Blood alcohol:

Forensic analysis by GC-MS and investigation of some pre-analytical factors that may influence the result

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by

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Declaration

I, **Frances Sewell**, declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature

30 November 2017

Date

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Abstract

Forensic laboratories are frequently called upon to determine blood alcohol concentrations, especially as a result of roadside testing. The reliability of the experimentally determined concentrations is being called into question due to the need to transport and store blood specimens – sometimes for months – before analysis can occur. Of specific concern are the length of time the specimens are stored and the temperature at which they are kept. Recently, the possible presence of certain micro-organisms has also been used as a defence as to why a blood alcohol concentration could be found to be elevated above the South African legal limit of 0.05 g/100 mL. Micro-organisms, such as Candida albicans, are known to ferment glucose to ethanol, and thus could potentially artificially increase the ethanol concentrations in contaminated blood specimens. In an effort to prevent this, blood specimens in South Africa are required to be stored with 1 g/100 mL sodium fluoride, and the laboratory processing the sample is required to show that this is the case.

In this study, a novel Isotope Dilution Gas Chromatography-Mass Spectrometry method for the determination of ethanol concentration in human blood was developed. Ethanol-d6 was used as internal standard, the analytes derivatized with pentafluorobenzoyl chloride, and the resulting esters detected in single ion monitoring mode. The method was validated based on figures of merit and the expanded measurement uncertainty was determined.

Following this, a method for the detection and quantitation of free fluoride in blood by means of fluoride ion selective electrode was developed. It was found that diluting specimens 20 times with Total Ionic Strength Adjustment Buffer II and deionized water sufficiently minimised matrix effects to allow aqueous calibration from 0.25 g/100 mL sodium fluoride to 3.00 g/100 mL sodium fluoride. The method was validated based on figures of merit and the expanded measurement uncertainty was determined. Using this method, the complexation effects of Fe^{3+}, Mg^{2+} and Ca^{2+} ions were investigated, and the effect of temperature on complexation of fluoride with Fe^{3+} was explored.

Throughout the study, the fluoride concentrations of specimens were ascertained utilising the in-house developed fluoride ion selective electrode method.

Blood specimens spiked at 0.02 g/100 mL, 0.05 g/100 mL and 0.3 g/100 mL ethanol were placed in evacuated tubes containing fluoride – at least 100 mg – as preservative, and stored at room temperature (12 ± 6 °C) and under refrigeration (4 ± 3 °C) for 29 weeks. Using the novel Gas Chromatography – Mass Spectrometry method, the ethanol concentration was monitored on a weekly basis to investigate the stability thereof, as well as its dependence on temperature. The expanded measurement uncertainty was used to gauge the significance of any changes observed.

The ethanol concentration of those specimens stored under refrigeration showed no significant deviation from the initial spiked concentration value for the 29 weeks; however, a decreasing trend was observed from week 25 for all three levels.

The specimens stored at room temperature exhibited a significant decrease in ethanol concentrations. This was particularly evident in the specimens initially spiked at 0.02 g/100 mL. At all three levels, the ethanol concentration was statistically stable for nine weeks. Upon termination of the study, the concentrations of the medium and higher levels showed a continuing decreasing trend, while the lower level was completely depleted.

Fresh blood specimens were then prepared at 0.02 g/100 mL and 0.05 g/100 mL ethanol, divided into evacuated tubes with and without fluoride, and inoculated at five levels of *Candida albicans*. These were then stored at room temperature and under refrigeration as before and the ethanol concentration as well as the *Candida albicans* cell count were monitored weekly for up to nine weeks.

Specimens stored under refrigeration exhibited statistically stable ethanol concentrations, with no significant deviation from the initial spiked values, while those stored at room temperature in the absence of fluoride showed a marked decrease in ethanol concentration. It was noted that the temperature at which specimens are stored has a greater impact on the ethanol concentration stability than the presence or absence of fluoride, although specimens containing fluoride exhibited greater stability than those that did not.

This study showed that the ethanol concentration of stored blood specimens does not increase over time as is currently being claimed, but rather decreases. This is to the advantage of the defendant. It is nevertheless recommended that all blood specimens for the determination of blood alcohol concentrations should be stored below 6 °C in the presence of at least 1 g/100 mL fluoride.

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Abbreviations

Full Form	Abbreviation
Alternating Current	ac
Analysis of Variance	ANOVA
AdenosineTriphosphate	ATP
Blood Alcohol Content	BAC
Centers for Disease Control and Prevention	CDC
Cumulative Distribution Function	CDF
1,2-cyclohexandiaminetetraacetic acid	CDTA
Chemical Ionisation	CI
Confidence Interval	CI
Certified Reference Material	CRM
Direct Current	dc
Decade	dec
Derivatised Ethanol	dEtOH
Derivatised Ethanol-d6	dEtOH-d6
Deionised Water	dH_2O
Electron Capture Detector	ECD
Electron Impact	EI
External Quality Control	EQC
Flame Ionisation Detector	FID
Fluoride Ion Selective Electrode	FISE
Refrigeration	Fr
Gas Chromatograph	GC
Gas Chromatography – Flame Ionisation Detection	GC-FID
Gas Chromatography – Mass Spectrometry	GC-MS
Headspace Gas Chromatography – Flame Ionisation Detection	HS-GC-FID
Internal Quality Control	IQC
International Organization for Standardization	ISO
Potassium Oxalate	KOx

Kolmogorov-Smirnov Test	KST
Limit of Detection	LOD
Limit of Quantitation	LOQ
Mass Spectrometer	MS
Measurement Uncertainty	MU
Nicotinamide Adenine Dinucleotide (oxidised)	NAD^+
Nicotinamide Adenine Dinucleotide (reduced)	NADH
National Metrology Institute of South Africa	NMISA
Phosphate Buffered Saline	PBS
2,3,4,5,6-Pentafluorobenzoyl Chloride	PFBCl
2,3,4,5,6-Pentafluorobenzoic Acid	PFB-COOH
Proficiency Testing	PT
Polytetrafluoroethylene	PTFE
Oxidation-Reduction	Red-Ox
Revolutions per Minute	rpm
Relative Standard Uncertainty	RSD
Room Temperature	RT
Signal-to-Noise	S/N
Sum of Squares due to Error	SSE
Sum of Squares due to Factor	SSF
Tricarboxylic Acid Cycle	TCA
Thermal Conductivity Detector	TCD
Total Ion Chromatogram	TIC
Total Ionic Strength Adjustment Buffer	TISAB
World Health Organization	WHO
Extracted Ion Chromatogram	XIC

Chapter 1

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1.1 Introduction

Blood alcohol analysis is one of the most called-for tests performed by forensic laboratories in South Africa. In fact, due to the sheer number of specimens that require testing, specimens are often stored for a period of time before being analysed. This has resulted in the integrity of specimens being called into question, specifically with claims that the blood alcohol concentration may not be stable from sampling to analysis. This could be due to microbial contamination or nonoptimal storage conditions. Since unfair prosecution could result, it is of paramount importance that the alcohol concentration remains stable, with no statistical increases or decreases.

1.2 Project Statement and Project Aims

In this study, an Isotope Dilution Gas Chromatography - Mass Spectrometry (GC-MS) method previously developed by the Forensic Toxicology Laboratory at the University of Pretoria will be adjusted and validated, and then compared with a currently employed Gas Chromatography – Flame Ionisation Detection (GC-FID) method. Following this, the GC-MS method will be used to investigate the effect of the storage conditions, temperature and time, as well as the effect of the presence of the micro-organism *Candida albicans*, on the stability of a specimen's ethanol concentration.

A Fluoride Ion Selective Electrode method to determine the NaF concentration in blood specimens will be developed and validated. This method will be employed to ensure that the fluoride concentrations of all specimens are above 1 g/100 mL throughout all investigations.

Throughout the study, specific emphasis will be placed on the **statistical significance** of all results obtained. Estimates for the measurement uncertainty of both the GC-MS method and the FISE method will be determined, and these MU values will be used to assess the significance of any changes in concentrations observed.

Finally, recommendations as to appropriate specimen storage conditions will be made, based on the results of the various storage studies carried out.

1.3 Blood Alcohol Concentration

According to the World Health Organization (WHO), approximately 3.3 million deaths are related to the misuse of alcohol worldwide each year.[1] South Africa, with an alcohol consumption rate of around 11 litres per capita, has the highest consumption rate in Africa, and the 29th highest in the world.[2]

Ethyl alcohol, or ethanol, is the alcohol present in the highest percentage in alcoholic beverages. For this reason, it is common to refer simply to the "alcohol content" of the beverage when meaning the ethanol content. After consumption, alcohol passes from the intestine into the bloodstream and is subsequently carried to the brain. Hence, the amount of ethanol in the bloodstream, or Blood Alcohol

Concentration (BAC), gives a good indication of the amount of alcohol consumed, and it is this concentration that is determined for forensic purposes, either in connection with road incidents or during post-mortem analyses. [3, 4]

Alcoholic intoxication can be categorised into various stages based on the amount of alcohol consumed and the signs and symptoms displayed. These categories include “subclinical”, wherein the person will display little or no behavioural differences and have a blood alcohol content of 0.01 to 0.05 g/100mL; “euphoric”, with a blood alcohol content of 0.03 to 0.12 g/100 mL, where the person will show increased confidence and some sensory-motor impairment; “confusion”, where the person will likely be disorientated and uncoordinated, as well as apathetic and lethargic, and have a blood alcohol content of 0.18 to 0.3 g/100 mL; and “coma”, where the person’s blood alcohol content will be 0.35 to 0.5 g/100 mL, and they will likely be completely unconscious.[3]

Forensic laboratories are often called upon to determine BAC levels in connection with criminal cases or as a result of roadside testing. The person whose blood is to be tested is transported to the nearest healthcare facility and a sample of blood is taken according to specified protocols. This blood sample is then stored under certain conditions before being transported to the forensic laboratory for analysis.[5]

In an ideal situation, specimens would be transferred directly from the healthcare facility to the forensic laboratory, and analysed immediately. However, this is rarely the case. Testing laboratories, especially in South Africa, are so inundated with specimens for analysis that severe backlogs result. Thus, blood specimens are commonly stored for a period of time before being analysed.

1.4 Ethanol Stability

In recent years the reliability of blood ethanol results has been called into question. Specific concerns are being raised about the stability of ethanol concentrations in stored blood specimens, especially when a result reflects a BAC of above the legal limit. [6] Should the ethanol concentration decrease it would only be to the benefit of the defendant, while if it were to increase they might be prosecuted unfairly. It is thus imperative for a reliable and accurate BAC result that the ethanol concentration remain unchanged from sampling to analysis – that is, with no *significant* increases or decreases.[3]

1.4.1. Ethanol Loss

Ethanol is a volatile organic compound and, as was shown by Brown et al.[7], has the potential to be lost from biological specimens by *evaporation*.[7] In order for evaporation to occur, the specimen container seal would have to be faulty, not properly in place or absent. Another possibility is that the ethanol could enter the headspace of the storage container and be lost upon opening. Clearly, storage conditions would play an important role since lower temperatures would reduce the probability of evaporation.

Another mechanism for the loss of ethanol is by *oxidation* to acetaldehyde. This was described by Smalldon and Brown[8] as an oxyhemoglobin-mediated oxidation which makes use of the oxygen in the air in contact with the blood as well as the oxygen in the blood itself.[8] It was found that the losses in ethanol due to oxidation were independent of ethanol concentration while highly dependent on specimen storage temperature. When specimens were refrigerated this loss was minimised or prevented altogether. Additionally, the presence of fluoride in the specimens did not prevent the loss of ethanol caused by ethanol oxidation to acetaldehyde.[8]

Finally, a decrease in ethanol could also be attributed to *microbial metabolism*. It is well known that certain micro-organisms, such as *Serratia marcescens* and *Pseudomonas* sp., are capable of using ethanol as a substrate for growth.[7, 9-11] Upon investigation, Harper and Corry[9] showed that unpreserved specimens containing large numbers of micro-organisms displayed rapid decreases in ethanol concentration.[9] The authors also observed that ethanol loss was greater when the headspace of the specimen container was larger. This suggested that aerobic, as opposed to anaerobic metabolism of ethanol was taking place. Sodium fluoride has been shown to be an effective preservative for preventing ethanol loss due to microbial action.[7, 10, 12, 13] Considering that blood specimens are collected in an aseptic manner, and stored with fluoride, the loss of ethanol by microbial metabolism does not seem the most likely mechanism of the three.

1.4.2. Ethanol Gain

An increase in ethanol can only be attributed to specimen contamination. This can either be by physical contamination or contamination with some micro-organism capable of producing ethanol.

For ante-mortem specimens, the only documented source of *physical contamination* is the use of ethanol-containing swabs to clean the draw site.[14-16] The additional ethanol then enters the blood being collected upon puncture and is drawn with the blood into the specimen collection tube. Although a significant increase in ethanol concentration due to this small amount of cleaning ethanol seems improbable, it is recommended that non-ethanol containing swabs be used. Indeed, in South Africa, according to the National Health Laboratory Service, as well as WHO guidelines on drawing blood, alcohol swabs, usually isopropyl alcohol, should be used for cleaning the draw site.[17, 18] A seemingly more likely form of contamination that would result in an increase in ethanol is contamination with some micro-organism. In this case, *microbial metabolism* would result in the formation of ethanol. There are many micro-organisms that have been shown to be able to produce small amounts of ethanol.[19] However, for significant amounts to be produced, conditions must be favourable. The micro-organism must be present in sufficient quantity, the temperature and oxygen conditions must be suitable, and adequate and correct substrate should be available for growth.[20] While the conditions in a specimen collection tube may be conducive to microbial growth in terms of

temperature and substrate, it is unlikely that sufficiently large quantities of an organism capable of producing ethanol would be present in a specimen.

1.5 Ethanolic Fermentation

In order for a micro-organism to survive, grow and replicate it must perform various processes collectively known as metabolism.[21] Metabolic reactions can be categorised as either catabolic or anabolic. Anabolism is the process whereby energy is used to build larger molecules from smaller ones, whereas in catabolism large molecules are broken down releasing energy.[21, 22] Catabolism produces the energy required for anabolism.

The first step in carbohydrate catabolism is glycolysis, a process whereby glucose is broken down, resulting in two pyruvate molecules, two nicotinamide adenine dinucleotide molecules in their reduced form (NADH), and four adenosine triphosphate (ATP) molecules, the latter being the primary energy source in cells.[21, 23] The glycolytic pathway producing pyruvate, also known as the Embden-Meyer pathway, is involved, with many steps, each of which is catalysed by a reaction-specific enzyme.[24, 25]

Upon completion of glycolysis, the presence or absence of oxygen, or some other electron acceptor, will determine the subsequent pathway the catabolism follows. An electron acceptor will result in respiration, while conditions where no electron acceptor is available will cause fermentation.[21] Respiration can thus be either aerobic, where oxygen is the electron acceptor, or anaerobic, where some other ion, such as nitrate or sulphate acts as electron acceptor. Fermentation is only anaerobic.[21]

The respiration pathway will result in the transfer of electrons from the NADH molecules to the electron acceptor, initiating what is known as the Kreb's Cycle, or Tricarboxylic Acid Cycle (TCA).[26] The pyruvate is oxidised forming carbon dioxide (CO_2) and water and a high yield of ATP is produced.

In the fermentation pathway, the electrons from the NADH molecules are returned to the pyruvate molecules, and NAD^+ is reformed. In anaerobic glycolysis, such as would occur in contracting muscle or due to lactic acid bacteria, the pyruvate is reduced to lactate, while ethanolic fermentation, such as that due to yeasts, results in the formation of ethanol and CO_2. While there are many other possible fermentation products depending on the organism present, the net result is always NADH reoxidised to NAD^+ and the production of fermentation products.[21, 25, 26]

During glucose fermentation only a small amount of ATP is produced, while many waste products are formed. Hence, respiration is the preferred pathway. However, in many environments only fermentation is possible.[21]

Figure 1 below shows the catabolism of glucose, and includes both respiration and fermentation. It can be seen that for every one glucose molecule two ethanol molecules are formed upon ethanolic fermentation.

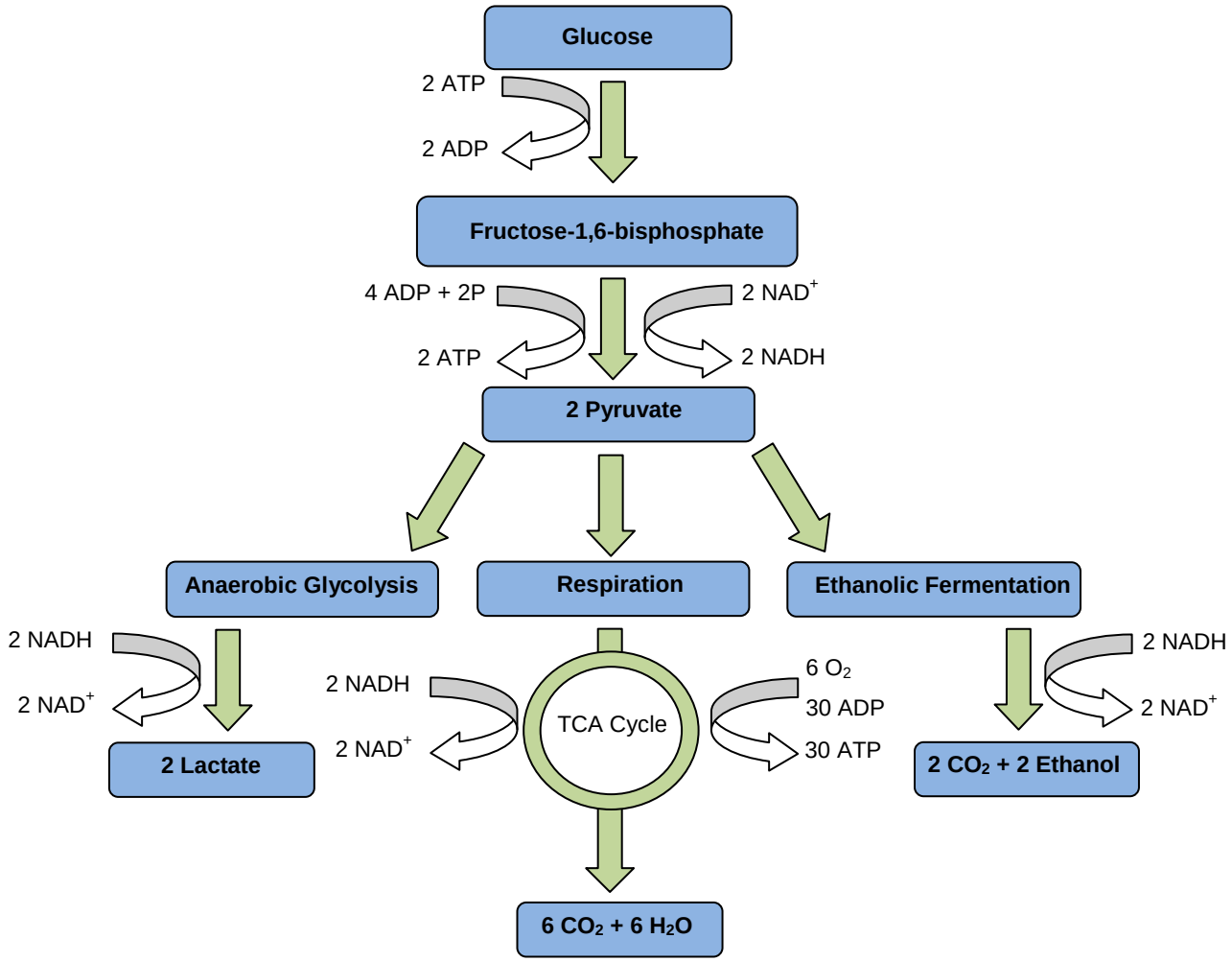

Figure 1: Catabolism beginning with glycolysis followed by the respiration and fermentation pathways[25]

There are various organisms that can ferment glucose to ethanol, all of which have the potential to colonise a blood specimen and affect the ethanol concentration.[27] It should be noted, however, that in healthy mammals, internal tissues such as the blood, muscles and brain are generally free of micro-organisms. It is the surface tissues that are continually in contact with the environment that have the greatest likelihood of being colonised.[28] Hence a micro-organism already being present in the blood specimen when it is drawn seems unlikely. Instead, environmental contamination seems more plausible.

Candida albicans is an opportunistic fungal pathogen that forms part of the human digestive tract microflora, but also colonises the rectum and vagina.[29] *Candida albicans* contamination of a specimen is often cited in court as a possible reason for an elevated BAC result as it is able to ferment glucose to ethanol.[30] Other skin colonisers that are capable of ethanolic fermentation include *Staphylococcus aureus* and *Staphylococcus epidermidis*, which, according to the Centers for Disease

Control and Prevention (CDC), are the most common bacterial contaminants of blood specimens, and are thought to enter the specimen through the needle once the skin is pierced.[31]

Other possible candidates include *Candida parapsilosis*, *Proteus mirabilis*, and *Escherichia coli*, none of which are part of the normal skin flora, but have been shown to form ethanol by glucose fermentation.[20, 32, 33] *Candida parapsilosis*, a yeast, may colonise on fingers and in body folds[34], *P. mirabilis*, a bacteria, has been known to colonise the skin of patients and healthcare workers in hospitals[35], and *E. coli*, also a bacteria, is naturally found in the intestinal tract but has been shown to colonise skin.[36]

Various studies have shown that microbial contamination can cause ethanol concentration to increase in blood specimens after sampling. One study performed by Yajima et al.[37] showed that the presence of *C. albicans* caused an increase in ethanol concentration, provided glucose was added, and that the ethanol production was proportional to the glucose concentration. It should be noted that no ethanol increase was observed in specimens that did not have added glucose.[37] This confirmed that *C. albicans* ferments the glucose to ethanol. Another study carried out by Blume et al.[38] indicated that various micro-organisms, including *C. albicans* and *E. coli*, were capable of producing ethanol in blood specimens. [38] The authors subsequently stressed the importance of proper specimen storage, demonstrating that the addition of a preservative inhibited the ethanol production for many of the micro-organisms in question. Chang et al.[39] provided further evidence that temperature plays an important role in ethanol concentration stability, showing it to be more stable at lower temperatures despite the presence of *C. albicans*.[39]

1.6 Specimen Preservation

With the high likelihood of blood specimens being stored for a period of time before analysis, specimen preservation becomes very important. It is essential that the integrity of the specimen be maintained for as long as possible after sampling.

For this reason, blood specimens for the analysis of ethanol concentration are usually collected into specimen vials containing a preservative as well as an anticoagulant. While there are many preservative-containing collection tubes on the market, the most commonly used are those containing a combination of sodium fluoride (NaF) and potassium oxalate (KOx). The NaF acts as a preservative by interrupting the glycolytic pathway in the fermentation mechanism[40], while the KOx prevents clotting by trapping the free calcium in the blood.[41]

In South Africa, the concentration of sodium fluoride in the stored blood specimens is required to be above 1 g/100 mL, or 1% (w/v), and the forensic laboratory processing the blood samples is required to show that sufficient sodium fluoride is present.[42] While there is no legally required KOx concentration, specimen collection tubes are generally prepared in such a way that the NaF and the

KOx are present in a 5:4 ratio, and any clotting observed in the specimen should be noted by the laboratory.[43]

The stability of ethanol concentrations in biological specimens using NaF as a preservative is well documented, dating back many years. Winek and Paul[12] showed that the ethanol concentration of specimens preserved with at least 1 g/100 mL NaF were stable for up to 14 days.[12] Chang and Kollman[39] showed that no ethanol was formed in specimens containing 1 g/100 mL NaF, while some ethanol was detected in specimens not preserved with NaF. These specimens were subjected to temperatures of 22 °C and 37 °C for up to 6 months.[39] In a more extensive study performed by Yajima *et al*.[37] where both ethanol and glucose levels were monitored, it was shown that when NaF was added at concentrations of 1 to 2 g/100 mL, no ethanol was produced and no change in glucose levels was detected. There are many other examples in literature supporting the storage of blood specimens with at least 1 g/100 mL NaF. It should, however, be noted that the majority of literature examples involving micro-organisms make use of blood obtained from a blood bank which contains dextrose – additional substrate for microbial growth.

Despite its effectiveness against most micro-organisms, NaF is claimed to be ineffective against *C. albicans*, with reports showing that even at ambient temperature, some ethanol was produced.[38, 39]

1.7 Analytical Techniques and Instrumentation

In order to determine the required ethanol and fluoride concentrations in the study, various analytical techniques and instrumentation were employed. What follows is a brief outline of each of these.

1.7.1. Determining Ethanol Concentration in Blood Samples

The determination of ethanol concentration in blood generally involves some means of extracting the ethanol from the complex blood matrix followed by some detection system. The most common technique used for blood alcohol analyses is Gas Chromatography with Flame Ionization Detection (GC-FID)[44, 45]; however, mass spectrometry is a powerful technique and has also been applied in the analysis of blood alcohol specimens.[44]

Gas Chromatography

The basic principle of chromatography is that analytes distribute between the mobile and stationary phases according to their physical and chemical properties. Compounds that have strong interactions with the stationary phase will be retained for longer than those that have weak interactions, since analytes only move while in the mobile phase. This difference in migration rates results in the separation of the analytes into bands that elute from the column at different times.[46]

The complex physical interactions between the analytes and the mobile and stationary phases results in

Gas Chromatography (GC) is an analytical technique employed by most laboratories to separate a broad range of compounds, provided they are thermally stable and sufficiently volatile. As with all chromatographic techniques, GC involves both a mobile phase and a stationary phase. In this case, the mobile phase is an inert carrier gas, often helium or nitrogen, and the stationary phase is chosen based on the polarity required for the application. Generally a capillary column is used, where the stationary phase coats the walls of a very small diameter tube. The coating is usually 0.25 μm thick and the column 0.32 mm in diameter. The column diameter is known as the column i.d..[46, 47] *Figure 2* shows separation achieved in a GC column.

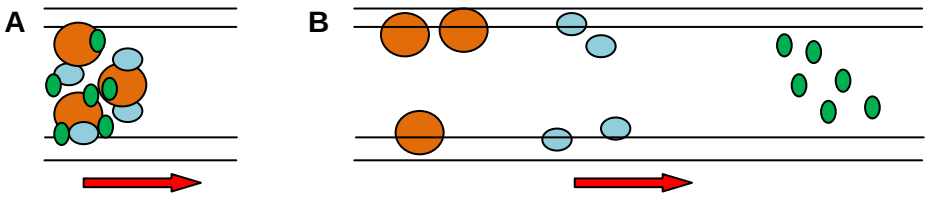

Figure 2: Separation of analytes in a GC column based on their interactions with the stationary phase due to physical and chemical differences. The analytes are represented by the circles of various sizes and the direction of carrier gas flow by the red arrow. The two sets of horizontal lines represent the walls of the capillary, housing the stationary phase.

The efficiency of a column is described by the number of theoretical plates, N_{th}. The more theoretical plates there are the more efficient the column. N_{th} can be used together with the length of the column, L, to derive a quantity known as theoretical plate height, H, as follows:[48, 49]

$$H = \frac{L}{N_{th}} \qquad \text{Equation 1}$$

The smaller the value of H, the more efficient the column. The van Deemter equation, *Equation 2*, allows the calculation of H, taking into account the different factors that influence column efficiency.

$$H = A + \frac{B}{u} + [C_s + C_m]u \qquad \text{Equation 2}$$

where A is the eddy diffusion coefficient, B is the longitudinal diffusion coefficient, C_s is the mass transfer coefficient for the stationary phase, C_m is the mass transfer coefficient for the mobile phase, and u is the linear velocity. The C_s and C_m terms are often combined into one coefficient, C, known as the resistance to mass transfer coefficient.[48, 49]

Eddy diffusion coefficient, A: Identical molecules travel differently through the column due to kinetic processes such as molecular dispersion, diffusion and mass transfer. In *Equation 2* above, the *A-term*

describes the eddy diffusion of molecules through the column. Molecules are transported by the mobile phase along the flow channels between the packing particles in the column. Since these flow channels differ depending on packing and particle shape, the speed of movement of molecules through the column as they travel along different flow channels will vary. This is accounted for by the eddy diffusion coefficient.[48, 49]

Longitudinal diffusion coefficient: The flowing mobile phase allows molecules to travel through the column. The longitudinal diffusion term, *B*, describes the slight differences in mean flow rate due to molecular diffusion of the mobile phase molecules.[48, 49]

Resistance to mass transfer coefficient: Analyte molecules are continuously moving between the mobile phase and the stationary phase in a dynamic equilibrium. This takes time due to the resistance to mass transfer between the two phases, and as such the concentration profiles of sample components in the two phases will always be slightly shifted. The effect of this is described by the *C-term* in *Equation 2*.[48, 49]

The effects of each of the terms present in the van Deemter equation can be used to optimise a chromatographic system. It should, however, be remembered that although the optimum plate height might be achieved, the optimum flow rate required might be unacceptably long. On the other hand, decreasing the flow rate will likely result in a decrease in peak resolution. The optimum chromatographic system may not have all its parameters optimised and compromises may need to be made. It is a delicate balance of each of the contributing factors described in *Equation 2*.[48, 49]

After optimisation of the chromatographic system, the unknown mixture is injected onto the column and the column is heated, usually employing temperature programming to improve separation of the analyte bands. The compounds interact with the stationary phase to varying degrees based on their vapour pressures and polarities, as well as the column temperature, and the carrier gas flow rate. In an analytical setting it is most useful to couple the GC to a detector in order to identify the analytes as they elute from the column. *Figure* 3 shows a GC system coupled to a detector.

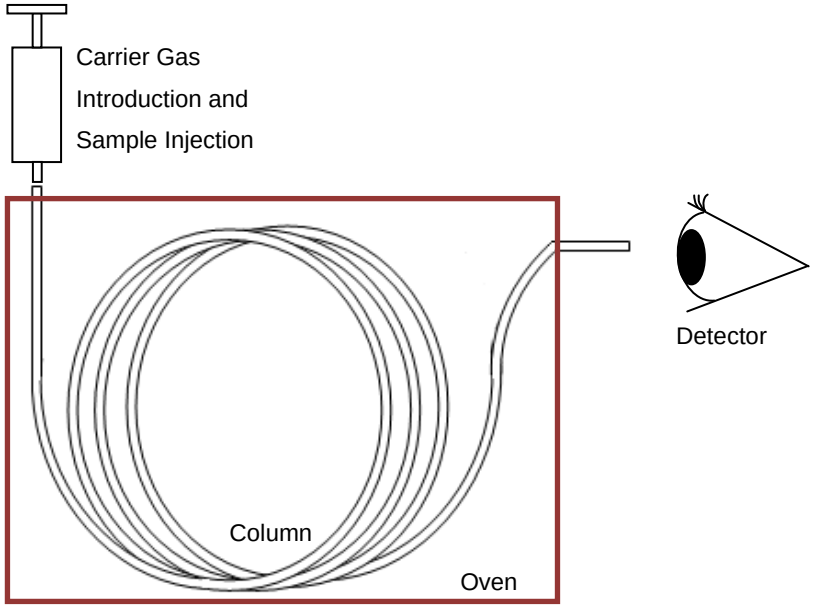

Figure 3: Schematic of GC system coupled to a detector

There are numerous detectors available on the market, including flame ionization detectors (FID), thermal conductivity detectors (TCD), mass spectrometers (MSD), and electron capture detectors (ECD).[46] The FID is most commonly employed in blood alcohol analyses.[44, 45]

Flame Ionisation Detection

The Flame Ionization Detector, seen in *Figure 4*, is the detector most commonly coupled to a GC system. It can be applied to hydrocarbon analytes and has a detection limit of 1 pg/s.[46] The effluent of the column enters a small air-hydrogen flame and is pyrolyzed, producing ions and electrons. These charge carriers are directed to a collector by a voltage applied between the burner tip and collector electrode. The resulting current is then measured by a high-impedance picoammeter.[46]

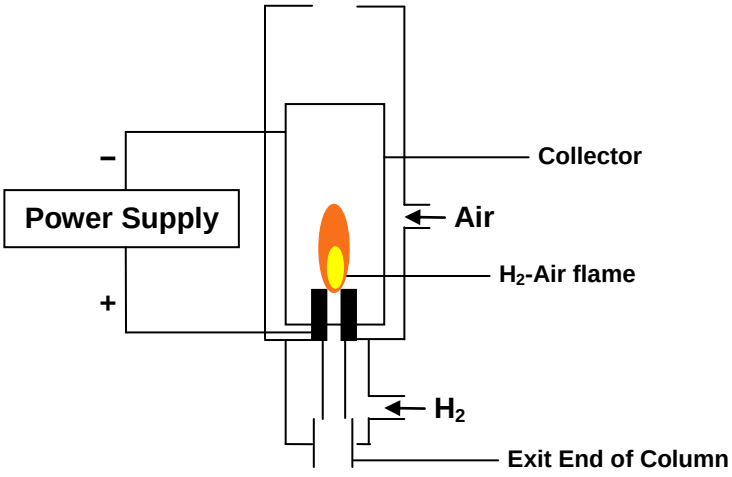

Figure 4: Schematic of a Flame Ionisation Detector[43,60]

The number of ions produced is approximately proportional to the number of reduced carbon atoms in the flame, hence the FID is sensitive to the number of carbon atoms entering the detector per unit of time. This makes the FID a mass-sensitive device as opposed to a concentration-sensitive device.[46]

The FID response is unaffected by changes in mobile phase flow rate, and has minimal noise, good sensitivity (~10^{-1} pg/s) and a large linear range (~10^{7}). It is also generally easy to use and rugged. Unfortunately, the sample cannot be recovered as it is destroyed in the flame.[46]

Mass Spectrometric Detection

A mass spectrometer is an instrument that fragments compounds into ions, and separates these ions based on their mass-to-charge ratios. The mass-to-charge ratio, m/z, of an ion is the ratio of its mass number, m, to the number of fundamental charges, z, on the ion. Very often, ions in mass spectrometry are singly charged, so the m/z of an ion is then equal to the mass of the ion.[46]

There are many different mass spectrometers available; however, they all have the same basic components as depicted in *Figure 5* below. The inlet system introduces a micro amount of the sample into the ion source, where it is bombarded by electrons, ions, molecules or photons and converted to gaseous ions, which are usually positive. This ionization can also be achieved by thermal or electrical energy.[46]

The gaseous ions produced are accelerated into the mass analyser where they are separated based on m/z ratio, and sent to the transducer, which produces an electrical signal from the ion beam.[46]

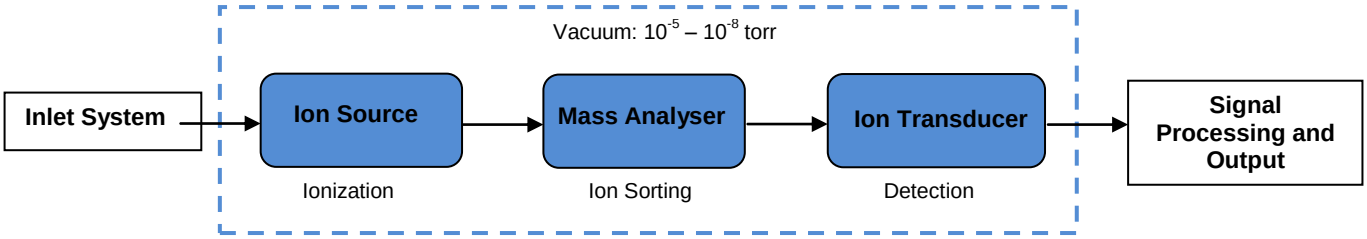

Figure 5: Basic components of a mass spectrometer[43]

Generally the flow rate of the GC capillary column is sufficiently low that the effluent can be fed directly into the ion source of a mass spectrometer, making GC-MS a powerful tandem tool for the analysis of complex unknowns.[46]

Ionization

The first step in any mass spectrometric analysis is the ionization of analytes upon elution from the column. There are a number of different ion sources available, and the mass spectra obtained are highly dependent on the method of ionization. For gaseous analytes, such as those eluting from a GC column, the two most common types of ion sources are the electron impact (EI) source and the chemical ionization (CI) source.[46]

Ionization sources are classified as either hard or soft sources. A hard ionization source transfers enough energy to the analyte molecules to excite them into a high energy state. Upon relaxation, bonds are broken and fragment ions, or daughter ions, are formed. These daughter ions have m/z ratios of less than that of the molecular ion. Conversely, soft ion sources cause little fragmentation, and so the spectra produced will contain the molecular ion and only a few other ion peaks. The mass spectra produced by hard ion sources are more repeatable than those produced by soft ion sources. An EI source, such as is depicted in *Figure 6*, is a hard ion source.[46]

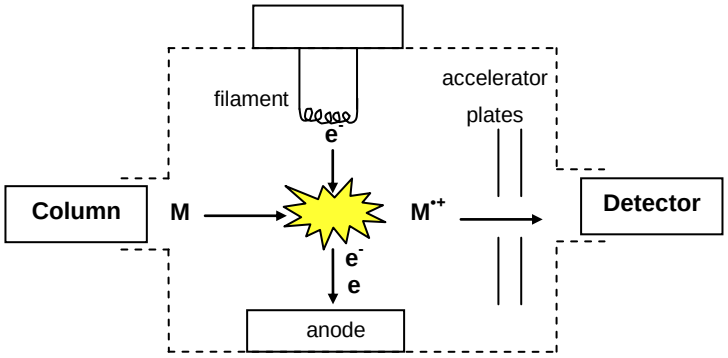

Figure 6: Schematic of an Electron Impact source[43,60]

In an EI source, the gaseous analyte molecules are bombarded with a beam of high energy electrons which are emitted from a heated tungsten or rhenium filament. The electrons are accelerated by a voltage of approximately 70 V applied between the filament and the anode. The paths of the molecules and electrons are perpendicular to each other, allowing collisions to take place near the centre of the source. Predominantly singly charged positive ions are produced by electro-static repulsion as the approaching electrons cause the molecules to lose electrons by the reaction:

$$M + e^- \rightarrow M^{\bullet +} + 2e^-$$

where M is the molecule and $M^{\bullet +}$ is the molecular ion produced.[46]

The ions produced are accelerated toward the detector by voltages applied across the accelerator plates.[46]

Detection

The most common mass spectrometer is the quadrupole mass analyser, shown in *Figure 7*. It is usually smaller, less costly and more rugged than other mass spectrometers.[46]

A quadrupole mass analyser consists of four parallel rods, with each pair of opposite rods connected electronically. One pair is attached to the positive side of a direct current (dc) source and

the other pair to the negative terminal. Variable alternating current (ac) voltages which are 180° out of phase are applied to each pair of rods.[46]

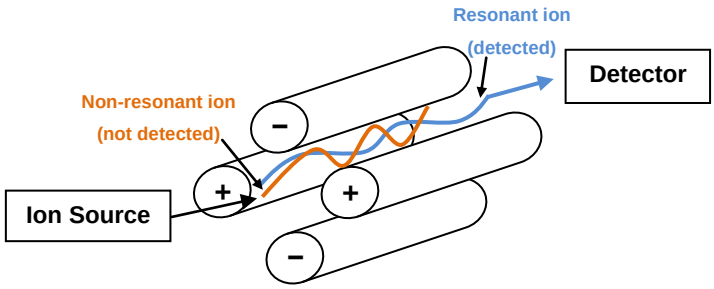

Figure 7: Quadrupole mass spectrometer [43,60]

A potential difference of between 5 and 10 V accelerates the ions to the space between the rods, and the ac and dc voltages are increased concurrently while keeping a constant ratio. In this way, certain m/z ratios are selectively allowed through to the transducer, while all others collide with the rods and are converted to neutral molecules.[46] Ions reaching the detector produce a mass spectrum, which is unique to the compound from which the ions were generated.

GC-FID Compared to GC-MS

Forensic laboratories currently perform blood alcohol analyses by Headspace GC-FID (HS-GC-FID). Literature, however, lists a few methods that couple Gas Chromatography with Mass Spectrometry (GC-MS). For example, Jones et al.[50] made use of GC-MS to determine endogenous ethanol concentration in blood[51]; Schuberth (1991) studied low molecular weight volatile organic compounds that may arise in drunk drivers' blood using GC-MS[52]; and Wasfi[53] developed a static headspace GC-MS method to analyse blood alcohol concentration.[53]

Gas Chromatography – Mass Spectrometry, which has long been considered the "gold standard" of analytical techniques[54], offers many advantages over the traditional GC-FID. Considering FID is applicable only to hydrocarbons, whereas MS can be applied to virtually any analyte, GC-MS is a much more versatile technique than GC-FID.[46] Furthermore, GC-MS has a greater power of identification than GC-FID, since FID relies only on retention time, while with MS, identification of unknowns is based on retention time as well as the unique mass spectrum obtained.[46, 55] This allows for less interference from other compounds with similar retention times as the analyte of interest.

