2014).

Method development and validation

The development of a method will be task specific, if a method has been developed for a specific customer purpose, the level of development and type of validation data may be different to a general purpose method, although all methods developed require some form of validation (Meyers, 2000). In a GC-FID method such as the method developed in this project, parameters are selected and optimised to the specified requirements of the analysis the method is developed to run. The specific requirements and validation experiments for this method are discussed below and demonstrate a validation plan for a specialist blood/urine alcohol detection method. Typically, validation of a method follows a similar pattern with the same parameters being verified. These parameters include but are not limited to; recovery, precision, accuracy, range, selectivity/specificity, limits of detection and working range. Potentially the most important of these parameters, and the parameters which should be monitored the most carefully are the reproducibility/repeatability and the accuracy (Higson, 2005).

The components of validation are outlined and explained by Harris (Harris, 2010) in the quantitative chemical analysis providing an overview of each part of the process. The specificity of a method is the ability of the analytical method to distinguish analytes from one another, the ideal separation is known as baseline separation (Harris, 2010). Baseline separation, as the name suggests is the return to the baseline of one peak before signal of the next peak begins (Harris, 2010). The linearity of a method is a measure of how effectively a calibration curve follows a straight line, the calibration curve is formed from known standard concentrations and it is suggested by Harris that a linearity of between 0.995 and 0.999 is considered adequate for most purposes (Harris, 2010).

The accuracy of a method is the closeness to the true value of a quantitation. A typical method of verification is the analysis of certified reference material (CRM), this method was used in the method developed in this research, a set is run after the calibration curve and a second is run after unknown samples verifying the accuracy of the analysis (Meyers, 2000). Meyers also notes that ISO guidelines define accuracy as a qualitative concept of the combination of precision and bias.

Precision is defined by Harris as the measure of how well replicate measurements agree with one another, which is generally demonstrated by standard deviation, Harris also suggested that there are more than one type of precision which are identified as; instrument or injection precision which is observed by injecting the same concentration of a sample a number of times (typically greater than or equal to ten separate times to monitor reproducibility and verifying the precision of the injection and instrumental variations (Harris, 2010) and intra-assay precision which is the analysis of homogenous aliquots several times in one day by a single analyst. It is expected that a higher variability will be expected due to the increased number of steps involved in this process (Harris, 2010).

The next measures of precision also cover reproducibility, these are defined by Harris as intermediate precision and inter-laboratory precision, intermediate precision is the preparation of samples by more than one analyst on separate days (Harris, 2010). Inter-laboratory precision is very similar however the different analysts are running samples in different laboratories (Harris, 2010) and results between laboratories are then compared.

The next factor of a validation is the range of the method. This is defined as the concentration interval at which precision, linearity and the accuracy are acceptable as per the specifications of the method (Harris, 2010). A reliable quantification can only be guaranteed within the calibration range, outside of these limits an accurate quantitation is no longer guaranteed, although it is still possible to achieve (Meyers, 2000). The final component of a typical validation is the limits of detection and quantitation, the limit of quantitation is the lowest concentration at which a quantitation can be achieved with a reasonable degree of accuracy (Harris, 2010), it is likely that this value will be the lowest calibrator used for the calibration curve given the calibration curve are the concentrations that are verified for accuracy.

Aims and method requirements

The method was required to analyse and quantify the concentration of ethanol in both blood and urine to the standard of an ISO accredited laboratory. As this method utilised a dual column, dual detector configuration with one column acting as a primary column, and the second acting as the confirmatory column it is expected that both ethanol and tertiary butanol should elute with no interference on at least one of the columns, calibration curves should provide a linearity (r^2) of at least 0.998 and are expected to be able to consistently quantify samples accurately and precision to a coefficient of variance (CV), also known as the relative uncertainty of no more than 2.5%. This method was developed and validated for commercial use according to an agreed validation plan. Upon validation, the method is intended for use with the analysis of road traffic act samples and the method will also be put forward for ISO accreditation. Prior to the validation process, the method was utilised to test the stability of blood, urine and LGC quality control samples in a room temperature stability test for up to one month and a two-cycle freeze thaw.

The objective of these studies was to investigate the effect of poor handling on sample stability. For example, the effect of unexpected breakdowns of a refrigerator and if samples are accidentally frozen in storage.

Materials and methods

Reagents and solvents

The following reagents were used in the experimentation

- Cerilliant ethanol standard solutions at concentrations of 10, 20, 50, 100, 200 and 400 mg/100ml.
- LGC European Reference Materials ethanol Certified Reference Material QC bottles at concentrations of 20, 80 and 200 mg/100ml.
- Fisher scientific anhydrous tertiary butanol.
- Fisher scientific sodium metabisulfite.
- Propan-1-ol.
- Propan-2-ol.
- Methanol.
- Acetaldehyde.
- Acetone.
- Distilled water.
- Defibrinated horse blood.
- Human blood (sourced in house from volunteers). Once opened this should be used within 30 days.
- B sample human blood sourced an external accredited laboratory.

Gases

- Helium for use as carrier gas.
- Hydrogen for use as FID fuel source.
- Blank air for use as FID flame oxygen supply.
- Nitrogen for use as a makeup gas.

Instrumentation and lab equipment

Headspace Gas chromatogram with Flame Ionisation Detector

- Shimadzu GC-2014 using a PC running Shimadzu GC solutions software with BAC 1 and BAC 2 dual column and FID -2014 configuration .
- HTA 200 H headspace auto sampler utilising a gas tight syringe.

Pipettes

- Gilson P1000 1ml adjustable volume pipette.
- Gilson 20-200 µl P200 adjustable volume pipette.
- Gilson 2-20 µl P20 adjustable volume pipette.
- Fixed volume 1ml pipette.
- Gilson positive displacement pipette 10 -100 µl M100 adjustable volume.

Sample preparation

Samples are prepared in duplicate using 100µl of analyte material in 1ml of internal standard in labelled 20ml headspace vials. The internal standard (ISTD) is prepared using 25 µl of tertiary butanol in 500 ml of distilled water; 2.5g of sodium metabisulfite antioxidant is also added. This solution is then inverted to homogenise the internal standard which can then be aliquoted into smaller bottles for easy storage. All samples should be prepared in a fume hood and in isolation of other solvents. Calibration curve standards are prepared by transferring 1ml of ISTD into 20ml headspace vials. 100 µl of each standard concentration is then spiked within the vials; caps are then crimped on and checked for movement, properly crimped caps should not move. The same procedure applies to the quality controls, 1 ml of ISTD into each vial following 100 µl of each concentration these samples are now ready for analysis but can be stored overnight and analysed up to 24 hours later if required, one set of QCs will be run after the calibration curve to ensure the accuracy of the calibration is acceptable and the second set will be run after sample analysis to demonstrate that the accuracy of the analysis is acceptable.

Headspace GC-FID procedure

Prepared samples were placed into headspace sample carousel, and conditioned according to the headspace sampling parameters, the sampler then injected the sample volume into the SPL inlet and data acquisition will begin, a peak area and height will be added into the calibration curve post run of each sample for calibration standards. Upon completion of calibration standards a judgement can be made on linearity and accuracy after quality control samples run and report formats along with an ASCII format of numerical data can be generated post run.

Uncertainty

The uncertainty measurement used within the experimentation was the expanded uncertainty, this measurement accounts for the measurement uncertainty (also known as the standard deviation), the uncertainty of the quality controls used (20, 80 and 200 mg) and applies a confidence interval of 95%. The calculation of this variable follows the basic formula:

Expanded uncertainty = $\sqrt{SD^2 + uncertainty\ of\ the\ quality\ control\ value^2}$ X the coverage factor (K) which in the experimentation was 2 or $\sqrt{sum\ of\ uncertainties^2}$ X K.

This provides the uncertainty of each set of quality control values calculated for a confidence interval of 95%. For biological samples, the standard deviation and Coefficient of variance (CV) also known as the relative uncertainty or relative standard deviation (Farrance, 2012) was used as measures of uncertainty as there is no unknown uncertainty attributed to samples making the expanded uncertainty unsuitable.

Instrument parameters

GC method parameters

Table 2: parameters for Gas chromatography analysis of ethanol	
Parameter	**Value**
Inlet Temperature (°C)	110
Injection mode	Split
Pressure (Kpa)	85
Column Flow (ml/min)	2.78
Linear velocity (cm/sec)	42.30
Purge flow (ml/min)	3.00
Split ratio	5.00
Oven temperature (°C)	40 isothermal
Detector temperature(°C)	280
Analysis time (minutes)	4

Headspace sampling parameters

Table 3: Headspace sampler parameters for analysis of ethanol		
Parameter	**Value**	
Oven temperature (°C)	60	
Syringe temperature (°C)	70	
Fill volume (ml)	1.75	
Sample volume (ml)	1.00	
Incubation time (minutes)	5.00	
Shaker time (minutes)	0.50 on	0.10 off
Sample speed (ml/min)	5.0	
Injection speed (ml/min)	80	

Headspace GC-FID procedure

Prepared samples were placed into headspace sample carousel, and conditioned according to the headspace sampling parameters, the sampler then injected the sample volume into the inlet and data acquisition will begin, a peak area and height will be added into the calibration curve post run of each sample for calibration standards. Upon completion of calibration standards, a judgement can be made on linearity and accuracy after quality control samples run and report formats along with an ASCII format of numerical data can be generated post run.

Uncertainty

The uncertainty measurement used within the experimentation was the expanded uncertainty, this measurement accounts for the measurement uncertainty (also known as the standard deviation), the

uncertainty of the quality controls used (20, 80 and 200 mg) and applies a confidence interval of 95%. The calculation of this variable follows the basic formula:

Expanded uncertainty = $\sqrt{SD^2 + uncertainty\ of\ the\ quality\ control\ value^2}$ X the coverage factor (K) which in the experimentation was 2 or $\sqrt{sum\ of\ uncertainties^2}$ X K.

This provides the uncertainty of each set of quality control values calculated for a confidence interval of 95%. For biological samples, the standard deviation and Coefficient of variance (CV) also known as the relative uncertainty or relative standard deviation (Farrance, 2012) was used as measure of uncertainty as there is no unknown uncertainty attributed to samples making the expanded uncertainty unsuitable.

Results

Method development

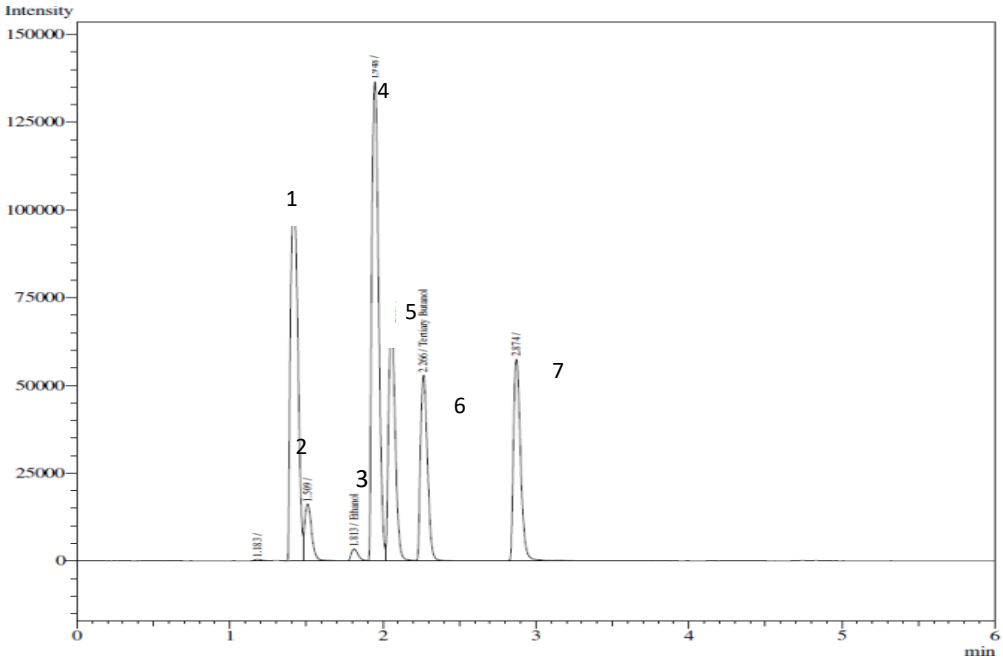
Development began with initial selection of columns and internal standard; the column installed on the instrument upon first encountering the system was a single Phenomenex ZB-1 which had a film thickness of 25µm, a column length of 30.0m metres, and an inner diameter of 25mm and a maximum temperature of 360^{o}C. A dual column configuration was selected using RTX- BAC columns with the BAC plus-1 as the primary analysis column and the BAC plus-2 as the conformational secondary column, the reason for this was to provide two different separations based on different factors to identify any co-elution and prevent peak interference with the target analyte or internal standard, ultimately each column has a different retention time or elution order per analyte (McShane, 2018).The first internal standard used was propan-2-ol, this however eluted too closely to ethanol with minimal baseline separation (Figure 17) meaning although there was no co-elution, it is a less suitable internal standard when compared to the other alternatives such as propan-1-ol and tertiary butanol. Propan-1-ol with the higher retention time allowed for a large separation between the two analytes, however the extended retention time made the method run time unnecessarily long, so tertiary butanol was selected as it provided the best balance of separation from ethanol whilst still allowing for a shorter total run time which increases efficiency and the turnover speed. All retention time values for ethanol and tertiary butanol were within the 2% identification window set within the analytical software.

Parameter selection is a vital part of method development; it will dictate factors such as retention time, run time and the column lifetime. The main parameters selected for the method were GC oven temperature; pressure and conditioning time were adjusted to find the optimum balance of run time and separation. Selectivity was also tested at the early stages to determine how efficient the separation of common organic compounds encountered within a forensic analysis.

System suitability:

The separation of a system suitability solution using the selected method parameters demonstrates the degree of separation that will be achieved between the components in the suitability solution. The suitability solution was prepared by adding 100 µl of methanol, acetaldehyde, acetone, isopropanol and 1-propanol analyte and 25 µl of tertiary butanol into a 1 litre bottle except for ethanol and homogenised. This system suitability solution was stored for use, this solution was diluted before use and 1ml was added to a 20 ml headspace vial, 100 µl of the 10 mg/dl Cerilliant standard ethanol was added at this point before the vial was sealed for analysis.

Column A



Column A peaks:

1: Acetaldehyde

2: Methanol

3: Ethanol

4: Acetone

5: Isopropanol

6: Tertiary Butanol

7:1-propanol

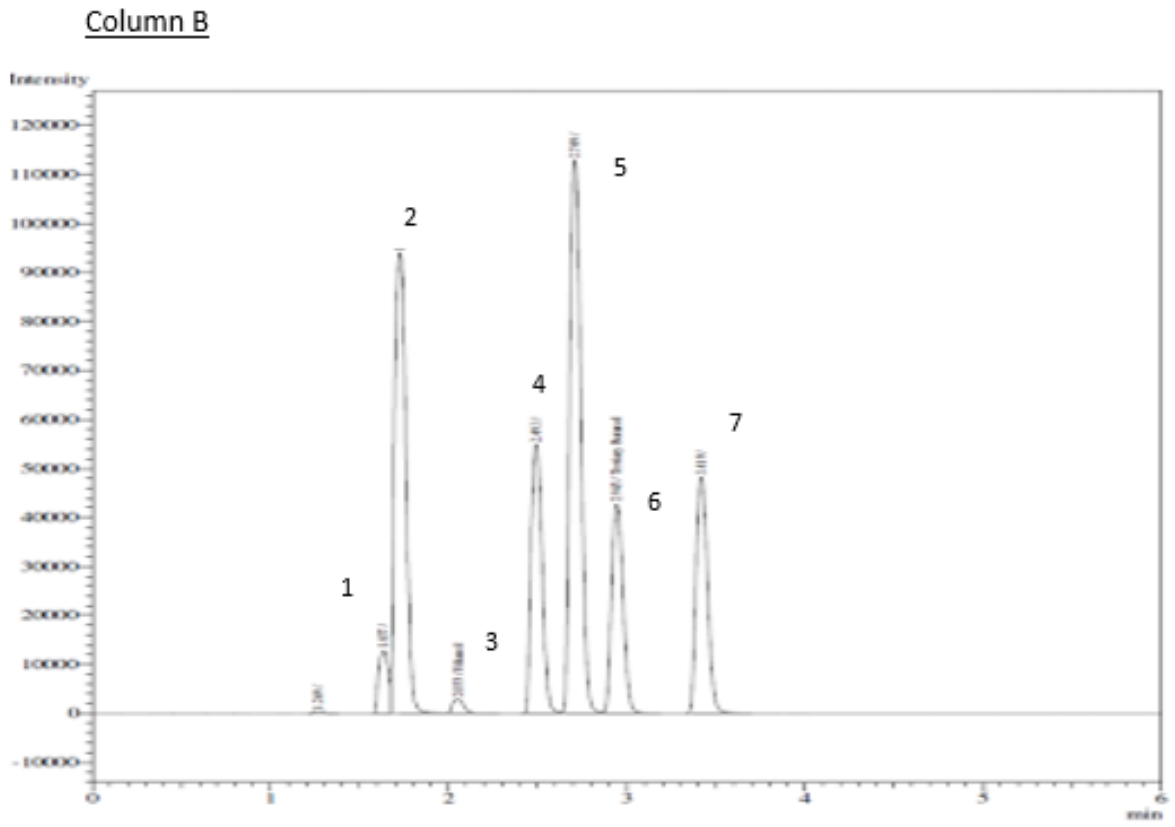


Figure 7: System suitability chromatogram demonstrating the selectivity of the method at the selected optimum pressure, conditioning time and oven temperature. Acetaldehyde co-elutes with methanol however the addition of sodium metabisulfite to the IS prevents its formation in casework samples. Column manufacturer material suggests a clear separation of all analytes; however, this uses unpractical conditions that would not be possible for accurate analytical quantitation. Retention time values for this analysis can be found in Table 11.

Pressure optimisation

Pressure optimisation was carried out based on a system suitability solution containing acetaldehyde, methanol, ethanol, acetone, isopropanol, tertiary butanol and propan-1-ol. Pressures were optimised by starting at 150kpa which decreased in 10 kpa increments until 90 kpa where an adjustment of only 5 kpa was carried out and the optimum of 85 kpa was identified. A test at a pressure below the identified optimum at 75 kpa was carried out to determine the effects of pressure on run time, peak shape and separation. It was determined that 85 kpa (Figure 9) provided the best run time whilst still having adequate separation on at least one column. As demonstrated, in figure 9 acetone co-elutes with ethanol and isopropanol on column A making samples with acetone difficult to quantify with this column. In the event of this occurrence it should be noted that acetone was detected in the sample and four samples should be prepared to obtain two sets of duplicate quantitative values with the second column with this being recorded in the data pack. It was also highlighted that lowering the pressure did not increase separation of acetone and ethanol on column A, the effect of the lower pressure was a relative retention time shift rather than the desired differential effect of separating ethanol and acetone.