While this work demonstrates the use of a GC-MS method for the quantitation of ethanol in blood specimens, its aim is not that all blood alcohol laboratories replace their GC-FID instruments with GC-MS setups. Rather, it strives to offer GC-MS as a viable alternative for those laboratories that do

not have dedicated instrumentation for BAC analyses, such as routine and clinical laboratories that test for a wide range of analytes.

Isotope Dilution

A commonly employed technique for quantitation using GC-MS is Isotope Dilution. In an Isotope Dilution method, the specimen is spiked with a known amount of an isotopically labelled form of the analyte, which functions as an internal standard. By using the ratio of the signals of the analyte and the isotopically labelled internal standard, the concentration of the analyte present in the sample can be calculated.[56, 57] The use of an internal standard in this fashion compensates for any losses in analyte content due to the specimen work-up procedure, since the internal standard has been subjected to the same process.

An analogy

Consider a pond of an unknown number of red fish. We wish to determine the number of red fish in the pond without removing them all and counting them. In order to do this, 10 blue fish are added to the pond.

After allowing a sufficient amount of time to ensure that the blue fish have completely mingled with the red fish, 20 fish are removed from the pond and examined for colour. Say out of these 20 fish, 2 are blue and the remaining 18 are red. This gives the ratio 1 blue fish to every 9 red fish, implying that since we added a total of 10 blue fish there are 90 red fish in the pond.[58]

Isotope Ratios

In chemical terms, a known amount of isotopically labelled substance (the internal standard) is added to the sample that is to be analysed. Doing this effectively “dilutes” the isotope ratio of the internal standard. This is depicted in *Figure 8* below. By measuring the resulting ratio of the isotopic composition it is possible to calculate the concentration of the analyte in the sample.[58, 59]

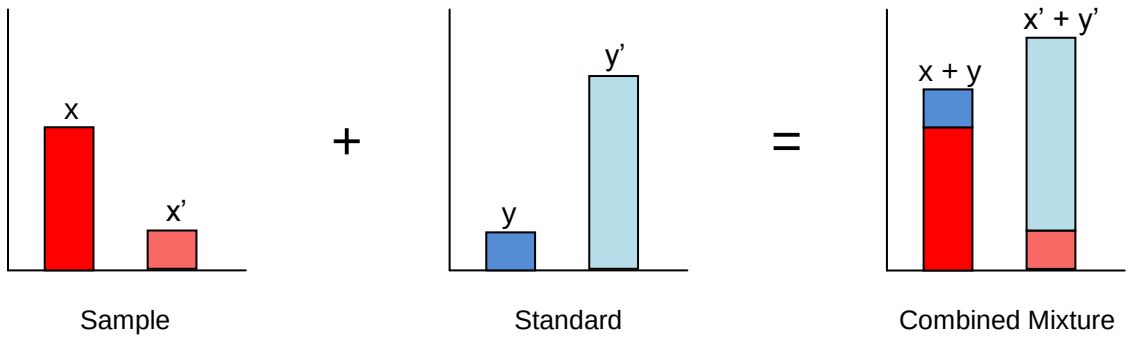

Figure 8: The basic principle of isotope dilution

In Isotope Dilution Mass Spectrometry, after addition of the isotope labelled internal standard to the sample and equilibration, the altered isotopic ratios are measured using mass spectrometry. The isotope ratio (R_m) of isotopes A and B is calculated by:[58]

$$R_m = \frac{A_x C_x W_x + A_s C_s W_s}{B_x C_x W_x + B_s C_s W_s} \qquad \text{Equation 3}$$

where A_x and B_x are the atom fractions of isotopes A and B in the sample, A_s and B_s are the atom fractions of isotopes A and B in the standard, C_x and C_s are the elemental mass concentrations in the sample and standard respectively, and W_x and W_s are the weights of the sample and standard respectively.

The concentration of the analyte in the sample can then be calculated using:[58]

$$C_x = C_s \left(\frac{W_s}{W_x}\right) \times \left(\frac{A_s - R_m B_s}{R_m B_x - A_x}\right) \qquad \text{Equation 4}$$

More often than not, however, analysts will opt not to employ these equations for isotope dilution calculations, and will rather prepare a calibration curve from mixtures of standards containing only the analyte of interest at various concentrations and a standard containing only the isotope labelled form of the analyte at a fixed concentration. These calibration curves will ultimately be obtained by plotting the ratio of the signals of the analyte standard to the isotopically labelled internal standard against the concentration of the analyte standard.[60]

1.7.2. Determining Fluoride Concentration in Blood Samples

As previously discussed, NaF is the preservative most often used for blood ethanol specimens, and in South Africa the NaF concentration of blood specimens is required to be above 1% (w/v). As such, all blood ethanol concentration results must be accompanied by the corresponding NaF concentration. Hence, laboratories analysing BAC also need a method for the quantitation of fluoride in blood.

Fluoride concentration can be determined by titration with lanthanum (III) nitrate,[61] although more common is the determination by fluoride ion selective electrode (FISE)[61-64]. The FISE measures the potential that develops across a membrane as a result of free fluoride in a sample, and this relationship can be described by the Nernst equation.[65] The level of free fluoride is subject to temperature, pH, total ionic strength and complexation.[65, 66]

Electrochemistry

In an oxidation-reduction reaction, or red-ox reaction, electrons are transferred from one species to another. The species gaining electrons is said to be reduced and is called the oxidizing agent, while the species losing electrons is said to be oxidised and is called the reducing agent. The loss of electrons causes an increase in oxidation state.[48, 67]

Electrochemical Cells

Oxidation-reduction reactions can occur in one of two ways. Either the reacting species are brought into physical contact with each other which allows for direct electron transfer, or the reaction occurs in what is known as an electrochemical cell, where no direct contact takes place.[48] In this case, two conductors called electrodes are immersed in electrolyte solutions which contain the oxidizing and reducing agents. Each electrode in its solution makes up half of the cell. The electrode where oxidation occurs is called the anode, while the electrode where reduction occurs is called the cathode.[48]

Since the electrodes would otherwise be isolated from each other, a salt bridge is used to maintain electrical contact between the two halves of the cell, while keeping the electrolyte solutions separate. The conduction of electricity in the cell is achieved by migration of positive ions through the salt bridge from one electrode to the other and negative ions in the opposite direction.[48] *Figure* 9 below depicts a general electrochemical cell. Ions flow through the salt bridge, while electrons move from the anode to the cathode.

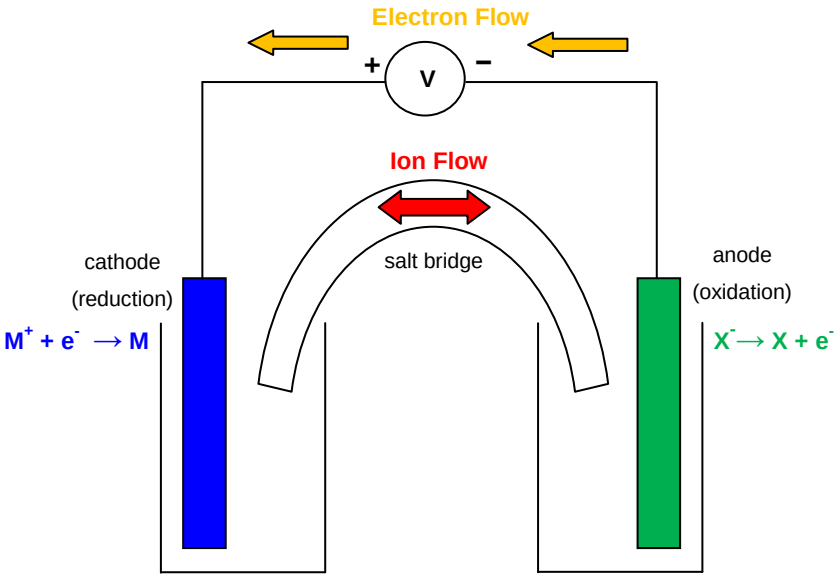

Figure 9: An electrochemical cell with a salt bridge[58,60,61]

There are two types of electrochemical cells. In *voltaic cells*, named for the Italian physicist Alessandro Volta, the cell reaction takes place spontaneously. Batteries are usually made up of

multiple voltaic cells connected in series, since voltaic cells store energy. Conversely, in an *electrolytic cell* an external power source is needed to drive the reaction forward. The direction of current in an electrolytic cell is the reverse of that in a voltaic cell, and the reactions at each electrode are also swapped. The cell shown in *Figure 9* is a voltaic cell, with oxidation occurring at the anode and reduction at the cathode.[48]

Electrode Potential

The potential of an electrode is a measure of its electron energy. Hence, the flow of electrons in an electrochemical cell occurs due to the potential difference between the electrodes, where this potential difference can be seen as the tendency of the red-ox reaction to proceed towards equilibrium. For a voltaic cell, this potential difference is called the cell potential, denoted E_{cell}, and under standard conditions (25 °C, 1 atm, reactants and products at 1 M) the standard cell potential, E^0_{cell}, is given as the difference between the cathode standard potential and the anode standard potential.[67]

$$E^0_{cell} = E^0_{cathode} - E^0_{anode} \qquad \text{Equation 5}$$

It is, however, rare to work under standard conditions. Hence, some means of calculating cell potentials under non-standard working conditions is necessary, and it is here that *Equation 6*, the Nernst equation, can be employed.[67]

$$E = E^0 - \frac{RT}{nF} lnQ \qquad \text{Equation 6}$$

where E is the electrode potential, E^0 is the standard electrode potential, R is the ideal gas constant ($R = 8.314\ J.K^{-1}mol^{-1}$), T is temperature in Kelvin, n is the number of moles of electrons in half-reaction for the electrode, F is the Faraday constant ($F = 96\,485\ C.mol^{-1}$), and Q is the reaction quotient.[67] Considering the reaction quotient, Q, is the ratio of the product of the concentrations of the products to the product of the concentrations of the reactants, the Nernst equation can also be used to relate cell potential to concentration.

Potentiometry

Potentiometry is an analytical technique in which the potential of an electrochemical cell is measured, often with the intention of determining the concentration of some ion. In order to do this an indicator,

or working, electrode, a reference electrode and a device capable of measuring and recording potential are required.[46]

All potentials determined are actually potential differences, since absolute values for individual half-cell potentials cannot be determined experimentally. As such, a reference electrode is needed. A reference electrode is a half-cell with an accurately known reference potential, E_{ref}, that is independent of the concentration of the analyte.[46]

The working electrode is immersed in the solution to be analysed and subsequently develops a potential, E_w, that depends on the activity of the analyte ions. A potential also develops at the liquid junctions at each end of the salt bridge connecting the reference and working electrodes. The net potential across the salt bridge, E_j, is then a contributing factor to the overall cell potential, E_{cell}, and hence to the accuracy and precision of the measurement. The potential of the cell can then be written as:

$$E_{cell} = \left(E_w - E_{ref}\right) + E_j \qquad \text{Equation 7}$$

In most cases, if the electrolyte of the salt bridge is appropriately chosen, the mobilities of the cations and anions through the salt bridge will be more or less equal, and the potentials that develop at each liquid junction cancel each other out. The net potential across the salt bridge, E_j is then negligible, and E_{cell} is simply the difference between E_w and E_{ref}. Potassium chloride is one such nearly ideal electrolyte for salt bridges.[46]

Most working electrodes used in potentiometric analyses are selective electrodes in that they respond selectively to one analyte in the presence of others.[46, 68]

The Fluoride Ion Selective Electrode

Working electrodes can be categorised as either metallic electrodes or membrane electrodes. The most common metallic electrodes consist of a pure metal electrode immersed in a solution of its own cation, or of an anion with which it forms a precipitate. Membrane electrodes have a membrane between the electrode surface and the solution containing the analyte. This membrane can be either crystalline or non-crystalline, and the measured potential is a kind of junction potential that develops across the membrane. Membrane electrodes are highly selective and for this reason are often called ion-selective electrodes.[46]

Properties of an ideal membrane include low solubility, electrical conductivity, and selective reactivity. It is important that the membrane not dissolve in the analyte matrix, and some electrical conductivity, which usually takes place in the form of migration of ions, is imperative. In addition, the membrane

should be selective for the analyte ion. As such, the membrane is usually solely comprised of, or at least contains some species that is able to selectively bind to the analyte.[46]

The most commonly known ion-selective electrode is a pH electrode, which is simply an electrode that is selective for hydrogen ions. This is the oldest type of electrode dating back to the early 1930s, and has a non-crystalline, glass membrane.[46]

The fluoride ion-selective electrode (FISE) contains a crystalline membrane, prepared by cutting a disk from a single crystal of lanthanum fluoride, LaF_3, doped with europium fluoride, EuF_2. Ionization at the interfaces between the membrane and the working electrode and the membrane and the analyte solution results in a charge on the membrane surface. This is shown by:[46]

$$LaF_3(s) \leftrightarrow LaF_2^+(s) + F^-(aq) \qquad \text{Equation 8}$$

This ionization means that one side of the membrane is in contact with a higher fluoride concentration than the other side, and a potential difference results. This potential difference is thus a measure of the difference in fluoride concentrations of the two solutions[46], and it is this potential that is measured by the ISE meter.

There are several commercially available configurations of the FISE; however, the most common and perhaps most convenient is the combination electrode. The reference electrode and working electrode are combined in one housing and separated by electrode filling solution. This can be seen in *Figure 10* below.[46, 65]

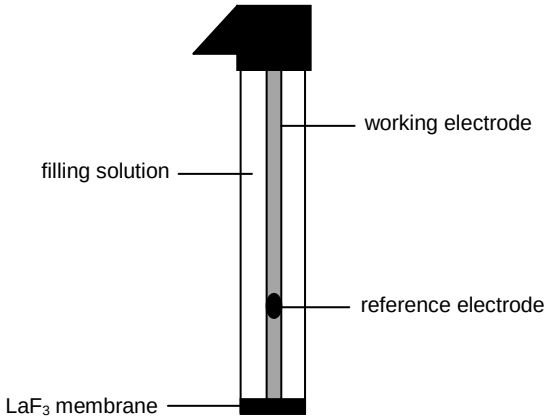

Figure 10: Combination FISE electrode[56]

The potential that develops across the membrane depends on the level of free fluoride in the analyte solution and is measured against a constant reference potential by an ISE meter. The electrode response due to free fluoride ions is governed by the Nernst equation in the following form:[65]

$$E = E_0 + S\ logA \qquad \text{Equation 9}$$

where E is the measured electrode potential, E_0 is the reference electrode potential, S is the electrode slope, and A is the fluoride ion activity in the analyte solution. The electrode slope is determined experimentally and depends heavily on temperature. For solutions between 20 °C and 25 °C the slope of the electrode is expected to be between -54 mV/dec and -60 mV/dec. These values are calculated as the difference between electrode responses of two samples whose concentrations are a decade apart. The fluoride ion activity is related to the free fluoride ion concentration, C_f, by the activity coefficient, γ, as given in *Equation 10*.[65]

$$A = \gamma \times C_f \qquad \text{Equation 10}$$

Ionic activity coefficients are variable and depend on the total ionic strength of the solution. Provided the background ion concentration is high and constant, the activity coefficient is also constant and activity is directly proportional to concentration. Thus, plotting the measured potential against the slope multiplied by the logarithm of the fluoride concentration affords a linear model for fluoride concentrations from 0.02 ppm to saturated, and fluoride concentrations instead of activities can be measured.[46, 65]

The only ions that interfere with electrode response are protons, H^+, at pH levels below 5, and hydroxide ions, OH^-, when the hydroxide level is at least one-tenth the level of the fluoride. Additionally, electrode response is highly dependent on temperature – all calibrator, control and sample solutions should be within ±1 °C of each other. Fluoride ion-selective electrodes respond only to free fluoride ions. This can pose a problem since in many sample matrices there are polyvalent cations, such as aluminium, silicon and iron that complex with fluoride.[46]

Fluoride Ion Selective Electrode analysis is a reasonably inexpensive and fairly rugged technique.[46, 69] It can be applied to many sample matrices, including water, blood, urine, saliva, beer, and canned foods, and sample work-up is usually simple. It is a non-destructive technique, with a wide dynamic range, that can easily be automated.[69] Hence, FISE analysis is ideal for determining fluoride concentrations in blood specimens for blood ethanol analysis.

Matrix Effects and How to Combat Them

Blood is a complex biological matrix consisting of leukocytes, or white blood cells; erythrocytes, or red blood cells; and thrombocytes, or platelets, suspended in plasma along with various plasma proteins.[70] Also present in blood are many ions, including aluminium (Al^{3+}), calcium (Ca^{2+}), iron (Fe^{2+} and Fe^{3+}), magnesium (Mg^{2+}), and zinc (Zn^{2+}), at varying concentrations.[71] Each of these ions has the potential to bind to the free fluoride, making it impossible to be detected by the FISE. Additionally, the pH of blood is usually between 7.35 and 7.45, while the optimal pH range for the FISE is between 5 and 7.[65, 72] All of these factors have the potential to influence the reliability of the final fluoride concentration result as determined by the FISE, and some means of combating them, or compensating for them, should be found.

The complexity of the matrix can be minimised by diluting the specimen several times, thereby minimizing protein interference.[63] The pH can easily be adjusted by the addition of a buffer, while the possibility of complexation due to the ions present in blood can be minimised by the addition of a decomplexation reagent. A decomplexation reagent is some compound that preferentially complexes with the interfering ion. In other words, the ion that would have complexed with the free fluoride complexes with the reagent instead, leaving the fluoride free to be detected by the FISE.[65]

Total Ionic Strength Adjustment Buffer (TISAB) solution is commonly added to specimen solutions in fluoride ion analysis.[73] The addition of TISAB maintains a high background ionic strength, and ensures that the pH of the solution is correct. It also contains a decomplexation reagent in order to prevent, or at least minimise, complexation of the free fluoride. A commonly employed buffer, TISAB II, contains 1,2-cyclohexandiaminetetraacetic acid (CDTA), which preferentially complexes Fe^{3+} and Al^{3+} in the sample. The CDTA forms a monomer with Fe^{3+} [74], and it is claimed that in the presence of 1 ppm fluoride, it will complex 5 ppm Al^{3+} or Fe^{3+}.[65] For higher levels of Al^{3+} or Fe^{3+}, TISAB IV, which contains sodium tartrate as decomplexation reagent, is recommended. The sodium tartrate is able to complex either Fe^{2+} or Fe^{3+}.[75]

A Note on Solubility

The solubility of NaF in water is 4.13 g/100 mL.[76] Considering the complexity of the blood matrix, it would stand to reason that the solubility of NaF in blood would be much lower than in water. Indeed, it does take more time and effort to solubilise anhydrous NaF in blood than in water. In addition, due to the opacity of blood it is difficult to gauge whether or not all of the NaF is in solution.

Typically, specimen collection tube suppliers recommend that the NaF/KOx tubes be filled to capacity and then inverted eight to ten times in order to mix.[77] It is unlikely that all the NaF will have dissolved after such a short mixing, and hence there exists the possibility of a concentration gradient throughout the sample. For analysis purposes, it is imperative that the specimen be homogeneous if the

concentration results of the aliquots are to be representative of the whole specimen. Thus it is recommended that specimens be well mixed before aliquoting for analysis.

1.8 Statistical Toolbox

Statistics is the science of learning from data.[78] All analytical results should thus be viewed through the lens of statistical analysis if they are to be interpreted fully and reliably. There are many statistical tools available for analysing and interpreting data, and some of these are outlined below.

1.8.1. Introduction to Terms

Error

A perfect measurement would yield the true value of the measurand every time. However, there will always be a difference between the true value and the measured value, called error. As such, no analytical measurement can be made completely free from error. This is most readily seen when multiple measurements are performed on the same sample, each yielding different values. In fact, it is extremely rare for the replicate measurements to yield the exact same value. This dispersion in measured values is due to the influences of measurement errors, specifically random errors.[79]

The two different types of error, random and systematic, can be seen in *Figure 11* below.

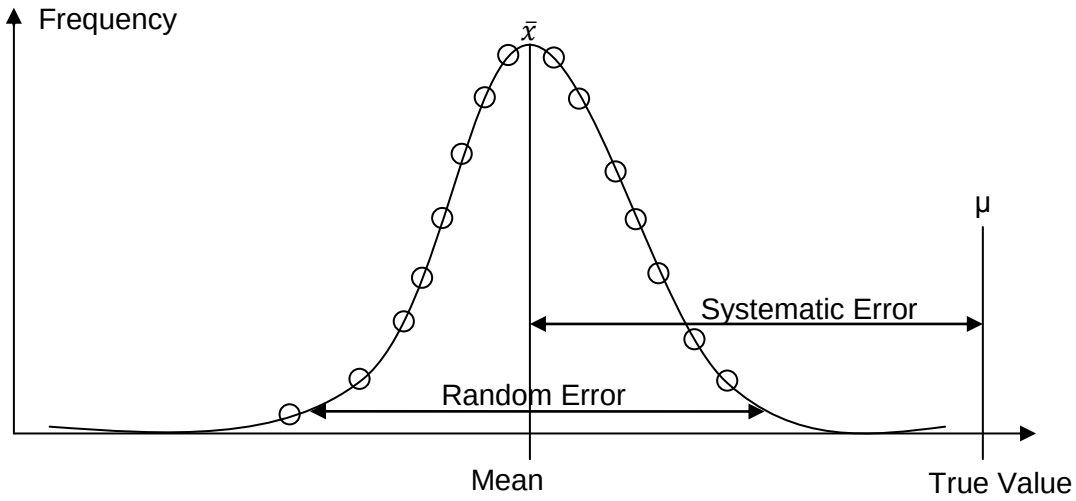

Figure 11: Random error and systematic error

Random errors are beyond the control of the experimental setup, and cause the measured values to fall on either side of the true value of the measurand. This means that if random errors were the only errors present, the average of many replicate measurements, $\bar{x}$, would equal the true value, μ, since the standard deviation of the mean would shrink to close to zero.[79, 80]

Systematic errors on the other hand cause the measurement value to be shifted in one direction away from the true value. That is, measurement results will always be larger or always be smaller than the

true value. The sources of this type of error should be established and eliminated as far as possible, and, pending that, the effects should be minimised by applying suitable corrections.[79, 80]

Bias and Precision

Analytical methods are most often assessed in terms of bias (or accuracy) and precision. It is possible for a method to be both accurate (displaying minimal bias) and precise, but also possible that a method be inaccurate and imprecise, or even a mixture of these two. This is better depicted using the target model.[79]

Bias would thus be considered the closeness in agreement between the true value and the average of the replicate measurements, and is caused by systematic errors.[80, 81]

Precision is the closeness in agreement between the individual replicate measurements, and is influenced by random errors.[80, 81]

Repeatability and Reproducibility

Repeatability and reproducibility are terms that are often confused, and are perhaps best differentiated in terms of precision.

Repeatability refers to the within-batch precision.[80] That is, the closeness in agreement between individual measurements within the same run under the same conditions.

Reproducibility refers to the between-batch precision.[80] That is, the closeness in agreement between individual measurements performed in different runs, usually on different days but could be by different analysts or even different laboratories.

Metrological Traceability

Metrological Traceability is an important property of a measurement result by which it can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty.[81, 82] This is especially important for testing laboratories that determine concentrations of analytes. All standard reference materials should have a known certified concentration value, accompanied by a measurement uncertainty. All certified reference materials used in the analysis process should then be accounted for in the measurement uncertainty calculations.

1.8.2. Statistics for Repeated Measurements

Mean

Throughout this work, wherever the mean of measurements was taken, the following equation was used:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$ Equation 11

where $\bar{x}$ is the mean, x_i are the individual measurements, and n is the number of measurements.[80]

Standard Deviation

Standard deviations were calculated as follows:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \quad (\text{for } n<30)$$ Equation 12a

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \mu)^2}{N}} \quad (\text{for } N\geq 30)$$ Equation 12b

where s and σ are the sample and population standard deviations respectively, x_i are the individual measurements, n and N are the number of replicate measurements in the sample set and population respectively, and $\bar{x}$ and μ are the sample mean and population mean respectively.[48, 80]

Pooled Standard Deviation

Standard deviations were pooled by means of:

$$s_{pooled} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)}}$$ Equation 13

where n_1 and n_2 are the number of measurements that contributed to the standard deviations s_1 and s_2.[80]

Propagation of Error

By its very nature, experimental work is open to many sources of error. In order to give an estimate of overall error – or measurement uncertainty – it is necessary to combine these errors into one single value. There are certain rules that govern how error propagates and these need to be applied in order to assess the overall error of a measurement model. The rules depend on the structure of the measurement model and can be summarised as follows:[80]

Linear Combinations

Consider a measurement model of the form:

$$y = K + Aa + Bb + Cc \qquad \text{Equation 14}$$

with y as the final result, calculated from the linear combination of measured quantities a, b, and c, and K, A, B, and C are constants.

If σ_a, σ_b, and σ_c are the standard deviations of the values a, b, and c, then the standard deviation of y, σ_y can be calculated by:

$$\sigma_y = \sqrt{(A\sigma_a)^2 + (B\sigma_b)^2 + (C\sigma_c)^2} \qquad \text{Equation 15}$$

Multiplicative Expressions

Consider a measurement model of the form:

$$y = \frac{Kab}{cd} \qquad \text{Equation 16}$$

where y is once again the final result, K is a constant, and a, b, c, and d are independent measured quantities, the relative standard deviation of y, $\frac{\sigma_y}{y}$, can be calculated by:

$$\frac{\sigma_y}{y} = \sqrt{\left(\frac{\sigma_a}{a}\right)^2 + \left(\frac{\sigma_b}{b}\right)^2 + \left(\frac{\sigma_c}{c}\right)^2 \left(\frac{\sigma_d}{d}\right)^2} \qquad \text{Equation 17}$$

In *Equation* 17, σ_a, σ_b, σ_c, and σ_d, are the standard deviations of the quantities a, b, c, and, d respectively.

General Functions

Consider the case where the measurement model is best expressed with y being some function of x, y = *f*(x). The standard deviation of y, σ_y, can be calculated by:

$$\sigma_y = \left|\sigma_x \times \frac{dy}{dx}\right| \qquad \text{Equation 18}$$

Very often, the measurement model will contain more than one of these function forms. In that case, more than one rule will be applied to calculate the overall uncertainty.

Normality

Due to the nature of analytical measurements, random errors will always be present. These errors cannot be controlled as such a variety, or spread, in results is observed when replicate analyses are performed. Plotting the frequency against the analyte concentration for a large number of replicate analyses will most often yield a bell-shaped curve similar to the one depicted in *Figure 11B*. The shape of this curve shows that there is a high probability of obtaining values close to the mean, while a low probability of obtaining values further away.[48, 80]

Such bell-shaped plots depict the mathematical model called the normal or Gaussian distribution. For a normal distribution the following properties hold: [80]

- approximately **68%** of the population values lie within **one standard deviation** of the mean
- approximately **95%** of the population values lie within **two standard deviations** of the mean
- approximately **99.7%** of the population values lie within **three standard deviations** of the mean

Many statistical tests, including those listed below in Section 1.8.3 assume data to be normally distributed, and while it cannot be said that all replicate values of a single analytical quantity are always normally distributed, it is most often the case. However, it is possible that the data be skewed either to the left or to the right, and wherever possible it is prudent to check for normality in data. This becomes especially important for pre-validations and mini-validations where small data sets are used.[48, 80]

Figure 12 shows how the distribution functions for skewed data compare to normally distributed data.

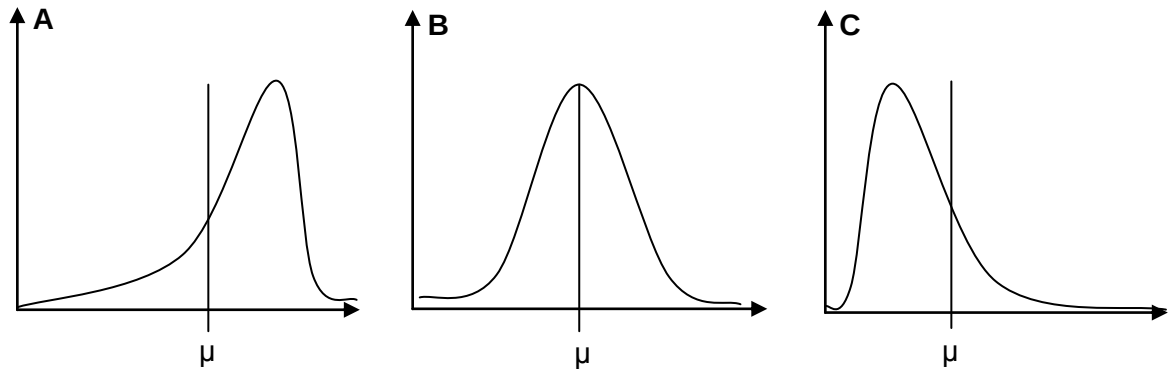

Figure 12 A: Left-skewed distribution; **B:** Normal distribution; **C:** Right-skewed distribution

The skewness factor, S, of a population can be calculated by:

$$S = \frac{n}{(n-1)(n-2)} \sum_i \left(\frac{x_i - \bar{x}}{s} \right)^3$$ Equation 19

where n is the number of replicate analyses, x_i are the individual replicate analyses, $\bar{x}$ is the mean of the population, and s is the standard deviation of the population.[83] When S is negative, the data is tailed to the left and a distribution similar to that in *Figure 11A* is obtained, whereas when S is positive, the data is tailed to the right and a distribution similar to that in *Figure 11C* is observed. When S is close to zero a distribution similar to that in *Figure 11B* is obtained and the data is said to be normally distributed.

There are various methods of testing normality for a data set. One rule of thumb says that if |S| is greater than 2 x $\sqrt{\frac{6}{n}}$, where n is the number of replicate analyses, then the data is considered to be significantly skewed, and hence is not normally distributed.[84] This is, however, only a rule of thumb. It is better to assess either the kurtosis of a data set (not discussed in this work), or to examine the cumulative distribution function (CDF) by means of the Kolmogorov-Smirnov Test (KST) for normality.[85]

The KST compares the sample CDF with the theoretical CDF, where the null hypothesis is that there is no difference between the two, while the alternative hypothesis states that there is a significant difference.

In order to perform the KST, the data set is first ordered in ascending order, and a standardised normal variable, z, is calculated for each value by:[80, 85]

$$z = \frac{(x - \mu)}{\sigma}$$ Equation 20

where x is the data point, μ is the population mean, and σ is the population standard deviation.

These z-values are then used to calculate theoretical normal cumulative distribution values for the data, assuming that the data is normally distributed, by means of:

$$F(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$ Equation 21

where x is the data point, μ is the population mean, and σ is the population standard deviation.

Following this, the experimental CDF values are calculated by:

$$CDF(i) = \frac{i}{n}$$

for 1 < i < n

Equation 22

where n is the number of replicates in the data set.

Plotting the sample data (x) versus CDF (y) and F (y) will yield curves similar to those depicted in *Figure 13*. The KST tests for a significant difference between the two curves, by comparing the largest difference (D_T) with a critical value (D_C) obtained from a table of Kolmogorov-Smirnov critical values.[85, 86]

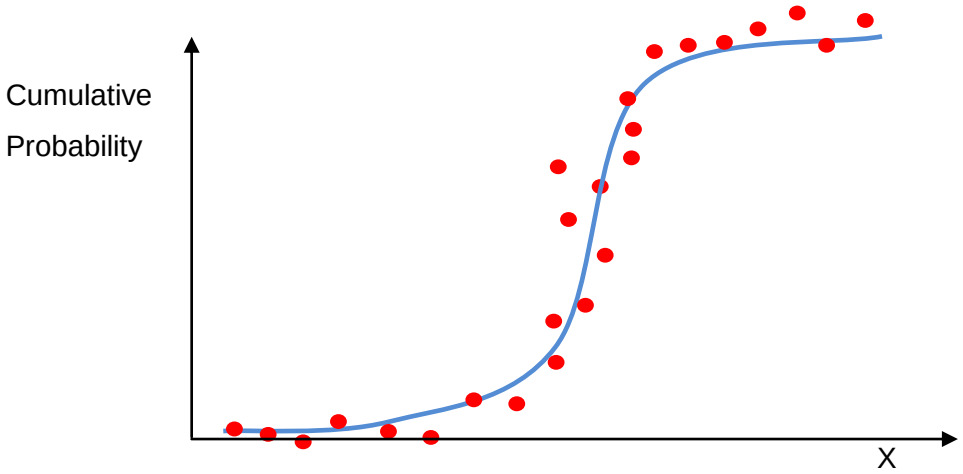

Figure 13: Plots of sample data versus CDF (red dots) and F (blue line)

Each difference, D_i, is calculated by:

$$D_i = |CDF_i - F(x_i)|$$

for 1 < i < n

Equation 23

where n is the number of replicates in the sample set. D_T is then assigned as largest of the differences, D_i.[85] When D_T is less than D_C the sample set is considered to be normally distributed.

Quality Control

Quality control is especially important in analytical laboratories, since results of a high standard should be consistently produced. As the name suggests, quality control methods allow a laboratory to monitor its performance for a certain assay and to ensure the quality of results being sent out to clients. It is essential that a laboratory check for time-dependent trends that may occur in results over and above the inevitable random errors.[80]

It is common practice that an internal quality control standard (IQC) be analysed every so often throughout each analytical run. An IQC is a standard of known composition and high stability, often prepared from a certified reference material (CRM) or purchased as such, that has ideally been through the same work-up procedure as the unknown specimens. Often more than one IQC at different concentrations is prepared and analysed. The concentrations are chosen such that they span the working range of the method. Provided the results for the IQC samples are within certain limits and display no time-dependent trends, the method is said to be *under control*. It is critical that the analytical process be halted as soon as quality control requirements are not met, since then it is likely that the method is giving erroneous results. In this way the precision of the method can be monitored.[80]

Each time an IQC specimen is analysed, the result should be added to the data set containing all previous IQC results of the same theoretical concentration, and it is usually plotted on what is known as a Levey-Jennings control chart. In order to use a Levey-Jennings control chart, at least 20 data points are required, and the mean and standard deviation (s) of the data should be calculated. The control chart is then constructed by plotting concentration on the y-axis and observation number on the x-axis. The x-axis is in terms of time, so it can also be labelled using day, run number or any other appropriate measure of time. Other than the control data, the mean and certain control limits should also be plotted, usually as horizontal lines. Let μ be the mean of the control data. Then the control limits are most often taken as $\mu \pm 2s$ or $\mu \pm 3s$, while $\mu \pm 1s$ is considered a warning limit. The analytical run is considered under control when an IQC result falls within the control limits and out of control when the limits are exceeded.[71, 80]

Since random error cannot be avoided in any run, it is possible that a result may fall outside the control limits and not be cause for alarm. One result exceeding the 2s limit is not necessarily grounds for rejection of the run and further rules need to be applied to properly interpret the control data.[71]

Usually, in order to interpret control chart data, the Westgard rules[87] are applied in what is known as the “Multirule Procedure”.[71] These rules are chosen such that the probability of a false rejection is low, and the probability for error detection is high. Use of the Westgard rules requires the charts to have control lines for $\mu \pm 1s$, $\mu \pm 2s$, and $\mu \pm 3s$, and hence can be applied to existing Levey-Jennings charts by simply adding further control limits.[71]

The following is a list of the Westgard rules and how they are applied. For simplicity, a short hand notation is adopted, whereby the control rules are abbreviated, for example 1_{2s} would denote one measurement exceeding the $\mu \pm 2s$ control limits.[71, 88]

Table 1: Summary of Westgard rules to be applied in the Multirule Procedure[71, 88]

Abbreviation	Rule	Action
1_{2s}	One measurement exceeds the μ ± 2s control limits	Warning rule; apply further rules
1_{3s}	One measurement exceeds the μ ± 3s control limits	Rejection rule sensitive to random error
2_{2s}	Two consecutive measurements exceed the same μ + 2s or μ - 2s control limit	Rejection rule sensitive to systematic error
R_{4s}	One measurement in a group exceeds the μ + 2s and another exceeds the μ - 2s control limits	Rejection rule sensitive to random error; only apply within-run, not between-run
4_{1s}	Four consecutive measurements exceed the same μ + 1s or μ - 1s control limit	Rejection rule sensitive to systematic error; only apply between-run, not within-run
10_{μ}	Ten consecutive measurements fall on the same side of the mean	Rejection rule sensitive to systematic error; only apply between-run, not within-run

In general, for detecting random errors the rules should be applied within-run, while for detecting systematic errors the rules should be applied between-run.

For every analytical run, controls should be included at each of the control concentrations and their results added to the control charts. If all control results fall within the ±2s limits, the run is said to be under control and the unknown specimen results can be reported. If one or more of the control results exceed these limits, the 1_{3s}, 2_{2s}, R_{4s} and 10_{μ} rules should be applied. When any one of these rules is violated, the run is out of control. The run must be rejected and the unknown specimen results cannot be reported. The source of error should then be identified and corrective action applied, before reanalysis.[71]

Another means of quality control is proficiency testing (PT). Homogeneous materials as similar as possible to specimens that would normally be tested are prepared by the organisation hosting the scheme, at various concentrations. Aliquots from the homogeneous materials are distributed to the laboratories taking part in the scheme, and each laboratory analyses the aliquots using its own analytical method. These External Quality Control (EQC) specimens should be treated identically to any unknown specimen that would usually be analysed, and are usually added to a routine run. The hosting body will then collate results from all participating laboratories and each laboratory will then receive a report containing all the data. These combined results allow laboratories to gain information on how their measurements compare with those of other laboratories, as well as how they compare with an external quality standard. In addition, most accreditation bodies require proof of participation in PT schemes.[80]

1.8.3. Significance Tests

One of the most important characteristics of an analytical method is that it be free from bias – that is, when the analytical quantity is assessed, the result obtained should not be statistically different from the true value. Since random errors will invariably be present in any measurement, it is necessary to determine whether the difference between the experimental result and the true value is significant or whether it is simply due to random error. In order to do this, significance tests are applied. Typically, these tests will make use of a null hypothesis, H_0, which assumes that the quantities being compared are in fact identical, and an alternative hypothesis, H_a, which assumes the opposite.[48, 80] Some of the most useful of these tests are explained below.

t-Test

It is often informative to compare an experimental mean with a known value, or indeed to compare two experimental means. This is frequently done by calculating a statistic known as a t-value, and comparing it with a certain critical value. The null hypothesis, H_0, is that the experimental mean and the known value, or the two means, are identical. In this case, should the t-value be less than the critical value, the null hypothesis would be satisfied.[80]

In order to assess whether the difference between an experimental mean, $\bar{x}$, and a true value, μ, is statistically significant, the t_{test} value is calculated as follows:

$$t_{test} = \frac{(\bar{x} - \mu)}{\frac{s}{\sqrt{n}}} \qquad \text{Equation 24}$$

where n is the sample size, and s is the sample standard deviation.[80]

The t_{test} statistic for two experimental means, $\bar{x}_1$ and $\bar{x}_2$, is calculated as follows:[80]

$$t_{test} = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad \text{Equation 25}$$

with n_1 and n_2 being the sample sizes of the sets that yielded the means $\bar{x}_1$ and $\bar{x}_2$, and s being the pooled standard deviation calculated by:[80]

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)} \qquad \text{Equation 26}$$

It is important to note that *Equation* 26 can only be applied if there is no significant difference between the standard deviations s_1 and s_2.

The absolute value of the calculated t_{test} value is then compared to the t_{crit} value, which can be found in a table of t critical values.[80]

F-Test

Often, especially when calculating pooled standard deviation, it is important to determine whether two standard deviations, or two variances, are the same. For this, a statistic denoted F_{test} is calculated and compared to a critical value, F_{crit}. Once again the null hypothesis, H_0, states that the standard deviations, or variances, are identical, while the alternative hypothesis, H_a, states the opposite. Should F_{test} be less than F_{crit}, the null hypothesis is supported. The F_{crit} value can be found in a table of critical F values, while F_{test} is calculated by:[80]

$$F_{test} = \frac{s_1^2}{s_2^2} \qquad \text{Equation 27}$$

where the subscripts 1 and 2 are assigned in such a way that $F_{test} \geq 1$.

Tests for Outliers

In many cases, it will be necessary to assess whether an experimental result belongs to a sample set or if it is an outlier. This is most commonly established by means of Grubbs' test for outliers, or Dixon's test for outliers. When assessing outliers in regression, it is more common to calculate Cook's Distance; however, Grubbs' test and Dixon's test may also be applied to the residuals. These three tests are described below.[80]

Grubbs' Test

Grubbs' Test for outliers is the test recommended by the International Organization for Standardization (ISO). It compares the difference between the suspect value and the sample mean with the standard deviation of the sample set. The null hypothesis, H_0, states that the suspect value does indeed belong to the sample population, while the alternative hypothesis, H_a, states that it is an outlier. The test statistic, G, is calculated as follows:

$$G = \frac{|suspect\ value - \bar{x}|}{s} \qquad \text{Equation 28}$$

where $\bar{x}$ is the sample mean, and s is the sample standard deviation. Both $\bar{x}$ and s are calculated with the suspect value *included*. Critical values can be found in literature and the suspect value is rejected if G is greater than the critical value.[80]

Dixon's Test

Another test for outliers is Dixon's test. This test, also sometimes called the Q-test, assesses the difference between the suspect value and the value closest in size to it with respect to the range of the measurements. Once again, the null hypothesis, H_0, states that the suspect value does indeed belong to the sample population, while the alternative hypothesis, H_a, states that it is an outlier. The test statistic, Q, is calculated as follows:[80]

$$Q = \frac{|suspect\ value - nearest\ value|}{largest\ value - smallest\ value} \qquad \text{Equation 29}$$

Critical values can be found in literature and the suspect value is rejected if Q is greater than the critical value.[80]

Cook's Distance

When generating regression data, it is common to use the least-squares method. This means that although the sum of the squares of the y-residuals is minimised, a large y-residual will have a large impact on both the gradient and intercept of the regression line generated. For this reason, it is important to be able to identify outliers in the regression data. By taking the y-residuals as a set of data, and identifying outliers therein, it is possible to identify outliers in the original regression data. That is, should a y-residual be identified as an outlier, the corresponding y-value will also be an outlier and can be omitted from the regression line.[80]

These residual outliers can be identified using Grubbs' test or Dixon's test; however, a more advanced method would be to calculate Cook's squared distance, CD^2, which is sometimes abbreviated to Cook's distance.[80]

Cook's distance is an "influence function", in that it measures the effect of rejecting the suspect regression point on the gradient and intercept. For a linear regression, CD^2 is calculated as follows:[80]

$$CD^2 = \frac{\sum_{j=1}^{n}\left(\hat{y}_j - \hat{y}_j^{(i)}\right)^2}{2s_{y/x}^2} \qquad \text{Equation 30}$$

where $\hat{y}_j$ is a predicted y-value when all the data points are included, $\hat{y}_j^{(i)}$ is the corresponding predicted y-value when the *i*th data point is excluded, and $s_{y/x}$ is the standard deviation of the regression line of y on x and is calculated with all data points included.[80]

A CD^2 value of greater than 1 warrants the exclusion of the suspect data point.

ANOVA

The t-test described above allows a way of testing for statistical differences between two experimental means, or between an experimental mean and a known value. However, very often it is necessary to compare more than two experimental means. In this case one usually assesses the significance of the variation due to a controlled or fixed effect, such as varying experimental conditions, analyst, or analytical method. Of chief interest then is to compare the within-batch variation with the between-batch variation. This is known as Analysis of Variance and is usually abbreviated to ANOVA.[48, 80]

As before, the null hypothesis, H_0, assumes that all experimental means are equal, while the alternative hypothesis, H_a, assumes that at least two means are different. When using ANOVA to test the variations between- and within-batch, the F-test is the principle statistical test employed, and a large F_{test} value in comparison to the F_{crit} value results in the rejection of the null hypothesis. The null hypothesis being true implies that the between-batch and within-batch variations are very similar, while rejection of the null hypothesis implies the between-batch variation is large with respect to the within-batch variation.[48, 80]

For the following explanation, let $\bar{x}_1, \bar{x}_2, \bar{x}_3, \ldots \bar{x}_I$ be the sample means of I sample sets, $s_1^2, s_2^2, s_3^2, \ldots s_I^2$ be the related variances, and $N_1, N_2, N_3, \ldots N_I$ be the numbers of measurements in each sample set. Define $\bar{\bar{x}}$ as the grand average; that is the average of all the data. It is calculated by summing all the data values and dividing by the total number of measurements.[48, 80]

Before the F_{test} variance ratio can be determined, several other quantities known as sums of squares need first to be calculated.

The sum of squares due to the factor (SSF) pertains to the between-batch variation and is calculated by:

$$SSF = N_1(\bar{x}_1 - \bar{\bar{x}})^2 + N_2(\bar{x}_2 - \bar{\bar{x}})^2 + N_3(\bar{x}_3 - \bar{\bar{x}})^2 + \cdots + N_I(\bar{x}_I - \bar{\bar{x}})^2 \qquad \text{Equation 31}$$

The sum of squares due to the error (SSE) pertains to the within-batch variation and is calculated by:

$$SSE = \sum_{j=1}^{N_1}(x_{1j} - \bar{x}_1)^2 + \sum_{j=1}^{N_2}(x_{2j} - \bar{x}_2)^2 + \sum_{j=1}^{N_3}(x_{3j} - \bar{x}_3)^2 + \cdots + \sum_{j=1}^{N_I}(x_{Ij} - \bar{x}_I)^2 \quad \text{Equation 32}$$

The sum of squares due to the error is also related to individual batch variances by:

$$SSE = (N_1 - 1)s_1^2 + (N_2 - 1)s_2^2 + (N_3 - 1)s_3^2 + \cdots + (N_I - 1)s_I^2 \quad \text{Equation 33}$$

The total sum of squares (SST) can be found simply by adding together the SSF and SSE.[48, 80]

In order to apply ANOVA reasoning, two assumptions need to be made. First, the variances of the populations are assumed to be equal, and second, the populations are assumed to be normally distributed.

Next, the number of degrees of freedom for each of the sums of squares must be found. The total sum of squares has N-1 degrees of freedom, and since SST is found by adding together SSF and SSE, the total degrees of freedom can be decomposed into the degrees of freedom for SSF and SSE.[48, 80]

There are I groups being compared, hence SSF has I-1 degrees of freedom, which leaves N-I degrees of freedom for SSE. This is expressed more concisely in the following formula.

$$SST = SSF + SSE$$
$$(N - 1) = (I - 1) + (N - 1) \quad \text{Equation 34}$$

By dividing the sums of squares by their corresponding degrees of freedom, quantities known as mean square values can be estimated.

The mean square due to the factor is given by:

$$MSF = \frac{SSF}{I - 1} \quad \text{Equation 35}$$

The mean square due to the error is given by:

$$MSE = \frac{SSE}{N - I} \quad \text{Equation 36}$$

The between-batch variance, σ_F^2, and the within-batch variance, σ_E^2, are related to MSF and MSE by:

$$MSE = \sigma_E^2 \qquad \text{Equation 37}$$

$$MSF = \sigma_E^2 + \sigma_F^2 \qquad \text{Equation 38}$$

Finally, the F_{test} statistic is calculated as the ratio of MSF to MSE:

$$F = \frac{MSF}{MSE} \qquad \text{Equation 39}$$

After calculating the F_{test} value, it should be compared to the F_{crit} value, obtained in a table of critical F values, and the hypotheses evaluated. [48, 80]

1.8.4. Method Validation

First and foremost, an analytical method must be fit-for-purpose. In other words, it must be able to perform the function for which it was intended and yield the analytical result required with a sufficiently small confidence interval. In order to assess a method's fitness-for-purpose, the method is validated, following which an estimate for the measurement uncertainty is obtained.[80]

A method should be validated if it is a new method, or if it is an existing method but some condition (e.g. instrumentation, matrix, laboratory) has changed. In addition, most accreditation bodies will require that a laboratory's methods be validated before granting them accreditation status.

Typically, method validation is done based on figures of merit, including linearity, limits of detection and quantitation, selectivity, specificity, bias, precision, and ruggedness. These are briefly outlined below.[80]

Linearity

It is common practice to plot the instrument response for several standards of known concentration against concentration, and then to use these plots to determine concentrations of unknown samples. The simplest model is linear; however, others can be applied provided the correct form is known. Generally it is assumed that, at least for certain concentration ranges, the models obtained in this fashion will be linear.[80]

In order to assess the extent of the linearity of the model, the product-moment correlation coefficient, r, can be calculated. A value for |r| close to 1 shows near-perfect correlation between the

experimental points and the best-fit linear regression line obtained. Often it is the r^2 value that is used to gauge linearity, and this should also be close to 1 to indicate a good linear fit.[80]

The value of r can be calculated by:[80]

$$r = \frac{\sum_i [(x_i - \bar{x})(y_i - \bar{y})]}{\{[\sum_i (x_i - \bar{x})^2][\sum_i (y_i - \bar{y})^2]\}^{\frac{1}{2}}} \qquad \text{Equation 40}$$

Regression Confidence Limits

Plotting the instrument response (y) against known concentration (x) will yield a regression model with the equation

$$y = a + bx \qquad \text{Equation 41}$$

where a is the y-intercept and b is the gradient.[80]

It is the gradient and intercept of this linear regression model that will be used to interpolate concentrations of unknown specimens. It is thus vital that the random errors in the gradient and intercept be determined.

In order to do this, the statistic $s_{y/x}$ must first be calculated. This statistic estimates the random errors in the y-direction. It should be noted that for these models it will be assumed that errors only occur in the y-direction; that is, the x-direction is free from errors entirely. $s_{y/x}$ is calculated by:[80]

$$s_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n - 2}} \qquad \text{Equation 42}$$

where y_i are the instrument response y-values, $\hat{y}_i$ are the "fitted" y-values calculated as the best-fit line y-values, and n is the number of different concentration points. This means that the n – 2 term is the number of degrees of freedom for the regression line.

The standard deviations of the intercept and gradient, s_a and s_b respectively, can now be determined by:

$$s_a = s_{y/x} \sqrt{\frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x})^2}} \qquad \text{Equation 43}$$

$$s_b = \frac{s_{y/x}}{\sqrt{\sum_i (x_i - \bar{x})^2}}$$ Equation 44

where x_i are the x-values or concentration values, $\bar{x}$ is the mean of the x-values, and n is the number of different concentration points.[80]

The confidence limits for the intercept can then be estimated by:

$$a \pm t_{(n-2)} s_a$$ Equation 45

and that for the gradient by:

$$b \pm t_{(n-2)} s_b$$ Equation 46

where a and b are the intercept and gradient respectively, $t_{(n-2)}$ is the t-value for n – 2 degrees of freedom, and s_a and s_b are the standard deviations calculated by *Equation 43* and *Equation 44*. The t-value can be obtained in a literature t-value table.[80]

Limits of Detection and Quantitation

The limit of detection gives an estimate of the lowest concentration of the analyte that the method can detect, while the limit of quantitation estimates the lowest concentration that the method can reliably quantify. Clearly low limits of detection and quantitation are desirable for an analytical method, since both give an indication of the sensitivity of a method.[80, 89]

Specificity and Selectivity

Specificity and selectivity are performance characteristics of analytical method, and are terms that are often confused, since both deal with the ability of the method to distinguish the analyte. However, selectivity is the ability of the method to correctly distinguish the analyte from other components present in the sample matrix without interference, while specificity is the ability of the method to correctly report a negative when the analyte of interest is not present.[71, 89, 90]

Bias

Also called trueness, bias is an indication of how closely the result obtained when the method is applied to a reference material agrees with the reference value. In order to assess this, the method should be applied several times to assess the analyte content of a reference material and the results investigated for significant differences from the known reference value.[80]

Precision

As stated previously, precision is a measure of the agreement between individual replicate measurements, and it is usually expressed as a standard deviation or a relative standard deviation. A reference material is analysed many times over varying experimental conditions, generally at two or more concentration levels, and a one-way ANOVA is applied to assess repeatability and reproducibility.[80]

Ruggedness

The ruggedness of a method is a gauge of the extent to which experimental conditions can vary before the results are significantly affected. These experimental conditions could include temperature, pH, instrumental conditions, and analyst, amongst others. The simplest way of assessing a method's ruggedness is to vary one condition at a time and monitor the result. In this way, it is possible to not only establish which factor influences the final result most significantly, but also, if necessary, to estimate ranges over which certain parameters may vary without significantly influencing the result.[80]

1.8.5. Measurement Uncertainty

By now it is clear that all analytical results are subject to variation due to random errors and method bias. As such, it is highly unlikely that the measurement result will be exactly equal to the true value. In fact, reporting only the result would be incorrect and virtually meaningless, since it gives no information on the range of possible values within which the true value is expected to lie.

Measurement uncertainty (MU) is a single, non-negative parameter that characterises the dispersion of values attributed to the measurand, and should take into account all uncertainty contributors. It is a symmetrical interval around the measured value within which the true value is expected with some level of probability to lie. Measurement Uncertainty thus gives an indication of the expected variability in the experimental result produced, which after all is only an estimate of the true value.[79-81, 91-93] It would seem pertinent now to make the distinction between "error" and "uncertainty". "Error" is the difference between a measured value and the true value, which in principle could be used to correct a measured result, and should as far as possible be eliminated from the result. "Uncertainty" is a range or interval which cannot be used to correct a measured result, and is always present.[92, 93]

There are two ways in which uncertainty can be expressed. Standard uncertainty, denoted *u*, expresses uncertainty as a standard deviation, whereas expanded uncertainty, *U*, defines a range that includes a large percentage of values within which the true value will lie. The expanded uncertainty is obtained by multiplying *u* with a coverage factor, *k*, which is chosen according to the level of confidence required. For example, a coverage factor of 2 would afford a confidence level of 95%, and would imply that there is a 95% probability that the true value would lie in the range $\bar{x} \pm U$, where $\bar{x}$ is the experimental result. When no information is given, k is usually assumed to be 2.[80]

In order to accurately report analytical results and subsequently to assess their statistical significance, it is essential that the MU be estimated. It is the responsibility of the reporting laboratory to evaluate the MU, or confidence interval, for each result and ensure that it is fit-for-purpose.[79, 91]

In evaluating the MU for a method, it is important that all sources of uncertainty are accounted for – both random and systematic, although systematic errors should be minimised as far as possible. Generally, the standard uncertainties, *u*, for each source of uncertainty are pooled as the square root of the sum of the squares, to yield the combined standard uncertainty, also denoted *u*. The confidence interval is then obtained by applying the appropriate converge factor, and acquiring *U*. [91]

Types of Uncertainties

Literature will categorise uncertainty components as either Type A or Type B depending on how they are evaluated.[91]

Type A components are those that can be determined from actual experimental data for which a standard deviation can be calculated. For example, the repeated analysis of IQC samples yields a standard deviation indicative of the precision of the method.[79, 81, 91]

Type B components, on the other hand, are those for which no experimental data can be obtained, and these must then be acquired by alternative means. An example of a Type B component would be the uncertainty associated with a CRM as given on the certificate of analysis. Type B components are literature values.[79, 81, 91]

Uncertainty Estimation

Although there is no universally accepted method for calculating MU, there are two generally accepted approaches.

The *bottom-up* approach is the "modelling" approach, which seeks to identify all potential sources of uncertainty at each separate stage of analysis and assign each one a value, expressed as standard deviations. These standard uncertainties are then combined to obtain the combined standard uncertainty. While this may seem to be a simple process, in reality it can become a quite complex exercise. Simple analytical processes may involve many individual steps, each of which has the potential to introduce error into the final result. Often, an overly optimistic uncertainty estimate is obtained, since some sources of error are neglected. It is thus imperative that *all* uncertainty sources be identified, and as such the calculation can become quite long-winded and involved.[79-81]

In contrast, the *top-down* approach is the "empirical" approach and makes use of data from validation, IQC analysis and PT schemes to estimate the overall uncertainties of measurements without trying to identify each individual uncertainty component. For this approach, the data used should be collected over a minimum period of six months, but this clearly will depend on the frequency of analysis. By taking data over a long period of time, the uncertainty estimate will include errors due to variation in

time, experimental conditions, analyst, reagents, and routine instrument maintenance, amongst other sources of error. The top-down approach is generally considered to be the more practical approach to uncertainty estimation.[79-81]

Other approaches to estimating MU involve combinations of the above top-down and bottom-up approaches to varying degrees.[81] It is, however, left to the discretion of the laboratory as to how the MU is calculated. According to the ISO 17025:2005 General requirements for the competence of testing and calibration laboratories[94], the only requirement is that testing laboratories have an adequate estimation for the measurement uncertainty of a method, and that all important components that contribute to the uncertainty are taken into account.

A Note on Significant Figures

It is important when performing calculations involving standard deviations to report final results to the correct number of significant figures. That is, both the final concentration result as well as the associated measurement uncertainty should be reported to the appropriate number of digits to properly express the precision of the final answer.

When calculating sums or differences, the result should contain the same number of decimal places as the number with the least decimal places. When calculating products or quotients, the result should be rounded off to contain the same number of significant figures as the initial number with the least number of significant figures. When calculating logarithms, the final result should contain the same number of decimal places as there are significant figures in the original number, while for antilogarithms there should be the same number of digits in the result as there are decimals in the original number.[48]

Finally, the expanded measurement uncertainty, U, should be expressed to at most two significant figures, and the number of decimal places in the measurement result should correspond to those in this uncertainty.[81]

Rounding should always be delayed until the final result is calculated and particular care should be taken. Incorrect rounding or rounding too soon can give inaccurate impressions of precision and can affect the statistical use of results.[81]

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Chapter 2

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2.1. Introduction

While Blood Alcohol Concentration (BAC) determinations are, for the most part, conducted by means of Gas Chromatography – Flame Ionisation Detection (GC-FID)[1, 2], clinical laboratories that analyse for a number of different analytes tend to make use of Gas Chromatography – Mass Spectrometry (GC-MS) for their various analyses.[3] These laboratories would not necessarily have access to a dedicated GC-FID instrument for BAC determinations, and as such a GC-MS method for the determination of ethanol concentration in whole blood would be more applicable to their needs.

Therefore, what is presented is the development and validation of a GC-MS method for the determination of BAC by means of isotope dilution. This method requires no dedicated instrumentation, such as a GC-FID, or specialised equipment. Separation is achieved on a mid-polar column installed on a GC-MS instrument that is used in a toxicology setting for the routine analysis of drugs of abuse.

Further, a full tutorial-style calculation for the expanded measurement uncertainty for the method is given, such that it could be followed for any analytical method in order to estimate the measurement uncertainty thereof.

2.2. Experimental

2.2.1. General Details

Certified Reference Materials

Aqueous ethanol Certified Reference Material (CRM) (20.909 ± 0.251 g/100 mL) was purchased from the National Metrology Institute of South Africa (NMISA) and is henceforth referred to as ethanol standard. (See Certificates of Analysis in ***Appendix E***)

Stable isotope labelled ethanol-d6 (99%) was obtained from Sigma-Aldrich, Midrand, South Africa.

Reagents and Solvents

Sodium hydrogen carbonate ($NaHCO_3$, 99%) and Sodium carbonate (Na_2CO_3, 99%) were purchased from Merck, Steinheim, Germany; pentafluorobenzoyl chloride (C_7ClF_5O, 99%) (PFBCl) was obtained from Sigma-Aldrich, Midrand, South Africa; sodium hydroxide pellets (NaOH, 97.0%) were acquired from Merck, Worli, Mumbai, and glycine ($C_2H_5NO_2$, 99.7%) from Merck, Darmstadt, Germany.

All solvents were analytical grade and were used without further preparation.

Dichloromethane (CH_2Cl_2, pesticide grade) and isopropanol (C_3H_8O, 99.9%) were obtained from Sigma-Aldrich, Steinheim Germany. Acetonitrile (CH_3CN, HPLC grade) was purchased from Sigma-Aldrich, Midrand, South Africa, while deionised water was sourced from Merck, Modderfontein, South Africa.

Whole blood

Throughout this work whole blood will be referred to as either “pooled blood” or “fresh blood”. Pooled blood shall refer to blood obtained from the Department of Health, Pretoria, which was prepared by pooling various blood alcohol specimens that were scheduled for destruction. Fresh blood shall refer to blank whole blood collected from healthy volunteers in evacuated tubes (Vacuette® tubes, Greiner Bio-One International, Frickenhausen, Germany) containing sodium heparin anticoagulant in accordance with ethical standards (See ***Appendix F***). This blood was also pooled prior to use, but contained only endogenous ethanol – that is, naturally occurring ethanol at a very low level.

Instrumentation and Equipment

An Agilent 7890A Gas Chromatographic system fitted with an Agilent 7683 Autoinjector and a 5975C Mass Selective Detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis.

The inlet temperature was set at 230 °C and a constant helium carrier gas flow rate of 2.0 mL/min was used. Sample injection (2 µl) was performed in split mode (20:1) onto a mid-polar fused silica column (ZB5-MSi, 15 m x 0.25 mm, d_f = 0.25 µm, Phenomenex, California, USA). The temperature program had an initial isotherm of 60 °C maintained for 1 minute, followed by a single ramp of 60 °C/min to a temperature of 320 °C which was then maintained for 1 minute. This resulted in a total chromatographic time of 6.33 minutes.

The MSD transfer line temperature was set at 280 °C, while the temperatures of the quadrupole and source were 150 °C and 230 °C respectively. A solvent delay time of 1 minute was set before the electron ionisation (EI) source was turned on and all mass spectra were recorded at 70 eV in selected ion monitoring (SIM) mode unless otherwise stated, in which case they were recorded in scan mode.

Processing of chromatographic and mass spectrometric data was performed using Agilent ChemStation software.

Selected ion monitoring mode

When collecting data in SIM mode, the characteristic qualifier ions 212 m/z and 167 m/z were monitored for the ethyl pentafluorobenzoate, while the quantifier ions for the ethyl pentafluorobenzoate and corresponding deuterated internal standard were 240 m/z and 245 m/z respectively.

Scan mode

When data was collected in scan mode, a mass range of 50 m/z to 500 m/z was monitored, and the quantifier ions 240 m/z and 245 m/z for the ethyl pentafluorobenzoate and corresponding deuterated internal standard respectively were extracted for quantitation purposes.

Sample preparation conditions

All experimental procedures were performed at room temperature (22 ± 6 °C) unless otherwise stated.

Preparation of calibrators and controls

Standard working solutions were prepared from the aqueous ethanol CRM (20 g/100 mL) according to *Table* 1, while the internal standard- and control working solutions were prepared according to *Table 2.*These were prepared afresh for each analytical run by serial dilution. $V_{Previous\ Std}$ denotes the volume required of the standard with the next highest concentration.

Table 1: Preparation of standard working solutions from aqueous ethanol stock solution

Working Solution	1	2	3	4	5	6	7
$V_{Previous\ Std}$ (µl)	200	250	667.4	749.2	728.6	785	350
V_{H2O} (µl)	800	750	332.6	250.8	271.4	215	650
V_{Final} (µl)	1000	1000	1000	1000	1000	1000	1000
C_{Final} (g/100 mL)	0.1001	0.5005	2.002	3.000	4.004	5.495	7.000

Table 2: Preparation of internal standard- and control working solutions

Working Solution	Internal Standard	Low Control	Medium Control	High Control
V_{Stock} (µl)	20*	10 **	25 **	150 **
V_{H2O} (µl)	1480	990	975	850
V_{Final} (µl)	1500	1000	1000	1000
C_{Final} (g/100 mL)	1.164	0.2000	0.5000	3.000

* 20 µl ethanol-d6 (99%, 17.66 mg)

** Prepared directly from 20 g/100mL aqueous ethanol stock solution

The respective working solution or control solution (**50 µl**) as well as internal standard solution (**50 µl**) was added to a reaction tube containing whole blood (**450 µl**).

Preparation of reagent blank and unknown specimens

To a reaction tube containing whole blood (**450 µl**) the respective working solution or control solution (**50 µl**) as well as internal standard solution (**50 µl**) was added."

The below sample preparation methods were then applied to reagent blanks, calibrators, controls and unknown samples.

2.2.2. Method Development

A. Supplied Method for Sample Workup

After thorough mixing and equilibration for 30 minutes, acetonitrile (**700 µl**) was added to each sample tube. These tubes were then vortexed for 30 seconds to allow precipitation of proteins, and centrifuged at 3000 rpm. The clear upper layer that resulted was transferred to a new tube, to which was added saturated $NaHCO_3$ solution (**1000 µl**), dH_2O (**500 µl**) and PFBCl solution (**1000 µl, 5% (v/v) in CH_2Cl_2**). The tubes were capped and placed on the multi-shaker for 3 hours. Following this, the tubes were centrifuged at 3000 rpm and the organic layer transferred to an amber GC vial. These sample solutions were dried under compressed air at 35 °C, reconstituted with CH_2Cl_2 (**100 µl**) and transferred to a glass insert, before being analysed by GC-MS.

B. Water Washes

The supplied method was followed; however, instead of transferring the organic layer directly to a GC vial it was placed in a new reaction tube and subjected to **a)** one wash with dH_2O (**1000 µl**); or **b)** three washes with dH_2O (**1000 µl each**). The organic layer was then transferred to an amber GC vial, dried under compressed air at 35 °C, and reconstituted with CH_2Cl_2 (**100 µl**). This was transferred to a glass insert and analysed by GC-MS.

C. Solid Base and Water Washes

The supplied method was followed with each of the following adjustments:

a) After shaking for 3 hours, a scoop of solid $NaHCO_3$ was added and the tube was vortexed for 30 seconds; or

b) After shaking for 3 hours, the organic layer was transferred to a new reaction tube. A scoop of solid $NaHCO_3$ and dH_2O (**500 µl**) were added and the tube vortexed for 30 seconds; or

c) After shaking for 2 hours, the aqueous $NaHCO_3$ layer was removed to waste, a scoop of solid $NaHCO_3$ added and shaking resumed for the final hour. The organic layer was transferred to a new tube and subjected to two dH_2O washes (**1000 µl**); or

d) After shaking for 2 hours, the aqueous $NaHCO_3$ layer was removed to waste, a scoop of solid Na_2CO_3 added and shaking resumed for the final hour. The organic layer was transferred to a new tube and subjected to two dH_2O washes (**1000 µl**).

Finally, the organic layer was transferred to an amber GC vial, dried under compressed air at 35 °C, and reconstituted with CH_2Cl_2 (**100 µl**). This was transferred to a glass insert and analysed by GC-MS.

D. Liquid Base and Water Washes

The supplied method was followed with each of the following adjustments:

Either, after 3 hours shaking, the organic layer was transferred to a new reaction tube and subjected to:

a) one wash with saturated $NaHCO_3$ solution (**1000 µl**); or
b) two washes with saturated $NaHCO_3$ solution (**1000 µl each**); or
c) one wash with saturated Na_2CO_3 solution (**1000 µl**); or
d) two washes with saturated Na_2CO_3 solution (**1000 µl each**); or
e) one wash with cold NaOH solution (**1000 µl, 1 M, approx. 15 °C**); or
f) two washes with NaOH solution (**1000 µl, 1 M**); or
g) one wash with saturated $NaHCO_3$ solution (**1000 µl**). Following this, the aqueous $NaHCO_3$ layer was removed to waste, and the remaining organic layer washed with dH_2O (**1000 µl**); or
h) one wash with saturated Na_2CO_3 solution (**1000 µl**). Following this, the aqueous Na_2CO_3 layer was removed to waste, and the remaining organic layer washed with two volumes of dH_2O (**1000 µl each**); or
i) one wash with saturated $NaHCO_3$ solution (**1000 µl**). Following this, the aqueous $NaHCO_3$ layer was removed to waste, and the remaining organic layer washed with dH_2O (**1000 µl**), and filtered through a Na_2CO_3 plug;

or, after 2 hours shaking, the aqueous $NaHCO_3$ layer was removed to waste,

j) a further measure of aqueous $NaHCO_3$ (**1000 µl**) was added and shaking resumed for 1 hour. The organic layer was transferred to a new reaction tube, and subjected to two washes with dH_2O (**1000 µl each**); or
k) aqueous Na_2CO_3 (**1000 µl**) was added and shaking resumed for 1 hour; or
l) aqueous Na_2CO_3 (**1000 µl**) was added and shaking resumed for 1 hour. The organic layer was transferred to a new reaction tube, and subjected to two washes with dH_2O (**1000 µl each**).

Finally, the organic layer was transferred to an amber GC vial, dried under compressed air at 35 °C, and reconstituted with CH_2Cl_2 (**100 µl**). This was transferred to a glass insert and analysed by GC-MS.

E. Reducing Derivatisation Reagent Concentration

The supplied method was followed; however, the recommended 5% (v/v) PFBCl solution was replaced with a 2% (v/v) PFBCl solution (**1000 µl**).

F. Introducing an Alternative Analyte

The supplied method was followed with each of the following adjustments:

a) After shaking for 3 hours, isopropanol (**250 µl**) was added and the tube thoroughly vortexed; or
b) After shaking for 3 hours, isopropanol (**750 µl**) was added and the tube thoroughly vortexed; or
c) After shaking for 3 hours, the organic layer was transferred to a new reaction tube, isopropanol (**1750 µl**) was added and the tube vortexed thoroughly; or
d) After shaking for 3 hours, the organic layer was transferred to a new reaction tube, isopropanol (**1750 µl**) was added and the tube vortexed thoroughly. The resulting solution was then washed with aqueous $NaHCO_3$ (**1000 µl**); or
e) After shaking for 3 hours, the organic layer was transferred to a new reaction tube, isopropanol (**1750 µl**) was added and the tube vortexed thoroughly. The resulting solution was then filtered through a Na_2CO_3 plug; or
f) After shaking for 3 hours, the organic layer was transferred to a new reaction tube, isopropanol (**1750 µl**) was added and the tube vortexed thoroughly. The resulting solution was then washed with 5 portions of NaOH solution (**1000 µl, 1 M**); or
g) After shaking for 3 hours, the organic layer was transferred to a new reaction tube and subjected to a wash with saturated aqueous glycine solution (**1000 µl**).

Finally, the organic layer was transferred to an amber GC vial, dried under compressed air at 35 °C, and reconstituted with CH_2Cl_2 (**100 µl**). This was transferred to a glass insert and analysed by GC-MS.

2.2.3. Method Validation

Method validation was performed based on figures of merit including linearity, limit of detection, limit of quantitation, selectivity, specificity, bias, precision, and ruggedness.

Linearity

Five response models of relative response (240/245) versus ethanol concentration were prepared on different days, using the working solutions outlined in *Table 1* and *Table 2*. From these, the 95% confidence interval of the correlation coefficient was determined and the linearity of the method assessed.

Limits of Detection and Quantitation

A response curve prepared using the working solutions outlined in *Table 1* was used to calculate the theoretical limit of detection (LOD) and limit of quantitation (LOQ) for the method. Following this, serial dilution of the low control working solution in *Table 2* allowed the preparation of eight further working solutions – each at a concentration half of that of the previous solution – ranging from 0.01 g/100 mL to 0.000078125 g/100 mL. Samples prepared from these working solutions were then

used to experimentally determine the LOD and LOQ for the method by means of signal-to-noise (S/N) ratios. Finally, the theoretical and experimental LODs and LOQs were compared and the most applicable was chosen.

Selectivity

Pairs of calibrators were prepared at the concentrations given in *Table 1* on five different days. After removing any outliers identified by the Grubbs' test, the 95% confidence intervals for the abundance ratios 240/212, 240/195, 240/167, 212/195, 212/167 and 195/167 were determined.

Specificity

Ten sets of duplicate blank samples were prepared using fresh blood from ten different volunteers, and assessed for ethanol content.

Bias and Precision

Five internal quality control (IQC) samples at three concentration levels (0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL) were prepared on five different days. Each group on each day was inspected for outliers, and, after removing any, these experimentally obtained ethanol concentrations were used to evaluate the bias and precision of the method as described below.

A bias correction regression line was obtained by plotting average experimental ethanol concentration versus theoretical ethanol concentration. From this, the method was assessed for multiplicative- and additive bias.

In order to evaluate the precision of the method, ANOVA was applied to the quality control data to determine the within- and between-group variances. The overall precision of the method was also estimated.

Ruggedness

a) *Derivatisation Time*

Twenty-five IQC samples were prepared at the medium control concentration level (0.05 g/100 mL), and after 2 hours of mixing, five samples were removed from the multi-shaker. Thereafter, five samples were removed every half-hour until a total mixing time of 4 hours was reached. Each group of samples was analysed by GC-MS for ethanol concentration and ANOVA was performed to establish whether deviating from the prescribed derivatisation time had a significant effect on the final ethanol concentration result.

b) *Derivatisation Reagent Concentration*

Fifteen IQC samples were prepared at each of the three control levels (0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL). Each set was then divided into three groups of five samples which were subjected to derivatisation reagent concentrations of 2% (v/v), 5% (v/v) and 10% (v/v)

respectively. ANOVA was then performed on the resulting groups of ethanol concentrations to determine whether variations in the derivatisation reagent concentration had a significant impact.

c) Drying Process

Six IQC samples were prepared at the medium control concentration level (0.05 g/100 mL). Upon drying under compressed air, three samples were allowed to dry, while three were removed from the drying manifold once only a small volume of solution remained. The t-test and F-test were applied to assess significant differences between the means and standard deviations respectively of the two sample sets.

d) Stability of Derivatised Analyte

One IQC sample was prepared at the medium control concentration level (0.05 g/100 mL), and placed on the autosampler tray. The sample was injected sixteen times, approximately once every hour, yielding sixteen concentration results spanning over approximately 16 hours on the autosampler tray. These concentrations were plotted, and their standard deviation compared to that of the samples used in the bias and precision evaluations.

2.2.4. Measurement Uncertainty Calculations

Control Charts

Over a period of 13 months, experimental concentration values were collected at the three IQC levels of 0.02 g/100mL, 0.05 g/100mL, and 0.30 g/100mL ethanol. This data was plotted on Levey-Jennings control charts along with the theoretical concentrations, the average concentrations and the confidence limits at 1, 2 and 3 standard deviations. Outliers were identified using both the Dixon and the Grubbs' tests, and while included on the plots for completeness, were excluded from the data sets in all further calculations.

Normality

Before proceeding, the normality of each of the three data sets was confirmed by comparing the plots of experimental concentration against experimental Cumulative Distribution Function (CDF) values with the plots of experimental concentration against theoretical CDF. (See ***Appendix D***)

Measurement Uncertainty

Finally, the expanded measurement uncertainty (MU) for the method was calculated using the IQC data at the three levels. A hybrid of top-down and bottom-up approaches was followed, based on the methodology of Gullberg[4] while including aspects from the SAC-SINGLAS Technical Guide on Measurement Uncertainty in Medical Testing.[5] The overall MU was calculated as the combined contributions of the ethanol CRM, pipettes, bias, and imprecision, and is reported as the expanded MU at both 95% and 99% confidence.

All concentration values were the mean value of two replicates.

2.3. Results and Discussion

2.3.1. Method Development

A. Supplied Method for Sample Work-up

Ethanol is a small, volatile organic molecule and thus, in order to make it more conducive to analysis by GC-MS, it is necessary to first derivatise the ethanol by means of PFBCl (structure **2**). This process is illustrated in *Figure 1* below. As such, it is the resulting ethyl esters of the analyte and internal standard – ethyl pentafluorobenzoate and ethyl pentafluorobenzoate-d5 – that are monitored (structures **3a** and **3b**).

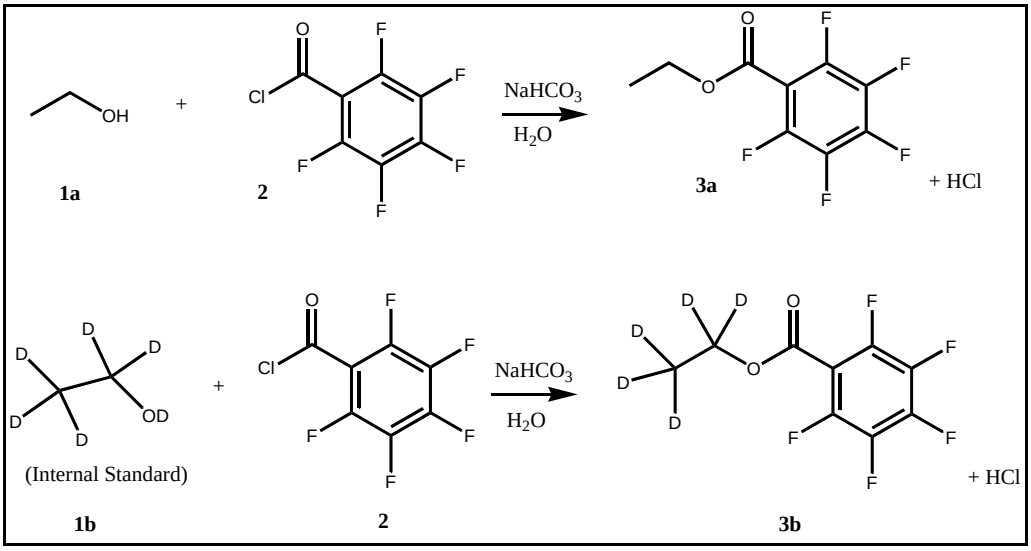


Figure 1: Reaction scheme for the derivatisation of ethanol and ethanol-d6

The ethyl esters typically eluted between 2.08 ± 0.12 minutes, and had characteristic ions at m/z 167, m/z 195, m/z 212, m/z 213, m/z 240 and m/z 245. A typical mass spectrum and extracted ion chromatograms (XIC) for the derivatised ethanol (dEtOH) and derivatised internal standard (dEtOH-d6) are shown in *Figure 2*.

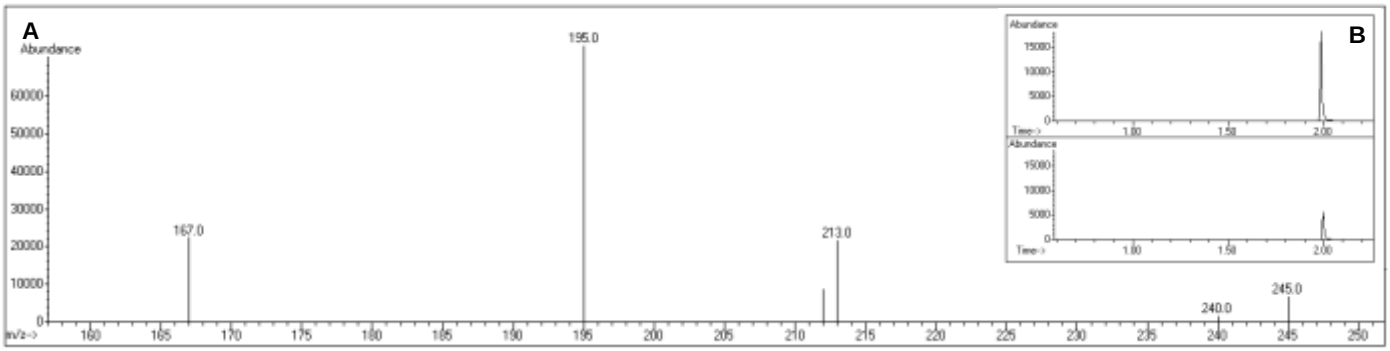
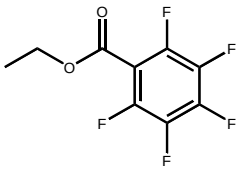
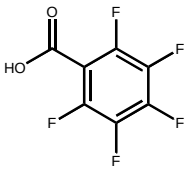
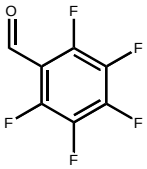
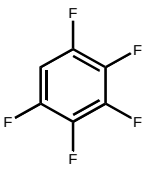
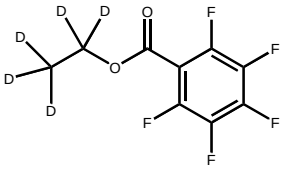
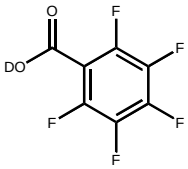


Figure 2 A: Mass spectrum for the analyte and internal standard ethyl esters obtained in SIM mode
B (inlaid): Extracted ion chromatograms for dEtOH (m/z 240) and dEtOH-d6 (m/z 245)

The fragments responsible for the characteristic ion signals shown in the mass spectrum are given in *Table 3*. It is important to note that although m/z 213, m/z 212, m/z 195 and m/z 167 are monitored as characteristic ions, these ions do not contain any part of the original analyte backbone. This is because ethanol is so small. Nevertheless, the presence of these ions in the mass spectrum in the correct ratios is required for a positive identification of ethanol at a specific retention time. That is, these four ions are considered qualifier ions. Conveniently, the molecular ions of both the derivatised analyte (m/z 240) and internal standard (m/z 245) are observed and can subsequently be used as quantifier ions.