Retention times at 150 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	0.842	0.197	Methanol	N/A	N/A
Methanol	N/A	N/A	Acetaldehyde	1.022	0.353
Ethanol	1.072	0.523	Ethanol	1.211	0.603
Acetone	1.151	0.635	Isopropanol	1.468	0.943
Isopropanol	1.214	0.725	Acetone	1.595	1.111
Tertiary butanol	1.337	0.899	Tertiary butanol	1.732	1.293
Propan-1-ol	1.692	1.404	Propan-1-ol	2.009	1.660

Table 4: Retention time data of the 150 kpa pressure optimisation run. The retention times for most analytes are too close together and may cause issues with co-elution or quantitation making this pressure sub-optimal for the method. Methanol and acetaldehyde co-eluted to an extent where a retention time value for methanol not being available on both columns.

Retention times at 140 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	0.897	0.198	Methanol	N/A	N/A
Methanol	0.951	0.270	Acetaldehyde	1.088	0.355
Ethanol	1.141	0.524	Ethanol	1.290	0.606
Acetone	1.226	0.637	Isopropanol	1.564	0.947
Isopropanol	1.293	0.727	Acetone	1.699	1.116
Tertiary butanol	1.424	0.901	Tertiary butanol	1.846	1.298
Propan-1-ol	1.803	1.407	Propan-1-ol	2.141	1.666

Table 5: Retention time data of the 140 kpa pressure optimisation run. The retention times for most analytes are too close together and may cause issues with co-elution or quantitation also making this pressure sub-optimal for the method. Methanol and acetaldehyde co-eluted to an extent where a retention time value for methanol not being available on column B.

Retention times at 130 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	0.959	0.198	Methanol	N/A	N/A
Methanol	1.019	0.273	Acetaldehyde	1.165	0.356
Ethanol	1.222	0.526	Ethanol	1.381	0.608
Acetone	1.312	0.640	Isopropanol	1.165	0.950
Isopropanol	1.385	0.730	Acetone	1.819	1.119
Tertiary butanol	1.525	0.905	Tertiary butanol	1.977	1.302
Propan-1-ol	1.931	1.412	Propan-1-ol	2.293	1.671

Table 6: Retention time data for the 130 kpa optimisation run, at this pressure the run time is still low, and peaks are still eluting too closely for analysis to eliminate coelutions.

Retention times at 120 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.030	0.196	Methanol	1.188	0.285
Methanol	1.098	0.275	Acetaldehyde	1.254	0.356
Ethanol	1.316	0.528	Ethanol	1.487	0.609
Acetone	1.413	0.641	Isopropanol	1.804	0.951
Isopropanol	1.492	0.732	Acetone	1.960	1.120
Tertiary butanol	1.643	0.907	Tertiary butanol	2.130	1.304
Propan-1-ol	2.080	1.415	Propan-1-ol	2.471	1.673

Table 7: Retention time data for the 120kpa optimisation run, at this pressure the run time is still low, and peaks are still eluting too closely for analysis to eliminate co-elution.

Retention times at 110 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.115	0.195	Methanol	1.285	0.288
Methanol	1.188	0.273	Acetaldehyde	1.356	0.359
Ethanol	1.425	0.528	Ethanol	1.610	0.613
Acetone	1.531	0.641	Isopropanol	1.955	0.959
Isopropanol	1.616	0.732	Acetone	2.124	1.129
Tertiary butanol	1.780	0.908	Tertiary butanol	2.310	1.314
Propan-1-ol	2.254	1.417	Propan-1-ol	2.680	1.686

Table 8: Retention time data for the 110 kpa optimisation run demonstrating a further shift when compared to 120 kpa.

Retention times at 100 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.215	0.196	Methanol	1.404	0.287
Methanol	1.296	0.276	Acetaldehyde	1.483	0.359
Ethanol	1.556	0.532	Ethanol	1.760	0.613
Acetone	1.671	0.645	Isopropanol	2.137	0.959
Isopropanol	1.765	0.737	Acetone	2.323	1.129
Tertiary butanol	1.944	0.914	Tertiary butanol	2.525	1.314
Propan-1-ol	2.464	1.426	Propan-1-ol	2.931	1.686

Table 9: Retention time data for the 100 kpa optimisation run demonstrating a further shift in retention times.

Retention times at 90 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.345	0.199	Methanol	1.552	0.285
Methanol	1.430	0.275	Acetaldehyde	1.640	0.357
Ethanol	1.717	0.531	Ethanol	1.946	0.611
Acetone	1.846	0.645	Isopropanol	2.362	0.955
Isopropanol	1.949	0.737	Acetone	2.567	1.125
Tertiary butanol	2.147	0.914	Tertiary butanol	2.791	1.310
Propan-1-ol	2.722	1.427	Propan-1-ol	3.239	1.681

Table 10: Retention time data for the 90 kpa optimisation run demonstrating a further shift in retention times and a slight increase in separation of analytes.

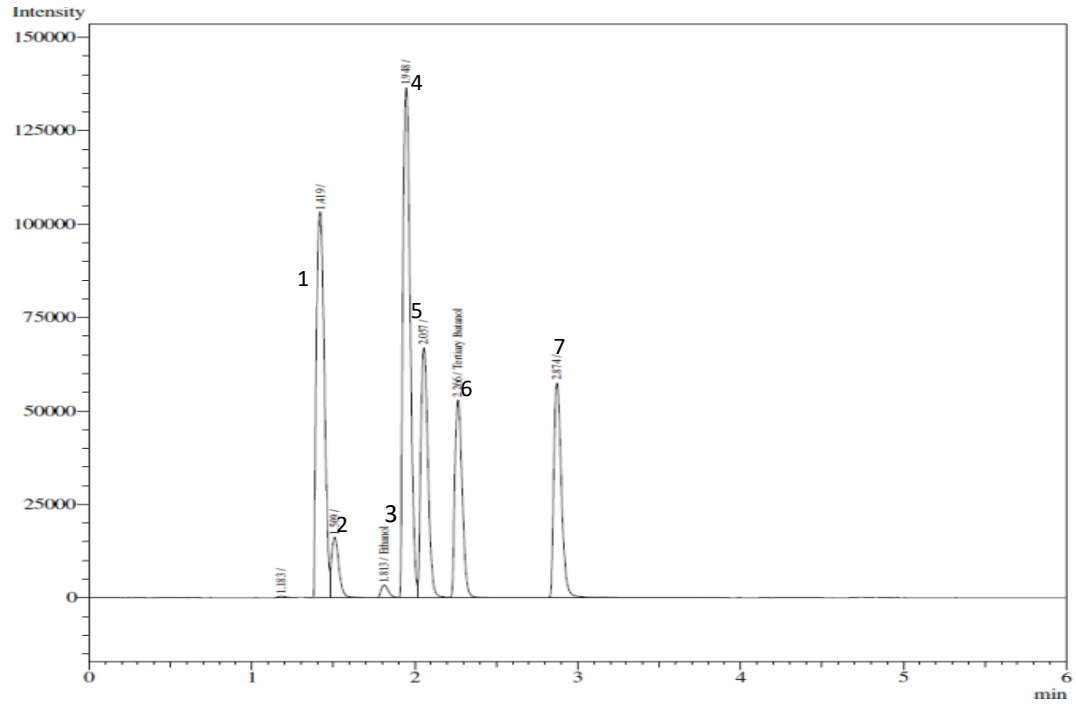
Retention times at 85 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.419	0.200	Methanol	1.637	0.290
Methanol	1.509	0.276	Acetaldehyde	1.729	0.362
Ethanol	1.813	0.533	Ethanol	2.053	0.618
Acetone	1.948	0.647	Isopropanol	2.493	0.964
Isopropanol	2.057	0.739	Acetone	2.709	1.135
Tertiary butanol	2.266	0.916	Tertiary butanol	2.945	1.321
Propan-1-ol	2.874	1.429	Propan-1-ol	3.419	1.694

Table 11: Retention time data for the 85 kpa optimisation run demonstrating a further shift in retention times and a slight increase in separation of analytes. This was determined as the optimum pressure.

85 kpa:

Column A



Column peaks:

1: Acetaldehyde

2: Methanol

3: Ethanol

4: Acetone

5: Isopropanol

6: Tertiary Butanol

7:1-propanol

Column B

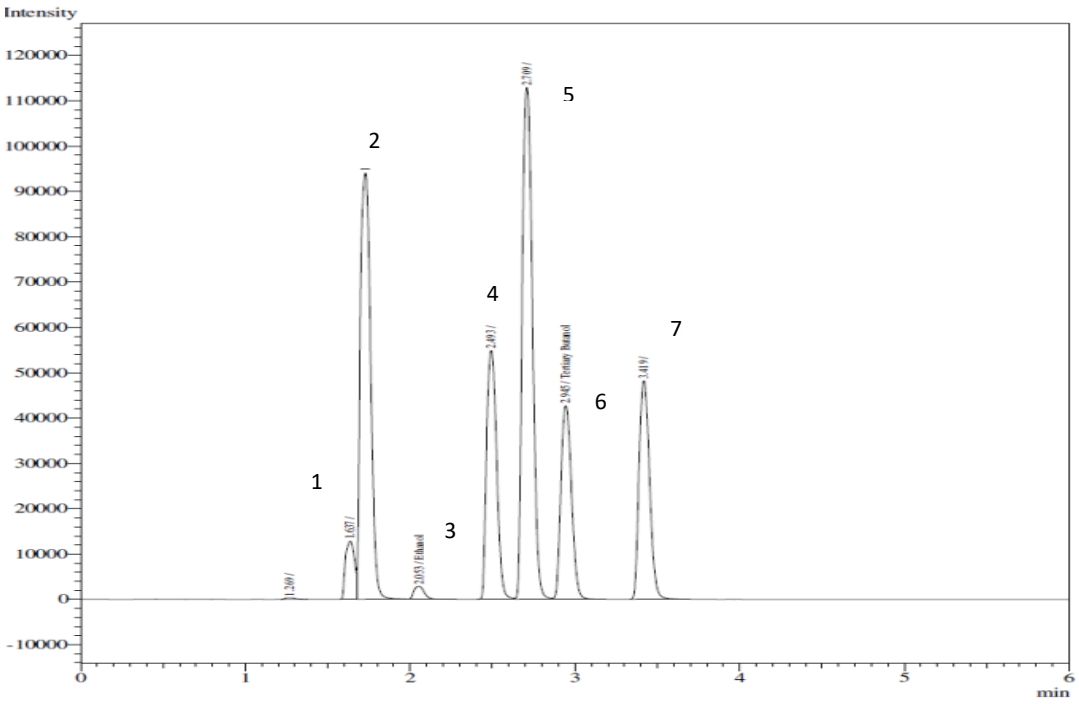


Figure 9: Chromatographic display for 85 kpa, despite an improvement in separation and an increased run time. This pressure was determined as the optimum for separation of key analytes (ethanol and tertiary butanol) whilst maintaining a relatively short retention time.

Retention times at 75 kpa:

Analyte	Retention time column A (mins)	Retention factor (K)	Analyte	Retention time column B (mins)	Retention factor (K)
Acetaldehyde	1.590	0.198	Methanol	1.830	0.292
Methanol	1.696	0.278	Acetaldehyde	1.933	0.365
Ethanol	2.040	0.537	Ethanol	2.300	0.624
Acetone	2.191	0.651	Isopropanol	2.802	0.979
Isopropanol	2.314	0.744	Acetone	3.046	1.151
Tertiary butanol	2.551	0.922	Tertiary butanol	3.315	1.341
Propan-1-ol	3.235	1.438	Propan-1-ol	3.848	1.717

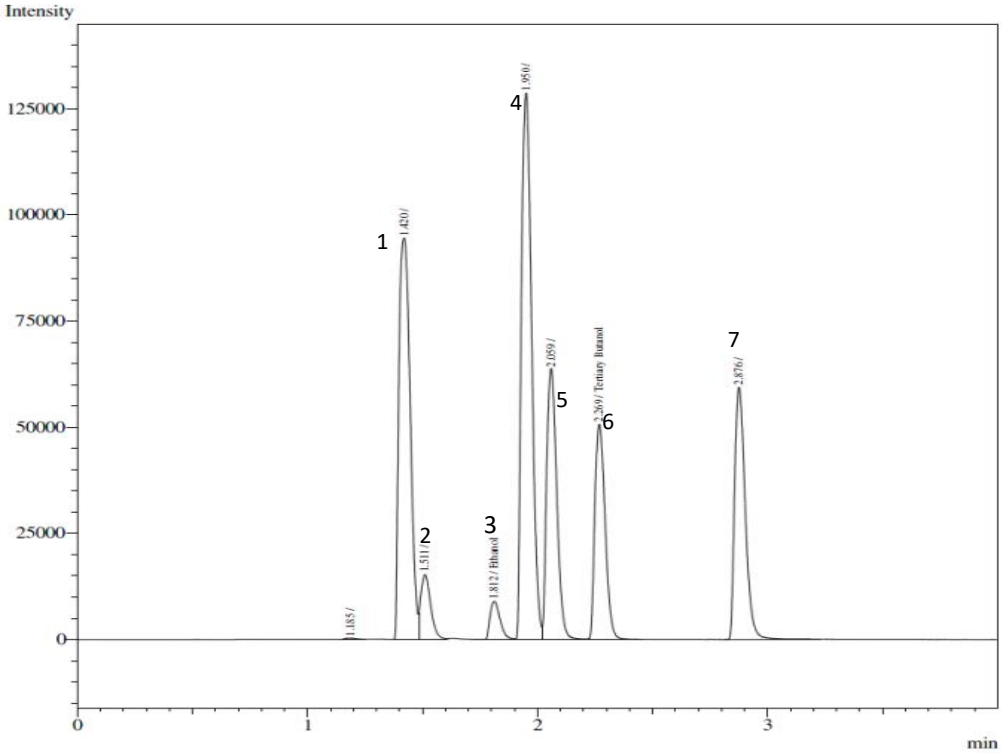
Table 12: Retention time data for the 75 kpa optimisation run demonstrating a further shift in retention times and a slight increase in separation of analytes.

Temperature selection

Temperature optimisation for this method was also carried out to determine if a higher temperature of 45 °C would improve the separation of analytes such as acetone on column A, as shown in figure 10 the peaks eluted even more closely resulting in a full co-elution, whilst not providing any improvement in separation of ethanol and acetone. The optimum temperature was 40 °C and this was selected for the method, pressure adjustment was also attempted to enhance the temperature selection to achieve the separation and run time balance.

40 °C

Column A



Column peaks:

1: Acetaldehyde

2: Methanol

3: Ethanol

4: Acetone

5: Isopropanol

6: Tertiary Butanol

7:1-propanol

Column B

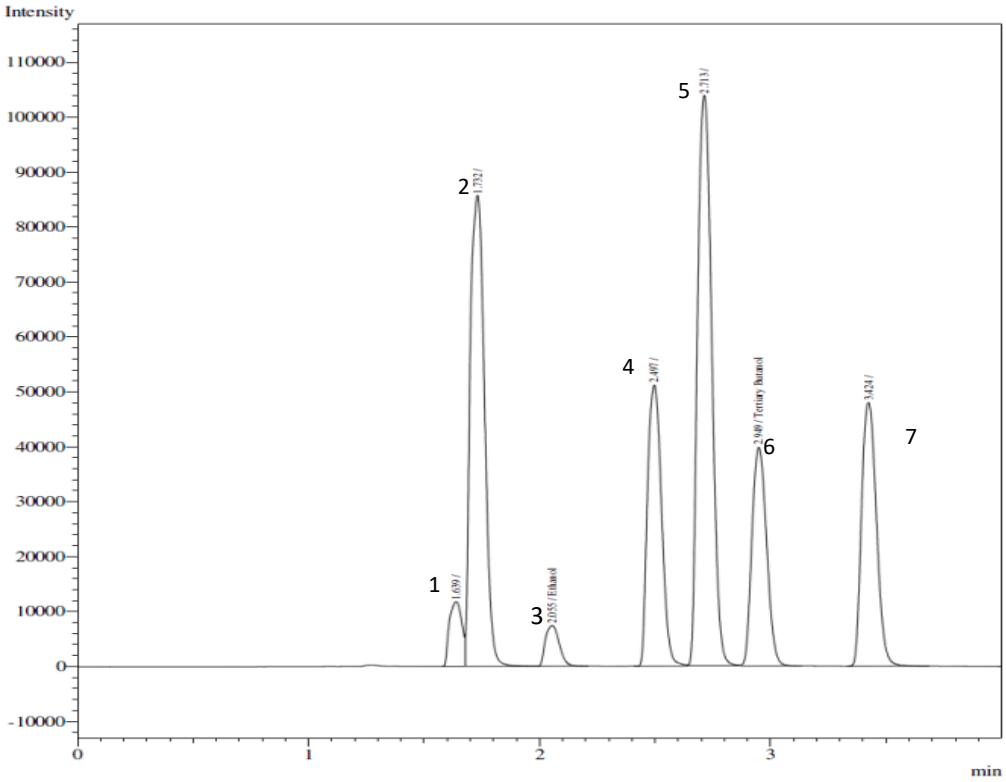
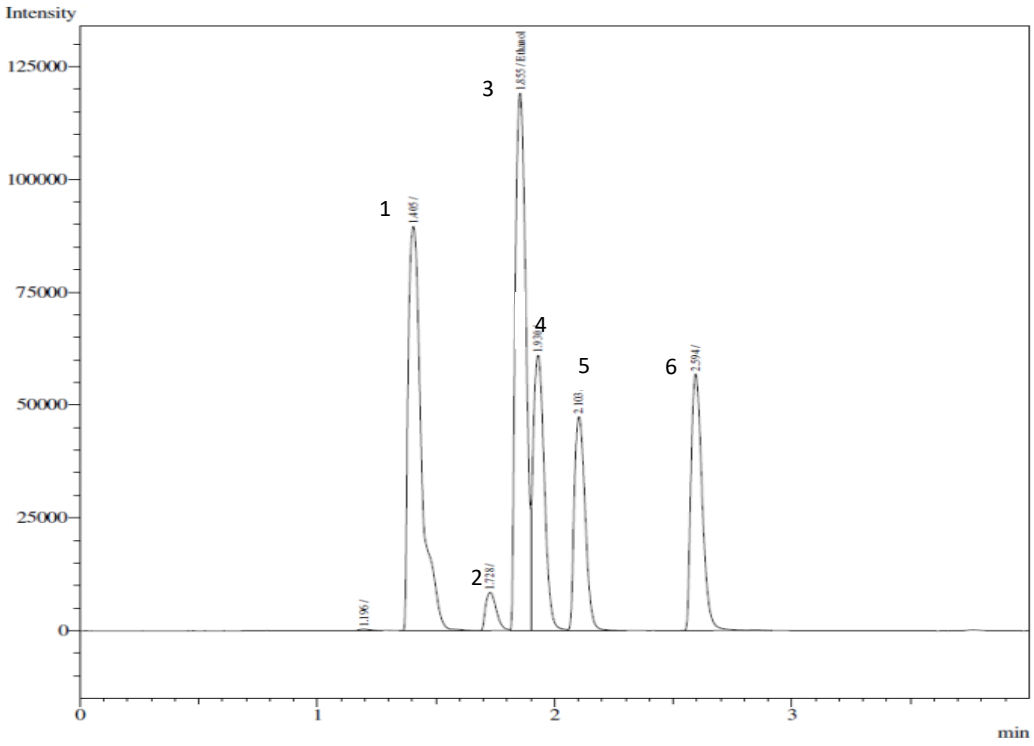


Figure 10: Chromatographic display of 40 °C GC oven conditions, this temperature was selected as the optimum allowing separation of key analytes (ethanol and tertiary butanol) whilst also allowing a moderately short run time.