Table 3: Structures of ions contributing to the signals in the mass spectra of dEtOH and dEtOH-d6

Ethanol	 **m/z 240**	 **m/z 212**	 **m/z 195**	 **m/z 167**
Ethanol-d6	 **m/z 245**	 **m/z 213**		

The supplied method yielded acceptable results for the quantitation of ethanol in whole blood; however, it was found to be harsh on the column of the GC, requiring the column to be replaced more often than is usual – or desired – in a routine laboratory setting. Closer inspection of the Total Ion Chromatograms (TICs) of samples prepared in this manner revealed large quantities of pentafluorobenzoic acid (PFB-COOH) at m/z 212 and typical retention times of 2.3 minutes to 2.6 minutes. This is illustrated in *Figure 3* below.

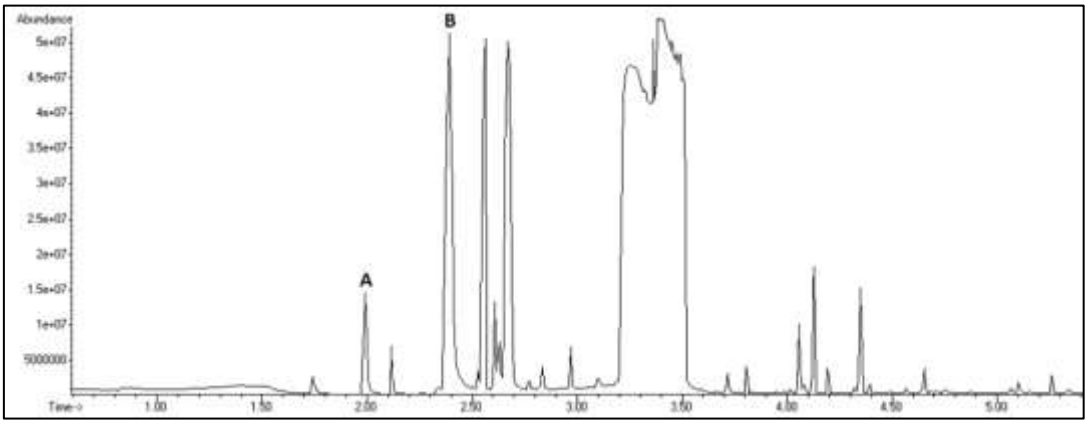


Figure 3: Typical TIC of sample prepared by the supplied method and analysed by GC-MS on scan mode, with **A:** dEtOH (m/z 240) and dEtOH-d6 (m/z 245); **B:** PFB-COOH (m/z 212)

Relatively few compounds are damaging to the stationary phase of a GC column. In fact, the only organic compounds that have been known to degrade stationary phases are perfluoroacids, and even then these need to be present at 1% or higher to do any real damage.[6] The use of PFBCl in such excess for the derivatisation of ethanol, results in high concentrations of PFB-COOH, which is detrimental to column condition. It was thus necessary to investigate adjusting the sample preparation procedure in order to minimise the PFB-COOH present upon injection onto the column.

The ideal method would not only decrease the PFB-COOH, but would also ensure that the dEtOH:dEtOH-d6 ratio remain constant – in other words, whatever alteration is made to the supplied method should not affect the final ethanol concentration result in any way. Hence, when considering each new method, it was necessary to evaluate not only the PFB-COOH:dEtOH-d6 ratio (212/245) but also the dEtOH:dEtOH-d6 ratio (240/245), and choose the method that caused the greatest decrease in 212/245 while keeping 240/245 as constant as possible.

The relative areas of the PFB-COOH (212/245) and dEtOH (240/245) of the adjusted method can be expressed as percentages of the respective relative areas of the supplied method, using *Equation 1*. In this way, the impact of the adjustment in sample preparation on the concentrations of PFB-COOH and dEtOH can be gauged.

$$Rel\ Area\ \% = \frac{Rel\ Area\ (adjusted\ method)}{Rel\ Area\ (supplied\ method)} \times 100 \qquad \text{Equation 1}$$

It should be noted that the relative areas used in *Equation 1* are mean values (n = 2), except where otherwise stated.

In order for the additional step in the sample preparation to be beneficial, it should result in a small PFB-COOH % while maintaining a dEtOH % as close to 100 as possible.

Potential alterations to the supplied method included introducing additional steps in the sample workup; the use of a base other than $NaHCO_3$ – or a base in a different form; adjusting the derivatisation step; and even the addition of another compound to consume the remaining PFBCl. Each of these is discussed below, in light of the requirements imposed upon the 212/245 and 240/245 ratios.

For each adjusted method, the relative area percentages are reported along with their corresponding standard deviations, calculated by means of *Equation 2*, according to the propagation of error.

$$\frac{s_{Rel\ Area\ \%}}{Rel\ Area\ \%} = \sqrt{\left(\frac{s_{AM}}{Rel\ Area\ (AM)}\right)^2 + \left(\frac{s_{SM}}{Rel\ Area\ (SM)}\right)^2} \qquad \text{Equation 2}$$

In *Equation 2*, the s values are the standard deviations of the relative area percentage, relative area of the adjusted method (AM), and relative area of the supplied method (SM) respectively, with n = 2. For each adjusted method the standard deviations of the relative percentages are reported at 95% confidence (k = 2) as "2s" values.

In some instances, data was obtained for only one sample prepared according to the supplied method. In such cases, where n = 1, it is assumed that there is no uncertainty in the relative areas of this sample, and only the uncertainty in the adjusted method contributes to the overall uncertainty of the calculated relative area %. *Equation 3* is thus used to determine the standard deviation in the relative area %, $s_{Rel\ Area\ \%}$.

$$\frac{s_{Rel\ Area\ \%}}{Rel\ Area\ \%} = \sqrt{\left(\frac{s_{AM}}{Rel\ Area\ (AM)}\right)^2} = \frac{s_{AM}}{Rel\ Area\ (AM)} \qquad \text{Equation 3}$$

Equation 2 will, however, be used throughout, unless otherwise explicitly stated.

B. Water Washes

Since benzoic acid ($C_7H_6O_2$) is soluble in water[7], it is reasonable to suggest that PFB-COOH might also be, hence it was hypothesised that washing the sample solution with sufficient portions of dH_2O should remove the majority of it. However, whether this would affect the final ethanol concentration result still needed to be determined.

Table 4 shows the relative area percentages, obtained by applying *Equation 1*, as well as the corresponding 2s-values, obtained by applying *Equation 2*, for the adjusted methods involving water washes.

Table 4: Relative areas of the PFB-COOH (212/245) and dEtOH (240/245) of the adjusted methods involving water washes expressed as a percentage of the relative areas of the supplied method, and the corresponding 2s-values (95% confidence)

Method	PFB-COOH (%)	$2s_{PFB\text{-}COOH}$ (%)	dEtOH (%)	$2s_{dEtOH}$ (%)
Ba	62.0	41.0	105.1	29.4
Bb	69.0	39.3*	76.7	28.8*

* Calculated according to *Equation 3*

The letter code provided under Section 2.2.2 will be applied in the following discussion.

Although method **Ba** does not significantly impact the dEtOH concentration – evidenced by the dEtOH % that is not statistically different from 100% – the PFB-COOH concentration is still unacceptably high.

Method **Bb** appears to have even less of an effect on the PFB-COOH concentration than method **Ba**. In addition, while the dEtOH concentration is not significantly different from the control, there is a greater chance of this adjusted method under-estimating the ethanol concentration of samples, as seen by the lower dEtOH % of 76.7%. At 95% confidence, more often than not, the dEtOH % will be lower than 100%.

Both methods **Ba** and method **Bb** show potential by decreasing the PFB-COOH concentration while not significantly affecting the final ethanol concentration result. However, neither method is considered to be sufficiently effective in removing the PFB-COOH and consequently other alternative methods needed to be investigated.

C. Solid Base and Water Washes

Considering the marginal effect of simply washing the sample solutions with dH_2O, the possible addition of a base in the work-up procedure was then investigated. In the supplied method, aqueous $NaHCO_3$ – a weak base – was utilised during the derivatisation process. It was theorised that perhaps the acidic PFB-COOH could be neutralised by the addition of an anhydrous base after derivatisation. Furthermore, the strength of base as well as the presence or absence of water was considered. A stronger base may be more effective in reducing the PFB-COOH, and subsequently washes with water could remove the salt of the PFB-COOH that was formed on adding the base. Once again, the effect on the EtOH concentration of each adjustment to the supplied method was assessed.

Table 5 shows the relative area percentages as well as the corresponding 2s-values for the adjusted methods involving solid base and water washes.

Table 5: Relative areas of the PFB-COOH (212/245) and dEtOH (240/245) of the adjusted methods involving solid base and water washes expressed as a percentage of the relative areas of the supplied method and the corresponding 2s-values (95% confidence)

Method	PFB-COOH (%)	$2s_{PFB\text{-}COOH}$ (%)	dEtOH (%)	$2s_{dEtOH}$ (%)
Ca	188.7	139.0	181.3	85.3
Cb	52.9	36.1	101.2	18.4
Cc	13.4	5.4	140.3	66.3
Cd	3.1	2.9	129.9	64.0

Due to the nature of the derivatisation procedure, PFBCl is present in excess in all samples. This excess forces the formation of the dimer at m/z 406, shown in *Figure 4* as a wide band at a retention time of approximately 3.2 - 3.6 minutes.

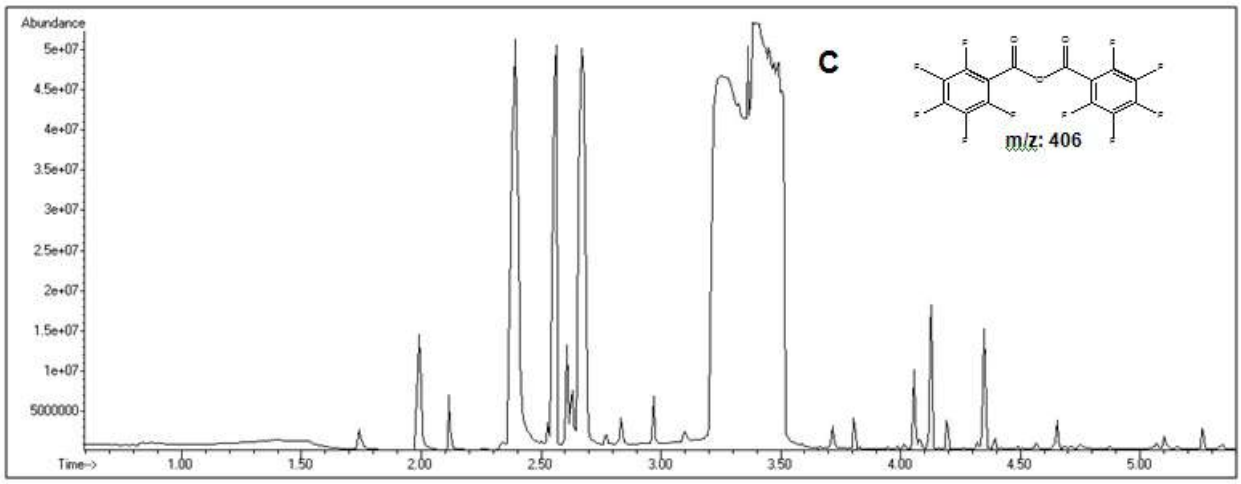


Figure 4: Typical TIC of a sample prepared by the supplied method and analysed by GC-MS on scan mode, with **C** the dimer peak (m/z 406)

Applying method **Ca** resulted in a very large PFB-COOH % with a large standard deviation of 139.0%. The large 2s-values observed, however, indicate a lack of repeatability for this adjusted method, making it impractical, while the substantial PFB-COOH % can be attributed to the hydrolysis of the dimer at m/z 406. For each dimer molecule that splits, one molecule of PFB-COOH is formed, as seen in *Figure 5* below.

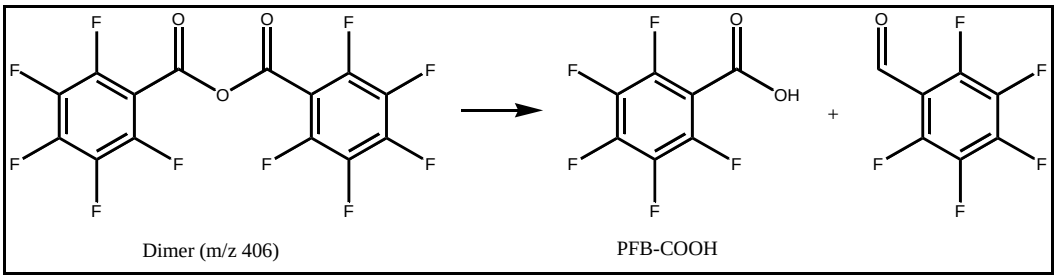


Figure 5: Hydrolysis of dimer m/z 406

Despite the dEtOH % not differing significantly from 100%, this method is not viable as the PFB-COOH concentration actually increases, and at 95% confidence this method more often than not will over-estimate the ethanol concentration.

Following this, method **Cb** includes the addition of a small volume of dH_2O as well as solid $NaHCO_3$. The dEtOH concentration is virtually unaffected, as evidenced by the dEtOH % of 101.2 ± 18.4%, and the PFB-COOH is substantially decreased. This appears to be a strongly viable alternative to the supplied method. However, it should be noted that adding solid $NaHCO_3$ powder to sample tubes can be messy, and the PFB-COOH 2s-value is still fairly sizable as compared to that of methods **Cc** and **Cd**. This results in the PFB-COOH concentration potentially varying anywhere between 16.8% and 89.0% of that of the supplied method.

Both methods **Cc** and **Cd** call for mixing to be halted after only 2 hours, pending the addition of a base, after which the mixing is resumed for the final hour. In both cases, the PFB-COOH is markedly decreased and exhibits small 2s-values. Method **Cc** makes use of $NaHCO_3$, the weaker base, while in method **Cd** Na_2CO_3, a stronger base, is added. Considering this, it is expected that method **Cd** be more effective in the removal of PFB-COOH than method **Cc**. This is seen in the very small PFB-COOH % of 3.1 ± 2.9% for method **Cd** in comparison to the 13.4 ± 5.4% of method **Cc**. Although both these methods effectively reduce the PFB-COOH concentration, and the dEtOH concentrations are not significantly affected, the large 2s-values for the dEtOH % are unacceptable. These large values show that the methods are not repeatable, and, in conjunction with the mean dEtOH %'s of well over 100%, would frequently over-estimate the ethanol concentration.

Method **Cb**, in which a scoop of solid $NaHCO_3$ and dH_2O (**500 µl**) were added, is the most viable of the four methods involving the addition of solid base with or without water washes.

D. Liquid Base and Water Washes

Since the only viable method of those involving the addition of a solid base involved a volume of dH_2O being present at the same time as the base, it was decided to investigate the use of aqueous solutions of various bases. Once more, the strength of the base was considered, with aqueous $NaHCO_3$ being the weakest base tested. Aqueous Na_2CO_3, a slightly stronger base, was also utilised, as well as aqueous NaOH, a very strong base.*Table 6* shows the relative area percentages as well as the corresponding 2s-values for the adjusted methods involving aqueous base and water washes.

Table 6: Relative areas of the PFB-COOH (212/245) and dEtOH (240/245) of the adjusted methods involving aqueous base and water washes expressed as a percentage of the relative areas of the supplied method and the corresponding 2s-values (95% confidence)

Method	PFB-COOH (%)	$2s_{PFB\text{-}COOH}$ (%)	dEtOH (%)	$2s_{dEtOH}$ (%)
Da	89.6	78.6	90.9	12.6
Db	23.7	3.1*	77.4	44.0*
Dc	NA	NA	NA	NA
Dd	212.3	86.1**	117.9	55.6**
De	18.0	12.3*	96.0	6.6*
Df	35.3	64.2*	89.2	26.5*
Dg	12.3	2.0*	94.2	9.8*
Dh	12.5	15.1*	88.4	7.7*
Di	21.2	38.1*	92.5	22.5*
Dj	2.9	6.0	79.9	43.3
Dk	NA	NA	NA	NA
Dl	95.3	10.2*	137.0	74.8*

* Calculated according to *Equation 3*

** Only one adjusted method sample analysed

NA: Not Analysed

Methods **Da** and **Db** involved one and two washes with aqueous $NaHCO_3$ respectively. Method **Da** did significantly decrease the PFB-COOH concentration, while not significantly affecting the dEtOH concentration; however, the 2s-value of the PFB-COOH % is considerable. In fact, it is nearly as large as the PFB-COOH % itself, showing a severe lack of repeatability. As was anticipated, including a second wash with aqueous $NaHCO_3$, as in method **Db**, decreased the PFB-COOH % even further. Moreover, the small 2s-value of the PFB-COOH % indicates a greater repeatability than in method **Da**. Unfortunately, while applying method **Db** did satisfactorily lower the PFB-COOH concentration, it also decreased the dEtOH concentration. Although the dEtOH % is not significantly different from that of the supplied method, method **Db** will tend to under-estimate the final ethanol concentration. In addition, the dEtOH % 2s-value is unacceptably large, once again suggesting a lack of repeatability in ethanol concentration results generated by this method.

Subsequently, methods **Dc** and **Dd** involved one and two washes with aqueous Na_2CO_3 respectively. Method **Dc** resulted in substantial quantities of a fluffy white precipitate upon reconstitution, and the samples could not be analysed without further method adjustment. One of the two samples prepared by method **Dd** reacted similarly, and was also not analysed. It was possible to analyse the second sample as it did not form the precipitate. However, it displayed a greatly elevated PFB-COOH % of 212.3 ± 86.1 %. This 2s-value was calculated in a similar fashion to that in *Equation* 3, except that the

values of the adjusted method, relative area (AM) and s_{AM}, were replaced with those of the supplied method, relative area (SM) and s_{SM} respectively. The fluffy white precipitate witnessed was most likely substantial quantities of PFB-COOH, produced upon hydrolysis of the dimer (m/z 406). Neither of these methods is deemed practical or beneficial.

Increasing the base strength once more, methods **De** and **Df** incorporated one and two washes with aqueous NaOH respectively. Applying method **Df** resulted in an apparently small PFB-COOH %. However, the large accompanying 2s-value implies the PFB-COOH % could vary anywhere between 0 and 99.5%, which is not a sufficiently consistent decrease in PFB-COOH concentration to warrant this adjustment to the supplied method. The NaOH solution used in method **De** was cooled to approximately 15 °C before use. One wash with this solution resulted in a significant – and satisfactory – decrease in PFB-COOH concentration, as well as a small corresponding 2s-value. In addition, the ethanol concentration was not significantly affected. Thus, method **De** would appear to be a viable alternative to the supplied method, despite the 2s-value for the dEtOH % being slightly larger than would be desired.

In methods **Dg** and **Dh**, washes with aqueous $NaHCO_3$ and Na_2CO_3 respectively were followed by one wash with dH_2O. Both methods substantially decreased the PFB-COOH concentration, yielding PFB-COOH %'s of 12.3 ± 2.0% and 12.5 ± 15.1% respectively. Considering the larger 2s-value for PFB-COOH % of method **Dh**, method **Dg** would be preferred. However, based purely on PFB-COOH % either method could be viable. The dEtOH % is not significantly affected by applying method **Dg**, and the small accompanying 2s-value lends confidence to the repeatability of the ethanol concentration results yielded by this method. Conversely, method **Dh** significantly decreased the dEtOH %, and thus the final ethanol concentration, rendering this method an unacceptable alternative to the supplied method.

Method **(Di)** mimicked method **(Dg)** but included passing the sample solution through a Na_2CO_3 plug. While the PFB-COOH % was substantially lowered, the corresponding 2s-value is larger than the PFB-COOH % itself. This indicates an unacceptable lack of repeatability. In addition, method **(Di)** appears to result in a higher concentration of PFB-COOH than method **(Dg)**, despite their similarities. This could be attributed to interaction of the dimer with the Na_2CO_3 as the sample solution filters through.

In method **Dk**, mixing was halted after only two hours, and the aqueous layer was replaced with a fresh volume of Na_2CO_3 solution, after which mixing was resumed for the final hour. Samples, however, formed a gel-like substance upon drying, and were hence not analysed.

Methods **Dj** and **Dl** were similar to method **Dk**, but the final hour of mixing was followed by two washes with dH_2O. Although method **Dj** resulted in a vast decrease in the PFB-COOH concentration and a satisfactorily small 2s-value for the PFB-COOH %, the large 2s-value for the dEtOH % along with the dEtOH % being substantially below 100, implies that this method would frequently under-

estimate ethanol concentrations. Method **Dl** did not significantly decrease the PFB-COOH concentration, and while the dEtOH concentration is not significantly affected, the 2s-value for the dEtOH % is unacceptably large. Neither method **Dj** nor method **Dl** was deemed fit-for-purpose.

Method **De** – utilising cold NaOH solution – and method **Dg** – applying one wash with $NaHCO_3$ followed by one wash with dH_2O – are the most feasible of the thirteen adjusted methods involving an aqueous base and dH_2O washes.

E. Reducing Derivatisation Reagent Concentration

Another way to decrease the PFB-COOH produced during derivatisation would be to minimise the excess PFBCl present in the sample solutions. Hence, the use of a lower concentration of the derivatising reagent was investigated, and for this purpose 2% (v/v) PFBCl was chosen. Only one of the two samples prepared in this manner exhibited peaks for the analyte and internal standard – and even then they were of low abundance. This is possibly due to the derivatisation reagent concentration being too low for proper interaction with the analyte molecules. This adjusted method resulted in a PFB-COOH % of 97.8 ± 88% and a dEtOH % of 116.4 ± 113.0%, where the 2s-values are calculated by *Equation 3*, with the values of the adjusted method, relative area (AM) and s_{AM}, replaced with those of the supplied method, relative area (SM) and s_{SM} respectively. In light of the large 2s-values, the ethanol concentration result being significantly increased, as well as the PFB-COOH concentration not being significantly lowered, this adjustment to the supplied method is not recommended.

F. Introducing an Alternative Analyte

Instead of minimising the excess PFBCl present by decreasing the derivatisation reagent concentration, the effect of introducing an alternative analyte was addressed. It was hoped that a compound with similar reactivity to that of the ethanol and ethanol-d6 already present in the sample, when added in excess, would consume the excess PFBCl before it was converted to the undesirable PFB-COOH. For this purpose isopropanol and glycine were selected. Both of these compounds are sufficiently larger than ethanol and, with the enhanced selectivity gained by use of the MS detector, would not interfere with the dEtOH and dEtOH-d6 signals.

Table 7 shows the relative area percentages as well as the corresponding 2s-values for the adjusted methods involving the addition of isopropanol or glycine.

Table 7: Relative areas of the PFB-COOH (212/245) and dEtOH (240/245) of the adjusted methods involving the addition of isopropanol or glycine expressed as a percentage of the relative areas of the supplied method and the corresponding 2s-values (95% confidence)

Method	PFB-COOH (%)	$2s_{PFB\text{-}COOH}$ (%)	dEtOH (%)	$2s_{dEtOH}$ (%)
Fa	373.1	120.0	90.5	2.8
Fb	NA	NA	NA	NA
Fc	NA	NA	NA	NA
Fd	NA	NA	NA	NA
Fe	NA	NA	NA	NA
Ff	-	-	-	-
Fg	1.8	3.6	90.4	136.0

The addition of a small volume (**250 µl**) of isopropanol as in method **Fa** resulted in a drastic increase in the PFB-COOH concentration, likely due to the hydrolysis of the dimer at m/z 406. Furthermore, the dEtOH concentration is significantly decreased, indicating the impracticality of this method.

Increasing the volume of isopropanol added to 750 µl and 1750 µl as in methods **Fb** and **Fc** respectively resulted in the formation of a fluffy white precipitate – most likely substantial quantities of PFB-COOH – rendering the samples prepared in this manner impossible to analyse without further adjustment to the method.

Despite the wash with aqueous $NaHCO_3$ included in method **Fd**, and filtering through Na_2CO_3 in method **Fe**, the white precipitate persisted upon drying, and the samples prepared by these methods were not analysed.

Method **Ff** involved washing the sample solution with aqueous NaOH until a clear solution was obtained, and no more white precipitate remained. However, upon analysis, almost no peaks were visible on the TIC. It is probable that the multiple washes with a strong base, while effectively removing the corrosive PFB-COOH, also hydrolysed the esters of interest.

Finally, in method **Fg,** the isopropanol was replaced with saturated aqueous glycine solution. Glycine, containing an amide, was expected to form a water-soluble ester allowing for an acid-base extraction of the excess PFBCl into the aqueous layer. The reaction of glycine with PFBCl is shown in *Figure* 6.[8]

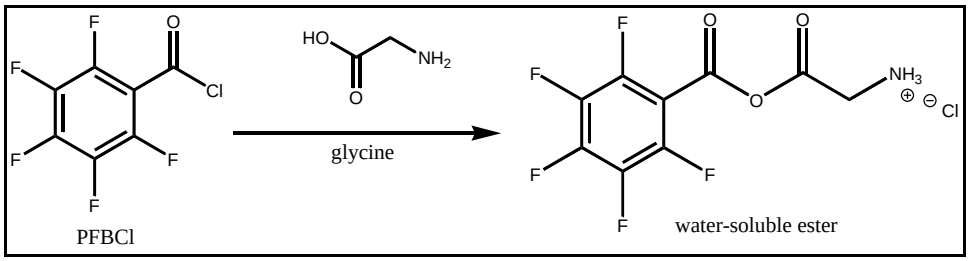


Figure 6: Reaction scheme of glycine with PFBCl

It is clear that the addition of the glycine solution to the sample removes the PFB-COOH most effectively. In addition, the dEtOH % is not significantly impacted. However, the large 2s-value of the dEtOH % indicates unacceptable unrepeatability in ethanol concentration results yielded by this method.

G. Summary of Viable Adjusted Methods

Very few of the many alternative methods investigated meet the rigid requirements of the ideal adjusted method. Those that effectively decreased the PFB-COOH concentration, tended to significantly affect the final ethanol concentration result, while those that did not affect the ethanol concentration failed to impact the PFB-COOH concentration sufficiently. The potentially viable methods are summarised in *Table 8*.

Table 8: Potentially viable methods for the removal of PFB-COOH

Method	PFB-COOH (%)	$2s_{PFB\text{-}COOH}$ (%)	dEtOH (%)	$2s_{dEtOH}$ (%)
Cb	52.9	36.1	101.2	18.4
De	18.0	12.3*	96.0	6.6*
Dg	12.3	2.0*	94.2	9.8*
Fg	1.8	3.6	90.4	136.0

* Calculated according to Equation 3

Although method **Fg** is the most effective in decreasing the PFB-COOH concentration, its lack of repeatability makes it an unacceptable alternative method for the accurate determination of ethanol concentration. Conversely, while method **Cb** affects the final ethanol concentration result the least, it does not sufficiently decrease the PFB-COOH concentration.

While method **De** yields a dEtOH % closer to 100, as well as a smaller dEtOH % interval, method **Dg** is more effective – and more repeatable - at removing PFB-COOH, and the dEtOH concentration result of method **Dg** is not significantly lowered.

Considering the above, method **Dg**, where a scoop of solid $NaHCO_3$ and dH_2O (**500 µl**) were added, is the most effective and viable alternative to the supplied method for the quantitation of ethanol in

blood samples. It is this method that was validated, and this method that was used to determine ethanol concentrations throughout all further studies.

2.3.2. Method Validation

It should be noted that this is a pre-validation. In other words, a small set of data collected initially is used to evaluate the applicability of the method and to determine whether or not it is fit-for-purpose. Following this, internal quality control data collected over at least 6 months will be used to more accurately assess the functionality of the method, and various validation parameters will be recalculated.

Linearity

Figure 7 shows five response models generated by the adjusted method. The eighth calibration point of model 2 was found to be an outlier by the CD^2 test and has therefore been omitted.

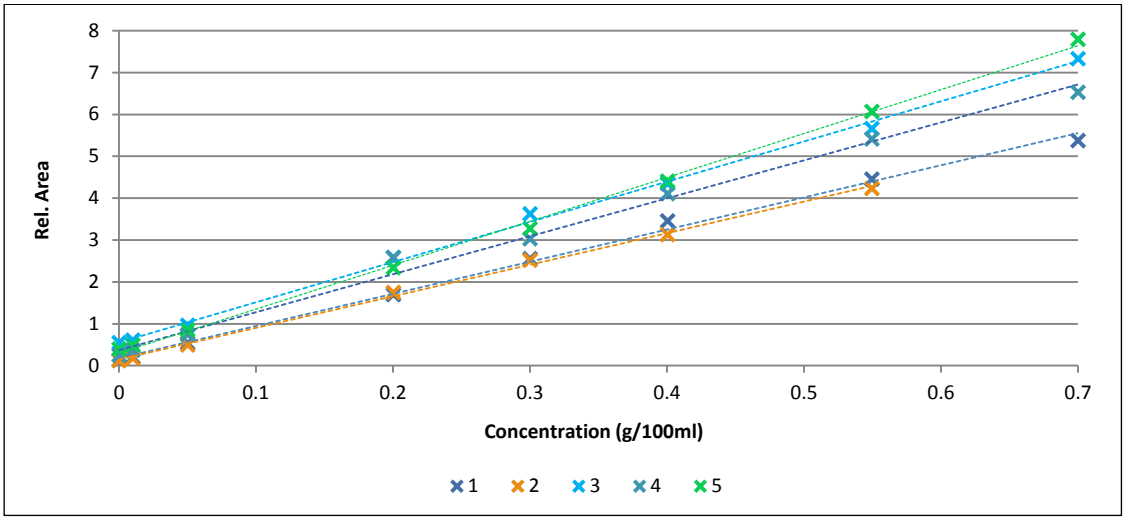


Figure 7: Five response models illustrating the linearity of the method

The gradients and correlation coefficients of the five response models are listed below in *Table 9*.

Table 9: Gradients and correlation coefficients of the five response models

Model	Gradient	Correlation Coefficient (r^2)
1	7.678	0.997
2	7.545	0.998
3	9.592	0.998
4	9.059	0.994
5	10.484	0.998

The average correlation coefficient for the method was calculated from those of the response models shown in *Figure 7*. The 95% confidence interval was also determined as a standard error by *Equation* 4, where s_r is the standard deviation, and n is the number of correlation coefficient values – in this case, n = 5.

$$Standard\ Error = \frac{s_r}{\sqrt{n}} \qquad \text{Equation 4}$$

The 95% confidence interval for the correlation coefficient was found to be $r^2 = 0.9971 \pm 0.0083$.
In general, in a forensic setting, an r^2 value of above 0.995 is required for a regression line to be considered sufficiently linear to function as a calibration graph.[9] The method therefore exhibits acceptable linearity to allow for linear response modelling.

It is, however, important to note the varying gradients obtained for the five regression lines. While this does not pose a problem, it simply means that it is necessary to prepare fresh calibration standards – and subsequently, a new calibration line – each day.

Limits of Detection and Quantitation

There are various ways in which the LOD and LOQ of a method can be determined. The most common is to make use of a typical calibration curve of the form y = bx + a, and calculate the LOD and LOQ by *Equation 5a* and *5b* and *Equation 6a* and *6b* respectively:

$$LOD_y = y_0 + 3s_{y/x} \qquad \text{Equation 5a}$$

$$LOD_x = \frac{(LOD_y - a)}{b} \qquad \text{Equation 5b}$$

$$LOQ_y = y_0 + 10s_{y/x} \qquad \text{Equation 6a}$$

$$LOQ_x = \frac{(LOD_y - a)}{b} \qquad \text{Equation 6b}$$

with LOD_y and LOQ_y the LOD and LOQ in terms of relative area (240/245) respectively, y_0 the y-intercept, $s_{y/x}$ the standard deviation of the regression of y on x, and LOD_x and LOQ_x the LOD and LOQ in terms of ethanol concentration respectively. Although the calibration curve is obtained

experimentally, the above calculations give a theoretical estimate of the LOD and LOQ of an analytical method.

Figure 8 depicts a typical calibration curve for the ethanol method, with 95% confidence intervals of 7.0607 ± 0.0935 and -0.0011 ± 0.0344 for the gradient and intercept respectively. Using these confidence intervals, the 95% confidence intervals for the theoretical LOD and LOQ were calculated to be 0.011 ± 0.00488 g/100 mL and 0.037 ± 0.00488 g/100 mL respectively.

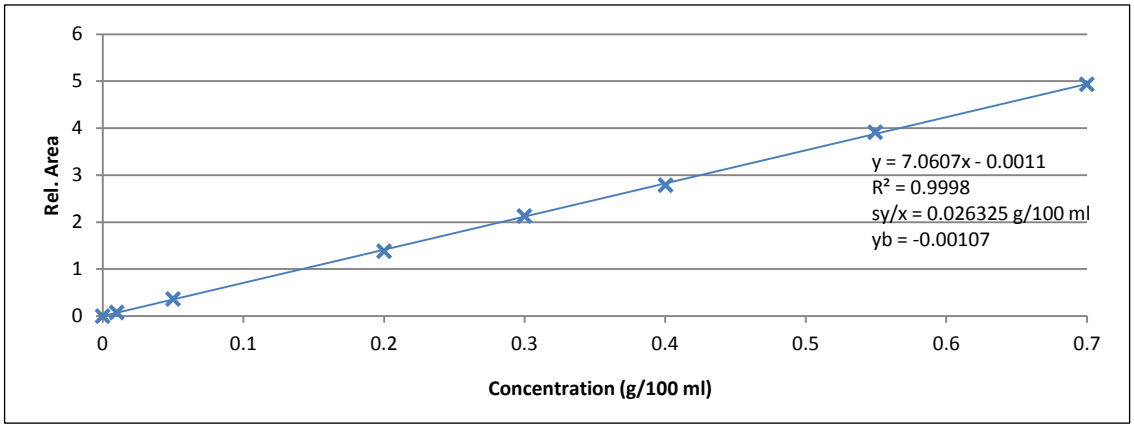


Figure 8: Typical calibration curve for the ethanol method

The intended application for this analytical method is to quantify ethanol concentration in whole blood for the purposes of determining whether the BAC of a patient is significantly elevated above the South African legal cut-off concentrations of 0.02 g/100 mL and 0.05 g/100 mL. While a theoretical LOD of 0.011 ± 0.00488 g/100 mL shows that it is possible to detect ethanol below the legal cut-offs, the large theoretical LOQ value of 0.037 ± 0.00488 g/100 mL seems to indicate that it will not be possible to accurately quantify ethanol concentration below 0.0324 g/100 mL. This is an unacceptably high concentration, meaning that the method could not be applied at the professional driver limit. Considering that the low concentration internal quality control solutions were prepared at 0.02 g/100 mL and showed fairly accurate concentration values (see *Bias and Precision* below), and the lowest calibrator is prepared at 0.01001 g/100 mL, this estimation of the LOQ – and therefore the LOD – is not justified.

Limit of Detection and Limit of Quantitation can also be estimated purely experimentally. Usually, the lowest calibrator is diluted down until a signal is no longer registered on the instrument. The LOD is then the lowest concentration that yielded a S/N ratio of 3:1, while the LOQ is the concentration that yielded a S/N ratio of 10:1. For chromatograms, the analyte signal intensity is compared to the intensity of the background noise.

In this case, it was the low control working solution that was diluted. A signal for ethanol could no longer be detected at the lowest spiked concentration of 0.000078125 g/100 mL; however, a S/N ratio of 3:1 was obtained at 0.00125 g/100 mL ethanol, and a S/N ratio of 10:1 at 0.0025 g/100 mL. Since these experimental estimates for the LOD and LOQ seemed more reasonable than those obtained by

theoretical calculations, the LOD and LOQ for the method were set at 0.00125 g/100 mL and 0.0025 g/100 mL respectively.

Selectivity

While the purpose of this method is to determine the concentration of ethanol in blood, it is necessary to first identify the analyte. Mass spectra are often described as “fingerprints”, implying that each compound will produce a unique fragmentation pattern, with the various ions occurring in reproducible abundance ratios. Although two mass spectra may exhibit the same ions, if the abundance ratios of these ions differ it is clear that the compounds responsible for the mass spectra are not the same. As such, a given peak on the TIC can only be attributed to dEtOH if it exhibits ions at m/z 240, 212, 195 and 167 in specific ratios.

Making use of calibrator samples, the abundance ratios of the ions listed above could be determined over the whole ethanol calibration concentration range (0.01 – 0.70 g/100 mL). The 95% confidence intervals for the ion abundance ratios were 0.223 ± 0.040 for 240/212; 0.070 ± 0.024 for 240/195; 0.223 ± 0.084 for 240/167; 0.314 ± 0.090 for 212/195; 1.00 ± 0.29 for 212/167; and 3.19 ± 0.60 for 195/167.

Specificity

Ten pairs of duplicate blank samples were analysed, and only one of these pairs displayed a small amount of endogenous ethanol, at an exceedingly low concentration of 0.0003 g/100 mL, which is below both the LOD and LOQ and can be considered to be negligible. The other eight sample pairs showed no ethanol content whatsoever. This speaks to the specificity of the method, showing that the blank blood matrix does not contribute to, or interfere with, the ethanol signal, and that the method is able to correctly report a negative when the analyte of interest is not present.

Bias and Precision

The experimental concentrations obtained over the five days at the three different concentration levels can be found in ***Appendix C***. Those indicated as outliers were not included in any calculations and are shown purely for completeness.

<u>*Bias*</u>

The non-weighted linear regression bias correction plot shown below in *Figure 9* was obtained by plotting the average experimental concentrations of the three internal quality control levels (Y) of 0.0215 g/100 mL, 0.0511 g/100 mL, and 0.2951 g/100 mL versus the theoretical concentrations (X) of 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL, and has the form $Y = BX + A$.

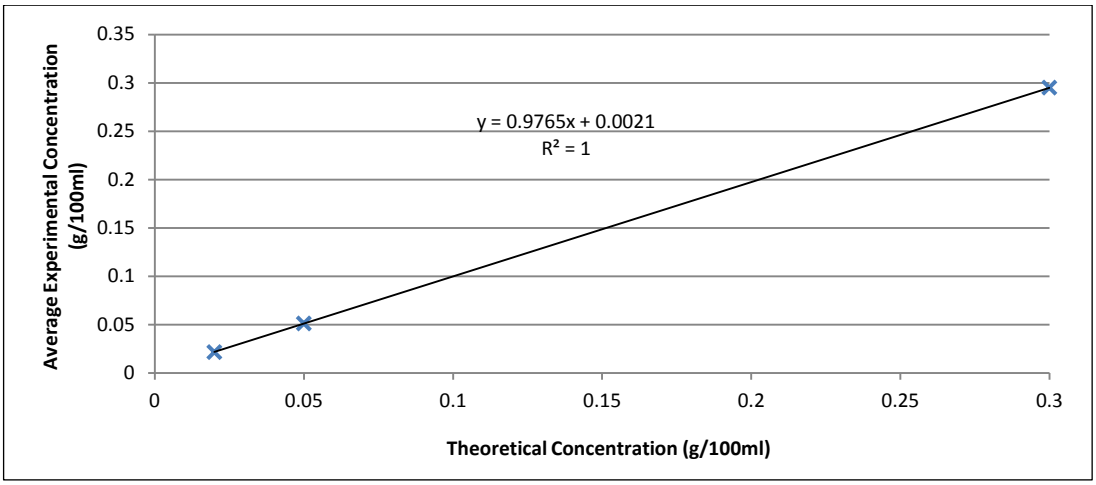


Figure 9: Bias correction plot obtained from the five days' ethanol validation data

This corrective function can be applied to experimentally obtained concentrations in order to correct for procedural bias by:

$$X_0 = \frac{Y_0 - A}{B} \qquad \text{Equation 7}$$

where *A* and *B* are the intercept and gradient respectively of the bias correction plot; Y_0 is the experimentally determined concentration; and X_0 is the experimentally determined concentration corrected for bias.

The gradient (*B*) of the corrective function represents multiplicative bias, while the intercept (*A*) represents additive bias. Should the gradient be significantly different from 1, the multiplicative bias would be considered significant. Similarly, the additive bias is significant when the intercept differs significantly from 0.