45 °C

Column A



Column peaks:

1: Acetaldehyde

2: Ethanol

3: Acetone

4: Isopropanol

5: Tertiary Butanol

6:1-propanol

Column B

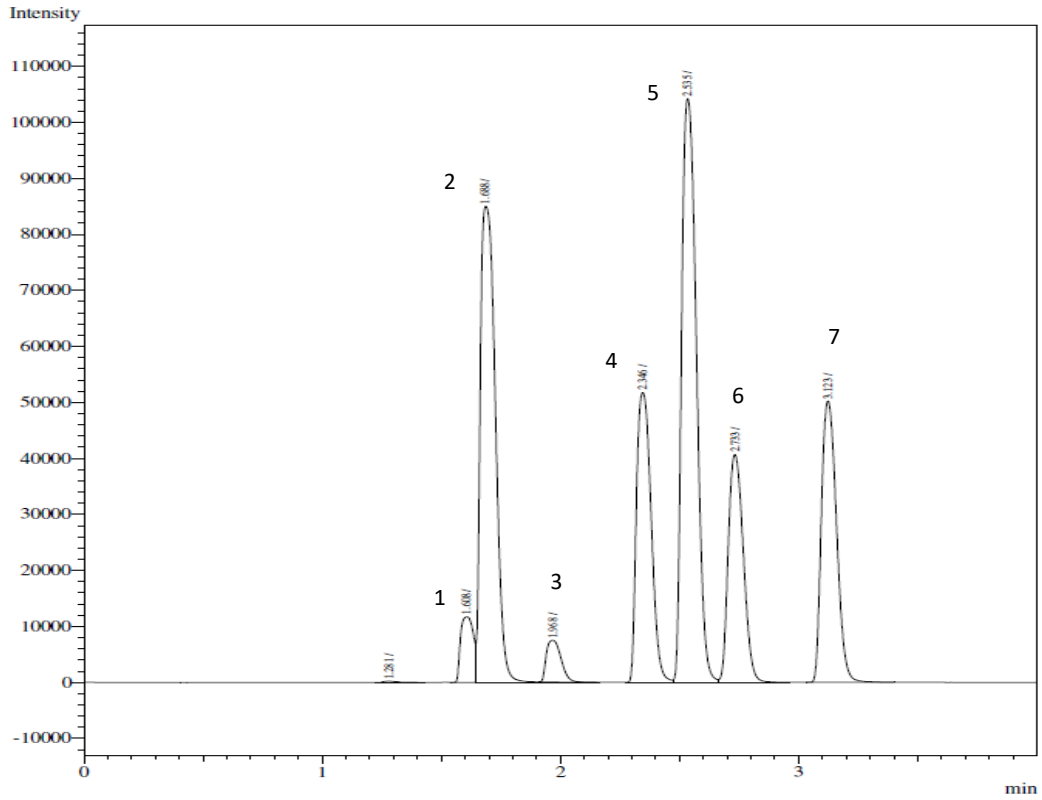


Figure 11: Chromatographic display of 45 °C GC oven temperature, column A shows an increased co-elution of methanol and acetaldehyde suggesting a differential change in retention times of analytes, the same is displayed in acetone and isopropanol, separation of acetone and ethanol does not appear to have improved when compared to 40 °C.

Conditioning time

Headspace conditioning time was assessed to review the impact of conditioning time on peak responses and to determine if there was a significant increase in peak area and height observed in samples conditioned for longer. Times of 5, 10, 15 and 20 minutes were selected, and a quantitation of the observed concentration was taken to determine if an increase in conditioning time contributed to an increase in quantitative value. The peak areas did not increase significantly according to the quantitation data with under a 1mg difference between a 5 and 20-minute conditioning time thus a 5-minute conditioning time was selected.

Table 13: Conditioning time summary data

	Concentration mg/dl		% difference from expected 20 mg/dl		Peak area		Peak height	
	Column A	Column B	Column A	Column B	Column A	Column B	Column A	Column B
5 minutes								
Ethanol	21.33	20.46	106.65	102.30	9880	11538	3139	2776
Tertiary Butanol	0.000	0.000	N/A	N/A	95958	113025	31005	25701
10 minutes								
Ethanol	21.36	21.15	106.80	105.75	10541	12750.3	3410	3035.2
Tertiary Butanol	0.000	0.000	N/A	N/A	102238	120607	32989	27561
15 minutes								
Ethanol	21.39	21.15	106.95	105.75	10165	12289	3303	2937
Tertiary Butanol	0.000	0.000	N/A	N/A	98433	116248	31793	26551
20 minutes								
Ethanol	21.40	20.91	107.00	104.55	10765	12802	3496	3092
Tertiary Butanol	0.000	0.000	N/A	N/A	104210	122566	33678	27909

Table 13: Quantitative data demonstrating the quantification of the QC 20 at a 5,10,15 and 20-minute conditioning time.

5 minutes

Column A Column B

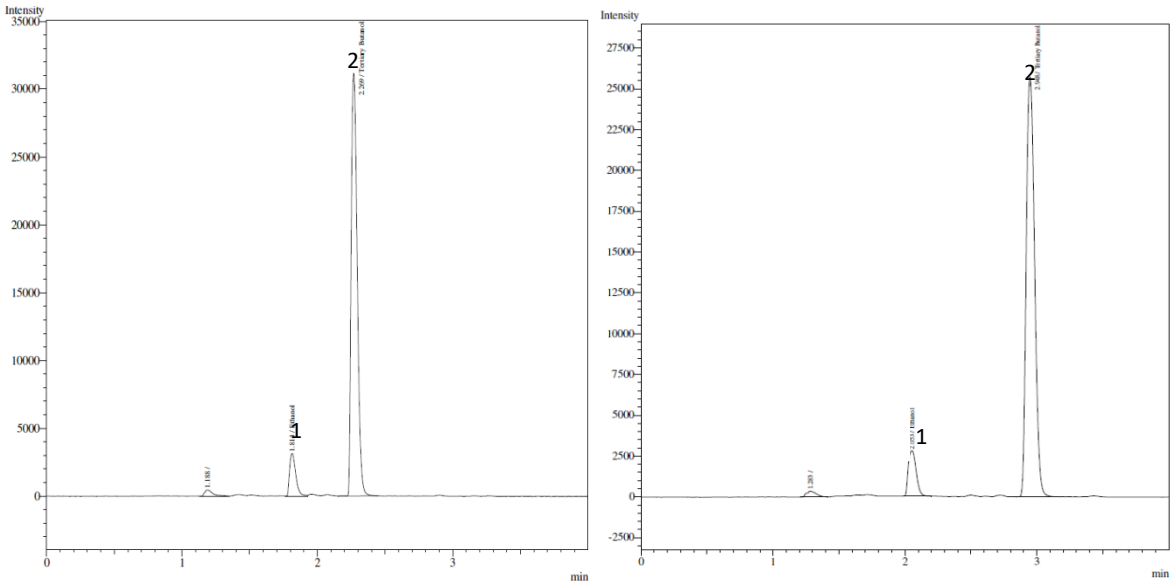


Figure 12: Chromatographic display for the 5-minute conditioning time. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

10 minutes

Column A Column B

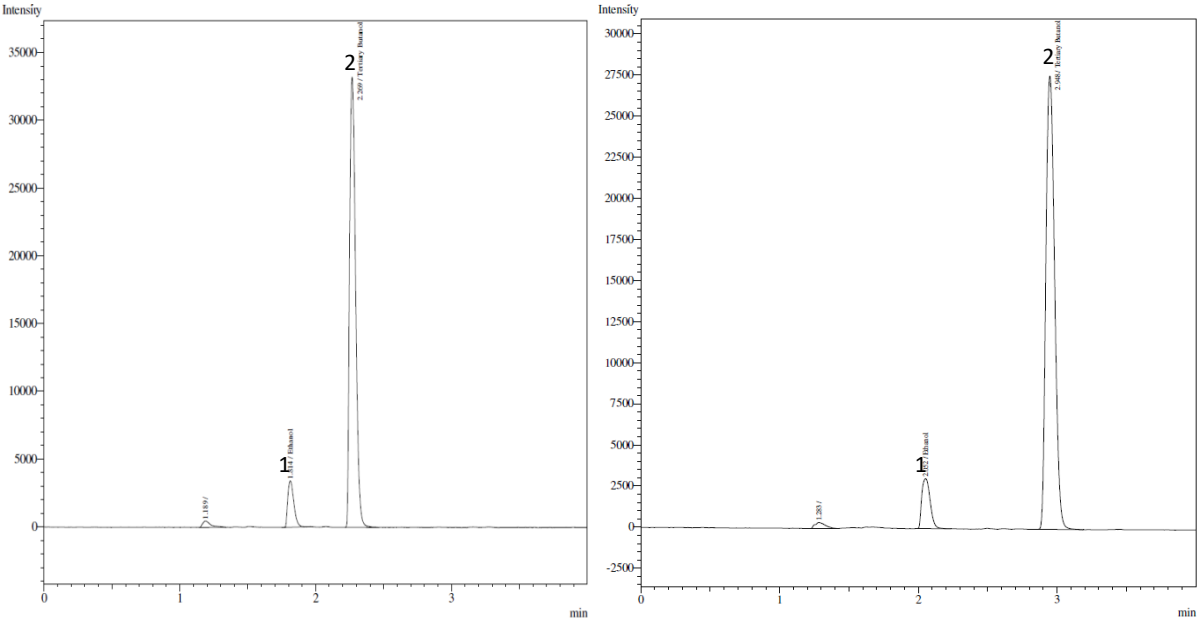


Figure 13: Chromatographic display for the 10-minute conditioning time. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

15 minutes

Column A

Column B

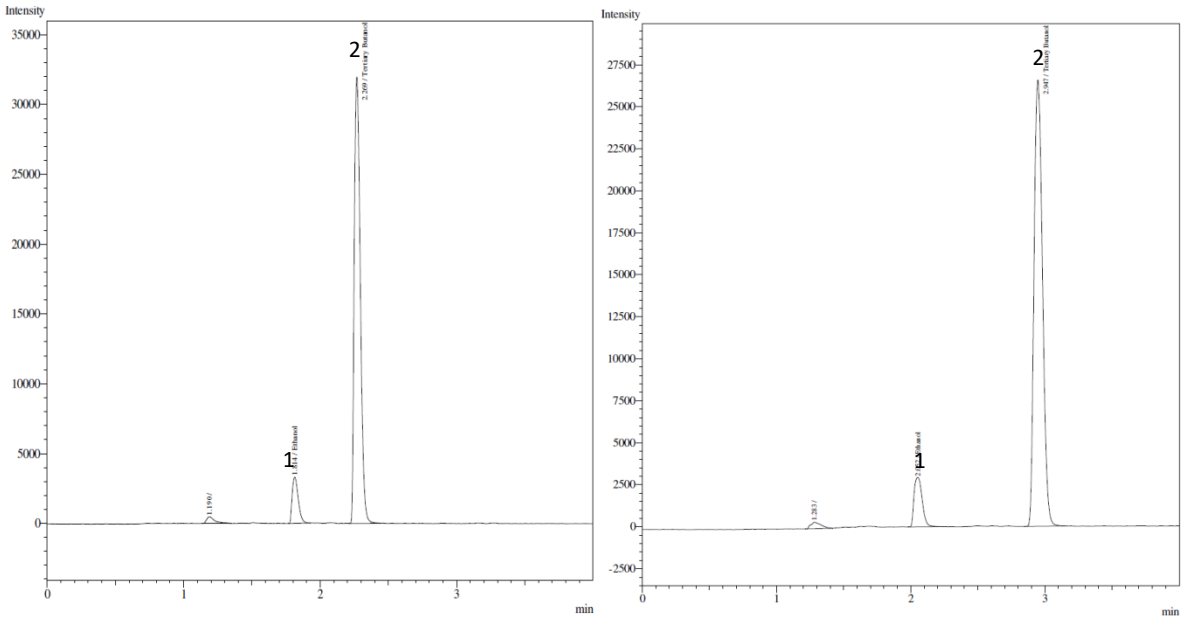


Figure 14: Chromatographic display for the 15-minute conditioning time. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

20 minutes

Column A

Column B

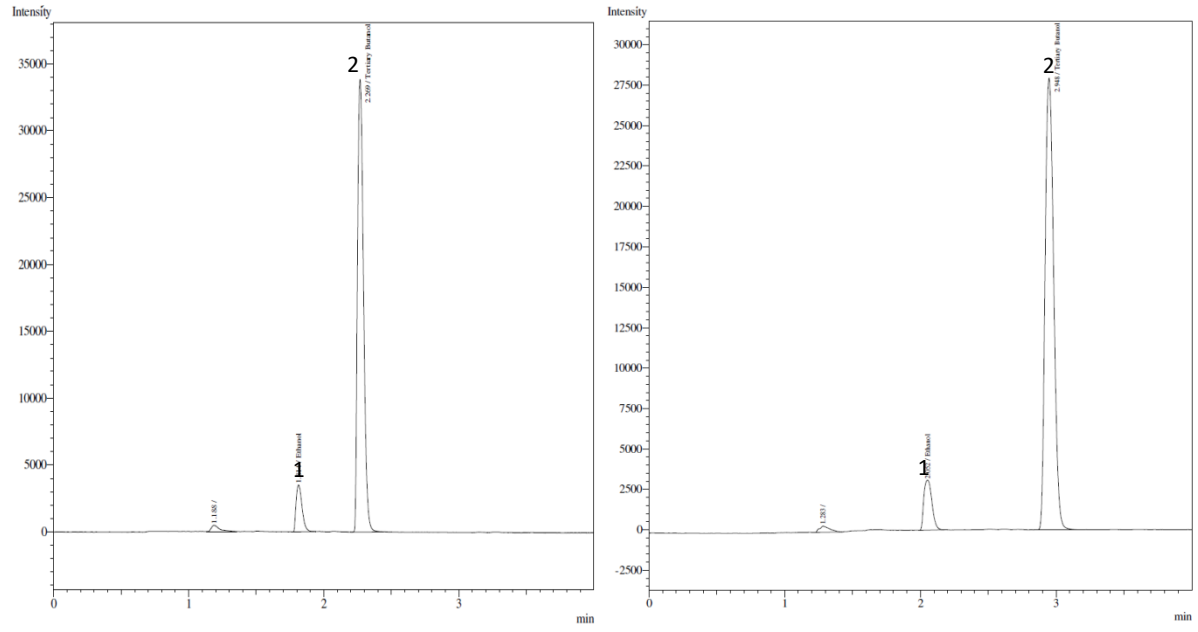


Figure 15: Chromatographic display for the 20-minute conditioning time. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

Optimum conditions

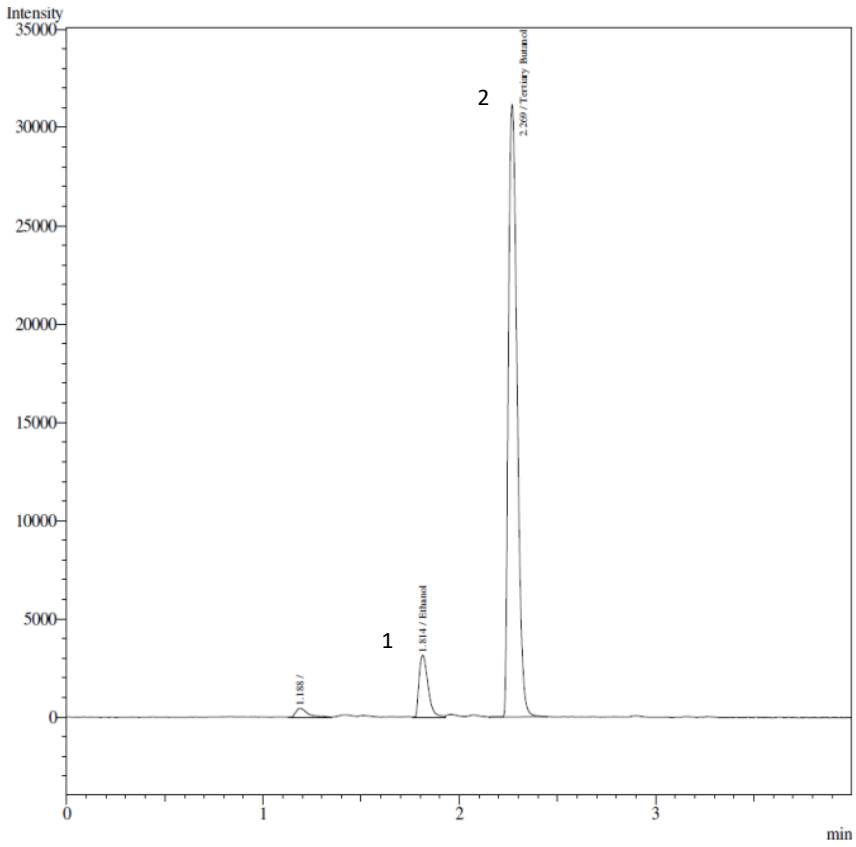
The optimum parameters selected for this method were 40°C GC oven temperature, a 5-minute conditioning time and an 85kpa pressure (Table 17). These parameters allowed for the best balance of run time separation and response, while there may have been better conditions for separation or run time individually, these conditions provided the best for both parameters. As demonstrated in the system suitability solution, acetaldehyde and methanol co-elute on both columns. The addition of an antioxidant, sodium metabisulfite in the internal standard solution prevents the build-up of acetaldehyde in analytical samples, methanol is also not typically analysed in road traffic blood alcohol analyses, so this co-elution is not anticipated to cause any issues with this method.