The 95% confidence intervals of the intercept and gradient of the bias correction line were determined to be $A = 0.0021 \pm 0.0024$ and $B = 0.976 \pm 0.014$ respectively. It was thus determined that the method exhibited no additive bias, since 0 falls within the confidence interval of *A* at 95% confidence. However, the 95% confidence interval of the gradient did not include 1, leading to the conclusion that the method does display multiplicative bias - that is, a bias that is dependent on concentration.

Considering the presence of significant multiplicative bias in the method, it is thus imperative that any concentration result obtained be corrected for bias by means of *Equation* 7.

Precision

Applying analysis of variance (ANOVA) to the multiple measurements performed at the three internal quality control levels, the within batch precisions were determined to be 6.51%, 3.53% and 5.53% for

the 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL levels respectively. Similarly the between-batch precisions were found to be 11.74%, 10.70% and 9.23% for the 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL concentrations. The ANOVA tables can be found in *Appendix C*.

It is worth noting that while all three ANOVA calculations yielded F test statistic values larger than the F critical values, and p-values much less than the 95% confidence α value of 0.05, the meagre sample size renders this albeit statistically significant result, practically insignificant. In other words, additional data at the three internal quality control levels should be generated for the method to increase the power of these tests, before any one result can be discarded.[10]

The within-batch, $u_{W,}$ and between-batch, $u_{B,}$ precisions at each concentration can be pooled to yield an overall precision estimate, u_T, by:

$$u_T = \sqrt{{u_W}^2 + {u_B}^2} \qquad \text{Equation 8}$$

Applying *Equation 8* to the above within- and between-batch precisions gave overall precision estimates of 13.43%, 10.20% and 10.76% for the 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL concentrations respectively.

Ruggedness

As part of "fit-for-purpose" testing, it is important to show that a method is rugged. That is, small deviations from the prescribed method should not significantly influence the final analytical result obtained, or alternatively, it should be determined how large the deviation is permitted to be before it has a significant impact.

a) Derivatisation Time

The supplied method – and subsequently the adjusted method – calls for sample solutions to be mixed thoroughly for 3 hours after addition of the derivatisation reagent. This being such a long period of time, it is not uncommon for the samples to be left mixing, while the analyst performs other tasks. It was thus important to determine whether mixing for longer or shorter than 3 hours would significantly affect the final ethanol concentration result, and if so, by how much the mixing time could be allowed to vary before this happened.

Removing groups of samples every half hour from the multi-shaker allowed information to be gained about the mixing time in half hour intervals on either side of the prescribed time of 3 hours. Hence, ANOVA was applied first to the control group (mixed for 3 hours) with the group that was mixed for half an hour shorter than prescribed. Groups were then added one at a time on either side of 3 hours and the ANOVA calculations repeated in order to obtain new F statistic values and thereby determine at what length of time the obtained ethanol concentration differed significantly

from that of the control samples mixed for 3 hours. *Table 10* shows the F test values (F_{test}) along with the corresponding F critical values (F_{crit}) obtained for each time interval.

Table 10: F test and F critical values for repeated ANOVA calculations for different derivatisation time intervals

Time Interval (hours)	F_{test}	F_{crit}
2.5 – 3.0	0.034101	5.317655
2.5 – 3.5	0.020065	3.885294
2.0 – 3.5	0.351544	3.238872
2.0 – 4.0	5.231343	2.866081

The F_{test} values are less than the F_{crit} values for the first three time intervals listed above. From this it can be deduced that mixing for up to one hour shorter than the prescribed time does not significantly affect the final concentration result, and neither, in fact, does mixing for up to half an hour longer. However, mixing for an hour longer than recommended results in an F_{test} statistic greater than the F_{crit} value, showing that the samples mixed for 4 hours exhibited concentrations that were significantly different from those mixed for only 3 hours.

The bias-corrected concentrations obtained for the different groups are shown in *Figure 10*. The 95% confidence interval around the theoretical value is also plotted, calculated as the theoretical ± 2s, where s is the standard deviation of the medium internal quality control samples prepared for the *Bias and Precision* investigation. From this, it can be seen that although none of the groups of samples differ significantly from the theoretical concentration, those that were mixed for 4 hours yielded the highest concentration results.

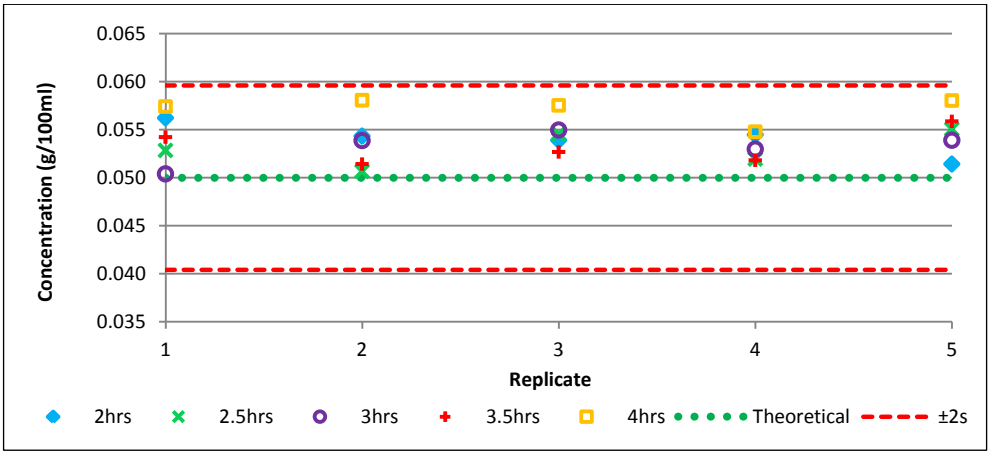


Figure 10: Ethanol concentrations of groups of samples with varying derivatisation times, together with the 95% confidence interval (±2s) around the theoretical concentration value (0.05 g/100mL)

b) Derivatisation Reagent Concentration

Applying ANOVA to the bias-corrected ethanol concentrations of the groups of samples prepared at the three IQC concentrations with three different derivatisation reagent concentrations yielded the F_{test} and F_{crit} values listed in *Table 11*.

Table 11: F test and F critical values for the ANOVA calculations performed on the groups of samples prepared at the three internal quality control concentrations with differing concentrations of derivatisation reagent.

Ethanol Concentration (g/100mL)	F_{test}	F_{crit}
0.02	6.159674	3.885294
0.05	2.485510	3.885294
0.30	2.021956	3.885294

At the medium and high concentration levels, the F_{test} values obtained are smaller than the F_{crit} values. This shows that varying the PFBCl concentration anywhere in the range 2% (v/v) to 10% (v/v) does not significantly affect the final ethanol concentration obtained. However, this is not the case at the low ethanol concentration level. The F_{test} value is larger than the F_{crit} value, signalling a significant difference between the groups. This warranted further investigation into the low control groups.

Comparing each group at 0.02 g/100 mL ethanol with each of the other low control groups in turn yielded the F statistic values shown in Table 12. It is clear from these values that no significant difference exists between the ethanol concentrations of the samples prepared using 5% (v/v) PFBCl and those prepared using 2% (v/v), or 10% (v/v). The significant difference arises when the concentrations of the samples prepared at 2% (v/v) PFBCl are compared with those prepared at 10% (v/v).

Table 12: F test and F critical values for the ANOVA calculations performed on pairs of low ethanol concentration groups at three different derivatisation reagent concentrations

PFBCl % compared	F_{test}	F_{crit}
2 and 5	3.833483	5.317655
5 and 10	2.136035	5.317655
2 and 10	13.563160	5.317655

From the above, it can be deduced that samples of lower ethanol content would be more susceptible to influence by variations in derivatisation concentration. In addition, provided the

concentration of the derivatisation reagent solution remained constant throughout the batch, and it was between 2% and 10% (v/v) PFBCl, the ethanol concentration results obtained from that batch would not be significantly different from those that would be obtained if they were prepared with a 5% (v/v) PFBCl solution.

The ethanol concentrations obtained for the various groups, as well as the 95% confidence intervals at each concentration level obtained from the standard deviations calculated in the *Bias and Precision* investigation, are shown in *Figure 11*, *Figure 12*, and *Figure 13*. At both the medium and high IQC levels, using a derivatisation reagent of 10% (v/v) PFBCl resulted in concentration values significantly elevated above the theoretical concentration value. Although small deviations in the derivatisation reagent concentration should not result in significant differences in ethanol concentrations, it is not recommended to use derivatisation reagent concentrations as high as 10% (v/v).

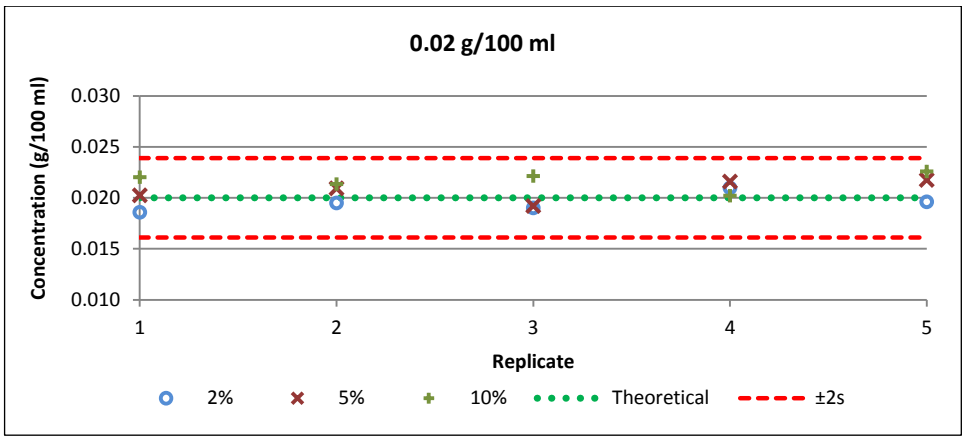


Figure 11: Ethanol concentrations of groups of samples prepared at 0.02 g/100 mL with varying derivatisation reagent concentrations, together with the 95% confidence interval (±2s) around the theoretical concentration value

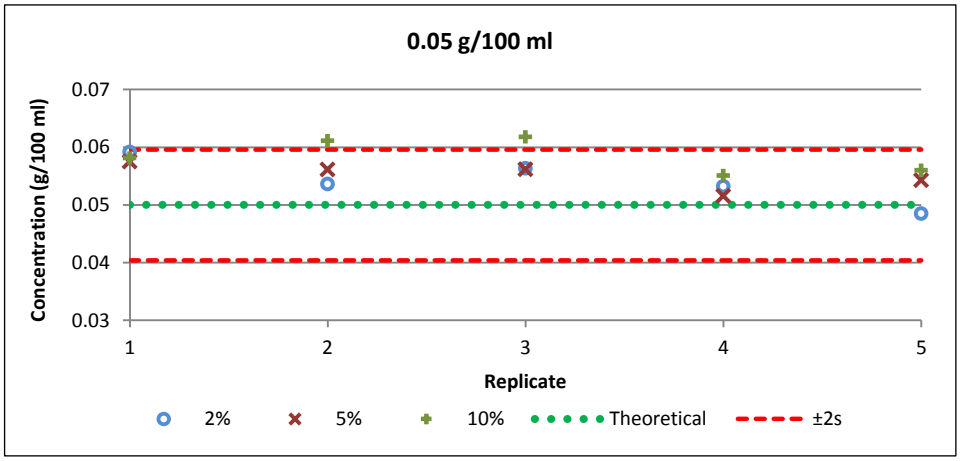


Figure 12: Ethanol concentrations of groups of samples prepared at 0.05 g/100 mL with varying derivatisation reagent concentrations, together with the 95% confidence interval (±2s) around the theoretical concentration value

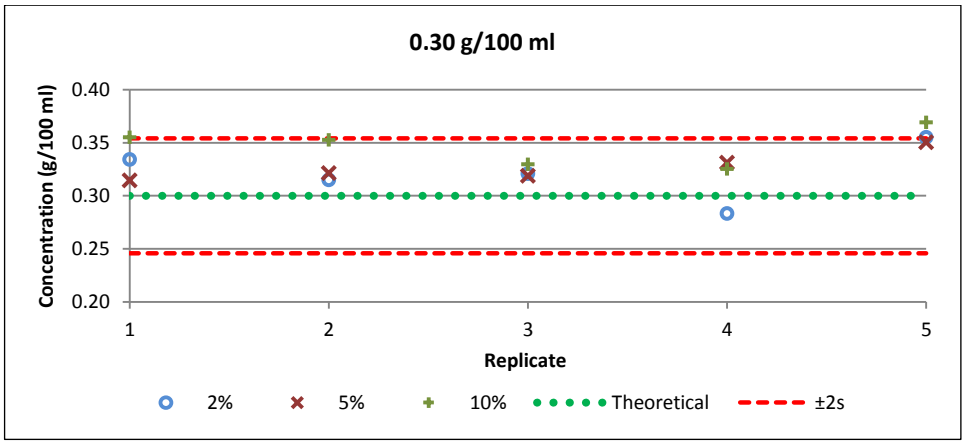


Figure 13: Ethanol concentrations of groups of samples prepared at 0.30 g/100 mL with varying derivatisation reagent concentrations, together with the 95% confidence interval (±2s) around the theoretical concentration value

It would thus appear from the above plots that a derivatisation reagent concentration between 2% (v/v) and 5% (v/v) would yield reliable experimental concentrations.

c) Drying Process

The concentrations of the samples that were dried completely had a mean of 0.05208 g/100 mL and a standard deviation of 0.000691 g/100 mL, while those that were removed from the drying manifold before drying to completeness had a mean of 0.05028 g/100 mL and standard deviation of 0.00103 g/100 mL.

Comparison of the means by the t-test resulted in a t_{test} statistic of 1.034, while the t_{crit} value for n = 3 is 4.303. This shows that there is no significant difference between the means of the two sets.

Comparison of the standard deviations by the F-test yielded an F_{test} value of 0.455, which was much smaller than the F_{crit} value of 19.0. This shows that there is no significant difference between the variations in the two sample sets.

Hence, whether samples are completely dry before reconstituting, or still contain a small amount of solvent, does not influence the ethanol concentration result obtained. The presence of the internal standard compensates for any volume differences.

d) Stability of Derivatised Analyte

Figure 14 shows the bias-corrected ethanol concentrations obtained for the repeated injection of a medium level internal quality control sample over a period of 16 hours, during which the sample stood on the autosampler tray. Also shown is the 95% confidence interval around the theoretical value of 0.05 g/100 mL, obtained from the standard deviation of the medium internal quality control samples in the *Bias and Precision* investigation.

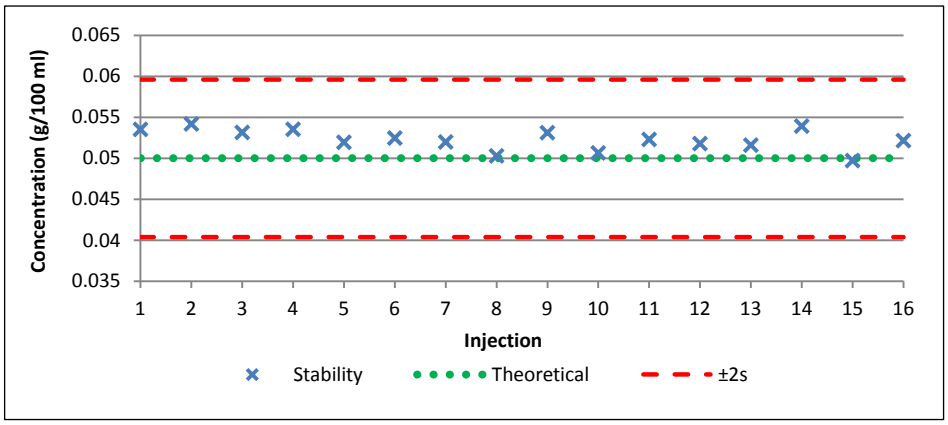


Figure 14: Stability of dEtOH on the autosampler tray for 16 hours

All 16 concentrations are well within 2 standard deviations of the theoretical value, and show hardly any variation in magnitude before the thirteenth injection, which corresponds to thirteen hours. After this, slightly larger deflections away from the average concentration are seen. It should thus be noted that the derivatised analyte concentration is statistically stable for at least up to 16 hours on the autosampler tray.

2.3.3. Measurement Uncertainty Calculations

Control Charts

Figure 15, *Figure 16* and *Figure 17* show the experimental concentrations obtained for the IQC samples prepared at 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL over a period of 13 months. These figures include outliers (indicated as red circles) as well as the confidence limits at 1, 2 and 3 standard deviations.

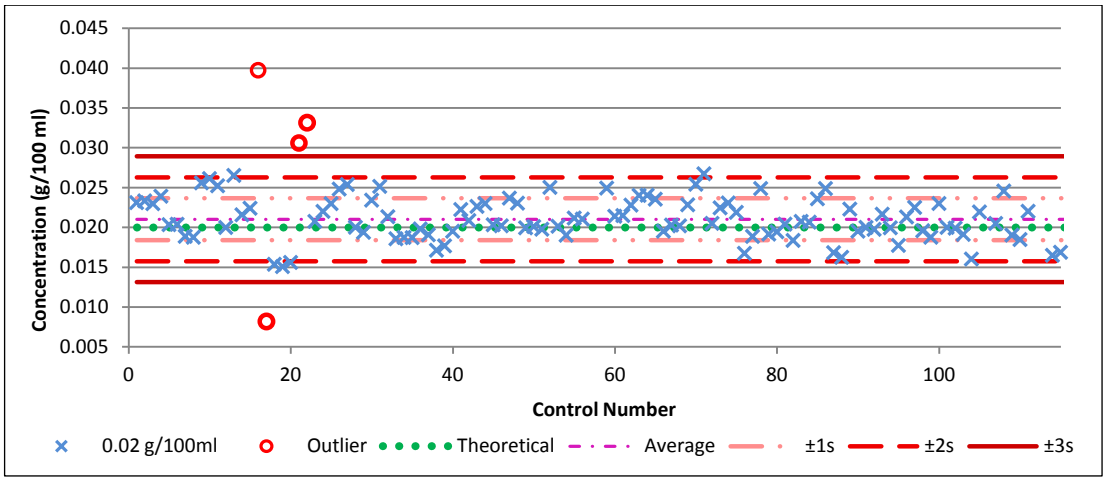


Figure 15: Levey-Jennings control chart for the internal quality control samples prepared at 0.02 g/100 mL ethanol, including outliers

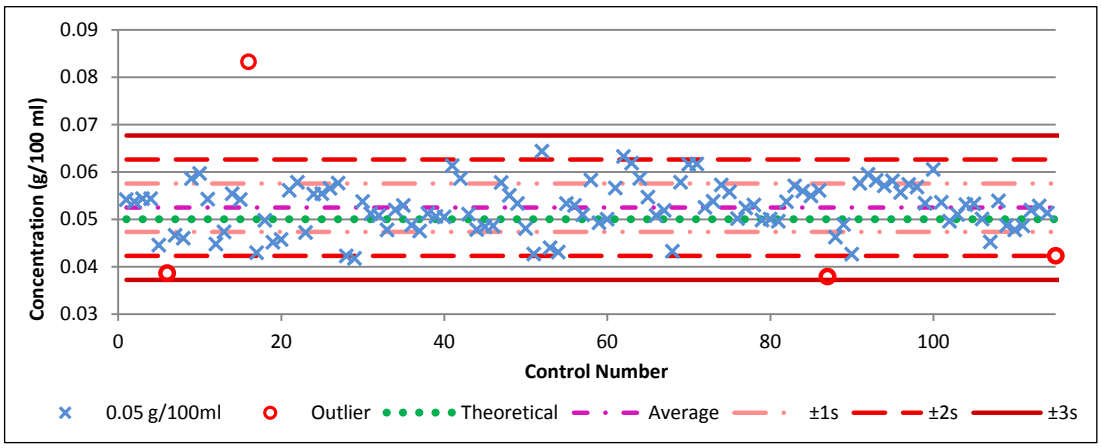


Figure 16: Levey-Jennings control chart for the internal quality control samples prepared at 0.05 g/100 mL ethanol, including outliers

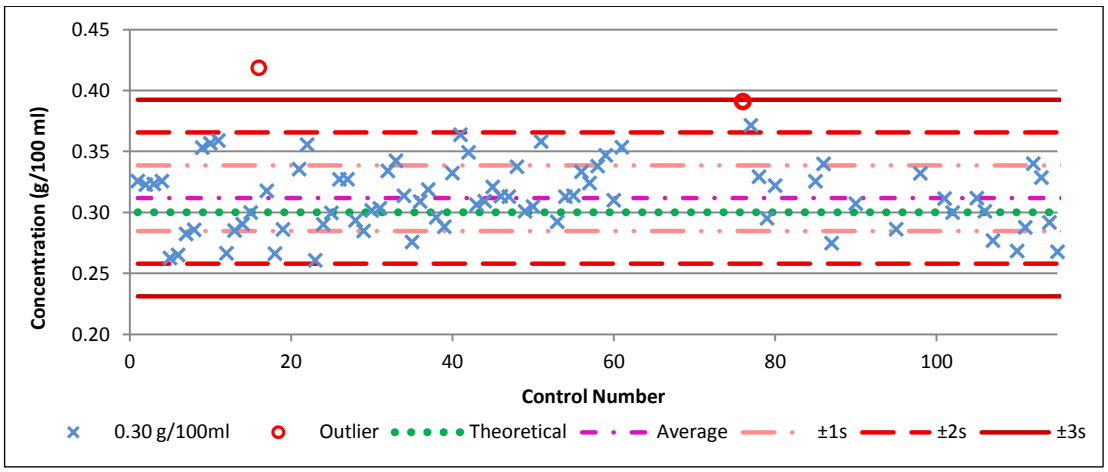


Figure 17: Levey-Jennings control chart for the internal quality control samples prepared at 0.30 g/100 mL ethanol, including outliers

Including the outliers in the above figures causes the y-axis scales to be compressed and makes the figures difficult to interpret. For clarity, the outliers in the above figures have been removed and the axes of the control charts adjusted where necessary, to yield *Figure 18*, *Figure 19* and *Figure 20*.

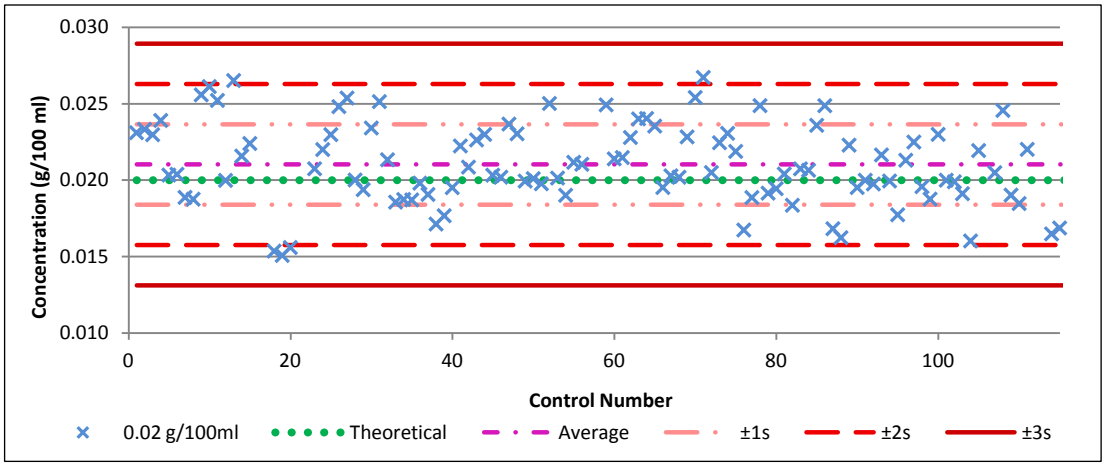


Figure 18: Levey-Jennings control chart for the internal quality control samples prepared at 0.02 g/100 mL ethanol

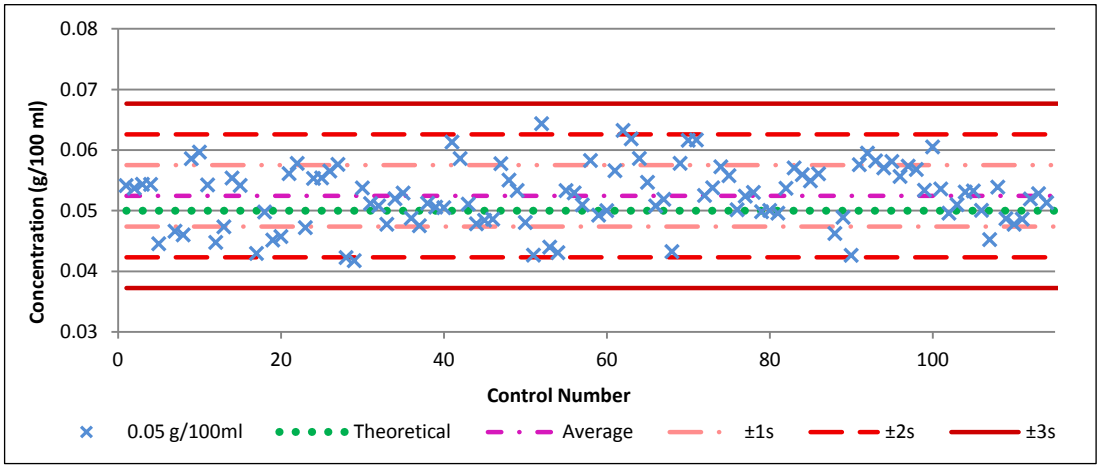


Figure 19: Levey-Jennings control chart for the internal quality control samples prepared at 0.05 g/100 mL ethanol

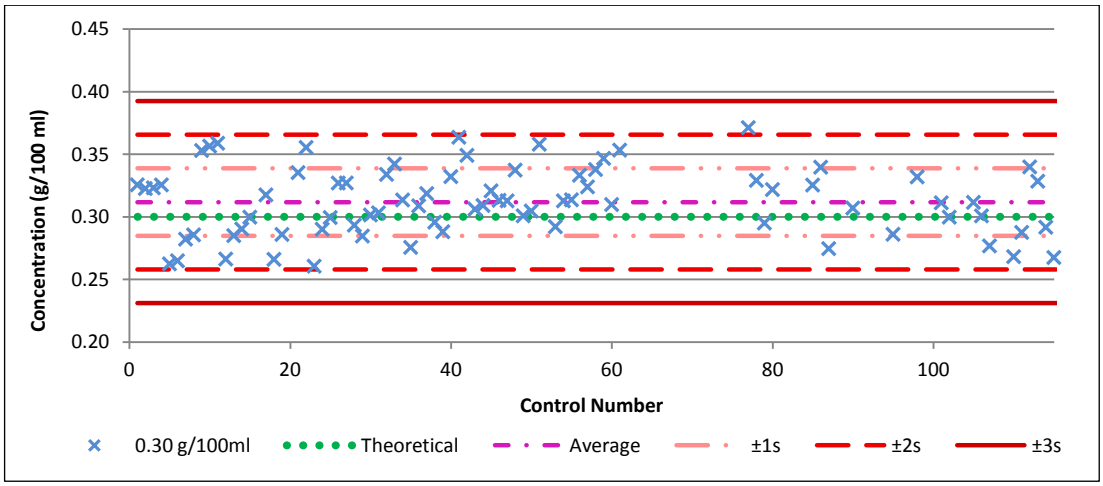


Figure 20: Levey-Jennings control chart for the internal quality control samples prepared at 0.30 g/100 mL ethanol

At each of the three levels, the experimental concentrations obtained exhibit no discernible patterns, appearing to be randomly scattered about the theoretical concentrations. Although some points do lie outside the 2s confidence interval, generally it is only individual points, and for these the Westgard Rules are not violated.[11] The only exceptions to this are points 18, 19 and 20 for the controls prepared at 0.02 g/100 mL, and points 28 and 29 for those prepared at 0.05 g/100 mL. While these sets of points violate the 2_{2s} rule, it should be noted that the samples in each of these sets were prepared in the same batches and analysed on the same days. As such, this should rather be considered to be a violation of the 1_{2s} rule, which simply acts as a warning to closely monitor the controls that follow. In addition, the concentrations of the samples that follow directly after each set lie within 2 standard deviations of the mean, giving credence to the fact that the sets do not constitute the start of a trend, and also that the method is still in control.

Points 91 to 101 for the samples prepared at 0.05 g/100 mL appear to violate the 10_{μ} rule; however, once again some of these samples were prepared in the same batches and analysed on the same days (91 and 92, 93 and 94, 95 – 98, 99 and 100). As such, the 10_{μ} rule is not violated.

Despite the positive bias observed at all three concentration levels, the three theoretical concentrations lie well within 1 standard deviation of the means. This speaks to the accuracy of the method, and shows that the observed bias need not be a cause for concern, but should rather merely be corrected for in calculation of the final concentration result, and accounted for in the uncertainty estimate.

Measurement Uncertainty

As of yet, there is no universally accepted way of estimating the measurement uncertainty for an analytical method. There are, of course, many documents in literature outlining just as many different methods of calculating this.[4, 5, 12-16] However, not one of these has been accepted as the “only correct way”. Indeed, the only stipulations laid out in the ISO 17025:2005 General requirements for the competence of testing and calibration laboratories[12] is that testing laboratories have and apply a method of calculating the measurement uncertainty and “when estimating the uncertainty of measurement, all uncertainty components which are of importance in the given situation shall be taken into account using appropriate methods of analysis”. As such, it falls to the individual laboratory to formulate an adequate estimate for the measurement uncertainty of a method. Especially in a clinical laboratory, this measurement uncertainty should be small enough to allow critical decisions to be made, while not being so small as to under-estimate the variability of the method.

Despite the myriad different approaches for calculating measurement uncertainty, common threads emerge. The majority of calculations include contributions from measurement bias and precision, and some form of estimate for the contribution from the reference materials used in the analytical method, often termed the traceability component. In addition, estimates tend to be either bottom-up or

top-down. The measurement uncertainty calculation presented below is an amalgamation of both top-down and bottom-up approaches.

The determination of blood alcohol concentration, as with any analytical measurement, is subject to the influence of several components, and as such, the measurement can be thought of as a function of these:

$$X_0' = f(X_1, X_2, \dots, X_N) \qquad \text{Equation 9}$$

where X_0' is the final ethanol concentration result corrected for bias, and X_1 ... X_N are the influencing components.

More often than not, and as in this case, the function *f* is not known; however, it is often assumed to have the following form:

$$X_0' = f_{X_1} \cdot f_{X_2} \cdot \dots \cdot f_{X_N} \qquad \text{Equation 10}$$

If each of the factors, $f_{X_1} \dots f_{X_N}$, is assumed to be independent then, by the rules for the propagation of error, the relative standard uncertainty (RSD) of the BAC measurement can be written in terms of the RSDs of the various components that influence the final concentration result.

$$RSD_{X_0'} = \sqrt{(RSD)_{X_1}^2 + (RSD)_{X_2}^2 + (RSD)_{X_3}^2 + \dots + (RSD)_{X_N}^2} \qquad \text{Equation 11}$$

Equation 11 can be rewritten as:

$$\frac{u_{X_0'}}{X_0'} = \sqrt{\left(\frac{u_{X_1}}{X_1}\right)^2 + \left(\frac{u_{X_2}}{X_2}\right)^2 + \left(\frac{u_{X_3}}{X_3}\right)^2 + \dots + \left(\frac{u_{X_N}}{X_N}\right)^2} \qquad \text{Equation 12}$$

where $u_{X_0'}$ is the standard uncertainty of the final concentration result corrected for bias and sample dilution, X_0', and u_{X_1} ... u_{X_N} are the standard uncertainties of the components X_1 ... X_N.

In the analytical determination of blood alcohol content, the main contributors to uncertainty in measurement are the certified reference solution of aqueous ethanol (CRM), sample dilution (dil), method bias and method imprecision (imp). As such, *Equation 12* can be simplified to:

$$\frac{u_{X_0'}}{X_0{}'} = \sqrt{\left(\frac{u_R}{R}\right)^2_{CRM} + \left(\frac{u_d}{d}\right)^2_{dil} + \left(\frac{u_{bias}}{X_0}\right)^2_{bias} + \left(\frac{u_{imp}}{X_0}\right)^2_{imp}} \qquad \text{Equation 13}$$

with R the concentration of the CRM as given in the manufacturer's certificate, d is the sample dilution factor, and X_0 is the ethanol concentration result after correcting for bias.

The final ethanol concentration result is calculated by taking the sample dilution into account as follows:

$$X_0{}' = f_{dil} \times X_0 \qquad \text{Equation 14}$$

where f_{dil} is the sample dilution factor and X_0 is the ethanol concentration result corrected for bias.

In order to determine the combined standard uncertainty of the final concentration result, $u_{X_0'}$, each of the terms in *Equation 13* needs to be evaluated individually.

Certified Reference Material Uncertainty Contribution

The certificate provided by the manufacturer for the certified aqueous ethanol solution indicated a concentration of 20.909 ± 0.251 g/100 mL at 95% confidence. This uncertainty in the reference material concentration needs to be accounted for in the overall measurement uncertainty estimate. Since this is an expanded uncertainty, with a coverage factor of k = 2, a normal distribution may be assumed, and the standard uncertainty, u_R, is found to be 0.1255 g/100 mL.

Sample Dilution Uncertainty Contribution

After aliquoting the blood specimen (**450 µl**), a volume of acetonitrile (**50 µl**) is added. Hence, the dilution factor, d, is calculated as:

$$d = \frac{V_{blood} + V_{CH_3CN}}{V_{blood}} = \frac{450\ \mu\text{L} + 50\ \mu\text{L}}{450\ \mu\text{L}} = 1.1111 \qquad \text{Equation 15}$$

Although the pipettes used to aliquot samples are calibrated, the internal volume of each pipette still has an uncertainty associated with it. This uncertainty is specific to the volume being pipetted, which is in turn dependent on temperature. As such, the uncertainty in the volume dispensed by each pipette should be considered to be the combination of uncertainties due to calibration (cal), temperature (temp), and repeatability (rep).

$$u_{Volume} = \sqrt{u_{cal}^2 + u_{temp}^2 + u_{rep}^2} \qquad \text{Equation 16}$$

Subsequently, by the rules of the propagation of error, the combined standard uncertainty for the dilution factor, u_d, is found by:

$$\frac{u_d}{d} = \sqrt{\left(\frac{\sqrt{u_{450\,\mu l}^2 + u_{50\,\mu l}^2}}{V_{450} + V_{50}}\right)^2 + \left(\frac{u_{450\,\mu l}}{V_{450}}\right)^2} \qquad \text{Equation 17}$$

Evidently, in order to determine u_d, the standard uncertainties $u_{450\,\mu l}$ and $u_{50\,\mu l}$ need to first be evaluated.

The full calculation for the combined standard uncertainty for the 50 µl volume, $u_{50\,\mu l}$, is given below.

Calibration

The certificate provided by the manufacturer indicated expanded measurement uncertainties of 0.26%, 0.03%, and 0.06% at 20 µl, 100 µl and 200 µl for the 200 µl pipette. Since none of these were the 50 µl volume in question, a plot of uncertainty versus volume was generated and the uncertainty for 50 µl was read off of this. (*See* ***Appendix B***)

Thus, the uncertainty at 95% confidence for a volume of 50 µl was found to be 0.15%. Assuming a rectangular distribution, the standard uncertainty due to calibration, u_{cal}, was calculated as

$$u_{cal} = \frac{0.0015 \times 50\ \mu\text{L}}{\sqrt{3}} = 0.0433\ \mu\text{L} \qquad \text{Equation 18}$$

Temperature

According to the calibration certificate furnished by the calibration facility, the pipettes used throughout the study were calibrated at 20 °C. The temperature of the laboratory in which the experimental procedures were carried out fluctuated within limits of 20 ± 4 °C. Since temperature affects volume, the uncertainties in the volumes dispensed by the pipettes needed to be accounted for, and this could be achieved by means of the coefficient of thermal expansion for water.[17] Once again assuming a rectangular distribution, the standard uncertainty due to temperature (u_{temp}) in the 50 µl volume was calculated as:

$$u_{temp} = \frac{0.00021℃^{-1} \times 50\ \mu L \times 4℃^{-1}}{\sqrt{3}} = 0.0242\ \mu L$$ Equation 19

Repeatability

In order to estimate the uncertainty due to variations in pipette filling, ten 50 µl volumes of water were dispensed and weighed. These yielded a standard deviation of 0.1560 µl, which gave an indication of repeatability and was used directly as the standard uncertainty, u_{rep}.

Finally, the contributions from calibration, temperature and repeatability were combined by *Equation 16* to give the combined standard uncertainty, $u_{50\ \mu l}$, as

$$u_{50\ \mu l} = \sqrt{(0.0433)^2 + (0.0242)^2 + (0.1560)^2} = 0.1637\ \mu L$$ Equation 20

All that remained to determine the overall uncertainty due to sample dilution was to estimate the combined standard uncertainty for the 450 µl volume, $u_{450\ \mu l}$. This was done in the same manner as $u_{50\ \mu l}$.

A plot of uncertainty versus volume was once again created (*See* ***Appendix B***), using the uncertainties indicated in the manufacturer's certificate (0.17%, 0.05%, and 0.04%) and the volumes 100 µl, 500 µl and 1000 µl. An uncertainty of 0.06% at 95% confidence for a volume of 450 µl was read off. This gave a standard uncertainty due to calibration of 0.1559 µl.

Weighing ten volumes of 450 µl resulted in a standard deviation of 0.843 µl, while the uncertainty due to temperature variations was found to be 0.2182 µl.

Combining these uncertainties, once again by *Equation 16*, yielded a combined standard uncertainty for the 450 µl volume of:

$$u_{450\ \mu l} = \sqrt{(0.1559)^2 + (0.2182)^2 + (0.843)^2} = 0.8846\ \mu L$$ Equation 21

Having determined estimates for both $u_{50\ \mu l}$ and $u_{450\ \mu l}$, *Equation 17* could now be employed to calculate the relative uncertainty due to sample dilution as:

$$\frac{u_d}{d} = \sqrt{\left(\frac{\sqrt{(0.8846)^2 + (0.1637)^2}}{450 + 50}\right)^2 + \left(\frac{0.8846}{450}\right)^2} = 0.0027\ \mu L \qquad \text{Equation 22}$$

With a sample dilution factor of d = 1.1111, u_d was found to be 0.002961.

Procedural Uncertainty Contribution

All aspects of sample analysis, from aliquoting and sample work-up to determining concentration from a linear regression response curve, introduce variability to the final result, and should therefore be accounted for in the uncertainty estimate. This overall procedural uncertainty can be estimated from the contributions of method bias and method imprecision.

Bias

The means of 77 experimentally obtained concentration values for internal control specimens prepared at the three internal quality control levels (0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL) were plotted against the theoretical concentrations of 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL. The resulting non-weighted regression line shown in *Figure 21* allowed correction for procedural bias (in a similar fashion to that of *Figure 9* and *Equation 7* obtained during method validation) by means of the correction function with the form $Y_0 = BX_0 + A$. The 95% confidence intervals for the intercept and gradient were $A = 0.0004 \pm 0.002707$ and $B = 1.0369 \pm 0.01538$ respectively.

Once again, in order to assess the method for significant bias, the confidence intervals for the gradient and the intercept were inspected more closely. Specifically, since the 95% confidence interval for *A* did include zero while the 95% confidence interval for *B* did not include one, it could be concluded that while the method did not exhibit additive bias, it did in fact exhibit significant multiplicative bias.