Table 17: Selected method parameters	
Parameter:	**Optimum selected**
Conditioning time	5 minutes
Oven temperature	40°C
Pressure	85kpa

Table 14: Demonstrating the selected optimum parameters used for the method

Separation of ethanol and internal standard using selected conditions:

Column A



Column B

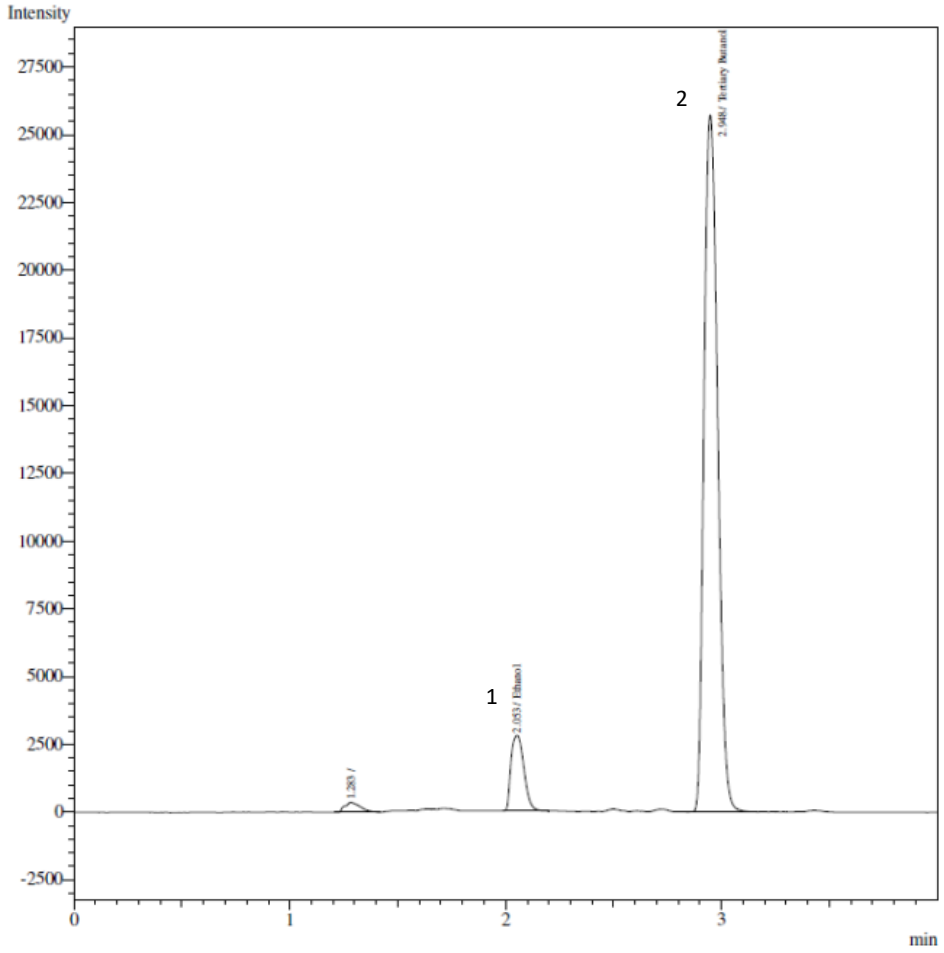
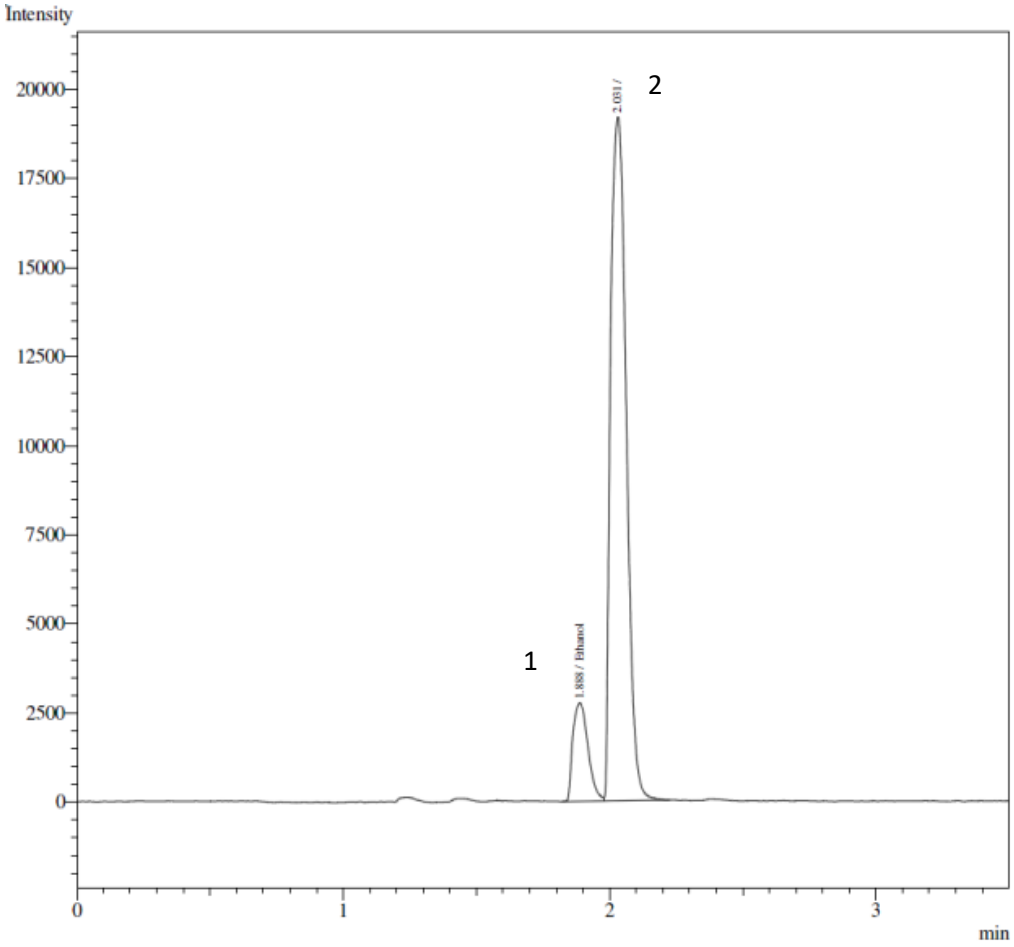


Figure 16: A chromatographic display demonstrating acceptable separation of ethanol and tertiary butanol, the internal standard on both columns. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

Acetone co-elution

A sample of ethanol spiked with acetone was analysed to determine the impact of co-elution on ethanol in isolation. Figure 18 demonstrates the degree of separation on column B which shows that it can be used to quantify ethanol without interference should acetone be detected in a sample. In the case of a low concentration of acetone it may not interfere with ethanol, however it is generally an interfering peak that results in interference on column A. A detection of a high amount of acetone usually results in a disparity of quantitation of ethanol between column A and column B and it will be clear that acetone has interfered with the quantitation, the amount of acetone required to interfere with an analysis is unknown, however a relatively low amount has the potential to interfere with the integrated peak area of ethanol effecting the quantitation. The purpose of this analysis was to demonstrate that acetone will elute closely with ethanol on one column in the absence of any other potential interferences including an internal standard. For this reason, a quantitation of ethanol was not achieved.

Column A


Intensity
20000
17500
15000
12500
10000
7500
5000
2500
0
1.888 / Ethanol
2.081 /
1
2
0
1
2
3
min

Column B

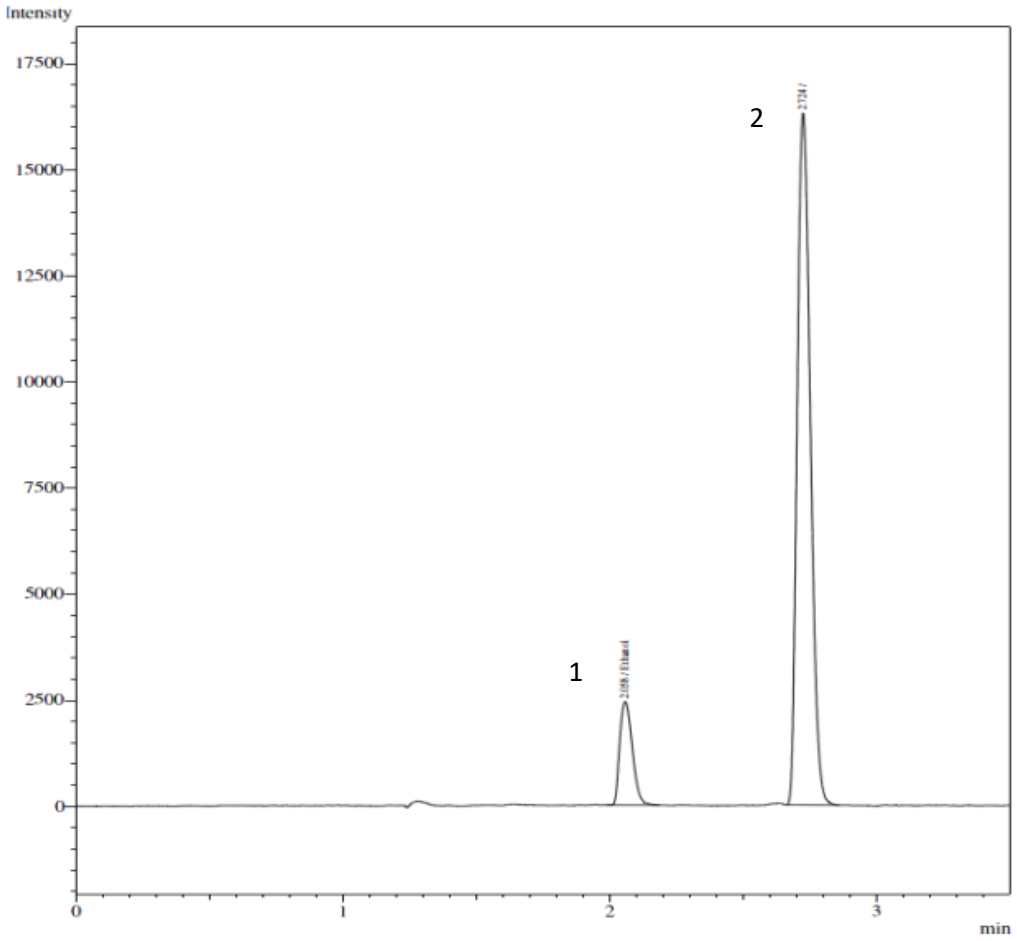


Figure 18: A chromatographic display of acetone and ethanol co-elution. Full separation is achieved with column B; however, baseline separation is not achieved on column A which will in some cases cause distortion of quantitative values obtained. For this reason, samples with acetone present are quantified on B column alone. Peak 1 represents ethanol with peak 2 representing the acetone.

Linearity

The linearity is automatically calculated by the GC software from the calibration curve created using a total of six calibration points, that is six separate concentrations ranging from 10 to 400mg/ using concentrations of 10,20,50,100,200 and 400 mg/dl. This value is represented as an r^2 value and the closer to a value of 1 this value is, the better the linearity. As demonstrated in figures, 19, 19.1 and table 20 linearity for this method was well in excess of the pass criteria featured in table 26 suggesting that the method accuracy and pipetting of samples was above the standard required of an analyst and analytical method. Samples of a concentration above 400 are analysed however the accuracy of the quantitation cannot be guaranteed.

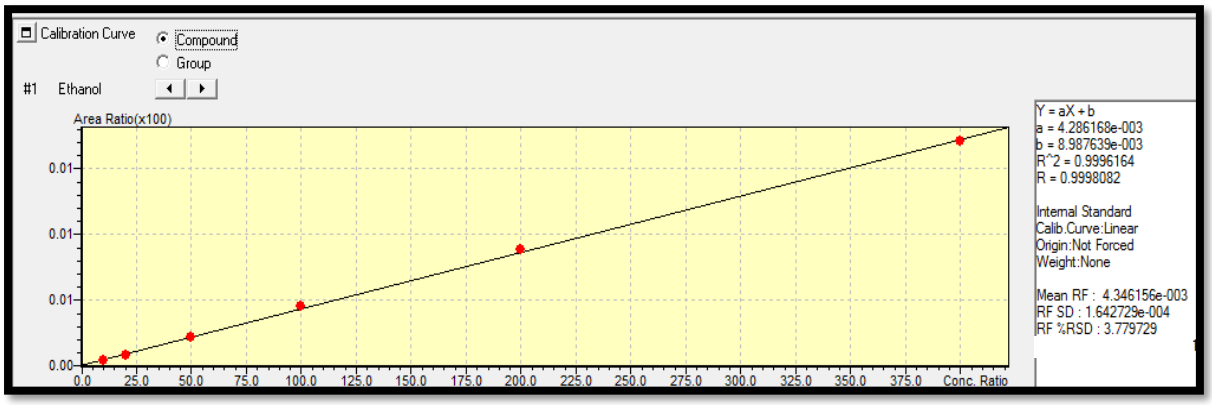


Figure 19: Representative calibration curve for column A shown in table15 . Concentrations of 10, 20, 50,100,200 and 400 mg/dl were used to calibrate the instrument, each calibration level was run in duplicate.

Level	Concentration (mg/dl)	Mean area ratio	Standard deviation	Relative standard deviation (%)	Area 1	Area 2
1	10	0.04	3.31	0.80	7,214	7,024
2	20	0.08	6.29	0.75	14,395	14,571
3	50	0.23	6.14	2.73	38,956	37,250
4	100	0.45	6.57	1.46	76,337	75,637
5	200	0.88	3.95	4.48	149,434	152,200
6	400	1.71	3.18	1.85	304,440	252,372

Table 15: The summary data given in the calibration curve table generated in figure 19. The attained areas of each calibrator are included demonstrating the pattern of the increasing peak area with increasing concentration.

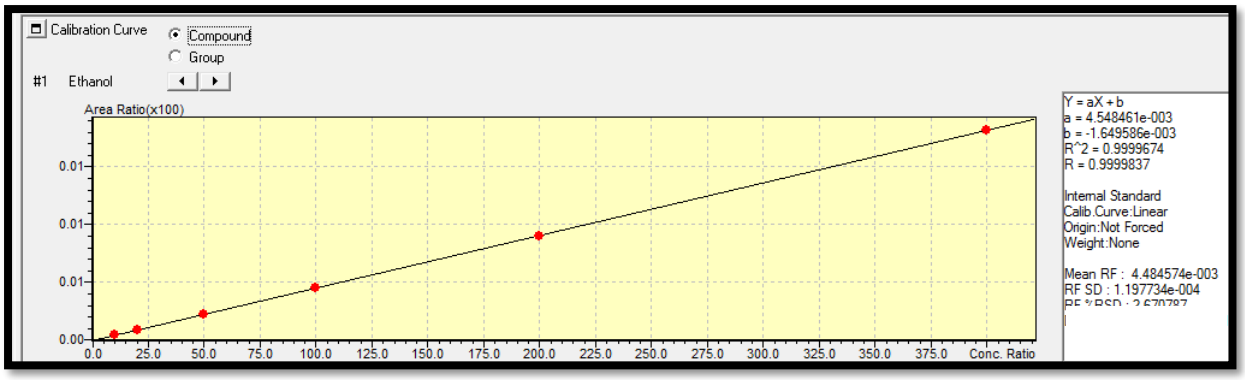


Figure 19.1: Representative calibration curve demonstrating a typical display for column B curves shown in table 16. Concentrations of 10, 20, 50,100,200 and 400 mg/dl were used to calibrate the instrument, each calibration level is run in duplicate.

Level	Concentration (mg/dl)	Mean area ratio	Standard deviation	Relative standard deviation (%)	Area 1	Area 2
1	10	0.04	2.02	0.47	5,712	5,610
2	20	0.09	2.99	0.34	11,253	11,338
3	50	0.23	5.62	2.46	30,035	28,883
4	100	0.46	6.03	1.32	58,954	58,560
5	200	0.90	3.28	3.64	115,661	117,791
6	400	1.82	3.37	1.84	234,821	201,862

Table 16: The summary data given in the calibration curve table generated in figure 19.1. The attained areas of each calibrator are included demonstrating the pattern of the increasing peak area with increasing concentration.

Table 20: Linearity data at the method development stage		
Date acquired	**Linearity Column A**	**Linearity Column B**
01/03/2018	0.9997	0.9997
04/03/2018	0.9996	0.9998
06/03/2018	**0.9996**	**0.9999**
28/03/2018	0.9999	0.9999
12/04/2018	0.9998	0.9998
24/04/2018	0.9997	0.9997
Average	0.9997	0.9998

Table 17: Linearity values for calibration curves of batches from the method development stage picked at random for inclusion, the data demonstrates that linearity at this stage was well in excess of the pass limit for the method linearity (0.998). (Linearity values shown in figure 19 and 19.1 are highlighted in bold).

Accuracy and precision

The accuracy and precision were recorded in quality control charts generated in Microsoft excel (figure 20, 20.1 and 20.2) which were prepared by using the mean as the centre line, the mean plus 3 and minus 3 standard deviations represents the control limits and plus or minus two standard deviations for the warning limits. Averages of QC results from batches were plotted, if a point exceeds the control limits then this should be assessed, and a possible cause determined as well as whether action should be taken to prevent these occurrences. If more than one point exceeds the warning limits it may suggest an issue which could need to be addressed however this may not be required if it is one point. A pattern of more than 4 increasing points or decreasing points consecutively may also be an indication of either an analyst skill issue or an issue with reliability of the method. At the method development stage only one set of QCs were used per batch compared to the later validation batches which have a set post calibration curve and a second post samples, providing two average values per concentration. It is for this reason that only half the number of points are used demonstrating the accuracy of the method over the same number of batches. Although there were more values available to add, the charts are representative of the precision and accuracy at the early stages of method development.

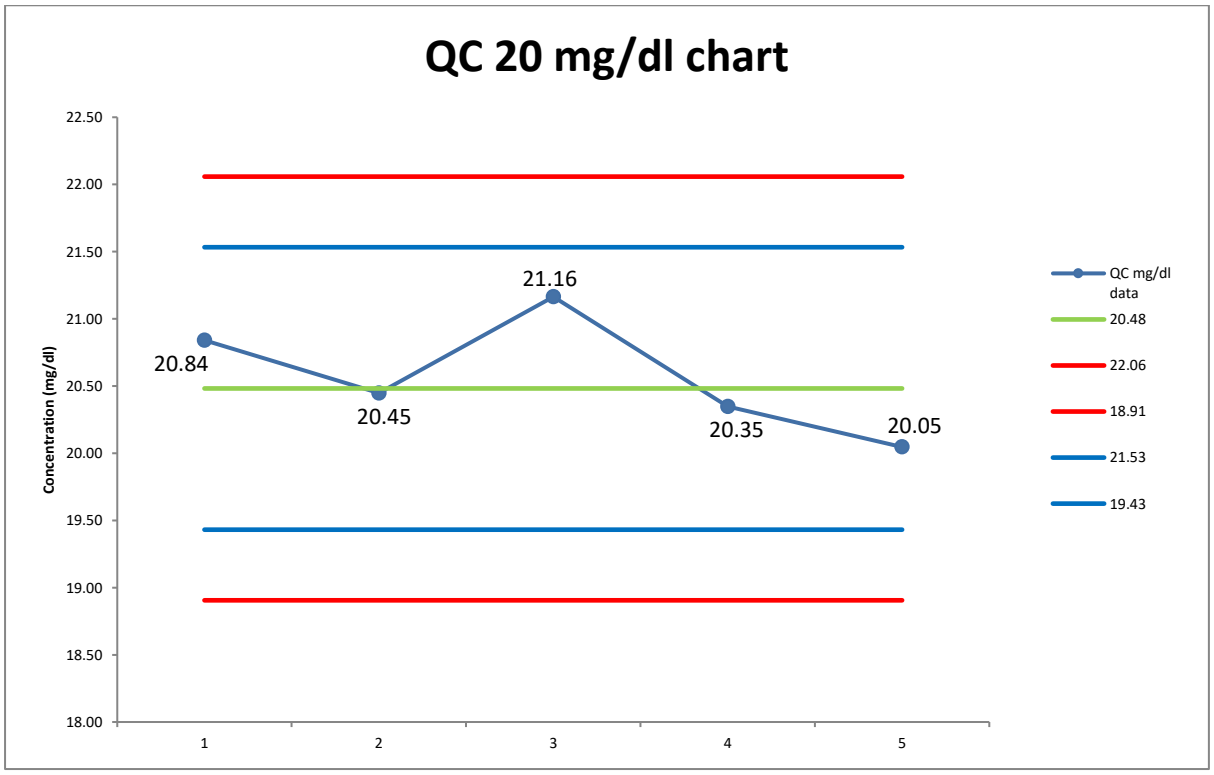


Figure 20: QC chart demonstrating precision of the low concentration quality control, all values were within 2 standard deviations of the mean showing that no corrective action was required with the method.

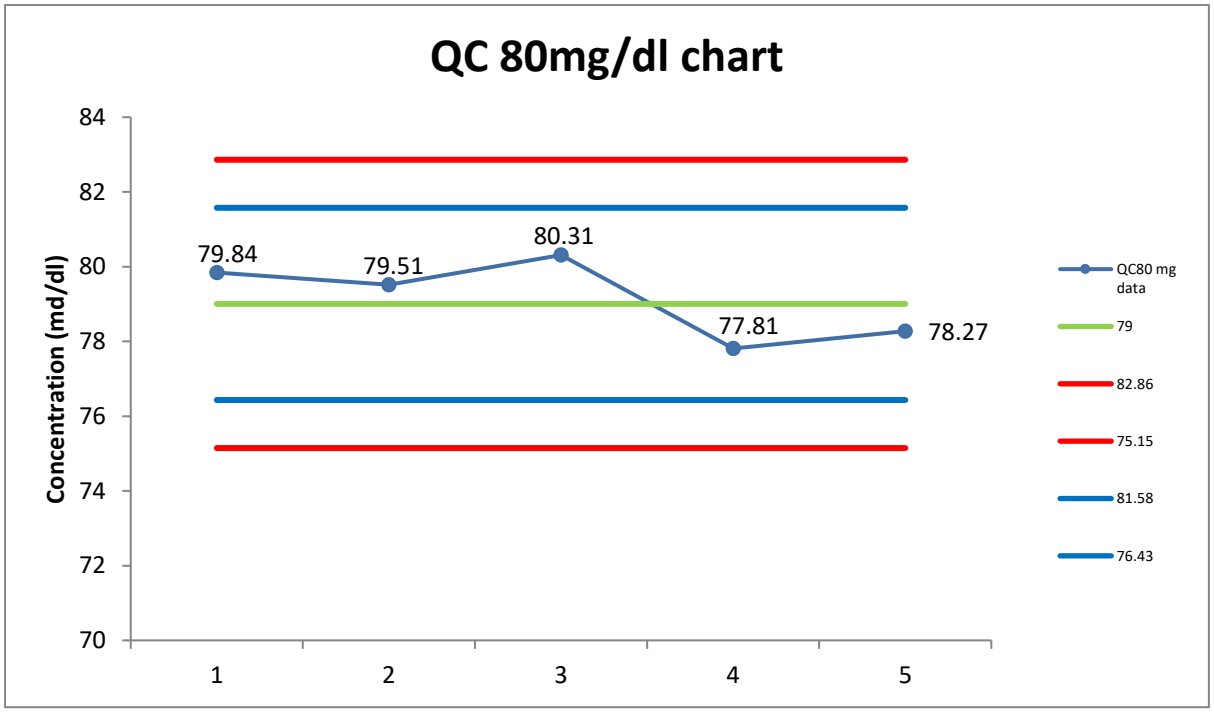


Figure 20.1: QC chart for the mid concentration quality control, this chart shows a similar pattern to the low concentration calibrator.

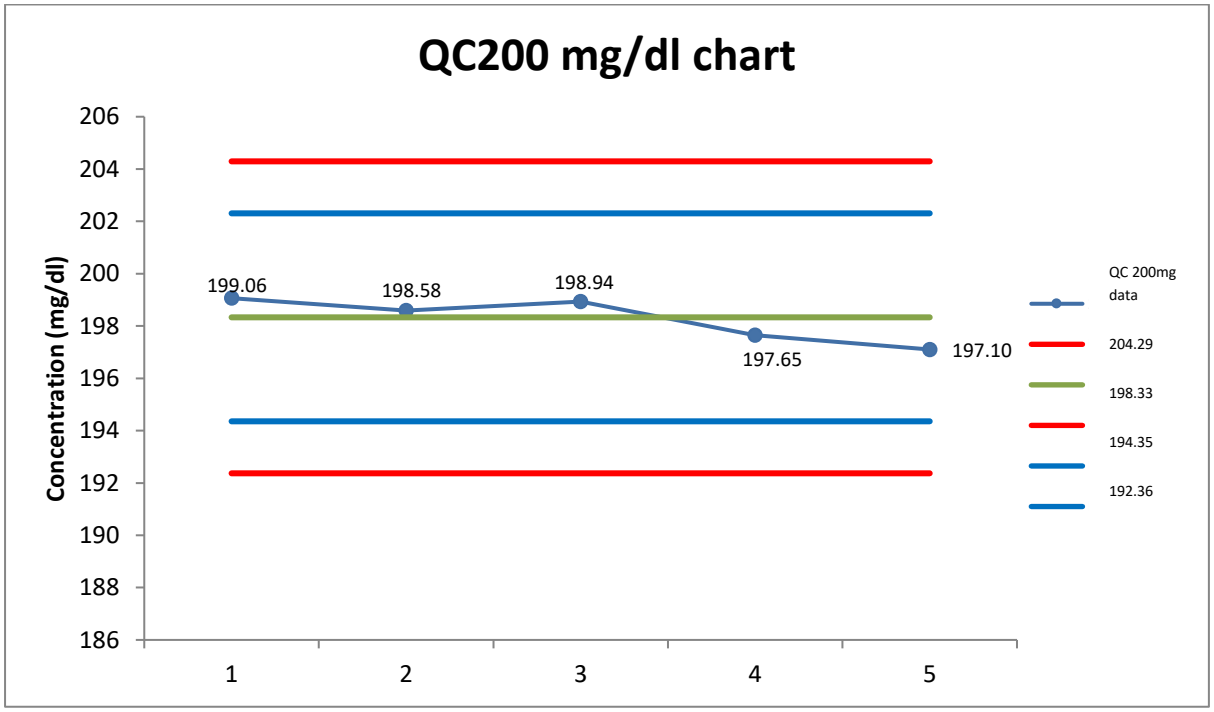


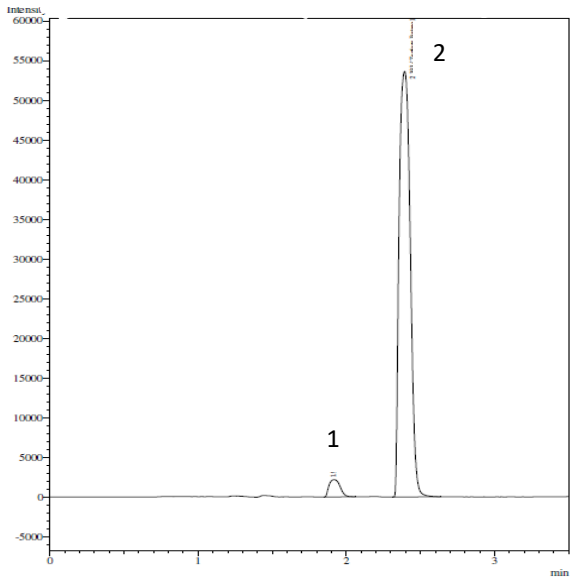
Figure 20.2: QC chart for the highest Quality control concentration, of the three concentrations the high concentration is shown to demonstrate the greatest accuracy.

Troubleshooting

During the method development the installation of the new BAC 1 plus and BAC 2 plus columns the insertion of the columns into the split was unequal, this is highlighted in the featured batch showing a large discrepancy between the column A and column B quantitation. This was rectified by removing the columns, verifying the lengths of each column and correcting the insert lengths before cutting and reinstalling the column at the splitter.

QC 20

Column A



Column B

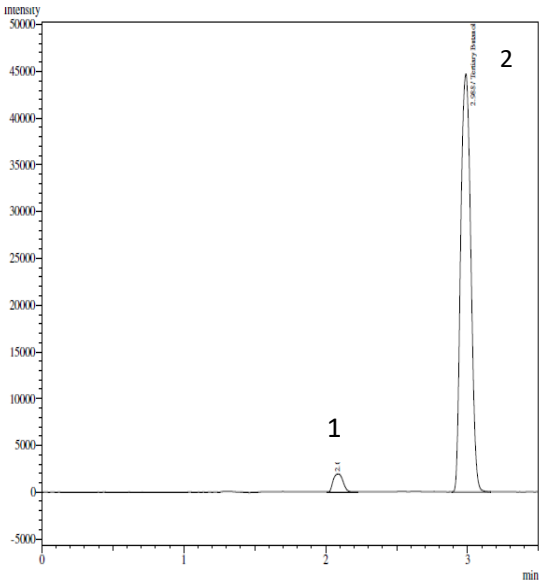


Figure 21: Chromatographic display of the failed batch QC 20 data. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

QC20 mg/dl	Concentration mg/dl		Retention time		Peak area		Peak height	
Analyte	Column A	Column B	Column A	Column B	Column A	Column B	Column A	Column B
Ethanol	19.648	39.066	1.918	2.088	11231	9143	2167	1909
Tertiary Butanol	0.000	0.000	2.393	2.988	278416	220781	53564	44667

Table 18: Quantitative data for the failed batch showing the discrepancy between column A and column B concentration.

QC 80

Column A Column B

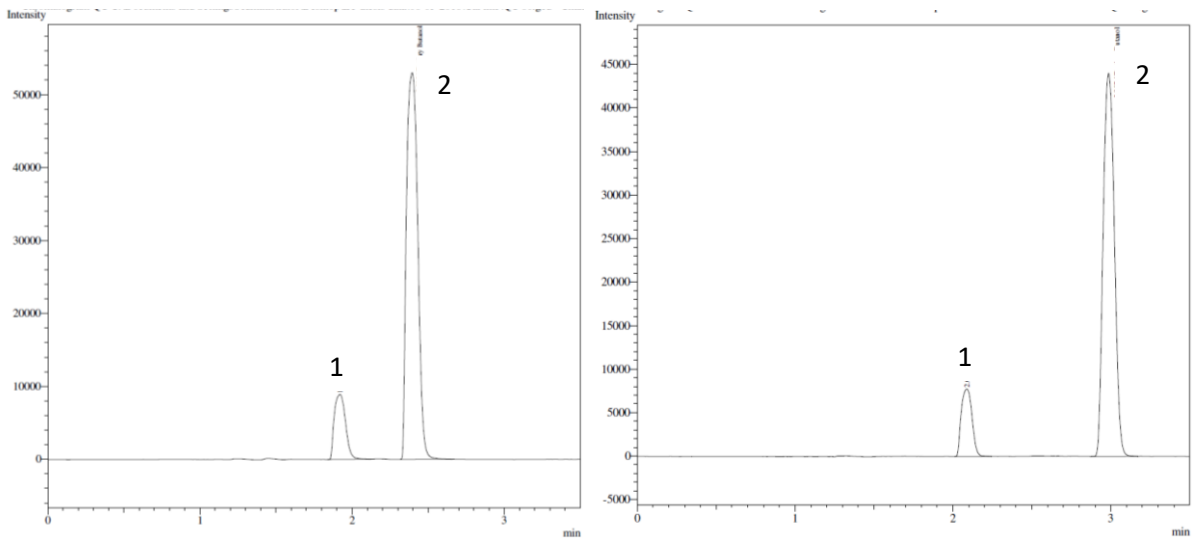


Figure 22: Chromatographic display of the failed batch QC 80 data Peak 1 represents ethanol and peak 2 the internal standard tertiary butanol.

QC 80 mg/dl	Concentration mg/dl		Retention time		Peak area		Peak height	
Analyte	Column A	Column B	Column A	Column B	Column A	Column B	Column A	Column B
Ethanol	79.670	154.807	1.920	2.088	45643	36749	8914	7756
Tertiary Butanol	0.000	0.000	2.395	2.987	273512	216904	52977	43953

Table 19: Quantitative data for the failed batch showing the discrepancy between column A and column B concentration.

QC 200

Column A Column B

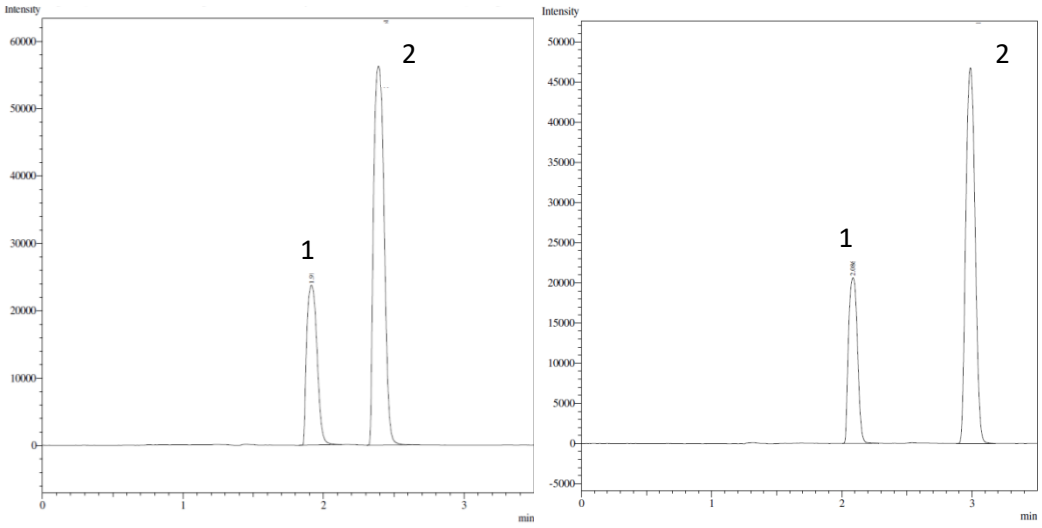


Figure 23: Chromatographic display of the failed batch QC 200 data Peak 1 represents ethanol and peak 2 the internal standard tertiary butanol.

QC 200 mg/dl	Concentration mg/dl		Retention time		Peak area		Peak height	
Analyte	Column A	Column B	Column A	Column B	Column A	Column B	Column A	Column B
Ethanol	197.749	383.799	1.914	2.806	121260	97796	23697	20595
Tertiary Butanol	0.000	0.000	2.391	2.987	291625	231363	56221	46669

Table 20: Quantitative data for the failed batch showing the discrepancy between column A and column B concentration.

Table 24: Summary of failed batch results									
	Concentration mg/dl		Expected concentration mg/dl	Retention time		Peak area		Peak height	
Analyte	**Column A**	**Column B**		**Column A**	**Column B**	**Column A**	**Column B**	**Column A**	**Column B**
Ethanol	19.648	39.066	20	1.918	2.088	11231	9143	2167	1909
Tertiary Butanol	0.000	0.000	0	2.393	2.988	278416	220781	53564	44667
Ethanol	79.670	154.807	80	1.920	2.088	45643	36749	8914	7756
Tertiary Butanol	0.000	0.000	0	2.395	2.987	273512	216904	52977	43953
Ethanol	197.749	383.799	200	1.914	2.806	121260	97796	23697	20595
Tertiary Butanol	0.000	0.000	0	2.391	2.987	291625	231363	56221	46669

Table 21: The summary of the failed batch data demonstrating the differences in column A and column B quantitation.

Quantitation of an unknown sample

A horse blood sample was run to demonstrate the accuracy of the instrumentation in detecting an unknown concentration of ethanol in a blood sample, the spiked amount was 20 mg/dl and the instrument demonstrated the ability to detect and quantify the unknown samples in blood, suggesting good accuracy within the stated limits.

Blood sample

Column A Column B

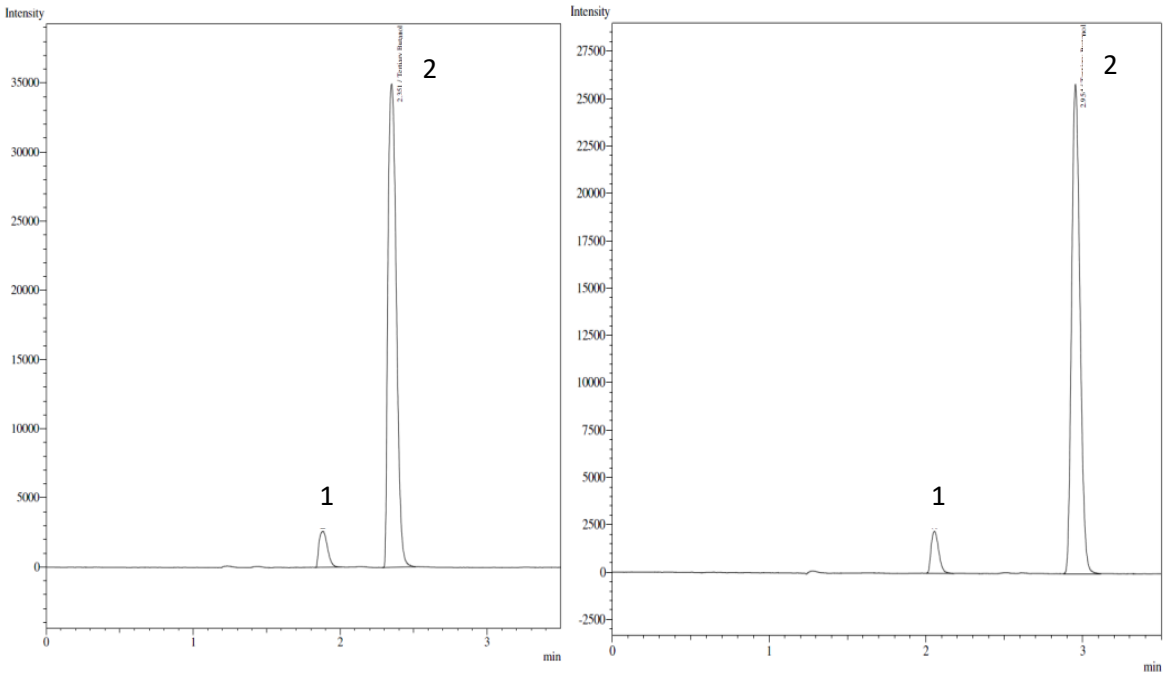


Figure 24: Chromatogram display demonstrating separation of analytes and clear peak shape in a blood sample. Peak 1 represents ethanol with peak 2 representing the internal standard tertiary butanol.

	Concentration mg/dl		Expected concentration mg/dl	Retention time		Peak area		Peak height	
Analyte	Column A	Column B		Column A	Column B	Column A	Column B	Column A	Column B
Ethanol	18.687	18.849	20	1.883	2.055	10372	7426	2602	2208
Tertiary Butanol	0.000	0.000	0	2.351	2.954	138209	95961	34912	25760

Table 22: A sample of 20 mg/dl was spiked into 1 ml of internal standard for analysis to verify the system's capability of quantifying true unknowns, it was also done to determine the suitability of the instrument to analyse blood samples. Quantitation was within the ± 2mg limit of the expected 20 mg/dl in both columns demonstrating good accuracy of quantitation in a blood sample.