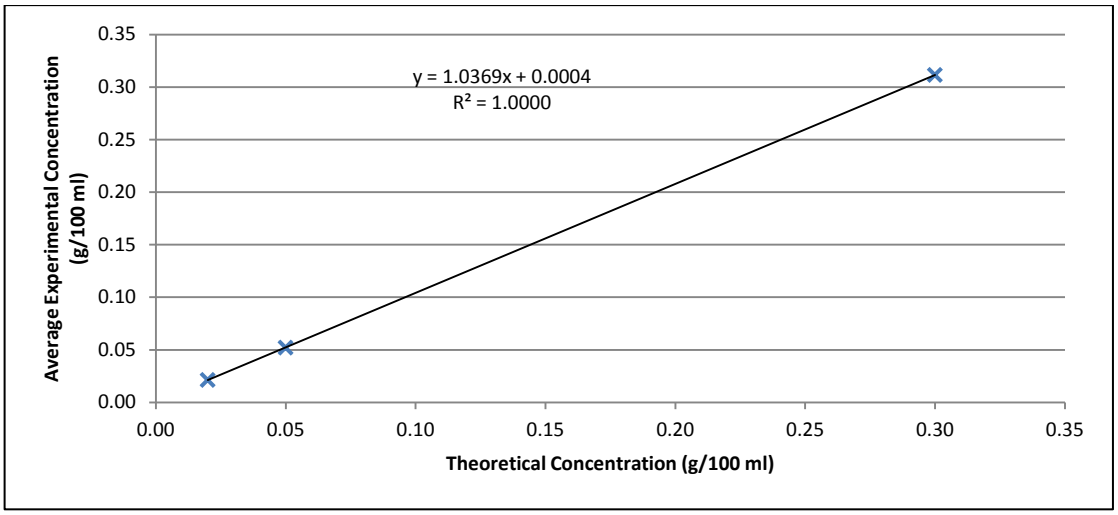


Figure 21: Bias correction plot obtained from 13 months' quality control data

Consequently, if this bias correction line is to be applied to an experimentally obtained concentration value, Y_0, the uncertainty introduced needs also to be considered. The uncertainty contribution due to bias correction is calculated by:[18]

$$u_{bias} = \frac{S_{Y/X}}{B}\sqrt{\left(\frac{1}{n}\right) + \left(\frac{1}{m}\right) + \frac{(Y_0 - \bar{Y})^2}{B^2 \sum_1^3 (X_i - \bar{X})^2}} \qquad \text{Equation 23}$$

where $s_{Y/X}$ is the standard deviation of the regression line of Y on X; n is the number of points on the line – that is the number of internal quality control concentrations; m is the number of replicates at each control level; and $(\bar{X};\bar{Y})$ is the centroid of the bias correction plot.

Substituting $S_{Y/X} = 0.00026$, $B = 1.0369$, $n = 3$, $m = 77$, along with the above average experimental concentrations yielded u_{bias} values of 0.0001919 g/100 mL, 0.0001722 g/100 mL, and 0.0002547 g/100 mL at the concentrations 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL respectively.

Imprecision

The second aspect of the procedural uncertainty contribution is method imprecision. Although there seems to be no consensus in literature as to how to estimate this component, it does seem logical to consider the standard deviation of experimental concentrations obtained over a long period of time. What follows are three methods of calculating the method imprecision, and a recommendation as to which seems the most apt.

Method 1

The 77 experimentally obtained ethanol concentrations at the three control levels are each the average of two replicates. Gullberg[4] proposes calculating the standard deviations of the differences between replicates at each concentration level by *Equation 24*, and then plotting these against theoretical concentration. The uncertainty at any concentration, between those of the low and high control, can then be estimated by means of the non-weighted linear regression obtained.

$$SD_G = \sqrt{\frac{\sum_{i=1}^{k} d_i^2}{2k}} \qquad \text{Equation 24}$$

Applying *Equation 24* to the differences between the replicates of the 77 internal quality control samples gave standard deviation values of 0.001927 g/100 mL, 0.004944 g/100 mL, and 0.02250 g/100 mL, which were then plotted against theoretical concentration to yield *Figure 22*.

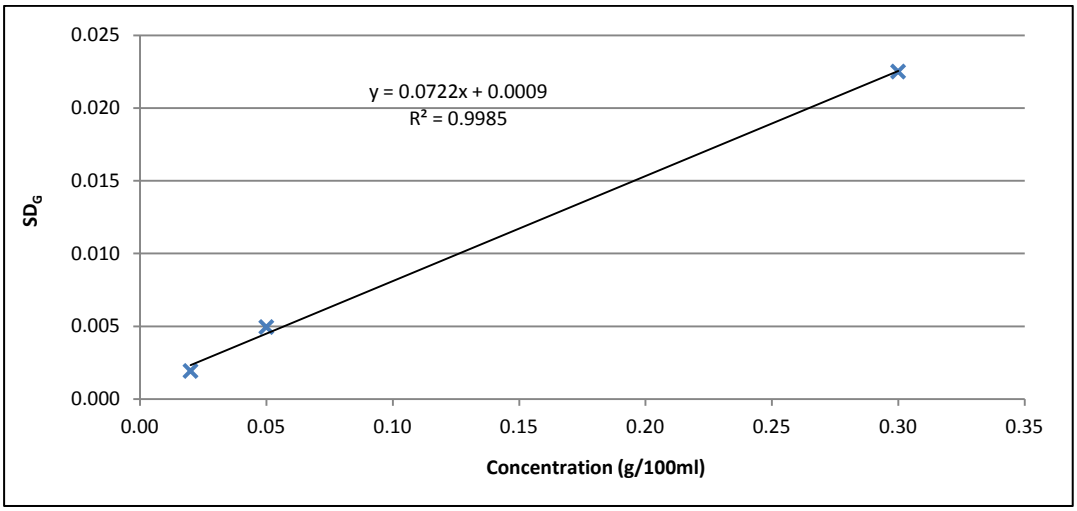


Figure 22: Standard deviation as calculated by Gullberg's method, SD_G, against theoretical concentration

Substituting the average experimental concentrations 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL into the regression equation from *Figure 22*, resulted in u_{imp} values of 0.002425 g/100 mL, 0.004645 g/100 mL, and 0.02338 g/100 mL.

Method 2

The SAC-SINGLAS Technical Guide on Measurement Uncertainty in Medical Testing[5] suggests a different approach, considering imprecision to be “an estimate of uncertainty due to random effects of the whole procedure over time”. The imprecision of a method is thus estimated by calculating the standard deviation (SD), or relative standard deviation (RSD), for results at suitable internal quality control concentrations over a period of time. Where more than two levels are used, the SD or RSD values are then combined to give an overall u_{imp} value by:

$$u_{imp} = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2 + \cdots + (n_k - 1)SD_k^2}{(n_1 - 1) + (n_2 - 1) + \cdots + (n_k - 1)}} \quad \text{Equation 25a}$$

$$u_{imp} = \sqrt{\frac{(n_1 - 1)RSD_1^2 + (n_2 - 1)RSD_2^2 + \cdots + (n_k - 1)RSD_k^2}{(n_1 - 1) + (n_2 - 1) + \cdots + (n_k - 1)}} \quad \text{Equation 25b}$$

where k is the number of levels, and n_1-n_k are the number of observations that contribute to the respective SD or RSD value. The usual rules for pooling standard deviations apply – that is, standard deviation values may only be pooled if they are found to not be statistically different. In order to test this, the F-test is applied.

For the 77 experimental concentration values at the three internal quality control concentration levels, standard deviation values of 0.002636 g/100 mL, 0.005314 g/100 mL, and 0.02657 g/100 mL were obtained for the concentrations 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL respectively.

Before these can be combined, it needs to be decided whether to use SD values or RSD values. The Eurolab Guide to the Evaluation of Measurement Uncertainty for Quantitative Test Results[13] offers some guidance in this respect. It suggests that RSD values be used when procedural standard deviation increases proportionally with concentration value. As this is clearly the case, it is RSD values that will be combined.

An additional check for this can be done by performing ANOVA tests on the three sets of 77 concentration values, as well as on the three sets of relative concentration values (relative to average concentration) to determine whether the standard deviation values of the three sets can be considered statistically the same and be subsequently combined. It should be noted that the standard deviation of the relative concentration values is the same as the relative standard deviation of the concentration values. The ANOVA results are summarised in *Table 13*.

Table 13: ANOVA results determining whether to use SD or RSD to estimate method imprecision

	F_{test}	F_{crit}
Concentration values	7818.8	3.035
Relative concentration values	7.98×10^{-14}	3.035

Applying ANOVA to the three sets of concentration values yielded an F_{test} value vastly greater than the F_{crit} value, while applying ANOVA to the relative concentration sets resulted in an F_{test} value much smaller than the F_{crit} value. This advocates the use of RSD values to estimate u_{imp} since, being significantly different, the SD values may not be pooled.

As such, dividing the SD values by the respective average concentration gave RSD values of 0.1235, 0.1020, and 0.08528. Combining these by *Equation 25b* resulted in an overall u'_{imp} value of 0.1048. This is a relative value, therefore u_{imp} for a specific experimental concentration value is calculated by:

$$u_{imp} = u'_{imp} \times Y_0 \qquad \text{Equation 26}$$

where u'_{imp} is the combined relative uncertainty and Y_0 is the experimental concentration.

Substituting the average experimental concentrations 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL into *Equation 26* gave u_{imp} values of 0.002236 g/100 mL, 0.005457 g/100 mL, and 0.03264 g/100 mL respectively.

Method 3

Finally, another way to estimate the method imprecision is to determine the standard deviations for a number of internal quality control levels, and plot this against theoretical concentration. This results in a linear regression model similar to that of Gullberg's, from which the standard deviation at any experimental concentration between the lowest and highest control levels can be estimated.

The standard deviations of the three sets of 77 internal quality control concentration values (which are each a mean of two replicates) obtained over a period of 13 months were 0.002636 g/100 mL, 0.005314 g/100 mL, and 0.02657 g/100 mL. The non-weighted linear regression curve obtained by plotting these against the theoretical concentrations 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL is shown in *Figure 23*.

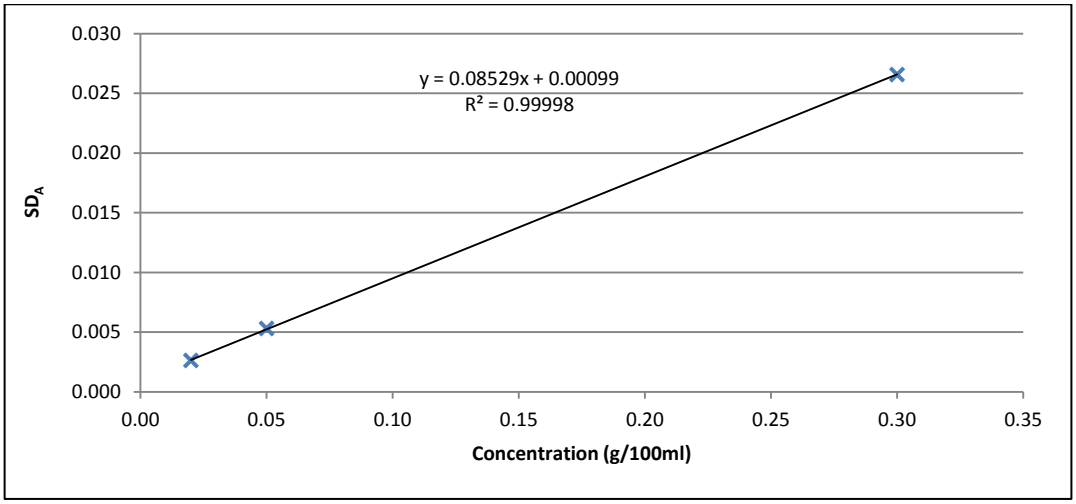


Figure 23: Standard deviation, SD_A, of the 77 average experimental ethanol concentration results against theoretical concentration

In order to evaluate the uncertainty due to imprecision, the equation of the regression line in *Figure* 23 is applied to the experimental concentration by:

$$u_{imp} = 0.08529Y_0 + 0.00099 \qquad \text{Equation 27}$$

Hence, applying *Equation 27* to the average concentration values 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL, resulted in u_{imp} values of 0.002806 g/100 mL, 0.005428 g/100 mL, and 0.02756 g/100 mL respectively.

Imprecision Summary

The u_{imp} values of the three methods at the three average concentration values are compared in *Table 14*.

Table 14: Summary of u_{imp} estimates obtained by the three methods for each of the average experimental concentrations

Concentration (g/100 mL)	M1 u_{imp} (g/100 mL)	M1 u_{imp} (%)	M2 u_{imp} (g/100 mL)	M2 u_{imp} (%)	M3 u_{imp} (g/100 mL)	M3 u_{imp} (%)
0.02134	0.002425	11.36	0.002236	10.48	0.002806	13.15
0.05208	0.004645	8.92	0.005457	10.48	0.005428	10.42
0.3115	0.02338	7.51	0.03264	10.48	0.02756	8.85

While all three methods would be considered acceptable ways of calculating the uncertainty component due to method imprecision, as was stated earlier, it falls to the individual laboratory to decide the most fitting means of estimating this quantity – provided the estimate employed is mathematically and statistically sound. Each laboratory must decide for itself, based on experience and on "knowledge of the performance of the method"[12], whether an uncertainty estimate is applicable to their specific situation.

The imprecision estimates afforded by Method 1 and Method 3 seem to be fairly comparable, with Method 1 giving a slightly smaller estimate. However, Method 1 calculates the within-batch imprecision over time, while Method 3 calculates the between-batch imprecision – the overall method performance. For this reason, it seems more applicable to estimate method imprecision from the range of concentration values obtained by the method over time. That is, from the standard deviations of average experimental concentration results at certain pertinent concentration levels. Both Method 2 and Method 3 make use of these standard deviations.

Pooling the relative standard deviations as in Method 2 provides a simple and convenient way of estimating the uncertainty due to imprecision at any concentration. It does, however, assume that the uncertainty is uniform over all concentrations. Examining the relative standard deviations of the three

internal quality control levels reveals that this is not the case. In fact, standard deviation is inversely proportional to concentration. As such, pooling the standard deviations in this manner and applying this estimate to all concentrations would result in an under-estimate at lower concentrations while over-estimating the uncertainty at higher concentrations.

Plotting the standard deviations against the control concentrations as is done in Method 3 accounts for the inverse relationship between concentration and uncertainty, and provides a less skewed estimate at concentrations other than those of the internal quality controls than does Method 2. There is also no manipulation of data, as the standard deviations of the control data are used directly. In addition, the regression line obtained in Method 3 exhibits vastly better linearity than that in Method 1, as evidenced by the correlation coefficient of 0.99998.

Despite being less convenient than Method 2, Method 3 was found to be the most applicable of the three. It was thus decided Method 3 would be employed to estimate the uncertainty due to imprecision. As such, u_{imp} was found to be 0.002806 g/100 mL, 0.005428 g/100 mL, and 0.02756 g/100 mL at the three average concentration values 0.02134 g/100 mL, 0.05208 g/100 mL, and 0.3115 g/100 mL respectively.

Having determined the uncertainty contributions for calibration, sample dilution, bias and imprecision, *Equation 28* given below can be used to estimate the standard uncertainty at a certain experimental concentration corrected for bias and sample dilution, X'_0.

$$\frac{u_{X_0'}}{X_0'} = \sqrt{\left(\frac{0.1255}{20.869}\right)^2_{CRM} + \left(\frac{0.0027}{1.1111}\right)^2_{dil} + \left(\frac{u_{bias}}{X_0}\right)^2_{bias} + \left(\frac{u_{imp}}{X_0}\right)^2_{imp}} \qquad \text{Equation 28}$$

It is interesting to note that the imprecision contributes 99.5% to the overall uncertainty. This means that variations in the bias contribution will have little if any effect on the overall measurement uncertainty.

Finally, the expanded uncertainty can be calculated by applying a coverage factor, k, suitable to the confidence level required, by:

$$U_{X_0'} = k \times X_0' \qquad \text{Equation 29}$$

The combined expanded uncertainties at 95% (k = 2) and 99% (k = 2.576) confidence for three example experimental concentrations are given in *Table 15*.

Table 15: Example ethanol concentrations corrected for bias and dilution, and their corresponding standard- and expanded measurement uncertainties at 95% and 99% confidence

Y_0 (Exp. Conc.) (g/100 mL)	X_0 (Y_0 Corr. For Bias) (g/100 mL)	X_0' (X_0 Corr. For Dil.) (g/100 mL)	$u_{X_0'}$ (g/100 mL)	95% Confidence		99% Confidence	
				$U_{X_0'}$ (g/100 mL)	$U_{X_0'}$ (%)	$U_{X_0'}$ (g/100 mL)	$U_{X_0'}$ (%)
0.0191	0.0180	0.0200	0.00275	0.00550 (0.0055*)	27.5	0.00709 (0.0071*)	35.0
0.0471	0.0450	0.0500	0.00533	0.01065 (0.011*)	21.3	0.01372 (0.014*)	28.0
0.2810	0.2706	0.3006	0.02677	0.05355 (0.054*)	17.8	0.06897 (0.069*)	23.0

* Expanded measurement uncertainty rounded up to two significant figures [5]

At first glance, these MU values seem quite large, especially when compared to that given by Gullberg for the HS-GC-FID method of 0.0012 g/100 mL at an ethanol concentration of 0.0809 g/100 mL.[4] However, the values given in *Table 15* allow distinction between the two legal limits, as well as the "states of drunkenness" as laid out in Garriot.[19] Since the aim of estimating measurement uncertainty is to determine the reliability of the results generated, these estimates, while conservative, indicate that the method is fit-for-purpose to provide sufficient distinction between blood ethanol concentrations.

It is common practice that once MU values are estimated, they are kept constant for a period of time, before being adjusted upon re-evaluation. Calculating the MU in the manner detailed above, means that the MU values are updated continually after each new set of internal quality control concentrations is obtained during routine batch analysis.

For many routine laboratories, 95% confidence is sufficient; however, where decisions need to be made that may result in prosecution, 99% confidence should be employed. As such, for the rest of this document, a 99% confidence level will be used.

In prosecution situations it is often useful to institute the concept of a "guard band". The measurement uncertainty of a method is applied by *Equation 30* at certain legal limit concentrations to ensure that fair conviction takes place.

$$X_{Prosecution} = X_{legal\ lim} + U_{X_0'} \qquad \text{Equation 30}$$

In this case, applying the measurement uncertainties in *Table 15* to the two South African legal limits of 0.02 g/100 mL and 0.05 g/100 mL resulted in prosecution levels of 0.027 g/100 mL and

0.064 g/100 mL respectively. This means that an ethanol concentration obtained by this GC-MS method can only be deemed above the legal limit if it is found to be above the prosecution level.

In practice, when a specimen concentration is determined, it is generally calculated as the mean of duplicate analyses. However, these analyses should not differ by more than what is known as the precision limit, R, if the mean is to be taken. At 99% confidence, this can be calculated by:[20]

$$R = 2.576 \times \sqrt{2} \times u_{X_0'} = 3.643 u_{X_0'} \qquad \text{Equation 31}$$

Applying *Equation 31* to the experimental concentrations in *Table 15* results in precision limits of 0.010 g/100 mL, 0.019 g/100 mL, and 0.0975 g/100 mL at the 0.02 g/100 mL, 0.05 g/100 mL and 0.030 g/100 mL levels respectively.

2.4. Conclusion

An alternative method was sought to accurately quantify ethanol in blood, since the supplied method was found to be damaging to the GC column. Pentafluorobenzoic acid was established to be the agent responsible for the column damage, and as such the alternative method would need to remove as much of this as possible while not affecting the final ethanol concentration result. Many adjustments to the supplied method were attempted, and finally it was decided that including a saturated $NaHCO_3$ wash followed by a dH_2O wash satisfactorily decreased the PFB-COOH concentration, while still allowing for the accurate determination of ethanol concentration.

The adjusted method was validated, and exhibited excellent linearity, although it is recommended that a response model be generated with each new batch. The LOD and LOQ were determined to be 0.00125 g/100 mL and 0.0025 g/100 mL respectively by S/N analysis. Acceptable limits for the ion abundance ratios were given and the concept of “identification before quantification” was introduced. The method was found to display excellent specificity, with the blank matrix not significantly contributing to the ethanol concentration result. Although exhibiting no additive bias, a small multiplicative bias was present. Additionally, the method demonstrated small within batch precisions, and between-batch precisions of around 10%, at the three internal quality control levels. The overall precisions at the medium and high control levels were approximately 10%, while that at the low control level was about 13%.

The method was found to be rugged, with derivatisation time and derivatisation reagent concentration hardly impacting the final concentration result. It was, however, recommended not to use PFBCl concentrations as high as 10%, and not to shake for longer than 3.5 hours.

It was found that drying samples to completeness, or removing them from the drying manifold with a small amount of solvent remaining, had little or no effect on the concentration result obtained. The internal standard successfully accommodates for this.

The derivatised analyte was found to be stable for at least 16 hours on the autosampler tray, implying that samples can safely be left on the instrument for the duration of a typical run without the ethanol concentration results being significantly affected.

Levey-Jennings control charts at the three internal quality control levels for a period of 13 months were given. These displayed no significant trends, and no Westgard rules were violated. A slight positive bias was observed in all three charts; however, the theoretical concentrations lay well within 1 standard deviation of the means. This emphasises the accuracy of the method, and shows that the observed bias need not be a cause for concern. The bias should merely be corrected for in the calculation of the final concentration result, and accounted for in the uncertainty estimate.

The calculation of the measurement uncertainty was tutorial in nature, designed to allow the reader to follow and apply it to their individual situation. It followed the approaches of Gullberg[4] and the SAC-

SINGLAS Technical Guide on Measurement Uncertainty in Medical Testing[5], with slight adjustments. The combined MU included contributions from calibration, sample dilution, bias and imprecision. The final expression obtained for this can be used to estimate the MU at any experimental ethanol concentration.

At the three internal quality control levels of 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL, the 99% confidence MU was determined to be 35%, 28%, and 23% respectively. These estimates were then used in the calculation of the prosecution levels at the two South African legal limits, which were found to be 0.027 g/100 mL for professional drivers and 0.064 g/100 mL for public drivers. Furthermore, the method was deemed fit-for-purpose since the measurement uncertainty allowed for distinction between the different “states of drunkenness”, and also between the South African legal limits.

Having arrived at an expression to estimate the MU for any ethanol concentration determined by the GC-MS method, further studies to investigate various claims made in court regarding the validity of blood ethanol concentration results can be performed. Of specific interest is the stability of ethanol concentrations in samples that are stored under various conditions for a period of time.

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Chapter 3

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3.1. Introduction

In South Africa, blood specimens are usually collected in evacuated sample collection tubes containing sodium fluoride (NaF) and potassium oxalate (KOx). The potassium oxalate prevents the sample from clotting[1], while the sodium fluoride is required to inhibit microbial growth.[2]

In accordance with South African case law, the blood specimen collection tubes are designed to have a final NaF concentration of at least 1% (w/v), or 1 g/100 mL when filled to capacity. A concentration of less than 1% (w/v) may not be sufficient to prevent ethanolic fermentation by various micro-organisms, and it is claimed that this could result in an increase in ethanol concentration.[3, 4]

Since the tubes are rarely filled completely, the NaF concentration will almost always be higher than 1% (w/v).[5] However, it is still necessary to show that sufficient fluoride is present to preserve the sample. That is, whenever a blood ethanol concentration is reported, the result must always be accompanied by the corresponding NaF concentration.

The most common way of determining fluoride concentration in biological matrices such as blood is by means of a Fluoride Ion Selective Electrode (FISE). The potential that develops across the membrane of the electrode is related to the free fluoride concentration by the Nernst equation.

The level of free fluoride in a sample is subject to temperature, pH, total ionic strength, and complexation reactions that may occur between free fluoride ions and various components present in the matrix.[6] Consequently, any analytical method developed for the determination of fluoride concentration by means of FISE needs to take each of these parameters into account to ensure accurate fluoride concentration results.

As is discussed below, a method for the determination of fluoride concentration in human whole blood by means of the FISE was developed, with specific emphasis on minimising matrix effects. The method was validated, and the measurement uncertainty calculated in full, following the same reasoning as with the ethanol GC-MS method measurement uncertainty calculation. From this, the minimum legal fluoride concentration was determined. Finally, some applications of the FISE method are presented.

3.2. Experimental

3.2.1. General Details

Certified Reference Materials

An aqueous solution of sodium fluoride (2.997 ± 0.075 g/100 mL) was purchased from the National Metrology Institute of South Africa (NMISA) and is henceforth referred to as NaF standard solution. (See Certificates of Analysis in ***Appendix E***)

Reagents and Solvents

Anhydrous sodium fluoride (NaF, 99.5%) was obtained from Merck, Steinheim, Germany. Total Ionic Strength Adjustment Buffer II with CDTA (TISAB II) and Optimum Results A filling solution were purchased from ThermoScientific, Chelmsford, MA, USA.

Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) was obtained from Boehringer Mannheim GmbH, Germany, while sodium tartrate ($C_4H_4O_6Na_2$, 99.5%) was purchased from Bio-zone Chemicals, Van Riebeeck Park, Gauteng, SA.

Iron (III) nitrate nonahydrate ($Fe(NO_3)_3{\cdot}9H_2O$, 99.99%) was obtained from Aldrich, Milwaukee, USA. Magnesium sulphate anhydrous ($MgSO_4$, 62-70%) was purchased from Saarchem, Wadeville, Gauteng, SA and calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, 99%) from Radchem, W. Germany.

Potassium oxalate monohydrate ($C_2K_2O_4{\cdot}H_2O$, 99.0%) was obtained from Sigma Aldrich, Steinheim, Germany.

Deionised water and concentrated hydrochloric acid (HCl, 32%) were purchased from Merck, Modderfontein, Gauteng, SA.

Preparation of TISAB IV

Total Ionic Strength Adjustment Buffer IV solution was prepared by placing 100 mL dH_2O in a 200 mL volumetric flask. Tris (hydroxymethyl) aminomethane (**48.423 g**) and sodium tartrate (**46.012 g**) were added, as well as 19.2 mL HCl (32%). The flask was swirled until the solids were completely dissolved, and the solution allowed to cool to room temperature (22 ± 6 ℃) before topping up to the mark with dH_2O. [6]

Whole Blood

Bovine blood, donated by the Roodeplaat Abattoir, Cullinan, was collected in evacuated tubes (Vacutainers) containing heparin anticoagulant, and was used in all initial studies. This blood was pooled prior to use and contained only endogenous fluoride.

Fresh human whole blood was collected from healthy volunteers in evacuated tubes (Vacuette® tubes, Greiner Bio-One International, Frickenhausen, Germany) containing sodium heparin anticoagulant in accordance with ethical standards (Ethical Clearance Number: **EC150618-013**; See ***Appendix F***). This blood was also pooled prior to use and contained only endogenous fluoride.

In this chapter, “blood” shall refer to fresh whole blood obtained from human donors, while the use of bovine blood will be explicitly stated.

Instrumentation and Equipment

A 9609BNWP Fluoride Combination Electrode (ThermoScientific, Chelmsford, MA, USA), filled with Optimum Results A filling solution, was used for analysis of control and specimen solutions. The solution to be analysed was placed into a 20 mL beaker (Lasec, Cape Town, South Africa) with a 3 mm polytetrafluoroethylene (PTFE) magnetic stirrer bar (Lasec, Cape Town, South Africa), and

agitated vigorously at 500 rpm during analysis. A polystyrene block was placed between the beaker and magnetic stirrer plate to prevent heat transfer to the sample. Additionally, an Orion Automatic Temperature Compensation Probe (927007MD) was placed in a volume of water as reference, and connected to the Orion Star A214 Benchtop pH/ISE Meter throughout to compensate for any fluctuations in temperature during analysis.

Electrode Setup

The electrode filling solution was topped up each day to just below the filling hole before using the electrode. Following this, the electrode slope was determined by analysing calibration solutions prepared at 0.250 g/100 mL and 2.50 g/100 mL from the NaF standard solution. If the difference between the millivolt readings was not between 54 and 60 mV, the electrode was deemed to not be functioning correctly and reparative action was taken.

Sample Preparation Conditions

All experimental procedures were performed at room temperature (22 ± 6 °C) unless otherwise stated.

Calibration Solutions

Each response model was prepared from seven calibration solutions (0.250, 0.500, 1.00, 1.50, 2.00, 2.50, 3.00 g/100 mL) with each point being the average of two replicates. Wherever anhydrous NaF was used to prepare calibration solutions, duplicate masses as close as possible to these theoretical values were weighed out.

3.2.2. Method Development

In each of the following experiments, response models were obtained by plotting potential against log of theoretical concentration. The response models from calibrators prepared in blood matrix were compared with those in aqueous medium in order to develop a method that performed identically in both matrices. Should this be possible, it would not be necessary to prepare matrix-matched response models when determining fluoride concentration in blood samples.

Calibration by Weighed NaF and 2x Dilution

Two sets of seven duplicate masses of anhydrous NaF were weighed into test tubes (2.80 – 30.05 mg).

To the first set of tubes, dH_2O (**1000 µl**) was added, while bovine blood (**1000 µl**) was added to the second set. The tubes were vortexed thoroughly for 2.5 minutes.

The calibrators were then transferred to beakers and TISAB II (**1000 µl**) was added to each. After stirring well to homogenise by means of the magnetic stirrer bar, the potential of each calibrator solution was determined.

Finally, seven duplicate calibrator solutions were prepared (**1000 µl** each) from the NaF standard solution. Total Ionic Strength Adjustment Buffer II (**1000 µl**) was added to each, and the solutions were analysed by means of the FISE.

Effect of TISAB

Two sets of seven duplicate masses of anhydrous NaF were weighed into test tubes (2.46 – 30.00 mg). Blood (**1000 µl**) was added to both sets of tubes, which were then vortexed thoroughly for 2.5 minutes. The blood calibration solutions were then transferred to beakers.

Total Ionic Strength Adjustment Buffer II (**1000 µl**) was added to each solution in the first set and TISAB IV (**1000 µl**) to each in the second set. After stirring well to homogenise, the potential of each blood calibrator was determined.

Two sets of seven duplicate calibrator solutions were prepared (**1000 µl** each) from the NaF standard solution. Total Ionic Strength Adjustment Buffer II (**1000 µl**) was added to each solution in the first set while TISAB IV (**1000 µl**) to each in the second set. After stirring well to homogenise, the potential of each aqueous calibrator was determined.

3x Dilution

Seven duplicate aqueous calibrator solutions were prepared (**1000 µl** each) from the NaF standard solution, and TISAB II (**1000 µl**) and dH_2O (**1000 µl**) were added to each. After stirring well to homogenise, the potential of each aqueous calibrator was determined.

Seven duplicate blood calibrators were prepared by spiking blood (**1000 µl**) with aqueous calibrator solutions (**1000 µl**) prepared as before from the NaF standard solution. A volume of dH_2O (**1000 µl**) was added to each, and the solutions were mixed well to homogenise. The potentials of each blood calibrator were then determined by means of the FISE.

10x Dilution

Seven duplicate aqueous calibrator solutions were prepared (**500 µl** each) from the NaF standard solution, and TISAB II (**2500 µl**) and dH_2O (**2000 µl**) were added to each. After stirring well to homogenise, the potential of each aqueous calibrator was determined.

Seven masses of anhydrous NaF were weighed into test tubes (2.49 mg, 5.01 mg, 10.02 mg, 14.99 mg, 20.05 mg, 24.95 mg and 30.02 mg). Bovine blood (**1000 µl**) was added to the tubes, which were then vortexed thoroughly for 2 min. The blood calibration solutions were transferred to beakers, and a portion (**500 µl**) of each solution was then pipetted into a second beaker. The resulting duplicate calibration solutions were diluted with TISAB II (**2500 µl**) and dH_2O (**2000 µl**), and analysed by means of the FISE.

20x Dilution

Seven aqueous calibrator solutions were prepared (**200 µl** each) from the NaF standard solution, and TISAB II (**2000 µl**) and dH_2O (**1800 µl**) were added to each. After stirring well to homogenise, the potential of each aqueous calibrator was determined.

Seven masses of anhydrous NaF were weighed into beakers (1.27 mg, 2.50 mg, 5.05 mg, 7.56 mg, 10.03 mg, 12.43 mg and 14.95 mg). Blood (**500 µl**) was added to each, and the contents of each beaker stirred vigorously for 45 minutes to dissolve the NaF. The calibrators were then diluted with TISAB II (**5000 µl**) and dH_2O (**4500 µl**), agitated for 15 minutes, and analysed by means of the FISE.

3.2.3. Method Validation

Method validation was performed based on figures of merit including linearity, limit of detection, limit of quantitation, selectivity, specificity, bias, and precision.

Linearity

Five calibration graphs of potential versus log of fluoride concentration were prepared on different days, using the seven aqueous calibration solutions. Each solution (**200 µl**) was diluted with dH_2O (**1800 µl**) and TISAB II (**2000 µl**) before recording the potential. From these graphs the 95% confidence interval of the correlation coefficient was determined and the linearity of the method assessed.

Limits of Detection and Quantitation

Ten sets of duplicate blank samples were prepared, using fresh blood from ten different volunteers, and assessed for fluoride content. These potentials were used to calculate the theoretical LOD and LOQ for the method.

Specificity

Ten sets of duplicate blank samples were prepared, using fresh blood from ten different volunteers, and assessed for fluoride content.

Bias and Precision

Five internal quality control samples at three concentration levels (0.250 g/100 mL, 1.00 g/100 mL, and 2.75 g/100 mL) were prepared on five different days. Each group on each day was inspected for outliers, and if found, these were removed. These experimentally obtained fluoride concentrations were then used to evaluate the bias and precision of the method as described below.

A bias correction regression line was obtained by plotting average experimental fluoride concentration versus theoretical fluoride concentration. From this, the method was assessed for multiplicative and additive bias.

In order to evaluate the precision of the method, the differences between the theoretical concentration and each replicate concentration for each of the five days was determined. Analysis of variance (ANOVA) was applied to these differences in order to determine the within- and between-group variances.

3.2.4. Measurement Uncertainty Calculations

Normality

Before proceeding, the normality of each of the three data sets was confirmed by comparing the plots of experimental concentration against experimental Cumulative Distribution Function (CDF) values with experimental concentration against theoretical CDF. (See ***Appendix D***)

Measurement Uncertainty

The expanded MU for the method was then determined using the internal quality control (IQC) concentration data obtained during method validation. The same methodology as was followed to calculate the MU for the ethanol method was applied, once again including aspects from both Gullberg[7] and the SAC-SINGLAS Technical Guide on Measurement Uncertainty in Medical Testing.[8] The overall MU was calculated as the combined contributions of the fluoride CRM, pipettes, bias, and imprecision, and is reported as the expanded MU at both 95% and 99% confidence.

3.2.5. Method Applications

Complexation Studies

Aqueous NaF calibration standards were prepared according to *Table 1* to a final volume of 10 mL.

Table 1: Preparation of NaF calibration standard solutions from aqueous NaF stock solution (3 g/100 mL)

Standard	1	2	3	4	5	6	7
V_{H2O} (mL)	9.1666	8.3334	6.6666	5.000	3.3334	1.6666	0.0000
V_{NaF} (mL)	0.8334	1.6666	3.3334	5.000	6.6666	8.3334	10.000
C_{Final} (g/100 mL)	0.250	0.500	1.00	1.50	2.00	2.50	3.00

Iron

An Fe^{3+} stock solution was prepared by dissolving 4.06248 g $Fe(NO_3)_3 \cdot 9H_2O$ in dH_2O to a total volume of 10 mL, giving a final concentration of 561.6 mg/mL. *Figure 1* illustrates the preparation of four sets of calibration standard solutions.

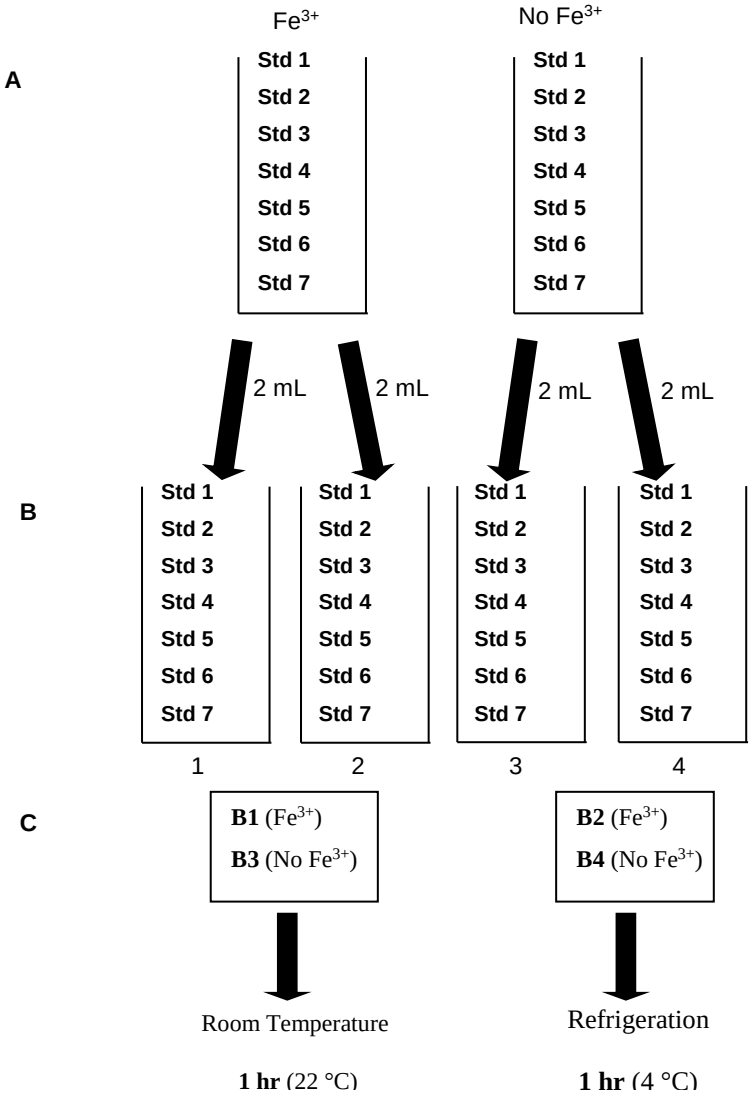

Figure 1: Preparation of four sets of calibrators for iron studies

Two sets of calibrators were prepared by aliquoting each of the calibration standards (**4 mL**) into beakers. Fe^{3+} stock solution (**80 µl**) was added to one set, and both sets of beakers thoroughly mixed (***Figure 1A***). Half of each standard solution (**2mL**) was transferred to separate beakers, resulting in four sets of calibrators (***Figure 1B***). One set of calibrators with Fe^{3+} and one set without were refrigerated for 1 hour, while the other two sets were kept at room temperature (***Figure 1C***).

Filters were prepared by lodging a small piece of cotton wool into the end of a Pasteur pipette, and these, along with the necessary test tubes and Pasteur pipettes for transferring solutions, were placed in the refrigerator with the calibrator solutions for 1 hour.

Cold solutions (***Figure 1C***) were filtered twice through the cotton wool plugs while kept cool, and then allowed to reach room temperature. Separate aliquots (**200 µl**) of each solution were placed in two beakers. To one beaker was added dH_2O (**1800 µl**) and TISAB II (**2000 µl**), while to the other only dH_2O (**3800 µl**) was added. After agitating for 15 minutes, each solution was analysed by means of the FISE. The potentials obtained were then used to prepare response models of potential versus log of fluoride concentration.

Separate aliquots (**200 µl**) of each room temperature solution (***Figure 1C***) were placed in two beakers. To one beaker was added dH_2O (**1800 µl**) and TISAB II (**2000 µl**), while to the other only dH_2O (**3800 µl**) was added. After agitating for 15 minutes, each solution was analysed by means of the FISE. The potentials obtained were then used to prepare response models of potential versus log of fluoride concentration.

The final Fe^{3+} concentration in those calibration solutions spiked with the Fe^{3+} was 5.616×10^{-1} mg/mL.

Magnesium

A Mg^{2+} stock solution was prepared by dissolving 0.70811 g $MgSO_4$ in dH_2O to a total volume of 10 mL, giving a final concentration of 14.30 mg/mL.The preparation of four sets of calibration standards is outlined in *Table 2*. The standard solutions that were prepared according to *Table 1* were used.

Table 2: Preparation of four sets of calibration solutions for magnesium studies

	Volume Std Solution (µl)	**Volume Mg^{2+} stock (µl)**	**Volume TISAB II (µl)**	**Volume dH_2O (µl)**
No Mg-No TISAB	250	0	0	4750
Mg-No TISAB	250	5	0	4745
No Mg-TISAB	250	0	2500	2500
Mg-TISAB	250	5	2500	2245

The final Mg^{2+} concentration in the calibration solutions in Set Mg-No TISAB and Mg-TISAB was 1.43×10^{-2} mg/mL.

After agitating for 15 minutes, each set of calibration solutions was analysed in duplicate by means of the FISE. The potentials obtained were then used to prepare response models of potential versus log of fluoride concentration.