Method validation criteria:

Validation plan:

Validation experiment	Experimentation required	Pass criteria	Attained performance	Result
LOD	Three samples of blood spiked at 5mg/dl using 50 µl of Cerilliant standard 10 mg/dl will be run to verify detection was achieved.	Detection of all three samples with clean integrated peaks and a signal – noise ratio of greater than 3:1 or appear as a clean peak on visual inspection.	Clear peaks were seen on all samples and both columns. The LOD for ethanol is 5 mg/dl.	PASS
LOQ	100 µl of Cerilliant standard 100 mg/dl will be diluted into 1 ml of internal standard in duplicate.	Samples should provide a reading within 2 mg of the expected 10 mg/dl concentration.	All observed concentration values were within 2mg of the 10 mg/dl expected concentration	PASS
Linearity	Linearity will be demonstrated within calibration curve data on each calibration curve produced.	The minimum linearity r^2 value will be 0.998.	All r^2 values in the validation exceeded 0.998.	PASS
Range	The range of the method is 10-400mg mg/dl. Quality control samples at 20, 80 and 200mg/dl demonstrating precision within the curve limits.	Quality control samples should have no more than two points which fall outside of the control limit of the mean ± 3 standard deviations and no more than 4 points outside of the warning limits± 2 standard deviations.	All Qc values featured in Quality control charts fell within 2 standard deviations of the mean.	PASS
Repeatability	A single analyst will prepare and run batches on three separate occasions on the same instrument. The batches will contain a standard curve featuring 6	All replicates for each Qc should fall within a 2.5% CV and within 2mg for 20 mg/dl and 80 mg/dl and 6 mg for 200mg.	All QC values were within the 2.5% CV, all values were within 2mg of the expected values with the exception of one	PASS

Repeatability continued	concentrations (10-400mg/dl) and two sets of QCs. QC samples will determine whether the batches were successful.			
Reproducibility	3 batches will be prepared by 3 different analysts on separate days and run on the same instrument. Each batch will contain the standard 6-point calibration curve with two sets of duplicate QCs (6 aliquots).	All replicates for each Qc should fall within a 2.5% CV and within 2mg for 20 mg/dl and 80 mg/dl and 6 mg for 200mg.	Qc values were within the 2mg/dl limit for QC 20 and 80 mg/dl and 6 mg of the 200 mg/dl.	PASS
Accuracy	Accuracy will be assessed with QC results obtained.	There should be no significant bias within samples, QC values should be within 2mg for QC 20 and 80 and 6 mg for QC 200mg.	Qc values were within 2 mg of the QC 20 and QC 80 and within 6mg of the QC 200 mg/dl.	PASS
Selectivity	Solutions of Acetaldehyde, methanol, ethanol, acetone, tertiary Butanol, propan-1-ol and propan-2-ol, first as single runs to determine retention order then as a mix to determine separation.	Baseline separation should be achieved between the primary analyte (ethanol) and all other selectivity components on at least one column.	Baseline separation of primary analyte and all other potential interfering peaks was achieved on at least one column.	PASS

Table 23: Validation outline indicating parameters for a successful validation, experimentation required to test the method and a judgement on if the method meets the requirements.

Limit of detection

Samples for limit of detection testing were prepared at 5mg/100ml using 50 µl of the 10mg/100ml Cerilliant standard in 1ml of ISTD. Although quantitative values were generated (Table 27) the values were outside the calibration curve and therefore accuracy of the quantitation cannot be assured. However according to the international conference on harmonisation (ICH) guidelines, it is plausible to determine detection limit by visual evaluation. The guideline for this approach is that limit of detection determined by an analysis of known concentrations of an analyte and establishing the minimum level with which the analyte can be reliably detected (ich.org, 2005). In this case 5 mg was selected and as demonstrated a relative degree of reliability of the quantitation was achieved and for this reason this method was used.

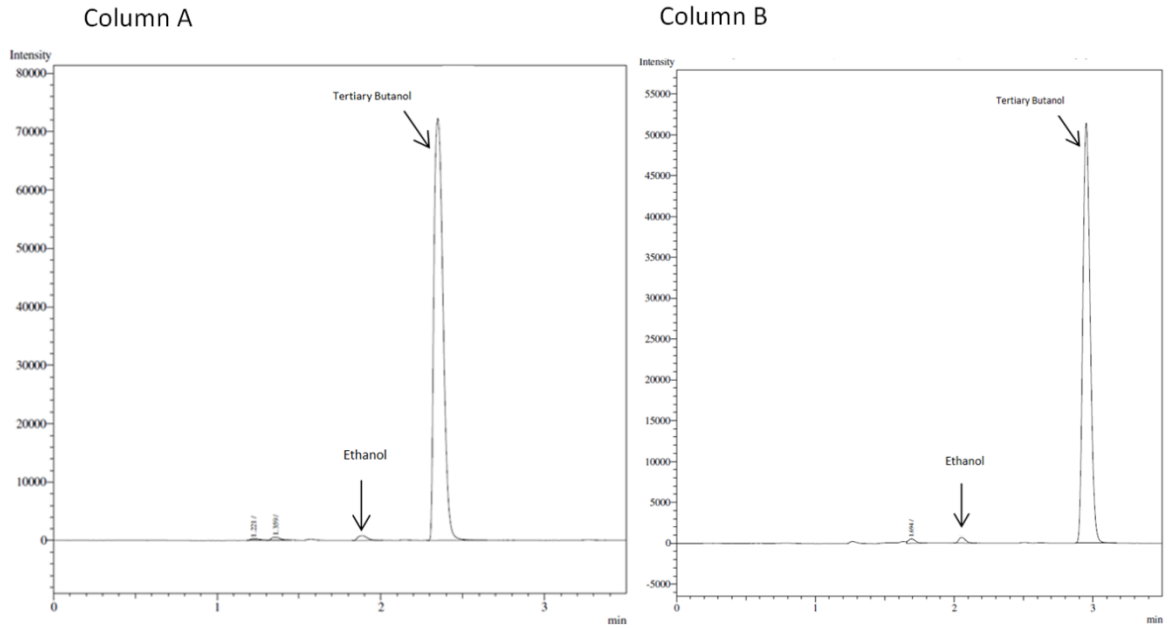


Figure 24: Chromatographic display of LOD experimentation demonstrating peak shape at 5mg/dl.

Expected concentration (mg/dl)	Observed concentration (mg/dl)	Peak area	Peak height	Matrix
5	6.4175	2261	606	Blood
5	6.2545	2052	556	Blood
5	5.9945	2205	595.5	Water
5	5.8995	2133	580	Water

Table 25: Quantitative data for LOD experimentation. 5 mg/dl is below the lowest calibrator (10mg/dl) and so accuracy cannot be guaranteed. LOD judgement was based on the appearance of a clean integrated peak.

Limit of quantitation

The limit of quantitation (LOQ) was the lowest concentration that can be accurately quantified, in the case of this method the lowest calibrator was 10mg/dl, this means that 10mg/dl is the lowest concentration of ethanol that can be accurately quantified and as such it was selected as the LOQ for the method. The samples were prepared as standard using the procedure for preparing the 10 mg/dl calibrator, the LOQ was run in triplicate to confirm an accurate quantitation.

Table 28: Limit of quantitation					
Sample number	Expected concentration (mg/dl)	Column A concentration (mg/dl)	Column B concentration (mg/dl)	Average observed concentration (mg/dl)	%Accuracy
10 mg sample 1	10	10.79	10.89	10.84	108.39
10 mg sample 2	10	10.77	10.91	10.84	108.41
10 mg sample 3	10	10.85	10.85	10.85	108.53
	Average	10.80	10.84	10.84	108.44

Table 26: LOQ observed concentrations are averages of column A and B for each value.

Linearity, accuracy and precision of method validation

Linearity data attained at the method validation stage		
Date acquired	**Linearity Column A**	**Linearity Column B**
19/06/2018	0.9999	0.9999
20/06/2018	0.9999	0.9999
26/06/2018	**0.9999**	**0.9999**
28/06/2018	0.9999	0.9999
05/07/2018	0.9997	0.9997
11/07/2018	0.9999	0.9999
Average	0.9999	0.9999

Table 27: Linearity of calibration curves from different analyses. The target minimal accepted R^2 value was 0.998, the method demonstrated a consistently higher linearity than this target with a typical value being 0.9999. Values from the representative calibration curve in figure 26 are highlighted in bold.

Representative linearity calibration curve

Column A

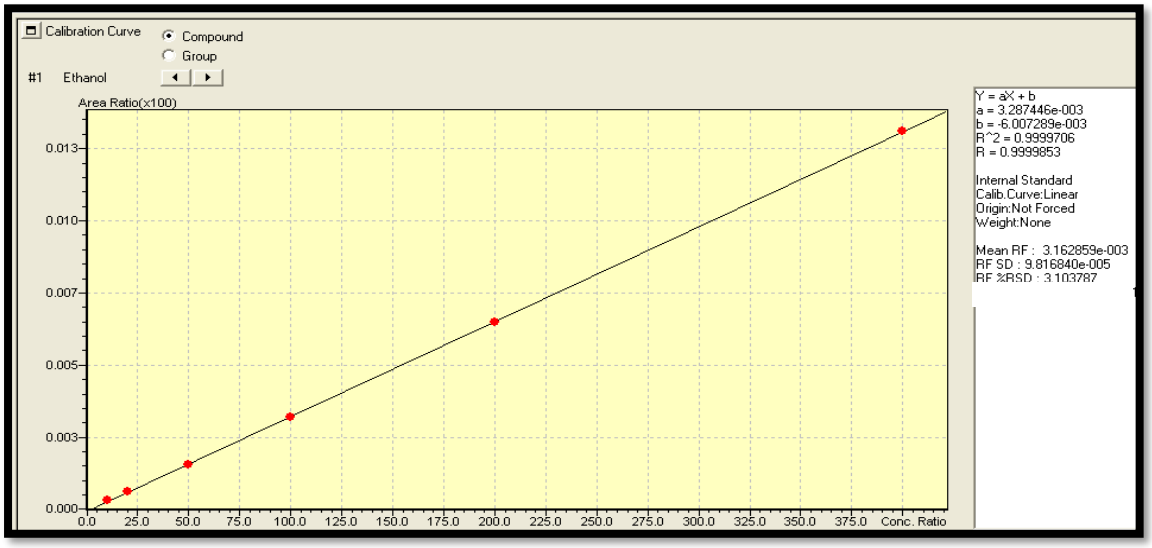


Figure 26: Representative calibration curve display for Column A data shown in Table 28, showing R^2 values, along with response ratios used to calculate concentration. Calibration curves are made up of 6 concentrations at 10, 20, 50,100,200 and 400mg/dl using a set of duplicates for each concentration.

Level	Concentration (mg/dl)	Mean area ratio	Standard deviation	Relative standard deviation (%)	Area 1	Area 2
1	10	0.03	7.46	2.40	5,969	5,730
2	20	0.06	5.52	0.92	10,914	11,100
3	50	0.16	1.81	1.16	28,331	29,384
4	100	0.32	3.06	0.96	59,720	57,889
5	200	0.65	1.40	0.22	120,593	122,470
6	400	1.31	3.59	0.27	244,187	232,538

Table 38: The summary data given in the calibration curve table generated in figure 26. The attained areas of each calibrator are included demonstrating the pattern of the increasing peak area with increasing concentration.

Column B

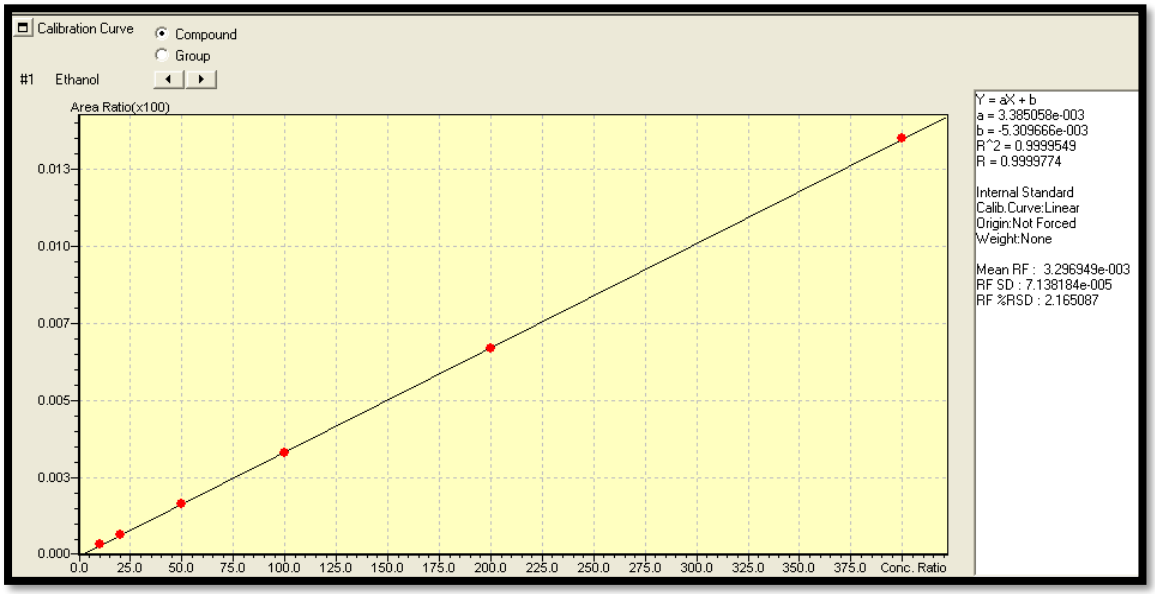


Figure 26.1: Typical calibration curve display for Column B data shown in Table 29, showing R^2 values, along with ratios used to calculate concentration. Calibration curves are made up of 6 concentrations at 10, 20, 50,100,200 and 400mg/dl using a set of duplicates for each concentration.

Level	Concentration (mg/dl)	Mean area ratio	Standard deviation	Relative standard deviation (%)	Area 1	Area 2
1	10	0.03	2.26	0.68	4,517	4,278
2	20	0.06	5.90	0.93	8,008	7,931
3	50	0.16	2.40	1.48	20,156	20,990
4	100	0.33	2.51	0.76	42,211	40,882
5	200	0.67	1.84	0.28	85,339	86,882
6	400	1.14	6.45	0.48	171,623	164,076

Table 29: The summary data given in the calibration curve table generated in figure 26.1. The attained areas of each calibrator are included demonstrating the pattern of the increasing peak area with increasing concentration.

Range

The range of the quality control values is a measure of how precise the quantitation is over an extended period; a quality control chart is used to verify this. From the chart, control limits were set to provide the maximum and minimum acceptable value for the method standard deviation and means, the control limits are set at the mean ± 3 standard deviations. Warning limits are similar however use a value of the mean ± two standard deviations. Results outside of these limits warrant further investigation. however, a decision in what action if any is to be taken. Figure 27, 27.1 and 27.2 demonstrate that in the analyses demonstrated a good inter-run accuracy and precision. Gross errors are not included within the charts. A set of 5 validation batches were selected for each chart to provide a representation of the accuracy of the method during the validation process and to provide a proportional comparison of the accuracy of 5 batches from the method development to the 5 selected for validation.

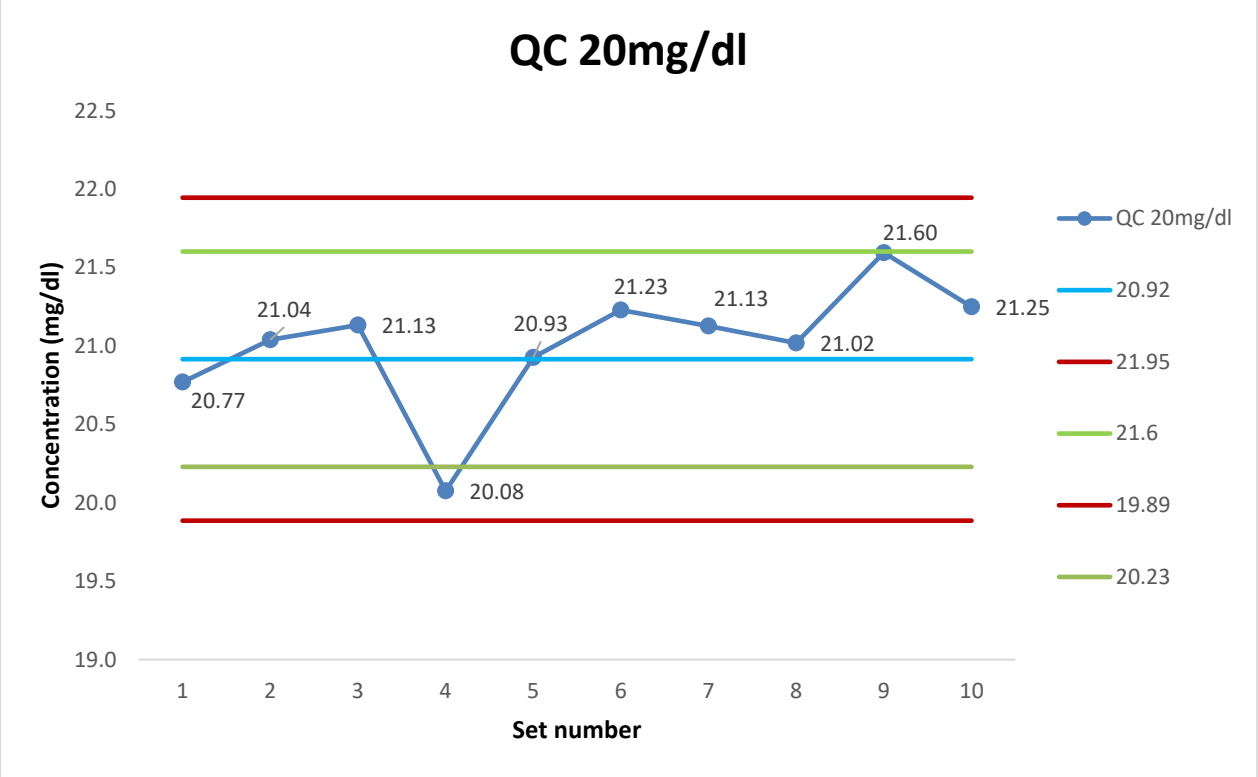


Figure 27: QC 20 method validation chart showing accuracy and precision of validation batches at a concentration of 20mg/dl.

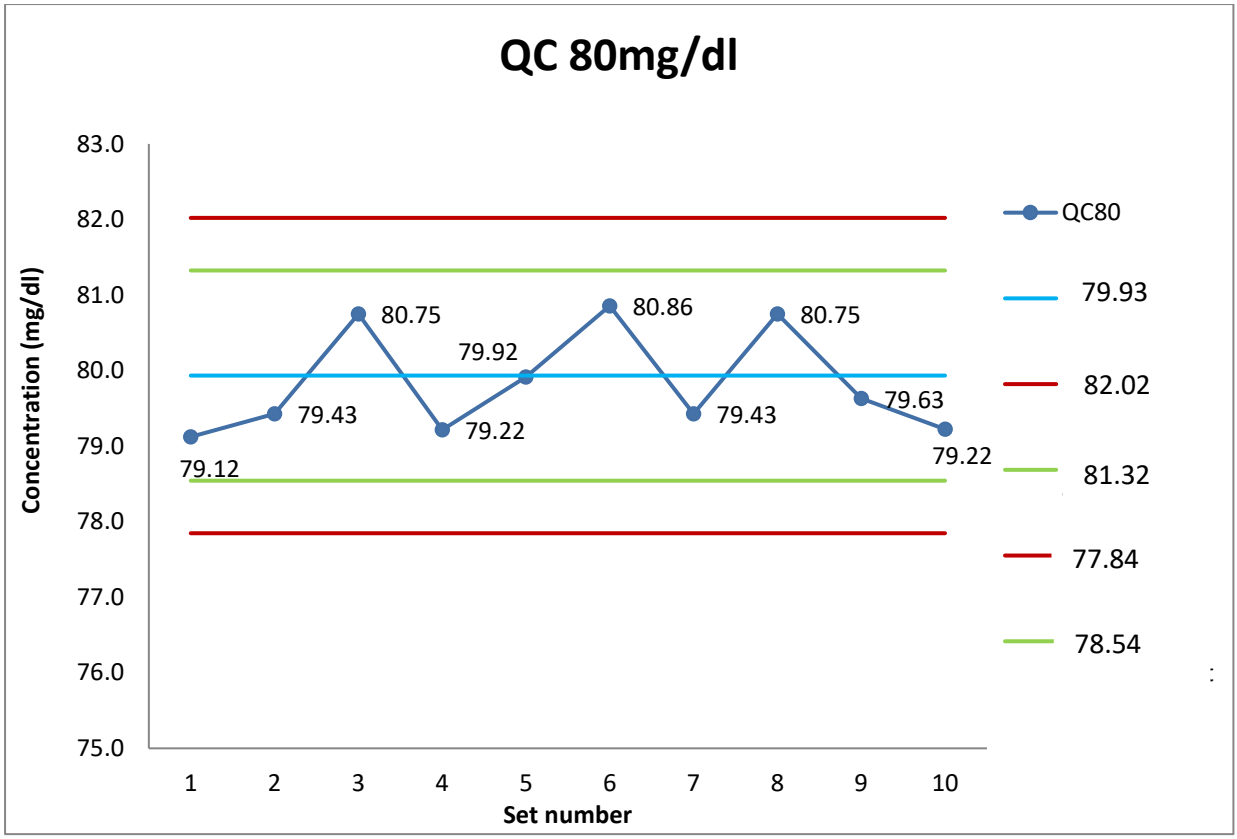


Figure 27.1: QC 80 method validation chart showing accuracy and precision of validation batches at a concentration of 80mg/dl.