Calcium

A Ca^{2+} stock solution was prepared by dissolving 1.93621 g $CaCl_2$ in dH_2O to a total volume of 10 mL, giving a final concentration of 52.78 mg/mL. The preparation of four sets of calibration

standards is outlined in *Table 3*. The standard solutions that were prepared according to *Table 1* were used.

Table 3: Preparation of four sets of calibration solutions for calcium studies

	Volume Std Solution (µl)	Volume Ca^{2+} stock (µl)	Volume TISAB II (µl)	Volume dH_2O (µl)
No Ca-No TISAB	250	0	0	4750
Ca-No TISAB	250	5	0	4745
No Ca-TISAB	250	0	2500	2500
Ca-TISAB	250	5	2500	2245

The final Ca^{2+} concentration in the calibration solutions in Set Ca-No TISAB and Ca-TISAB was 5.278×10^{-2} mg/mL.

After agitating for 15 minutes, each set of calibration solutions was analysed in duplicated by means of the FISE. The potentials obtained were then used to prepare response models of potential versus log of fluoride concentration.

Oxalate Investigation

An oxalate stock solution with a final concentration of 149.98 g/100 mL was prepared by dissolving 14.998 g potassium oxalate in dH_2O to a total volume of 10 mL.

Aqueous calibration standards were prepared in duplicate using the solutions in *Table 1*. To the standard solution (**200 µl**) was added dH_2O (**1800 µl**) and TISAB II (**2000 µl**). After thoroughly mixing, the solutions were analysed by means of the FISE, and a response model was prepared by plotting potential versus log of fluoride concentration.

An aqueous control solution (**5 mL**) was prepared at 1.00 g/100 mL from the stock NaF solution, and eight samples were prepared as outlined in *Table 4* below.

Table 4: Preparation aqueous samples with and without oxalate

Sample	Volume Control Solution (µl)	Volume Oxalate Solution (µl)	Volume TISAB II (µl)	Volume dH_2O (µl)
1 - 4	250	0	2500	2250
5 - 8	250	5	2500	2245

Sodium fluoride/potassium oxalate blood collection tubes are prepared in such a way that there is twice as much KOx as NaF.[9] Spiking control solutions 5 to 8 with 5 µl of the oxalate stock solution resulted in a final KOx concentration of 3 g/100 mL, more than the usual.

Samples 1 to 8 were agitated for 15 minutes and analysed by means of the FISE.

3.3. Results and Discussion

3.3.1. Method Development

Although it is considered good practice to perform matrix-matched calibrations, sourcing fluoride-free whole blood proved to be challenging, not to mention the added inconvenience – and lack of metrological traceability – of weighing anhydrous NaF in order to prepare the calibrator solutions. For this reason, a method that would allow the determination of fluoride concentration in blood samples while calibrating in aqueous medium would be ideal.

Since literature suggested matrix effects could be minimised by sample dilution[10-12], various sample dilutions were investigated. The performance of each method was assessed by comparing the response model obtained in blood matrix with that in aqueous medium.

Calibration by Weighed NaF and 2x Dilution

Blood samples for alcohol analysis are drawn into evacuated tubes containing anhydrous NaF, which must then be dissolved into the blood matrix. In an effort to mimic this, blood calibration solutions were prepared by weighing out anhydrous NaF and dissolving it in blood. Additionally, the FISE User Guide[6] recommends a 1:1 TISAB II or IV volume to sample volume ratio, and hence a 2x dilution was taken as the starting point for this investigation.

To ensure that the actual process of weighing and dissolving NaF in the medium of choice did not play a role, an additional response model was prepared by dissolving NaF in dH_2O. *Figure 2* shows the response models obtained for the CRM calibration standards, the standards prepared by dissolving anhydrous NaF dissolved in blood and water.

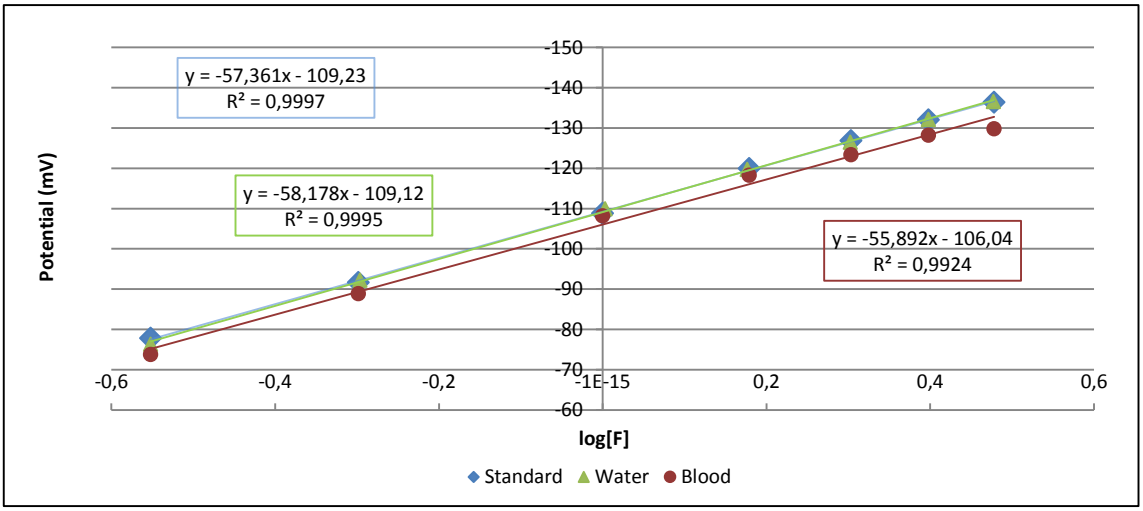

Figure 2: Response models obtained at 2x dilution of the aqueous standard solution, and calibrators prepared by dissolving anhydrous NaF in water and blood

The response model obtained for anhydrous NaF dissolved in dH_2O, lies virtually on top of that for the aqueous standard solution, showing that weighing and dissolving anhydrous NaF in water yields the same response as the standard solution. Hence, the weighing and dissolving process, while lacking metrological traceability, is not a contributing factor to any discrepancies seen when dissolving NaF in blood matrix.

The response model for the anhydrous NaF in blood matrix exhibited a levelling off, starting at 2.00 g/100 mL, which suggests that the blood matrix suppresses the analyte signal. The FISE measures only free fluoride. The high iron content of the matrix may be artificially lowering the fluoride signal by complexing with the free fluoride present.

It is clear that the matrix has an effect on the FISE response and that the matrix effects need to be minimised as far as possible. This could perhaps be achieved through the use of a different TISAB solution or by changing the ratio of TISAB volume to sample volume.

Effect of TISAB

Total Ionic Strength Adjustment Buffer solution is not only added to samples in order to buffer the pH and keep the background ionic strength high. It is also intended to decomplex fluoride by preferentially binding with ions such as iron, magnesium and calcium.

Total Ionic Strength Adjustment Buffer IV is claimed to complex more than 100 ppm iron in the presence of as little as 1 ppm fluoride. In addition, with sodium tartrate as the active complexing agent which can bind to two Fe^{3+} ions, [13] it was theorised that it would be more effective as a complexing agent than the TISAB II, containing CDTA which can only bind to one Fe^{3+}. [14]

Response models were thus prepared in both aqueous and blood medium with no TISAB added, and also with TISAB II and with TISAB IV.

Figure 3 shows the response models obtained in aqueous and blood medium when no TISAB was added. The blood response model exhibits a slope that lies outside the acceptable range for optimum electrode functioning of -54 to -60 mV/dec, as given in *Electrode Setup*[6], as well as poor linearity when compared to the aqueous standard response model. This shows that there are certainly matrix effects at play in blood samples that are not observed in the aqueous standard solutions.

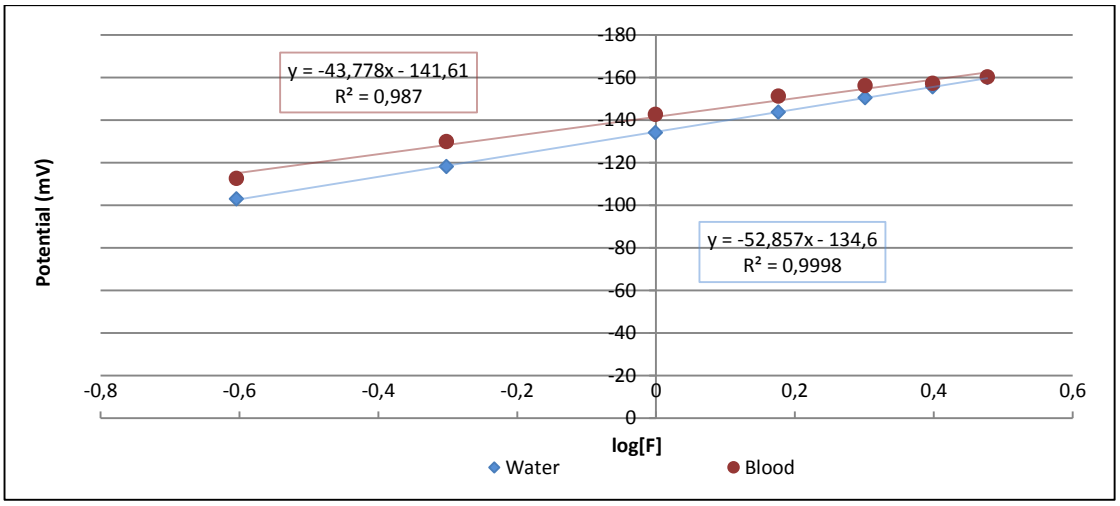

Figure 3: Response models obtained for the aqueous standard and blood calibration solutions prepared with no TISAB solution

Figure 4 shows the response models obtained in aqueous and blood medium when TISAB IV was added. The blood response model displays a slope outside the accepted range. In addition, a levelling off similar to that seen in *Figure 2* is observed, once again beginning at 2.00 g/100 mL. This shows that despite the addition of what should be the stronger complexing agent, TISAB IV is not sufficient to combat the effect of the blood matrix on the free fluoride concentration.

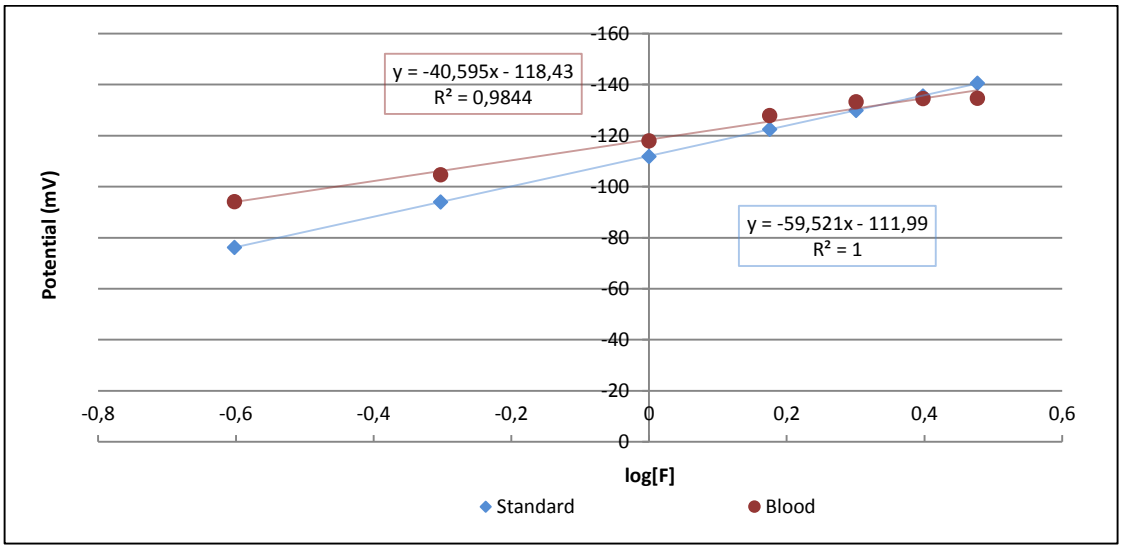

Figure 4: Response models obtained for the aqueous standard and blood calibration solutions prepared with TISAB IV

Adding TISAB II to samples resulted in the response models seen in *Figure 5*. The blood response model also exhibits poor linearity, the slope is still outside the acceptable range and once again a levelling off is observed starting at 2.00 g/100 mL.

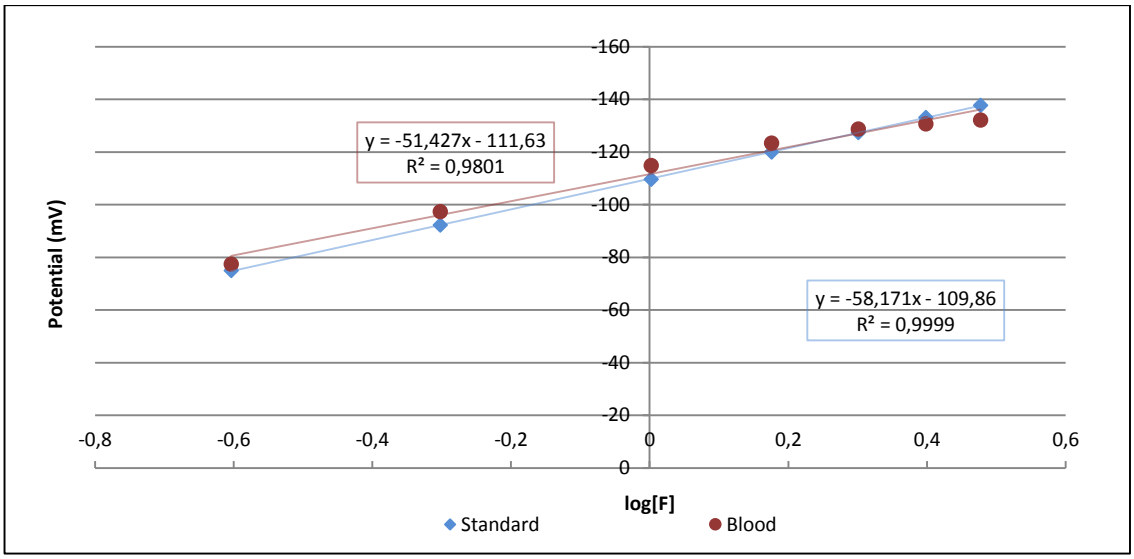

Figure 5: Response models obtained for the aqueous standard and blood calibration solutions prepared with TISAB II

Nevertheless, the gradient and intercept of the blood response model in *Figure 5* are much closer to those of the corresponding aqueous response model than seen previously, where no TISAB or TISAB IV were used. This would seem to indicate that the TISAB II is better at suppressing matrix effects than TISAB IV, although a greater dilution factor is possibly required to allow blood samples to more closely mimic aqueous samples.

3x Dilution

Response models with TISAB II were then prepared by diluting calibrators three times, while still keeping the TISAB volume at half the total volume of the analysed solution. These are shown in *Figure 6*.

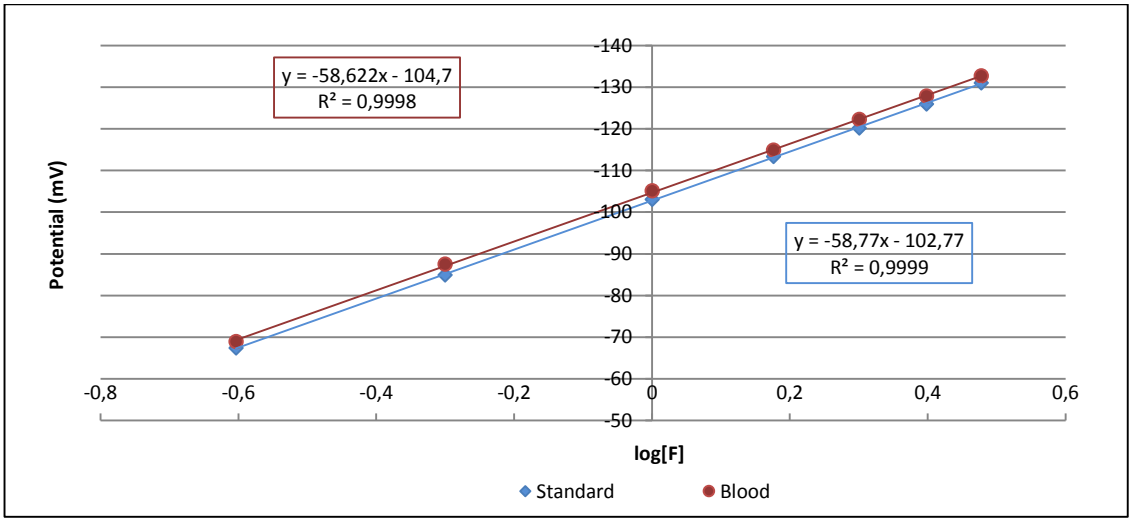

Figure 6: Response models obtained for the aqueous standard and blood calibration solutions prepared with TISAB II and diluted three times

The 95% confidence intervals for the gradient and intercept of the blood response model in *Figure 6* were *-58.7698 ± 0.6948* and *-102.771 ± 0.2577* respectively. Similarly, the 95% confidence intervals

for the gradient and intercept of the aqueous response model were *-58.6219 ± 0.9148* and *-104.702 ± 0.3392* respectively.

There is thus no statistical difference between the gradients of the blood and aqueous response models since the confidence intervals overlap. However, the confidence intervals of the intercepts do not overlap. This indicates a significant difference between the intercept of the blood response model and that of the response model prepared in aqueous medium.

As such, despite the vastly improved linearity and gradient of the three times diluted blood response model, it still cannot be considered to be the same as the corresponding aqueous response model since there is a statistical difference between the intercepts. Attempting to quantify fluoride in a blood sample by means of an aqueous response model after preparing samples in this manner would result in an over-estimation of fluoride concentration. Further dilution is required.

10x Dilution

Calibrators were then diluted ten times, while keeping the TISAB II volume at half the total volume of the analysed solution. The resulting response models in aqueous and blood medium are given in *Figure 7.*

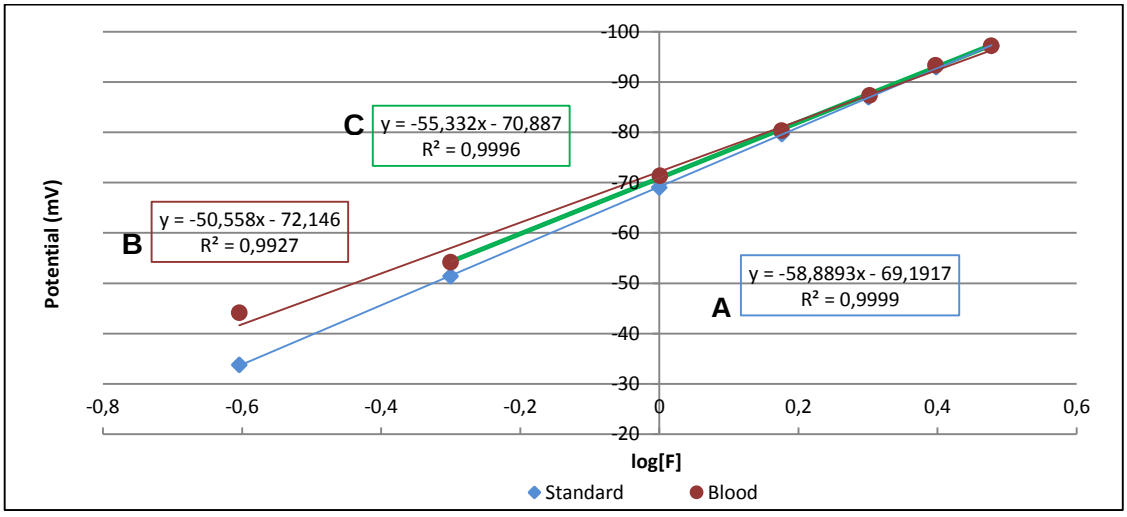

Figure 7: Response models obtained for the aqueous standard (**A**) and blood calibration (**B**) solutions prepared with TISAB II and diluted 10 times. Regression line **C** is obtained by excluding blood calibration point 1 ([F] = 0.250 g/100 mL)

The slope of the blood response model (***Figure 7B***) lies outside the acceptable range for electrode operation and is larger than that for the aqueous model. Hence, preparing samples by diluting 10 times and making use of an aqueous curve to determine fluoride concentration in blood would then result in an over-estimation, especially at lower concentrations. Clearly this is unacceptable, particularly for a measurement where the concentration is required to be above a certain level.

Calibration point 1, at fluoride concentration 0.250 g/100 mL, was determined to be an outlier by the CD^2 test, and so was omitted yielding regression line **C** (***Figure 7C***) with a gradient of *-55.3316 ± 1.6184* and an intercept of *-70.8868 ± 0.5109* at 95% confidence. Neither the gradient nor

the intercept of the aqueous response model lie within these confidence limits, denoting a significant difference still between the blood and aqueous response models. Ten times dilution of sample solutions is evidently still insufficient to suppress matrix effects.

20x Dilution

Calibration solutions were then diluted twenty times. This resulted in the response models in *Figure 8*.

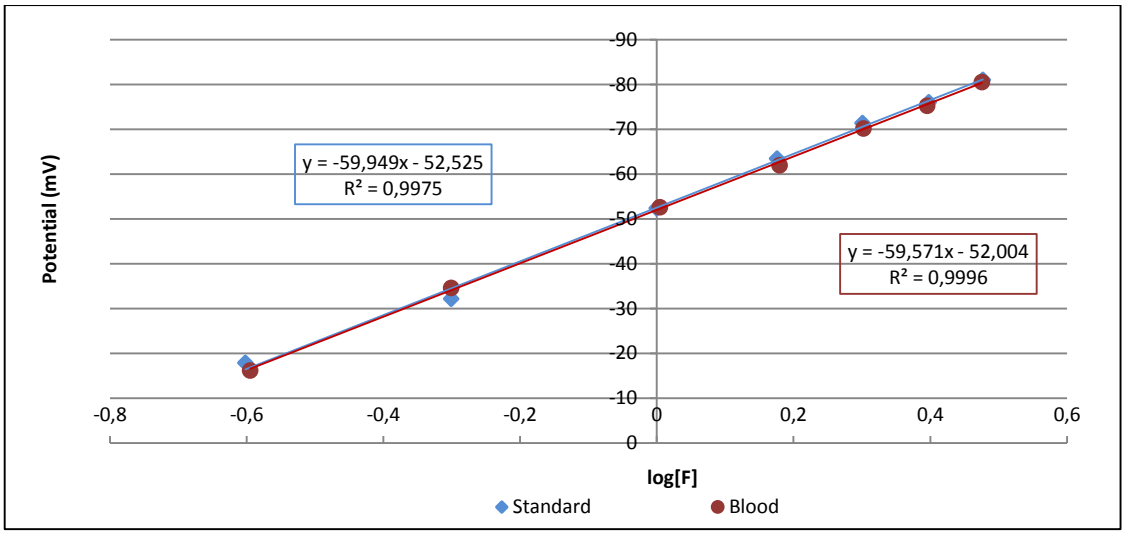

Figure 8: Response models obtained for the aqueous standard and blood calibration solutions prepared with TISAB II and diluted 20 times.

The aqueous response model exhibited gradient and intercept 95% confidence intervals of *-59.9489 ± 3.4317* and *-52.5253 ± 1.2713* respectively. The blood response model gradient (-59.5708) and intercept (-52.0039) lie well within the confidence intervals of the aqueous curve. Thus, 20 times dilution of blood calibration solutions results in a statistically identical response model to that obtained when treating aqueous calibration solutions in a similar manner.

In other words, diluting blood samples 20 times with dH_2O and TISAB II will allow the fluoride concentration to be determined from an aqueous response model, provided samples and calibrators are treated in the same manner.

This is especially powerful since a certified reference material can be used to produce the response model, ensuring metrological traceability remains intact. In addition, making use of a solution certified at a certain fluoride concentration is much more reliable than weighing off various masses of NaF to prepare calibration solutions. Finally, dH_2O and NaF CRM are much more readily obtained than fresh whole blood.

Blood samples were henceforth diluted 20 times with dH_2O and TISAB II, before analysis and response models were prepared by diluting aqueous NaF standard in the same way.

3.3.2. Method Validation

This validation is based only on a small set of data. This is mainly due to the fact that it is not practical to prepare matrix-matched controls each time the method is performed. In addition, what follows is a justification for the use of aqueous calibrations in order to estimate fluoride concentration in blood samples, provided the sample and calibrators are sufficiently diluted. This validation study emphasises the method's fitness for purpose.

Linearity

Figure 9 shows five response models generated by the FISE method. Point 2 is not an outlier in any of the response models, as determined by performing the Grubbs' test on the residuals, as well as the CD^2 test. Despite this, in each of the five models point 2 gives consistently large values. This would seem to indicate a problem with the calibration solution, since all five curves were prepared from the same seven stock calibration solutions.

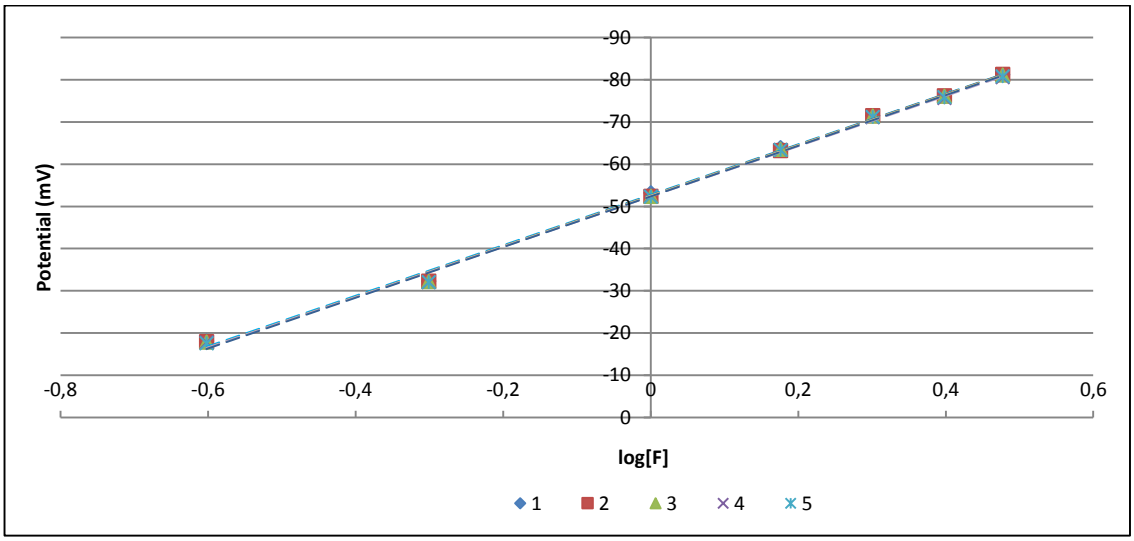

Figure 9: Five aqueous response models illustrating the linearity of the FISE method

The gradients, intercepts and correlation coefficients for the five response models are given in *Table 5* below.

Table 5: Gradients, intercepts and correlation coefficients for the five fluoride response models

Model	Gradient	Intercept	Correlation Coefficient (r^2)
1	-59.794	-52.764	0.997
2	-59.952	-52.568	0.998
3	-59.949	-52.525	0.998
4	-59.606	-52.333	0.998
5	-59.942	-52.340	0.998

The average correlation coefficient for the method was calculated from those listed in *Table 5*, and the 95% confidence interval was calculated in the same fashion as for that in the ethanol method validation. Hence the 95% confidence interval for the correlation coefficient was found to be $r^2 = 0.9975 \pm 0.000084$, and the method thus exhibits good linearity for linear regression response modelling. Additionally, the gradients and intercepts of the response models are nearly identical on the five days, meaning that it could be possible to prepare a response model on a weekly rather than daily basis.

Limits of Detection and Quantitation

As was stated in *Chapter 2*, the most common way of estimating LOD and LOQ values is based on the signal of the blank, y_0, and $s_{y/x,}$ the standard deviation of a typical regression line of y on x. This is done by means of *Equation 1a* and *1b* and *Equation 2a* and *2b* below.

$$LOD_y = y_0 + 3s_{y/x} \qquad \text{Equation 1a}$$

$$LOD_x = \frac{(LOD_y - a)}{b} \qquad \text{Equation 1b}$$

$$LOQ_y = y_0 + 10s_{y/x} \qquad \text{Equation 2a}$$

$$LOQ_x = \frac{(LOD_y - a)}{b} \qquad \text{Equation 2b}$$

When the x-axis is in concentration units, the signal of the blank corresponds to the y-intercept since zero concentration is at x = 0. However, when the linear regression model calls for a semi-log graph, as in the case of the FISE, the x-axis is in log-concentration units. This means that x = 0 is not zero concentration. Hence, the y-intercept cannot be used as an estimate for the signal of the blank. In this case, the potential of twenty blank samples was determined, and the average of these was used as y_0.

In addition, due to the nature of the responses obtained from the FISE, the signs in *Equation 1a* and *Equation 2a* need to change. A more positive potential indicates a lower concentration, and since we wish to estimate the concentration of fluoride *above* the signal of the blank that can be reliably detected, the appropriate multiple of $s_{y/x}$ needs to be *subtracted* from y_0 in order to yield a more negative potential and thus a higher concentration. Hence, *Equation 1a* and *Equation 2a* become:

$$LOD_y = y_0 - 3s_{y/x}$$ Equation 3

$$LOQ_y = y_0 - 10s_{y/x}$$ Equation 4

Finally, to obtain a concentration value for LOD and LOQ, the antilog needs to be applied to LOD_x and LOQ_x as follows:

$$LOD_{[F]} = 10^{LOD_x}$$ Equation 5

$$LOQ_{[F]} = 10^{LOQ_x}$$ Equation 6

Figure 10 shows a typical response model for the FISE method. Making use of this calibration data, the theoretical $LOD_{[F]}$ and $LOQ_{[F]}$ were subsequently found to be 0.00075 g/100 mL (95% CI: 0.000386 - 0.00145 g/100 mL) and 0.00106 g/100 mL (95% CI: 0.000545 - 0.00205 g/100 mL) respectively.

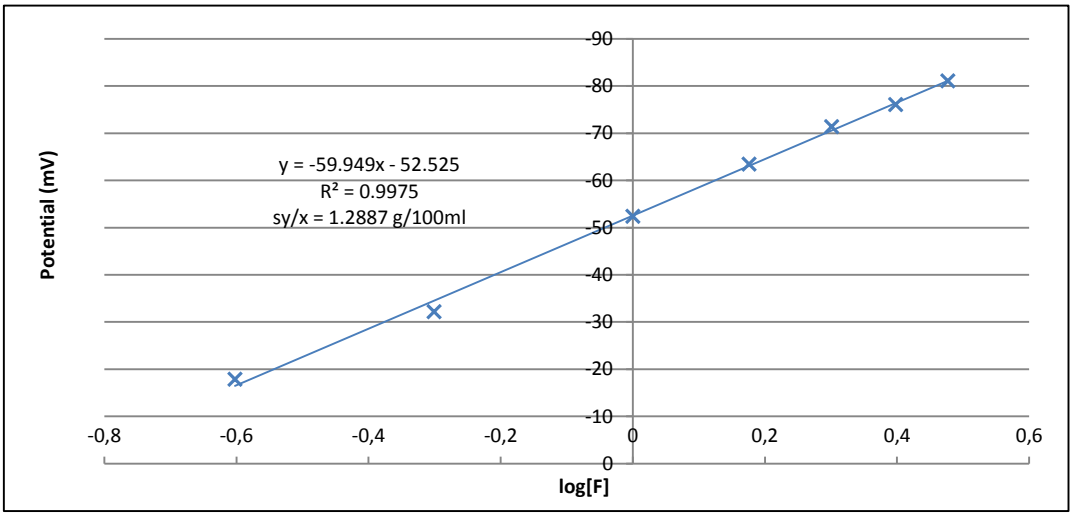

Figure 10: Typical response model for the FISE method

It is very important to note that the transformation of a logarithmic curve to a linear curve results in a non-symmetrical confidence interval. As such, it is imperative that confidence intervals be reported as a range of values. The above confidence interval ranges were determined by first calculating the upper and lower limit for LOD_y, and converting these potential values to log of concentration values by means of the regression equation in *Figure 10*. Finally, the antilog of these concentration values was taken, yielding an upper and lower limit for the $LOD_{[F]}$ and $LOQ_{[F]}$ values.

Specificity

The analysis of ten duplicate blank samples yielded a 95% confidence interval for the average fluoride concentration of 0.000552 ± 0.000401 g/100 mL. Each individual potential value obtained, as well as the average potential value was well outside the calibration range, and the concentrations calculated from these potentials were very small. In fact, all concentrations were below the average LOQ, with all but one below the average LOD and one below the lower limit of the LOD. It is clear from this virtually negligible average blank concentration that the method is highly specific for free fluoride in blood.

Bias and Precision

The experimental fluoride concentrations obtained on the five days at the three internal quality control concentrations can be found in ***Appendix C***. Those indicated as outliers were not included in any calculations and are shown purely for completeness.

Bias

Plotting the means of 25 experimental concentrations at each of the three levels (Y) (0.302 g/100 mL, 0.979 g/100 mL and 2.408 g/100 mL) against the theoretical concentrations of 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL rendered the bias correction plot in *Figure 11*. It has the form $Y = BX + A$, and can be applied to experimentally obtained concentration values in order to compensate for procedural bias by:

$$X_0 = \frac{Y_0 - A}{B} \qquad \text{Equation 7}$$

with A and B the intercept and gradient respectively, and Y_0 and X_0 the experimentally determined concentration before and after bias correction.

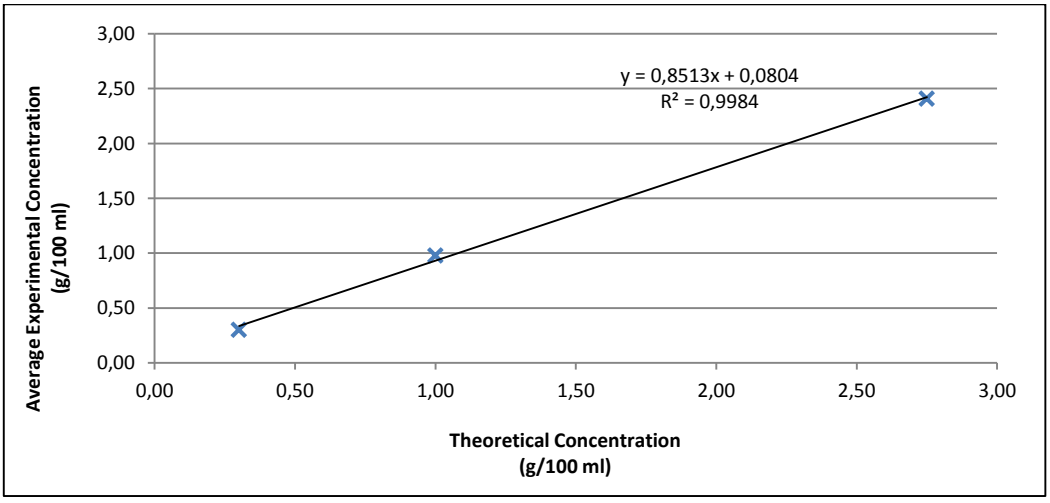

Figure 11: Bias correction plot obtained from the five days' fluoride validation data

The 95% confidence intervals for the gradient and intercept were *0.8513 ± 0.4271* and *0.0804 ± 0.7254* respectively. Since the gradient is not statistically different from 1 and the intercept is not statistically different from 0, it can be said that the method exhibits no multiplicative or additive bias.

Precision

On each of the five days, fluoride was weighed off in order to prepare the quality control samples at each of the three concentration levels. This means that samples prepared on the same day have the same theoretical concentrations; however, the theoretical concentrations differ day to day. For this reason the differences in experimental concentration and theoretical concentration were considered for the ANOVA calculations employed to estimate method precision. These differences and the ANOVA tables can be found in ***Appendix C***.

The within-batch precisions were found to be 1.09%, 1.02% and 1.93%, while the between-batch precisions were 0.176%, 0.415% and 2.77% for the 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL internal quality control levels respectively.

The ANOVA calculations once again yielded F test statistic values larger than the F critical values, and p-values much smaller than the 95% confidence α value of 0.05. This is likely to be due to the small sample size, and should not result in the discarding of any one result until more information is obtained. [15]

As before with ethanol precision data, the within-batch precision, u_w, and the between-batch precision, u_B, at each fluoride concentration was pooled to give an overall precision estimate, u_T, by *Equation 8*.

$$u_T = \sqrt{{u_W}^2 + {u_B}^2} \qquad \text{Equation 8}$$

The u_T values obtained were 1.11%, 1.10% and 3.37% for the 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL concentrations.

3.3.3. Measurement Uncertainty Calculations

Considering the difficulties involved in preparing blood control samples, as well as the method development work showing that 20 times dilution of blood specimens sufficiently suppresses matrix effects to allow aqueous calibration, no further blood internal control specimens were prepared. Instead, the control concentration data obtained during method validation was used in the MU calculations that follow.

The main components that contribute to the uncertainty in the fluoride concentration are the certified reference solution of aqueous sodium fluoride (CRM), sample dilution (dil), method bias and method imprecision (imp).

Once again assuming independence of the various contributing components, the RSD of the fluoride measurement can be written in terms of the relative uncertainties of the components as:

$$\frac{u_{X_0'}}{X_0'} = \sqrt{\left(\frac{u_R}{R}\right)^2_{CRM} + \left(\frac{u_d}{d}\right)^2_{dil} + \left(\frac{u_{bias}}{X_0}\right)^2_{bias} + \left(\frac{u_{imp}}{X_0}\right)^2_{imp}} \qquad \text{Equation 9}$$

with X_0' the final fluoride concentration, R the concentration of the CRM as given in the manufacturer's certificate, d the sample dilution factor, and X_0 the fluoride concentration result after correcting for bias.

X_0' is calculated by correcting the fluoride concentration for dilution by applying the sample dilution factor, f_{dil}, as seen in *Equation 10*.

$$X_0' = f_{dil} \times X_0 \qquad \text{Equation 10}$$

Before it is possible to determine the combined standard uncertainty of the final concentration result, $u_{X_0'}$, each term in *Equation 9* must first be calculated.

Certified Reference Material Uncertainty Contribution

The concentration of the aqueous sodium fluoride CRM as given on the certificate provided by the manufacturer was 2.997 ± 0.075 g/100 mL at 95% confidence. Hence, assuming a rectangular distribution with k = 2, the standard uncertainty due to CRM, u_R, was found to be 0.0375 g/100 mL.

Sample Dilution Uncertainty Contribution

The calibrated pipettes used in sample aliquoting contribute to the uncertainty in the final concentration result. To all calibrators and specimen aliquots (**200 µl**) was added TISAB II (**2000 µl**) and dH_2O (**1800 µl**). Unlike in the ethanol preparation method, calibrators and specimens are diluted in an identical manner, resulting in a dilution factor, f_{dil}, of 1. This is shown in *Equation 11* below.

$$f_{dil} = \frac{d_{cal}}{d_{sam}} = \frac{\frac{200\ \mu l + 2000\ \mu l + 1800\ \mu l}{200\ \mu l}}{\frac{200\ \mu l + 2000\ \mu l + 1800\ \mu l}{200\ \mu l}} = \frac{20}{20} = 1 \qquad \text{Equation 11}$$

In *Equation 11*, d_{cal} and d_{sam} are the dilution factors for the calibrators and sample being assessed for fluoride content respectively.

From this, the relative uncertainty in the dilution factor, u_{dil}, can be written as:

$$\frac{u_{dil}}{f_{dil}} = \sqrt{\left(\frac{u_{d_{cal}}}{d_{cal}}\right)^2 + \left(\frac{u_{d_{sam}}}{d_{sam}}\right)^2} \qquad \text{Equation 12}$$

with the terms $u_{d_{cal}}$ and $u_{d_{sam}}$ representing the uncertainties due to dilutions in the calibrators and sample respectively.