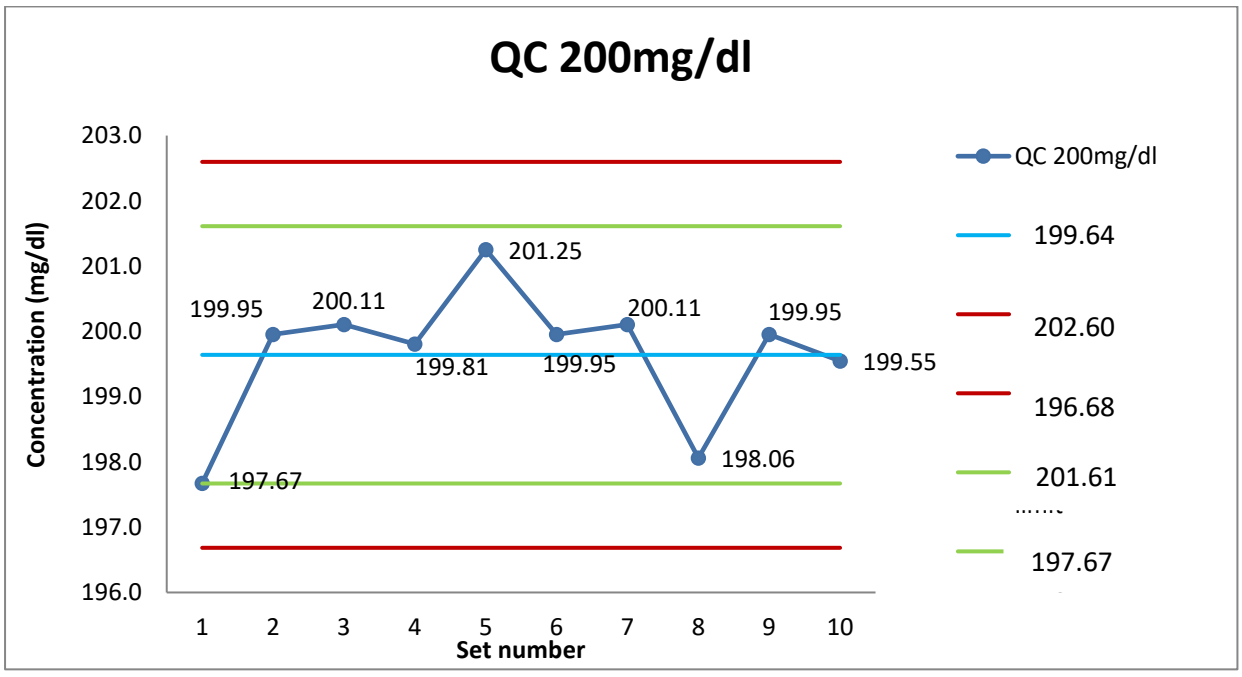


Figure 27.2: QC 200 method validation chart showing accuracy and precision of validation batches at a concentration of 200mg/dl.

Range of sample concentrations provided by external forensic laboratory:

An accredited forensic laboratory provided 8 B samples to test on the developed GC method to use in the validation, the values shown are from the A sample vials and a small difference between the A sample values and B sample values are to be expected as were observed in table 36, this was the first full batch of all samples analysed.

Sample:	Obtained concentration (mg/dl)
Blood sample 1	**37**
Urine sample 2	**484**
Urine Sample 3	**277**
Urine sample 4	**127**
Blood sample 5	**219**
Blood sample 6	**139**
Blood sample 7	**305**
Urine sample 8	**195**

Table 30: Values from A sample analysis carried out by an independent forensic laboratory using an independently developed and validated method. Table 36 demonstrates the first batch containing all the samples and shows that similar values were attained by this developed method.

Repeatability

Repeatability was tested by analysing the same samples in three batch analyses on three separate days featured in tables 33,35 and 37. Differences in concentration were observed due to the nature of the metrological ethanol evaporation caused by repeated opening of sample vials. QC data was included in tables 34,36 and 38 to demonstrate that inter-run precision has not been lost despite this occurrence. The purpose of a repeatability test is to verify that the method can accurately quantify known concentrations using calibration standards regularly and to verify the accuracy of the pipetting of the analyst. Replicates for the quality control values should fall within a specified limit within the validation plan, in this case the limits of a 2.5%CV for any concentration and a 2mg/dl from the 20mg/dl and 80mg/dl quality controls and a 6mg/dl limit for the 200mg/dl.

Repeatability batch 1

Repeatability batch 1 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	31	N/A	0.22	0.70	31	37	83.8
Urine sample 2	499	487	6.43	1.30	493	484	101.9
Urine sample 4	129	128	1.33	1.03	128	127	100.8
Blood sample 5	213	211	0.78	0.37	212	219	96.8
Blood sample 6	131	132	0.69	0.53	132	132	100.0
Blood sample 7	309	310	1.79	0.58	309	305	101.3
Urine sample 8	208	196	6.15	3.04	202	195	103.6

Table 31: Analytical values for batch 1 repeatability, all values are an average of two duplicates. Urine sample 3 was not analysed within this batch due to initial batches of the validation running limited samples to minimise ethanol loss with repeated familiarisation batches which occurred at this stage with the new pipette system and so is not included. Blood sample 1 is a single replicate due to a file corruption of the duplicate.

QC values for repeatability batch 1							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
QC20 mg/dl	21	21	0.31	1.48	21	20	103.3
QC80 mg/dl	79	81	0.49	0.61	80	80	99.5
QC200 mg/dl	201	198	1.27	0.64	200	200	100.2
QC20 mg/dl post sample	21	21	0.28	1.41	21	20	102.6
QC80 mg/dl post sample	80	81	0.80	1.02	81	80	100.84
QC200 mg/dl post sample	202	201	1.66	0.84	199	200	99.4

Table 32: Analytical QC values for batch 1 repeatability. The term “post sample” refers to the second set of QC samples which are run after samples to verify that the intra-run accuracy of the analysis.

Repeatability batch 2

Repeatability batch 2 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	33	33	0.48	1.44	33	37	89.9
Urine sample 2	497	497	1.64	0.33	497	484	102.6
Urine sample 3	284	281	1.61	0.57	283	277	102.0
Urine sample 4	129	126	1.46	1.14	127	127	100.3
Blood sample 5	212	213	1.19	0.56	212	219	97.0
Blood sample 6	132	133	0.77	0.58	132	132	100.3
Blood sample 7	304	308	2.62	0.86	306	305	100.2
Urine sample 8	201	198	1.57	0.79	200	195	102.3

Table 33: Analytical values for batch 2 repeatability.

QC values for repeatability batch 2							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
QC20 mg/dl	21	21	0.16	1.48	21	20	105.7
QC80 mg/dl	80	79	0.23	0.61	79	80	99.3
QC200 mg/dl	200	200	0.46	0.64	200	200	100.0
QC20 mg/dl post sample	21	21	0.06	1.41	21	20	105.1
QC80 mg/dl post sample	81	80	0.97	1.02	81	80	100.9
QC200 mg/dl post sample	201	200	0.99	0.84	200	200	100.1

Table 34: Analytical QC values for batch 2 repeatability.

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Repeatability batch 3

Repeatability batch 3 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Average concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Urine sample 2	493	475	8.98	1.86	484	484	100.0
Urine sample 3	281	280	0.63	0.22	280	277	98.9
Urine sample 4	125	127	1.23	0.97	126	127	100.9
Blood sample 7	290	292	2.52	0.85	291	305	103.5
Urine sample 8	196	197	0.58	0.30	196	195	99.3

Table 35: Analytical values for batch 3 repeatability. Due to ethanol loss resulting from a larger headspace in the sample vials samples 1, 5 and 6 were no longer suitable for accurate quantitative analysis and so were not analysed in this batch.

QC values for repeatability batch 3							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
QC20 mg/dl	22	21	0.64	2.96	22	20	103.3
QC80 mg/dl	80	79	0.66	0.83	80	80	99.5
QC200 mg/dl	200	199	4.50	2.30	199	200	100.2
QC20 mg/dl post sample	21	21	0.30	1.46	21	20	102.6
QC80 mg/dl post sample	80	79	0.63	0.80	79	80	98.4
QC200 mg/dl post sample	195	201	3.43	1.73	198	200	99.4

Table 36: Sample data including QC quantitation for batch 3 of repeatability. All samples passed and meet the validation criteria on page 52-53

Reproducibility

Reproducibility was tested by having three separate analysts prepare a batch on different days using the same samples, as with repeatability results will not be identical, partly due to expected ethanol loss and partly due to different analysts preparing each batch.

Analyst	Linearity (r^2) column A	Linearity (r^2) column B
1	0.99997	0.99995
2	0.99999	0.99999
3	0.99990	0.99997

Table 37: Linearity results taken from the calibration curves from each of the analyst batches. All curves are in good agreement and exceed the minimal criteria of 0.998.

Concentration data for reproducibility experimentation

Reproducibility batch 1 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	33	33	0.48	1.44	33	37	89.9
Urine sample 2	497	497	1.64	0.33	497	484	102.6
Urine sample 3	284	281	1.61	0.57	283	277	102.0
Urine sample 4	129	126	1.46	1.14	127	127	100.3
Blood sample 5	212	213	1.19	0.56	212	219	97.0
Blood sample 6	132	133	0.77	0.58	132	132	100.3
Blood sample 7	304	308	2.62	0.86	306	305	100.2
Urine sample 8	201	198	1.57	0.79	200	195	102.3

Table 38: Concentration data obtained by analyst 1.

QC values for reproducibility batch 1							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
QC20 mg/dl	21	21	0.16	1.48	21	20	105.7
QC80 mg/dl	80	79	0.23	0.61	79	80	99.3
QC200 mg/dl	200	200	0.46	0.64	200	200	100.0
QC20 mg/dl post sample	21	21	0.06	1.41	21	20	105.1
QC80 mg/dl post sample	81	80	0.97	1.02	81	80	100.9
QC200 mg/dl post sample	201	200	0.99	0.84	200	200	100.1

Table 39: Reproducibility quality control values obtained by analyst 1.

Reproducibility batch 2 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	33	32	0.55	1.71	32	37	87.5
Urine sample 2	484	491	2.36	0.48	488	484	100.8
Urine sample 3	278	284	3.47	1.23	281	277	101.5
Urine sample 4	127	125	1.04	0.83	126	127	99.5
Blood sample 5	213	208	3.04	1.44	210	219	96.1
Blood sample 6	128	131	1.68	1.30	130	132	98.1
Blood sample 7	303	296	4.09	1.36	300	305	98.2
Urine sample 8	195	197	1.40	0.72	196	195	100.3

Table 40: Analyst 2 quantitative values for the second reproducibility analysis, all relative uncertainty values are within the prescribed limits for a successful validation.

QC values for reproducibility batch 2							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
QC20 mg/dl	21	20	0.54	2.60	21	20	104.4
QC80 mg/dl	79	81	0.17	0.81	21	20	104.9
QC200 mg/dl	200	197	0.68	0.85	80	80	99.8
QC20 mg/dl post sample	21	21	1.65	2.08	80	80	99.4
QC80 mg/dl post sample	78	81	1.36	0.69	198	200	99.2
QC200 mg/dl post sample	198	192	2.96	1.52	195	200	97.4

Table 41: Reproducibility concentration data obtained by analyst 2. The post sample QC200 shows a high combined uncertainty and a concentration value close to the lower limit allowed for analysis. This sample was a gross error however and the high degree of uncertainty is accounted for by this error, a typical analysis like this would be re-run if running forensic samples however for validation purposes the error is due to analyst error.

Reproducibility batch 3 samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	Insufficient volume	Insufficient volume	Insufficient volume	Insufficient volume	Insufficient volume	Insufficient volume	Insufficient volume
Urine sample 2	489	489	2.36	0.48	489	484	101.0
Urine sample 3	278	275	3.47	1.23	277	277	99.9
Urine sample 4	125	127	1.04	0.83	126	127	99.0
Blood sample 5	203	200	3.04	1.44	201	219	92.0
Blood sample 6	120	118	1.68	1.30	119	132	90.2
Blood sample 7	293	288	4.09	1.36	291	305	95.3
Urine sample 8	192	196	1.40	0.72	194	195	99.6

Table 42: Reproducibility data obtained by analyst 3. All relative uncertainty values are within the allowed limits according to the validation plan on page 52-53. Differences in ethanol content between batches can be partially attributed to expected ethanol loss.

QC values for reproducibility batch 3							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% difference
20 mg/dl	21	20	0.50	2.42	21	20	104.1
80 mg/dl	80	79	0.34	0.34	21	20	106.0
200 mg/dl	198	198	0.92	1.16	79	80	99.4
20 mg/dl post sample	21	21	0.51	0.65	79	80	98.3
80 mg/dl post sample	79	78	0.69	0.35	198	200	99.0
200 mg/dl post sample	197	198	1.13	0.57	197	200	98.7

Table 43: Quality control values for reproducibility batch 3 demonstrating all values are within the prescribed limits for the validation. QC 20 appears to have a high combined and relative uncertainty however they still meet pass criteria and can be attributed to differences in analyst skill with the method and sampling equipment.

The results of the reproducibility experimentation show that the QC values passed with three separate analysts on three separate days according to the criteria on page 52-53. This demonstrated that the method could accurately quantify QC samples. Blood and urine samples also show a consistent quantification despite some expected ethanol loss between the analyses which is accounted for due to the QC values all achieving a pass.

The effect of storage conditions on sample stability:

The aim of the stability and storage studies was to assess the accuracy of analysis of samples following a potential malfunction with the storage equipment. The study is broken down into several stages, firstly a 72-hour quality control sample stability study to identify if a refrigerator breakdown overnight would have a significant impact on concentration, extending up to 72 hours. A stability study in blood, urine and again quality controls of up to one month. The aim was to determine if poor sample handling samples would lead to an effect on ethanol concentration value by testing at three points, zero hours, two weeks and one month. The final stage of study involved the experimentation on the effects of freezing on urine, blood and quality control samples. The aim of this was to determine if 2 freeze - thaw cycles had an effect on ethanol concentrations observed. These studies also served to determine if there was a difference in ethanol loss observed between three separate matrices. Storage stability samples were 0.5ml aliquoted into 1.5ml Agilent these were then and sealed in separate vials. one vial per sample per time point was used due to sample limitation. Quality control samples are aliquoted as standard and so a fresh aliquot was used per time point. All quality control samples passed in each batch featured within the stability study.

Bench top stability of quality control samples

The bench top stability study was carried out to verify whether quality control samples left at room temperature are stable over a period of 72 hours should they be mishandled or in the event there is a malfunction with the refrigeration unit. A long-term stability test was also carried out for up to one month to verify the results of this experimentation, as shown in Table 46 where the average concentration at zero, 24 and 72 hours are shown. The table shows that all the QC samples still pass at 72 hours demonstrating that the quality control samples are stable at room temperature for at least 72 hours. The extended stability tests will identify how stable quality controls are at room temperature for up to one month.

Stability of QCs up to 72 hours at room temperature quantitative results:

72-hour benchtop QC stability study						
	Zero hours		24 hours		72 hours	
Expected concentration (mg/dl)	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
20	20	20	21	21	22	21
80	79	80	77	79	80	78
200	199	200	198	198	199	197

Table 44: Bench top stability study results for QC ethanol samples for up to 72 hours. 72 hours of room temperature exposure had no effect on ethanol concentration.

Stability of Blood samples

Blood samples were left at room temperature for two weeks and 1 month to observe the effect of room temperature storage on the concentration of ethanol in the samples. A zero-hour control was taken to identify the concentration before stability testing began. Of the samples used, blood sample 1 had a decrease of approximately a 33% decrease (10 mg from 30) between zero hours and one month. Sample 5 and 6 did not exhibit a significant drop in ethanol. The ethanol loss observed in sample 1 could be attributed to the sample being opened multiple times since receiving the sample or in a large headspace for evaporation to occur compared to sample 5 and 6 this could account for the higher ethanol loss observed, however the exact cause of this is unknown. All quality control samples achieved a pass and were within acceptable parameters.

Summary data of stability of blood samples						
Sample	**Zero hours (mg/dl)**		**2weeks (mg/dl)**		**1 Month (mg/dl)**	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
Blood sample 1	33	32	25	25	23	23
Blood sample 5	203	200	201	195	198	198
Blood sample 6	120	118	119	117	116	118

Table 45: A summary of column A and B concentration values obtained in the blood stability study. Only sample 1 exhibited a significant decrease in ethanol concentration was between zero-hours and two-weeks and one month.

Zero hours stability of blood samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	33	32	0.55	1.71	32	37	87.5
Blood sample 5	203	200	3.04	1.44	201	219	92.0
Blood sample 6	120	118	1.68	1.30	119	132	90.2

Table 46: Zero-hour stability quantitation values for blood samples 1, 5 and 6 used in the stability storage study. Values shown are taken from the most recent batch of which they were sampled and analysed.

2-week stability of blood samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	25	25	0.23	1.21	25	33	76
Blood sample 5	201	195	2.85	1.44	198	201	99
Blood sample 6	119	117	1.24	1.05	118	119	99

Table 47: Two-week blood sample stability quantitation again showing a higher concentration decrease in blood sample 1 when compared to that of blood sample 5 and 6 with the quantitation being almost 33% lower after two weeks.

1-month stability of blood samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Blood sample 1	23	23	0.01	0.05	23	33	70
Blood sample 5	198	198	0.21	0.11	198	201	99
Blood sample 6	116	118	1.56	1.33	117	119	98

Table 48: 1-month stability of blood quantitative values, only a slight decrease in ethanol was exhibited between the two-week and 1-month time point suggesting that any differences between the zero hour and two -week values were partially attributable to expected ethanol loss from sample vials being opened. The degree of ethanol loss exhibited from sample 1 was also more like the other samples between the aliquoted samples at two weeks and a month which may suggest that the sample vial was the source of this ethanol loss.

Stability of urine samples and quality control samples:

Urine samples and aliquots of the quality controls were also tested in the same manner as the blood samples were carried out, again as with the blood samples ethanol volumes within the samples remains relatively constant between two weeks and a month, a possible cause for the lack of a notable change could be the headspace of the vials becoming saturated with ethanol meaning no more ethanol can evaporate to fill the headspace above the sample it is not beyond reason to suggest this as a cause of the low ethanol loss experienced in the aliquots. All quality control samples passed and were within acceptable parameters.

Zero hours stability of urine samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Urine sample 3	278	275	3.47	1.23	277	277	99.9
Urine sample 4	125	127	1.04	0.83	126	127	99.0
Urine sample 8	192	196	1.40	0.72	194	195	99.6

Table 49: Zero- hour urine stability values, all %CVs were within the acceptable range verifying the validity of the analysis.

2-week stability of urine and quality control samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Urine sample 3	278	275	1.63	0.59	276	277	100
Urine sample 4	125	124	0.60	0.48	125	126	98
Urine sample 8	196	195	0.78	0.40	196	194	100
QC 20	21	20	0.40	1.93	21	20	103
QC80	79	78	0.39	0.49	78	80	98
QC200	196	198	0.93	0.47	197	200	98

Table 50: Values for the benchtop stability study of urine and QC samples at 2 weeks. All values are averages of duplicate dual column analyses. All retention time values for both internal standard and ethanol were within the set 2% window for identification. All % CV values were within the 2.5% limit verifying that there were no errors with sample preparation.

1-month stability of urine and quality control samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Urine sample 3	281	279	1.21	0.43	280	277	101
Urine sample 4	127	127	0.53	0.42	127	126	100
Urine sample 8	195	197	0.92	0.47	196	194	101
20 mg stability sample	21	21	0.40	1.90	21	20	105
80 mg stability sample	80	80	0.44	0.55	80	80	100
200 mg stability sample	200	198	1.03	0.52	199	200	100

Table 51: Values for the benchtop stability study of urine and QC samples at 1 month. All values are averages of duplicate dual column analyses. All retention time values for both internal standard and ethanol were within the set 2% window for identification. All %CV values are also within the selected 2.5% value.

The results of the storage stability show that blood samples are stable at room temperature for up to one month showing a slight decrease in ethanol which could be attributed to ethanol loss to evaporation, the effect of this loss is relatively small however and is possibly attributed to analytical variance. The QC samples left at room temperature appear to be stable for up to one month with differences in values quantified being attributable to analytical variances and all values attained would still pass should they have been run as QCs in an analysis demonstrating the stability. Urine samples exhibit a slight increase in ethanol at the one-month time point, however similarly to the blood samples it is only a slight increase, and this could also be attributed to analytical variance, it is possible however that this could have been a result of bacterial activity although the increase is not sufficient to attribute the values to this.

Samples of venous blood sourced for freeze-thaw stability study

Samples of venous blood were sourced by a trained phlebotomist. Two volunteers drank a small quantity of alcohol, 1 pint of beer (3.8%) was consumed within one hour, samples were run as unknown analytical samples. These were retained for use in the freeze thaw study, ethical approval for this was not required. All quality controls used for freeze thaw study batches were within the pass range and all batches passed within pre-determined acceptance limits. It was decided that these samples would be used instead of the B samples from previous work due to the fact that the B samples provided which were several months old. This sampling followed by rapid freeze is a better representation of a scenario in which sampled blood for BAC analysis may be frozen inadvertently before analysis could take place. All quality control samples passed and were within acceptable parameters

Venous blood-quantitation sourced in house for freeze-thaw analysis					
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)
Venous Blood 1	21	21	0.22	1.05	21
Venous Blood 2	37	37	0.21	0.56	37

Table 52: zero- hour quantitation of venous blood before a freeze-thaw cycle.

Freeze-thaw stability study

Data obtained from a two-cycle freeze thaw stability experiment demonstrated that the freeze-thaw process did not contribute to a significant reduction in ethanol concentrations in blood, urine and in the QC samples. This suggests that samples were not significantly adversely affected by the freeze-thaw cycle. Table 53 and 54 show the quantitative data obtained in the analyses. Cycle 1 and 2 concentrations were within 2% of each other which was within the normal range for precision under normal analytical conditions. Any deviations between samples were potential analytical variance and not something symptomatic of a freeze – thaw cycle. Both sample cycles were removed and thawed at the same time cycle 1 samples were thawed and then allowed to come to room temperature before being sampled. Cycle 2 samples were allowed to thaw and come to room temperature but were then refrozen and thawed the next day for sampling in the same way.

Freeze thaw blood and QC samples

Freeze-thaw stability of blood and QC samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
VB 1 cycle 1	17	17	0.11	0.65	17	21	81
VB 1 cycle 2	20	20	0.31	1.56	20	21	95
VB 2 cycle 1	33	33	0.12	0.36	33	37	89
VB 2 cycle 2	31	32	0.23	0.73	32	37	86
20 mg cycle 1	20	20	0.33	1.64	20	20	100
20 mg cycle 2	20	20	0.26	1.29	20	20	100
80 mg cycle 1	80	81	0.55	0.69	80	80	100
80 mg cycle 2	80	78	0.98	1.25	79	80	100
200 mg cycle 1	199	197	1.48	0.74	198	200	99
200 mg cycle 2	195	198	1.44	0.73	197	200	99

Table 53: Freeze thaw stability values for both blood and quality control samples. Each sample had a separate aliquot for each freeze-thaw cycle. All retention time values for both internal standard and ethanol were within the set 2% window for identification. All %CV values are also within the selected 2.5% value. Both cycles were run in one batch, cycle 2 samples were taken out and thawed to room temperature the day before the run to enable two freeze thaw cycles to occur before analysis.

Freeze-thaw stability of urine samples							
Sample	Replicate 1 concentration	Replicate 2 concentration	Standard deviation	%CV (Relative Standard deviation)	Concentration (mg/dl)	Target value (mg/dl)	% Accuracy
Urine 3 cycle 1	281	281	0.54	0.19	281	277	101
Urine 3 cycle 2	276	279	1.43	0.52	278	277	100
Urine 4 cycle 1	128	1271	0.36	0.28	128	126	102
Urine 4 cycle 2	124	126	1.11	0.89	125	126	99
Urine 8 cycle 1	196	198	0.88	0.45	197	194	102
Urine 8 cycle 2	198	193	2.21	1.13	196	194	101

Table 54: Freeze thaw stability values for urine samples showing both cycle 1 and 2 for comparison of ethanol concentrations. Although there were differences between the runs it is not a large enough difference to infer any major effect of freeze-thaw on ethanol concentrations, this difference could be attributed to sample preparation or analytical variances which occur regardless of the analyst skill.

Discussion

It was determined that a reliable method was required to accurately quantify the content of alcohol (ethanol) in both blood and urine samples, and to provide separation of the key components found within a sample. As this method utilises two columns and detectors the standard was set that at least one column must show a full separation of ethanol and any potential interfering substances such as acetone. The same requirement was set for the internal standard tertiary-butanol, which was selected partially as it elutes in between propan-1-ol and propan-2-ol making it the most suitable for the purpose of this method. It provides a good balance between separation of analytes and run time. Tertiary butanol was also selected due to the fact it is suitable for post -mortem analysis given it is not found as a result of post- mortem bodily changes (Corry 1977). Development testing on method conditions began upon installation of the selected columns, the Restek BAC plus 1 and the BAC plus 2. These columns were selected for their ability to resolve organic solvents such as alcohols (Restek, 2018). The method was expected to be able to detect and quantify ethanol at a variety of concentrations ranging from 10 mg/dl up to 400mg/dl. The limit of detection was 5 mg/dl. In order to achieve these goals, several parameters were optimised and tested.

Tables 2 and 3 show the selected optimum parameters, the headspace conditioning time, the GC oven temperature, run time and the pressure. Compared to methods developed by other researchers (Chun *et al.*2016*,* Tiscione *et al.*2011*)* this method uses a much shorter conditioning and run time. The current conditioning time is 5-minutes with a run time of 4 minutes. By contrast, Tiscione *et al*'s method utilised an Agilent D1888 headspace sampler with a GC utilising a Dean's switch FID and DB-ALC1 type column which used a 20-minute conditioning and 13.5-minute cycle time. The current method developed is 4 times shorter which enables savings in both sample turnover and cost of materials such as gases. The method developed also ran an isothermal oven temperature program which reduces the run time as the heating and cooling cycle is eliminated. This allows for quicker injection of the next sample after the previous sample has been completed. This was especially valuable when large volumes of samples were being analysed. 40^{o}C is also a moderately low operating temperature and this helps to preserve the column lifetime. Tiscione *et al.* also utilised a program of consecutive conditioning so that the next sample is being conditioned while the GC analysis takes place for the sample ahead of it. The rate limiting step then becomes GC run time. Using a ramped temperature program requires a longer time frame for the oven to heat and cool thus adding to the GC run time thus adding to the GC run time. The method described in this project also utilised a conditioning time lag of approximately two minutes rather than the full 5 minutes.

The R^2 value for linearity demonstrated by Tiscione *et al'*s method was 1.000 whereas the average attained in the validation process of this method was 0.9999, both methods far exceed the 0.998 requirement*.* The method developed by Chun *et al.* is still sufficient for the analytical standards with an average linearity of 0.999, the linearity of Chun *et al*'s method was also tested in two matrices, deionised water and brain tissue. Sample preparation of brain tissue was carried out by diluting human brain tissue 1:4 in deionised water and homogenised, this was then used in system suitability testing by adding the same solution used to the homogenate (Chun *et al.*, 2016).

Separation and system suitability are also comparable between the two other studies and the method described in this project, Tiscione *et al.* demonstrated with their method that there was a separation of ethanol from the surrounding compounds, in this case the elution order was different due to different column types being used and was lacking analytes such as acetaldehyde and tertiary butanol. It was highlighted however from the chromatograms that on this analysis acetone and n-propanol

eluted closely with insufficient baseline separation in a similar manner to ethanol and acetone featured in this method's development stage (table, 11) which demonstrated the difficulty of analysing ethanol in samples with acetone present. The second detector used in Tiscione *et al*'s method was a mass spectrometer which provided a similar retention time and chromatography. The mass spectra provided fragmentation patterns of each analyte tested. This method also utilised n-propanol as the internal standard suggesting an analysis of ethanol with the presence of acetone will be difficult due to possible co-elution of ethanol and the n-propanol internal standard which may interfere with the quantitation of ethanol. This contrasted with the results of this project, which relied on separation of acetone and ethanol from a second column with differing chemistry with differential polarities and two identical detectors.

A study by Schlatter *et al.* utilising a Thermo Finnigan model GC FID system coupled with Uptibond 1 premium type column had a total run time of 20 minutes. This run time achieved clear baseline separation of ethanol and acetone, and tertiary butanol however, acetaldehyde and methanol still elute closely but to a lesser extent than in this method, although as stated these are typically not analysed in road traffic blood alcohol concentration analysis and so full baseline separation is not vital (Schlatter, 2014). This method was derived from another method (Pontes *et al.*2009) which utilised the same Thermo Finnigan type GC FID but with a CPWAX 57CB type column and tested the separation of analytes in different matrices including blood, vitreous humour, urine and cell culture media. This method obtained a different elution order and separation pattern to the previous methods described. Pontes *et al*'s method demonstrated acetone eluted before ethanol and had full baseline separation (Pontes *et al.* 2009), the separation mixture also contained acetonitrile which was not included in the system suitability solution tested in the method developed in this project. The method does however include tertiary butanol and propan-2-ol which are not included within Pontes *et al*'s method. This means that although acetone has a clearer separation it is difficult to fully compare the separation profiles of the two methods due to different analytes being included in the experimentations.

Lewis *et al.* utilised the same BAC plus 2 type column used in this validation plan and achieved a similar co-elution of ethanol and acetone. This suggested that a limitation of the column is an inability to separate acetone and ethanol using analytically viable parameters despite the column being advertised as being able to clearly separate the two compounds. This also demonstrates that it is not a method limitation and is more likely attributed to the column than the parameters selected. It is for this reason that optimum conditions were selected, and it was decided that in the event of an acetone being present in a case sample the second column (the BAC plus 1) will be used for quantification by running two sets of duplicates,(Lewis *et al*, 2004). Lewis *et al.* used a flow rate of 20 ml/min whereas this method used a flow rate of almost 10 times lower at 2.78 ml/min and despite this the separation of acetone did not improve markedly. This suggests that there is a proportional effect of pressure on acetone and ethanol rather than a differential effect. Chun *et al.* also used a BAC plus 2 column in their method and used a flow rate of 16.8ml/min yet still achieved the same level of separation of acetone and ethanol showing an almost identical chromatogram of a system suitability solution to the one featured in figure 17, given that three separate and independent methods all exhibit the same limitation it is unlikely due to specific limitations of this method.

The current method was developed and validated according to the pre-determined validation plan which is in line with UKAS guidelines for best practice in ISO17025 accreditation. The method was successfully validated, and the second objective of the experimentation which was stability studies

was then addressed. The outcome of these studies showed that blood, urine and quality control samples are all stable at room temperature for at least one month. Although they were all stable for up to one month, it is still advisable that samples are not kept at room temperature for longer than is required for the samples to reach room temperature and the analytical samples to be prepared for analysis. Further, it was determined that urine and QC samples that had been deliberately frozen still provided accurate quantitative results although again it is not advised that samples are deliberately frozen. These studies provided a perspective of samples that were mishandled to identify how samples may behave should there be a storage malfunction. Of blood samples tested, three out of the four samples tested exhibited a decrease of approximately 5 mg/dl which could possibly mean that blood samples are less stable when frozen regarding blood alcohol concentrations, the difference between cycle 1 and cycle 2 freeze-thaw was less pronounced and so the results are inconclusive. A loss of ethanol could not have occurred between the sampling of the zero-hour batch and the preparation of freeze-thaw aliquots due to these being taken at the same time and further investigation would need to be carried out in order to identify the exact reason for this result.

A potential limitation of this method which is currently unexplored is the suitability of this method to analyse post-mortem samples as well as other biological matrices such as vitreous humour. Post-mortem samples are more difficult to analyse and a study by Sylvester *et al.* demonstrated this showing different alcohol concentrations depending on where the blood was sampled from on the body, as well as a difference between blood and vitreous humour (Sylvester *et al.,* 1998). Further investigation into this would be required to determine the method suitability for post-mortem analysis with regards to acetone formation and post-mortem samples exhibiting acetone. It is also suggested that ethanol is produced as a product of putrefaction which may artificially raise the alcohol content of a sample fluid, in addition substances such as acetaldehyde an n-propanol may also be produced post-mortem (Corry, 1977). The method has been validated to accurately quantify samples of ethanol however and so the method should be suitable to test post-mortem samples accurately and the limiting factor of these samples will be the uncertainty of sampling techniques rather than a limitation of the method. It is also shown that acetone can be separated on one column and so the method is anticipated to be accurate enough to analyse post-mortem samples.

Potential future expansions and improvements which could be made to this method include the possibility of changing the carrier gas to nitrogen which would reduce the running cost of the method as nitrogen is a cheaper gas to source compared to helium. The method is currently operating a moderately low linear velocity which may be suitable for the use of nitrogen as a carrier gas, this could also be provided using a nitrogen generator further reducing running costs of the method. In addition to this, a different secondary column could be utilised such as the CPWAX 57CB which was shown to resolve ethanol and acetone more effectively than the current BAC 2 type column installed. This would solve the current limitation of acetone and ethanol co-eluting on one column enabling a reduction in sample volume required for analysis of samples containing acetone. These improvements would require the method to be re-validated however and so the assessment of the benefits compared to the time required to re-validate the method would be required before any changes were made to the current method. Further experimentation that could be performed using this method include using smaller sampling sizes of 10, 5 and 1 µl of sample and accordingly smaller internal standard samples at 100, 50 and 10 µl respectively to investigate the viability of this method using reduced sample volume and IS solution. This method is also expected to undergo inter-laboratory PT testing in 2019 which was not possible to date.

To conclude, the method developed and validated in this thesis demonstrated a similar performance to other validated blood alcohol GC-FID methods including methods utilising the same column types. These studies also provided evidence that the BAC 2 column shared the same limitations and verified that the optimum parameters selected were valid and that it would have proven to be unnecessary and an inappropriate usage of laboratory time attempting to achieve full separation on this column. This study also highlighted that the method linearity is well within industry standards and this also demonstrates that the degree of accuracy of calibration range is above what is acceptable for forensic analysis of blood and urine alcohol thus fulfilling the stated method purpose and objectives. The initial blood and urine alcohol values attained by this method show similar values to the values provided by the external accredited laboratory, demonstrating that blood and urine samples can be accurately quantified by the method. This method also meets the requirement set out by the UKAS code of conduct that a method should demonstrate compatibility of results obtained compared to other analyst results using different equipment/methods. These UKAS guidelines for the validation of measurement-based methods state that the validation plan should ensure that parameters are tested a) using a competent analyst with experience in the field of work of the study to be able to make decisions based on the observations made as the study progresses; and b) using equipment that is within specification, calibrated and functioning correctly (Renninson, 2011). Both are satisfactorily met by the validation plan with repeatability and reproducibility demonstrating that both the analyst undertaking the validation is competent and that the equipment is to standard when used by different analysts.

The guidelines also outline the functional and performance characteristics which a method should exhibit which include the competence of the analyst, which has been demonstrated In addition to a number of other considerations such as; environmental constraints, sample size and handling, sample homogeneity, the ability of the sampling process to provide a representative sample of the exhibit, the efficiency of recovery of the analyte during sample preparation for analysis, the ability to detect the presence or absence of analyte in a sample, minimum quantity of the analyte that can be reliably detected (LOD) and accurately quantified (LOQ), the identification/measurement of the analyte relates to the analyte alone and is not influenced by an interfering substance or compromised by a matrix, the results are reliable, consistent and include an uncertainty measurement, compatibility of results obtained by other analysts using different equipment and methods and the limitations of applicability (Renninson, 2011). These guidelines were met and addressed in either the sample preparation stage with sample size, homogeneity and handling being addressed in the sample preparation stage and the recovery of analytes during sample preparation being demonstrated with consistent accurate QC values which also shows that the method is both accurate and precise. QC charts plotted for all QC concentrations demonstrate that none of the representative values fall outside of the control limits. QC results as well as the %CV values were also within acceptable limits according to the validation plan which further demonstrated the accuracy and precision of the method. An uncertainty measurement was also included in all results attained in accordance with the guidelines on best practice with the relative uncertainty being selected for use in unknown samples and both relative and expanded uncertainties being used for QC samples which have certificates of analysis allowing for the expanded uncertainty to be calculated.

The limits of detection and quantitation were tested and shown in accordance with the codes of conduct and acetone, the major interfering substance encountered in the development and validation process was separated from ethanol on one column as required by the method objective and the code

of practice. All validation parameters achieved a pass thus the objective of validating a method capable of accurately quantifying and detecting ethanol in both blood and urine was successfully achieved. Stability of samples was also investigated, and matrix stability studies verified the stability of samples for analysis whilst also highlighting that samples may be stable, but it is imperative that samples are stored correctly to avoid the risk of samples being exposed to unfavourable conditions which may lead to inaccuracies in the analysis. The method that has been developed can accurately detect ethanol in a range of 5mg/dl-400mg/dl and this method will be used to provide casework analytical services in UK Road Traffic Act cases.

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