Assuming all significant errors to be in the y-direction, the only volumes that contribute to the uncertainty are those of the sample. [16] Hence, $u_{d_{cal}}$ is zero, and by *Equation 11* and *Equation 12* we have:

$$\frac{u_{dil}}{1} = \sqrt{\left(\frac{u_{d_{sam}}}{d_{sam}}\right)^2} \qquad \text{Equation 13}$$

$$u_{dil} = \frac{u_{d_{sam}}}{d_{sam}}$$

Additionally, from *Equation 11*, d_{sam} can be written as follows:

$$d_{sam} = \frac{V_{blood} + V_{TISAB\ II} + V_{H_2O}}{V_{blood}} = \frac{200\ \mu l\ + 2000\ \mu l + 1800\ \mu l}{200\ \mu l} = 20 \qquad \text{Equation 14}$$

The volumes in *Equation 14* are all subject to error due to the uncertainty in the certified internal volume of the pipette, and the temperature of the solutions differing to the temperature at which the pipette was calibrated. Hence, the combined uncertainty in the 200 µl volume, $u_{200\ \mu l}$, was calculated by:

$$u_{200\ \mu l} = \sqrt{{u_{cal}}^2 + {u_{rep}}^2 + {u_{temp}}^2} \qquad \text{Equation 15}$$

with the terms representing the uncertainty contributions due to CRM, repeatability and temperature. The full calculation of each of these terms for the 200 µl volume is given below.

Calibration

The expanded measurement uncertainty at 95% confidence for the 200 µl volume as indicated on the manufacturer's certificate was 0.06%. Thus, assuming a rectangular distribution the standard uncertainty due to pipette calibration, u_{cal}, was calculated as:

$$u_{cal} = \frac{0.0006 \times 200\ \mu L}{\sqrt{3}} = 0.0693\ \mu L \qquad \text{Equation 16}$$

Temperature

The calibration certificate provided by the calibration facility states that the pipettes were calibrated at 20 °C. The laboratory in which these experiments were conducted fluctuated within limits of 20 ± 4 °C. Making use of the coefficient of thermal expansion for water, [17] the effect of the temperature on the internal volume of the pipette can be accounted for. With a rectangular distribution, the standard uncertainty due to temperature, u_{temp}, was calculated as:

$$u_{temp} = \frac{0.00021℃^{-1} \times 200\ \mu L \times 4℃}{\sqrt{3}} = 0.0970\ \mu L \qquad \text{Equation 17}$$

Repeatability

To assess the variability in pipette filling, 10 volumes of dH_2O (**200 µl**) were dispensed and weighed. The standard deviation of these masses was 0.3228 µl, and this was used directly as the standard uncertainty due to repeatability, u_{rep}.

The uncertainties due to calibration, temperature and repeatability were then combined to yield the combined standard uncertainty, $u_{200\ \mu l}$, as follows:

$$u_{200\ \mu l} = \sqrt{(0.0693)^2 + (0.0970)^2 + (0.3228)^2} = 0.3441\ \mu L \qquad \text{Equation 18}$$

In the dilution of specimens and calibrators, 1800 µl of dH_2O and 2000 µl TISAB II were used. The 1800 µl volume was dispensed as separate volumes of 1000 µl and 800 µl. Similarly, the 2000 µl was dispensed as two volumes of 1000 µl. Hence, in order to estimate the uncertainty in the volumes of TISAB II and dH_2O, the uncertainties in the subvolumes 800 µl and 1000 µl needed first to be evaluated. These uncertainties would then be combined by *Equation 19a* and *Equation 19b*:

$$u_{1800\ \mu l} = \sqrt{{u_{1000\ \mu l}}^2 + {u_{800\ \mu l}}^2}$$ Equation 19a

$$u_{2000\ \mu l} = \sqrt{{u_{1000\ \mu l}}^2 + {u_{1000\ \mu l}}^2}$$ Equation 19b

with $u_{1000\ \mu l}$ and $u_{800\ \mu l}$ the uncertainties in the 1000 µl and 800 µl volumes respectively.

The standard uncertainties $u_{1000\ \mu l}$ and $u_{800\ \mu l}$ were calculated following the same procedure as for $u_{200\ \mu l}$.

For the 1000 µl volume, the manufacturer's certificate indicated an uncertainty of 0.04%. Applying *Equation 16* yielded the standard uncertainty due to pipette calibration, u_{cal} to be 0.2309 µl. Weighing 10 volumes of dH_2O (**1000 µl**) gave a standard uncertainty due to repeatability, u_{rep}, of 1.055 µl, while applying *Equation 17* resulted in a standard uncertainty due to temperature, u_{temp}, of 0.485 µl. Pooling these by *Equation 15*, $u_{1000\ \mu l}$ was found to be 1.1839 µl.

As with the 50 µl volume required in the ethanol uncertainty calculation, 800 µl was not one of the volumes listed with an uncertainty on the certificate provided by the manufacturer. The plot of uncertainty versus volume prepared for the 1000 µl pipette in the ethanol calculation was used once more to determine the uncertainty in the 800 µl volume. This was read off the plot as 0.024%, and the standard uncertainty, u_{cal} for the 800 µl volume was calculated by *Equation 16* to be 0.1109 µl. Weighing 10 volumes of dH_2O (**800 µl**) resulted in a standard deviation, and a u_{rep} value, of 0.837 µl. By *Equation 17*, u_{temp} was found to be 0.388 µl. Pooling these uncertainties gave a combined standard uncertainty, $u_{800\ \mu l}$, of 0.9292 µl.

These standard uncertainties in the 800 µl and 1000 µl volumes were then pooled by *Equation 19a* and *Equation 19b*, to yield the uncertainties $u_{1800\ \mu l}$ and $u_{2000\ \mu l}$ as 1.505 µl and 1.674 µl respectively.

The relative standard uncertainty due to dilution could then be calculated by:

$$\frac{u_d}{d} = \sqrt{\left(\frac{u_{(V_{200})}}{V_{200}}\right)^2 + \left(\frac{u_{(V_{200}+V_{2000}+V_{1800})}}{V_{200}+V_{2000}+V_{1800}}\right)^2}$$

$$= \sqrt{\left(\frac{0.3441}{200}\right)^2 + \left(\frac{\sqrt{(0.3441)^2 + (1.6474)^2 + (1.505)^2}}{4000}\right)^2}$$ Equation 20

Equation 20 was evaluated to give $\frac{u_d}{d}$ = 0.001812, and since d = 1, the uncertainty contribution due to sample dilution, u_d was also 0.001812.

Procedural Uncertainty Contribution

Each step in the sample analysis procedure, from aliquoting to determining concentration from the linear regression calibration introduces variability into the final result. These should thus be accounted for in the uncertainty estimation, and this can be done by evaluating the uncertainty due to method bias as well as that due to method imprecision. Together, these estimates give the overall procedural uncertainty.

Bias

Since the method validation data was used in the measurement uncertainty calculations, the bias correction plot in *Figure 11* still applies – that is, the plot of average experimentally obtained fluoride concentration (*Y*) versus theoretical fluoride concentration (*X*). The 95% confidence intervals for the gradient and intercept were *B = 0.8513 ± 0.4271* and *A = 0.0804 ± 0.7254* respectively, and these were used to correct for bias by:

$$X_0 = \frac{Y_0 - A}{B} \qquad \text{Equation 21}$$

The uncertainty contribution due to bias correction, u_{bias} , is calculated by:[16]

$$u_{bias} = \frac{S_{Y/X}}{B}\sqrt{\left(\frac{1}{n}\right) + \left(\frac{1}{m}\right) + \frac{(Y_0 - \bar{Y})^2}{B^2 \sum_1^3 (X_i - \bar{X})^2}} \qquad \text{Equation 22}$$

where $s_{Y/X}$ is the standard deviation of the regression line of Y on X; n is the number of points on the line – that is the number of internal quality control concentrations; m is the number of replicates at each control level; and $(\bar{X};\bar{Y})$ is the centroid of the bias correction plot.

Applying *Equation 22* to the mean experimentally obtained fluoride concentrations, with $s_{Y/X}$ *= 0.05999*, *B = 0.8513*, *n = 3*, and *m = 25*, yielded u_{bias} values of 0.061 g/100 mL, 0.045 g/100 mL, and 0.070 g/100 mL at the concentrations 0.302 g/100 mL, 0.979 g/100 mL and 2.41 g/100 mL.

Imprecision

Following the same methodology as with the ethanol uncertainty calculation, the standard deviations of the three sets of 25 internal quality controls (0.015 g/100 mL, 0.031 g/100 mL, and 0.14 g/100 mL) were plotted against the theoretical concentrations of 0.300 g/100 mL,

1.00 g/100 mL and 2.75 g/100 mL. This yielded *Figure 12*, a curve that had a better parabolic fit than linear. This could possibly be attributed to the small number of points plotted. If there were more concentrations with corresponding standard deviations, perhaps a linear fit could be accomplished. For our purposes however, the second-order equation obtained is sufficient to allow the estimation of standard deviations at concentrations between 0.300 g/100 mL and 2.75 g/100 mL.

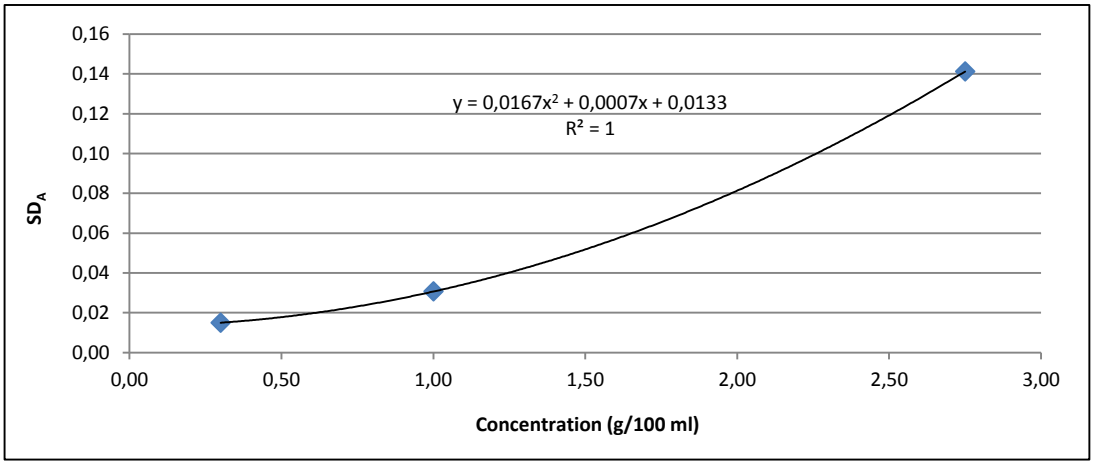

Figure 12: Standard deviation, SD_A, of the 25 average experimental fluoride concentration results against theoretical concentration

Hence, the uncertainty due to method imprecision at an experimental concentration of Y_0 was calculated by:

$$u_{imp} = 0.0167\,{Y_0}^2 + 0.0007\,Y_0 + 0.0133 \qquad \text{Equation 23}$$

Applying *Equation 23* to the three mean experimental fluoride concentrations, 0.302 g/100 mL, 0.979 g/100 mL, and 2.41 g/100 mL, resulted in u_{imp} values of 0.015 g/100 mL, 0.033 g/100 mL, and 0.14 g/100 mL.

Subsequently, the uncertainty contributions due to calibration, sample dilution, method bias and method imprecision can be combined by *Equation 9* in order to estimate the overall standard uncertainty at a certain experimental concentration, X'_0, corrected for bias and sample dilution.

$$\frac{u_{X'_0}}{X'_0} = \sqrt{\left(\frac{0.075}{2.997}\right)^2_{CRM} + \left(\frac{0.001812}{1}\right)^2_{dil} + \left(\frac{u_{bias}}{X'_0}\right)^2_{bias} + \left(\frac{u_{imp}}{X'_0}\right)^2_{imp}} \qquad \text{Equation 24}$$

Finally, $u_{X_0'}$ can be converted to an expanded uncertainty by:

$$U_{X_0'} = k \times u_{X_0'} \qquad \text{Equation 25}$$

The combined expanded uncertainties at 95% (k = 2) and 99% (k = 2.576) confidence for three example experimental fluoride concentrations are given in *Table 6*.

Table 6: Example fluoride concentrations corrected for bias and dilution, and their corresponding standard- and expanded measurement uncertainties at 95% and 99% confidence

Y_0 (Exp. Conc.) (g/100 mL)	X_0 (Y_0 Corr. For Bias) (g/100 mL)	X_0' (X_0 Corr. For Dil.) (g/100 mL)	$u_{X_0'}$ (g/100 mL)	95% Confidence		99% Confidence	
				$U_{X_0'}$ (g/100 mL)	$U_{X_0'}$ (%)	$U_{X_0'}$ (g/100 mL)	$U_{X_0'}$ (%)
0.336	0.300	0.300	0.0617	0.1235 (0.12*)	41.2	0.1591 (0.16*)	53.0
0.932	1.00	1.00	0.0561	0.1122 (0.11*)	11.2	0.1445 (0.14*)	14.5
2.42	2.75	2.75	0.1617	0.3234 (0.32*)	11.8	0.4165 (0.42*)	15.1

It can be seen from the values given in *Table 6* that the relative expanded uncertainty is very large at low concentrations, while seeming to become more or less uniform at the higher concentrations. Although the expanded MU of 14.5% at 1.00 g/100 mL is also larger than is usually expected for an analytical method of this nature, this conservative estimate for the MU allows one to be absolutely certain sufficient fluoride is present in a sample.

The idea of a "guard band" that was introduced in *Chapter 2* is also applied here; however, instead of an upper limit, a lower limit is placed on the fluoride concentration. That is, since it is required that the fluoride concentration of a blood sample for ethanol analysis be *at least* 1 g/100 mL, the expanded measurement uncertainty is used to determine the minimum legal concentration of fluoride. Any concentration found to be below this "lower limit" can be said to be statistically below the required concentration. This lower limit can be calculated by:

$$X_{Minimum} = X_{legal\ required} - 2.576u_{X_0'} \qquad \text{Equation 26}$$

Applying *Equation 26* with $X_{legal\ required}$ = 1.00 g/100 mL and $u_{X_0'}$ = 0.0561 g/100 mL, a minimum legal fluoride concentration of 0.855 g/100 mL was obtained. Hence, any fluoride concentration

determined by this FISE method is significantly lower than the legally required 1 g/100 mL only if it is below 0.855 g/100 mL.

One of the advantages of this method of calculating the MU for a method is supposed to be the fact that the MU is recalculated with each batch of controls that is prepared. However, since blood controls are not prepared with each fluoride analysis, this will not be possible for this method. Hence it is recommended that blood controls be prepared periodically, perhaps every three months, in order to confirm that the method is still under control.

The precision limits for duplicate analyses could be calculated in a similar fashion to those for the ethanol analytical method, by means of:[18]

$$R = 2.576 \times \sqrt{2} \times u_{X_0}{}' = 3.643 u_{X_0}{}' \qquad \text{Equation 27}$$

Applying this to the concentrations in *Table 6* yielded precision limits of 0.225 g/100 mL, 0.204 g/100 mL, and 0.589 g/100 mL at the 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL concentration levels respectively.

3.3.4. Method Applications

Complexation Studies

The whole blood matrix contains many ions including aluminium, cadmium, calcium, copper, iron, and magnesium. Of these, iron is found in the highest abundance, as Fe^{3+}. Following this, calcium, Ca^{2+}, and magnesium, Mg^{2+}, are present at concentrations smaller by a whole order of magnitude. The other ions are present at such low concentrations they did not warrant investigation.

The ions Fe^{3+}, Ca^{2+} and Mg^{2+} all have the potential to complex with free fluoride, thereby artificially lowering the measured fluoride concentration. However, the extent to which the free fluoride is removed from the system needed to be explored further.

Iron

The average Fe^{3+} content of whole blood is $(4.730 \pm 0.880) \times 10^{-1}$ mg/mL. [19] It is possible for this Fe^{3+} to complex with the free F^- in the blood, forming solid iron trifluoride, FeF_3. However, whether or not this complexation of the fluoride would be sufficient to significantly affect the measured fluoride concentration still needed to be investigated.

In addition, for most salts solubility decreases with decreasing temperature. [20] This would imply that, in blood specimens stored under refrigeration, not only the NaF but also any FeF_3 that is formed would be less soluble than in blood specimens at higher temperatures, i.e. room temperature. The challenge then came in measuring such solubility changes with the newly validated FISE method,

since all solutions, including samples, calibrators and electrode filling solution need to be at the same temperature during analysis.

Initially, the electrode filling solution was cooled with the samples to approximately 4 °C; however, the KCl began to crystallise and precipitate out of solution, and the filling solution became unusable. This meant that all analyses would need to take place at room temperature for accurate measurements.

Simply allowing the chilled test calibration solutions to return to room temperature before analysing them would defeat the purpose, since any fluoride complexes (NaF or FeF_3) that had become insoluble due to lower temperature would surely go back into solution upon warming. It was thus decided to filter the cold solutions to remove any precipitate formed before warming to room temperature. In this way, the fluoride concentration obtained would be an indication of the amount of free fluoride that was still in solution even at the lower temperature.

Upon cooling the calibration solutions, there was no visible change in those solutions without Fe^{3+}. However, in those that did contain Fe^{3+}, a white precipitate was seen in all seven of the solutions, in increasing quantities as NaF concentration increased. Furthermore those solutions at 0.250 g/100 mL and 0.500 g/100 mL fluoride had a large quantity of yellow precipitate. The majority of this yellow precipitate was easily removed by filtering through the cotton wool plug, while the white precipitate was still visible in the solutions at 2.50 g/100 mL and 3.00 g/100 mL fluoride, even after filtering a second time.

After filtering, the solutions at 0.250 g/100 mL and 0.500 g/100 mL fluoride were yellow, and those at 2.50 g/100 mL and 3.00 g/100 mL were milky, while the other three were clear. This is shown in *Figure 13*.

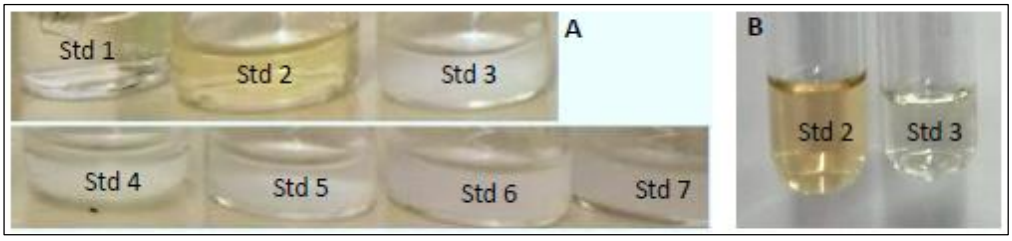

Figure 13: Cooled calibration solutions containing Fe^{3+} **A:** Before filtering; and **B:** After

For the rest of this discussion, *Fr* shall denote those solutions that were cooled and filtered, *RT* those solutions that were kept at room temperature throughout, and *Fe* those solutions that were spiked with the Fe^{3+} stock solution. Hence, the four sets of calibration solutions (Fr-Fe, Fr-no Fe, RT-Fe, RT-no Fe) afforded a total of eight response models, since from each set a response model with TISAB II and a response model without TISAB II were prepared. These models are depicted below.

Figure 14 and *Figure 15* show the response models obtained when no Fe^{3+} solution was added. The 95% confidence intervals for the intercepts and gradients of the four models are given in *Table 7*.

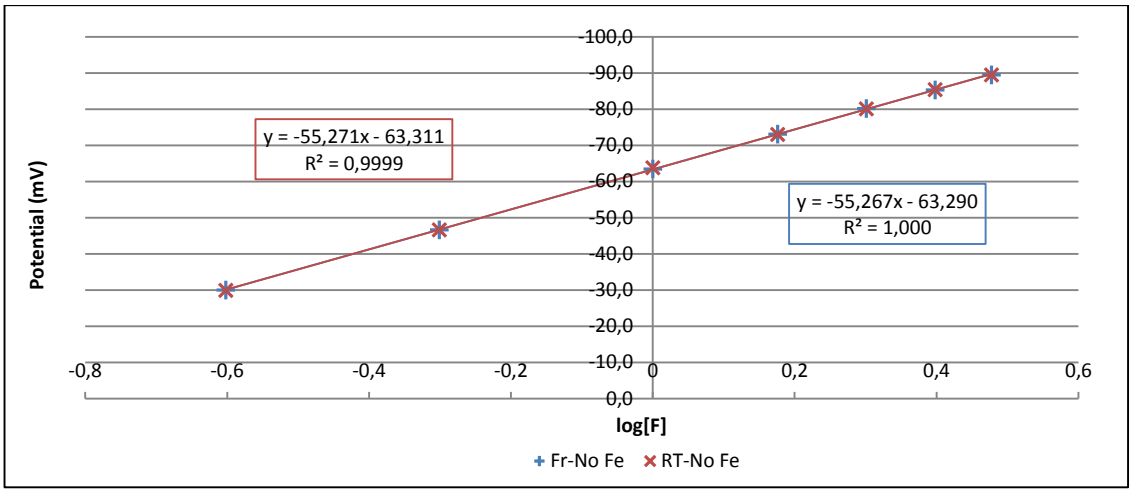

Figure 14: (CONTROL) Response models obtained from Fr and RT calibration solutions with no Fe^{3+} and no TISAB II added

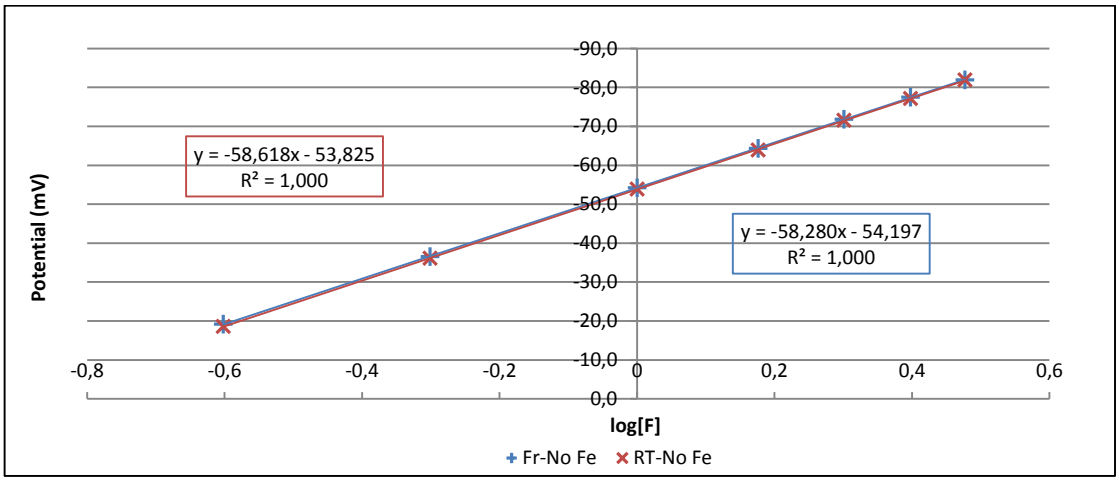

Figure 15: (CONTROL) Response models obtained from Fr and RT calibration solutions with TISAB II but no Fe^{3+} added

Table 7: Regression data for the response models with no Fe^{3+} added

Model	TISAB II added	Gradient	Gradient CI (95%)	Intercept	Intercept CI (95%)
Fr-No Fe	No	-55.267	0.392	-63.290	0.145
RT-No Fe	No	-55.271	0.670	-63.311	0.248
Fr-No Fe	Yes	-58.280	0.340	-54.197	0.126
RT-No Fe	Yes	-58.618	0.347	-53.825	0.128

It is clear that while the addition of TISAB II to aqueous calibration solutions that contain only NaF is not necessary for a linear response, it does significantly impact both the gradient and intercept. This just serves to emphasise the need to treat calibrators, controls and samples in an identical manner.

In addition, the cooling and filtering process has no effect on the free fluoride concentration, provided the solution analysed is pure aqueous NaF. This is evidenced by the fact that there are no significant differences in the gradients or intercepts of the chilled calibrators as compared to the room temperature calibrators when no Fe^{3+} is added. The only exception to this, however, is the intercepts of the models prepared with TISAB II. There is a significant difference at 95% confidence between the intercepts of the model prepared with cooled, filtered calibration solutions compared to that prepared with calibration solutions that were kept at room temperature. In fact, the Fr-No Fe response model had a more negative intercept than the RT-No Fe response model. Considering the statistically identical gradients of the two curves, this would seem to indicate a consistently higher free fluoride concentration in the cooled calibration solutions. This is counter-intuitive since, if anything, the free fluoride concentration would be expected to be lower in the cooled, filtered calibrator solutions than in those kept at room temperature. Despite this, any subsequent decreases in fluoride concentration observed upon addition of Fe^{3+} can confidently be attributed to the complexation of free fluoride by the added Fe^{3+}.

Figure 16 and *Figure 17* show the response models obtained when Fe^{3+} solution was added, while the 95% confidence intervals for the intercepts and gradients of the three models are given in *Table* 8. No regression data is given for the Fr-Fe calibration standards with TISAB II.

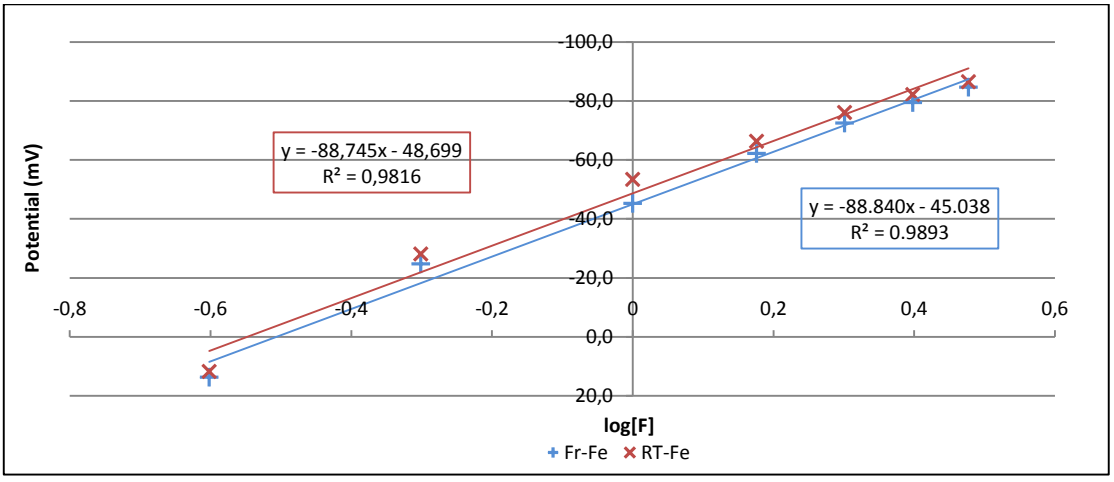

Figure 16: Response models obtained from Fr and RT calibration solutions with Fe^{3+} but no TISAB II added

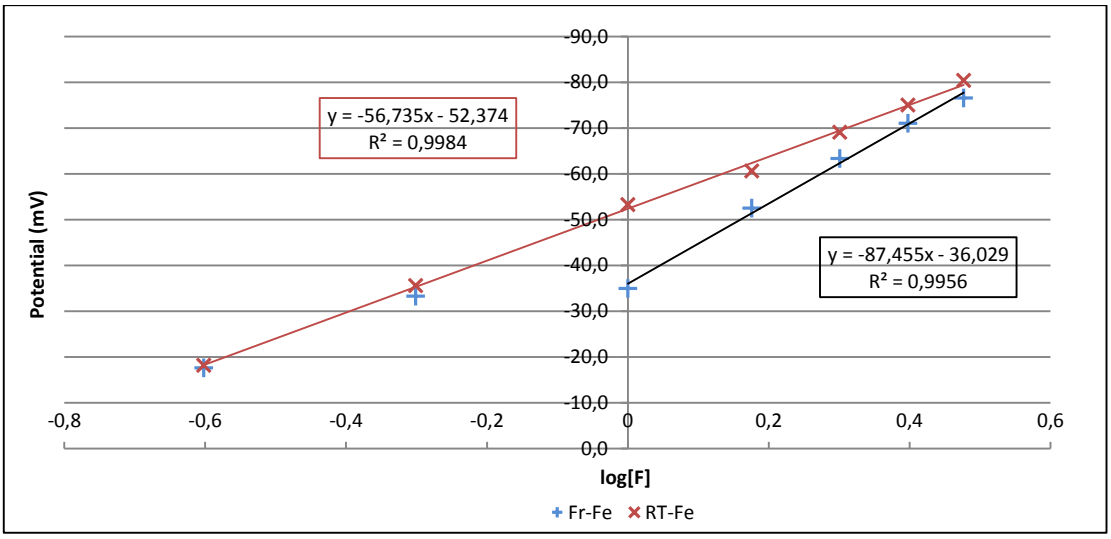

Figure 17: Response models obtained from Fr and RT calibration solutions with Fe^{3+} and TISAB II added

Table 8: Regression data for the response models with Fe^{3+} added

Model	TISAB II added	Gradient	Gradient CI (95%)	Intercept	Intercept CI (95%)
Fr-Fe	No	-88.840	10.606	-45.038	3.929
RT-Fe	No	-88.745	13.955	-48.699	5.170
RT-Fe	Yes	-56.736	2.632	-52.374	0.975

Although the response models in *Figure 16* are statistically identical and fairly linear, both gradients lie outside the acceptable range for optimal functioning of the FISE. Comparing the response models in *Figure 16* with those in *Figure 14* – also without TISAB II – it is clear that the addition of Fe^{3+} significantly impacted the free fluoride concentration. Furthermore, while the filtering process could have removed some of the insoluble FeF_3 that may have formed in the cooled calibration solutions, the effect of this is not noticeable when comparing the Fr-Fe and RT-Fe response models without TISAB II. This is most likely due to the similar formation of FeF_3 in the solutions kept at room temperature, and it being insoluble without the addition of a decomplexation agent, the fluoride is permanently complexed. Thus, despite the Fr-Fe solutions being filtered, the free fluoride concentrations of the Fr-Fe and RT-Fe solutions upon analysis are comparable.

The RT-Fe response model obtained by adding TISAB II to the calibration solutions shown in *Figure 17* exhibits satisfactory linearity and a gradient that lies within the acceptable range specified in the FISE user manual. Additionally, the gradient and intercept are very similar to those in *Figure 15*, which were prepared with TISAB II but contained no Fe^{3+}. This shows that the TISAB II is able to decomplex the fluoride by preferentially complexing with the Fe^{3+}, thus freeing the fluoride for analysis.

It should be noted that the response models in *Figure 16* and *Figure 17* were generated on a different day to those in *Figure 14* and *Figure 15*, and will thus have slightly different gradients and intercepts due to varying experimental conditions.

Up to now, the response models discussed have been more or less standard linear curves. The Fr-Fe response model obtained by adding TISAB II to the calibration solutions, however, exhibits a jump at a concentration of 1.00 g/100 mL.

In forming FeF_3, Fe^{3+} and F^- react in a 1:3 ratio – that is, 3 moles of fluoride are required for every 1 mole of Fe^{3+} in order to form 1 mole of FeF_3. Thus, fluoride is the limiting reagent. At concentrations below 1.00 g/100 mL, there is insufficient fluoride to be complexed by the Fe^{3+}, and hence the points at 0.250 g/100 mL and 0.500 g/100 mL of the RT and Fr response models lie almost on top of each other.

From 1.00 g/100 mL, there is sufficient fluoride present to form FeF_3, which is removed by the filtering process and is not afforded the opportunity to go back into solution upon warming to room temperature or to be decomplexed by the TISAB II. This has a greater effect at lower concentrations of fluoride, explaining the decreasing deviation from the RT-Fe points from 1.00 g/100 mL to 3.00 g/100 mL.

Additionally, the regression line obtained from the Fr-Fe points at concentrations of 1.00 g/100 mL to 3.00 g/100 mL mimics the response models seen in *Figure 16* that had Fe^{3+} but no TISAB II added. It exhibits a similar gradient and intercept to these response models. This would seem to imply that without TISAB II, free fluoride is removed from the system by complexation and can no longer be detected by the FISE. The addition of TISAB II can successfully combat the complexation effects of iron, provided the FeF_3 is present to decomplex. The filtration process removed the FeF_3, thereby effectively removing the free fluoride from the system, preventing decomplexation.

From the response models shown it can be deduced that this concentration of iron does complex free fluoride, but this effect can be mitigated by the addition of TISAB II. Additionally, at lower temperatures the complexation has a greater effect since the FeF_3 is less soluble. However, provided solutions are allowed to warm to room temperature and TISAB II is added, this effect is also sufficiently diminished.

While this set of experiments was performed in aqueous medium, the iron concentration was chosen to mimic that of the Fe^{3+} content in human whole blood. Thus from this it can be seen that blood specimens should be sufficiently diluted and, most importantly, TISAB II should be added to all blood specimens in order to combat the complexation of free fluoride by the iron present in the blood.

The enhanced complexation seen at lower temperatures does beg the question as to whether the effective fluoride concentration is indeed that which is measured. That is, NaF is added to blood specimens since free fluoride is a preserving agent. The concentration of this fluoride is required to

be 1.00 g/100 mL, but if the fluoride is complexed by iron, and other free ions, present in the blood matrix, the possibility exists that the effective free fluoride concentration is in actual fact *lower* than the specimen is initially spiked at, and hence lower than the decomplexed concentration determined by this method. There is some comfort in the fact that since blood specimen collection tubes are rarely filled to capacity, the initial fluoride concentration of most blood specimens is usually much greater than the required 1.00 g/100 mL.

Magnesium

The concentration of Mg^{2+} in the average whole blood specimen is $(1.25 \pm 0.18)\ x10^{-2}$ mg/mL. [21] It has the potential to complex with fluoride, forming MgF_2. As such, for the investigation of the effect of Mg^{2+} on the free fluoride concentration, aqueous NaF calibration solutions were spiked with Mg^{2+} stock solution to a final concentration of $1.43\ x10^{-2}$ mg/mL Mg^{2+}.

Four response models were then generated as indicated in *Table 2*, and will subsequently be denoted No Mg-No TISAB, No Mg-TISAB, Mg-No TISAB, and Mg-TISAB, where “Mg” indicates magnesium and “TISAB” refers to TISAB II. These response models are shown in *Figure 18*.

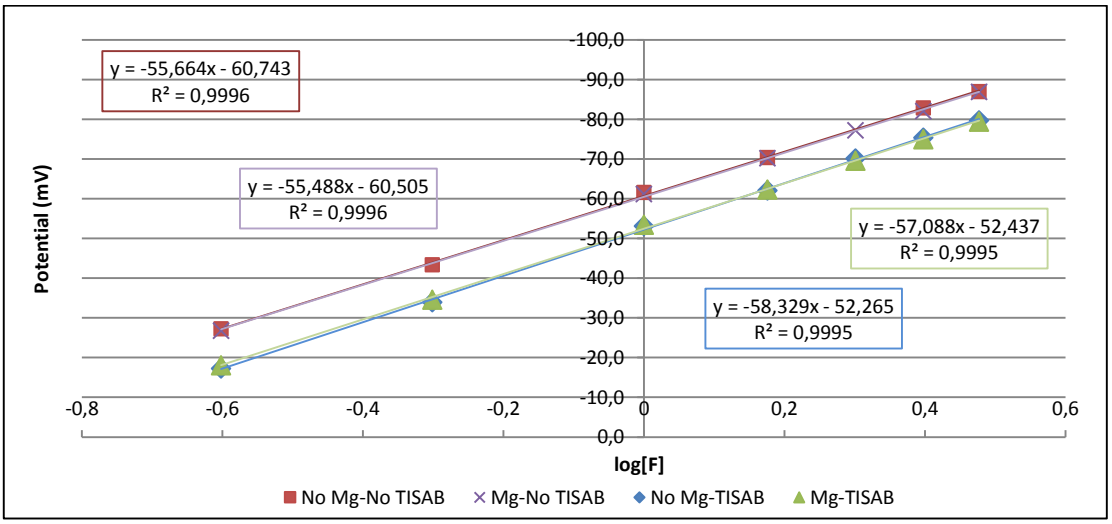

Figure 18: Response models obtained for Mg^{2+} complexation studies

Calibration point 5 ([F] = 2.00 g/100 mL) for the No Mg-No TISAB regression model was determined to be an outlier by applying Dixon’s Q-test to the residuals of the regression line obtained when the point was included. This point was thus omitted from the regression model. The 95% confidence intervals for the intercepts and gradients of the four models are given in *Table 9*

Table 9: Regression data for the response models of the Mg^{2+} studies

Model	Gradient	Gradient CI (95%)	Intercept	Intercept CI (95%)
No Mg-No TISAB	-55.664	1.603	-60.743	0.611
Mg-No TISAB	-55.488	1.440	-60.505	0.549
No Mg-TISAB	-58.329	1.571	-52.265	0.582
Mg-TISAB	-57.088	1.525	-52.438	0.565

As can be seen from the regression data in *Table 9*, regardless of the presence or absence of Mg^{2+}, the two regression lines obtained without the addition of TISAB II are not significantly different, as are those obtained when TISAB II is added. This shows that if the Mg^{2+} does complex any of the free fluoride present in the samples, it is not of a sufficiently high concentration to significantly affect the effective fluoride concentration.

Examining the mole ratios, Mg^{2+} reacts with F^- in a ratio of 1:2, meaning that for every 1 mole of Mg^{2+} 2 moles of F^- is required. A 1 mL aliquot of the calibration solution spiked to a final concentration of 1.43×10^{-2} mg/mL Mg^{2+} contains 5.884×10^{-7} mol Mg^{2+}. Hence if all of the Mg^{2+} is used in complexing free fluoride, twice as many moles of fluoride will be complexed – that is, 1.177×10^{-6} mol F^-. Converting this mole value to mass units results in 2.235×10^{-5} g of free fluoride that is complexed. At the lowest concentration calibration standard, with a concentration of 0.250 g/100 mL or 0.250×10^{-2} g/100 mL F^-, this is only 0.894%. It is then not surprising that the addition of Mg^{2+} at the above concentration does not significantly influence the effective free fluoride concentration.

Calcium

The average whole blood specimen contains $(4.88 \pm 0.40) \times 10^{-2}$ mg/mL Ca^{2+}, [21] which can complex free fluoride, forming CaF_2. As with the FeF_3 and MgF_2, this could artificially reduce the effective free fluoride concentration in blood specimens. In order to examine this further, aqueous NaF standard solutions were spiked to a final Ca^{2+} concentration of 5.278×10^{-2} mg/mL.

As before, four response models were then generated as indicated in *Table 3*, and will subsequently be denoted No Ca-No TISAB, No Ca-TISAB, Ca-No TISAB, and Ca-TISAB, where “Ca” indicates calcium and “TISAB” refers to TISAB II. These response models are shown in *Figure 19*.

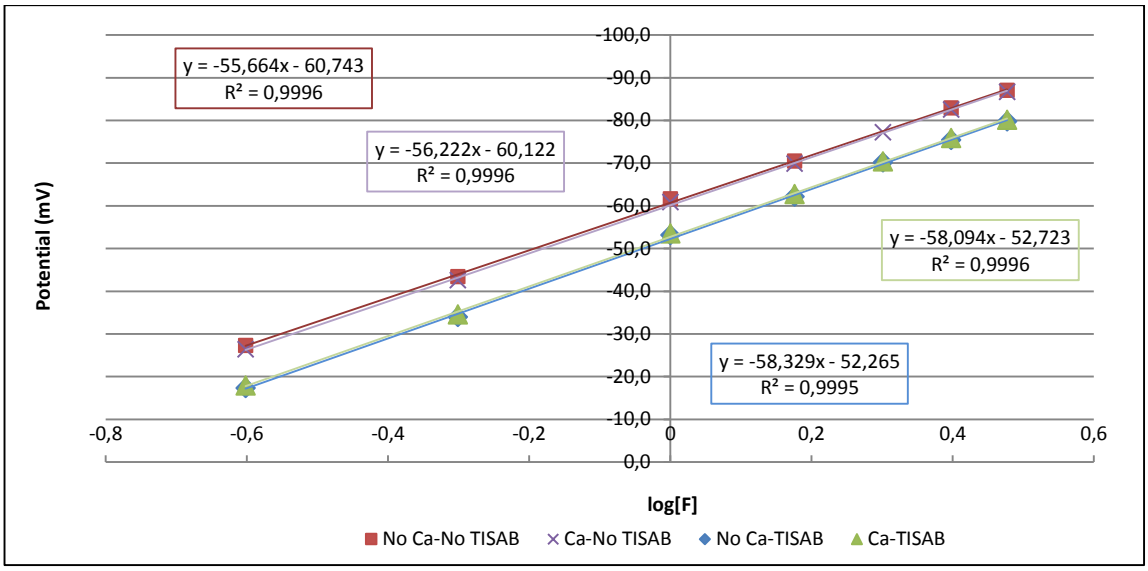

Figure 19: Response models obtained for Ca^{2+} complexation studies

Calibration point 5 ([F] = 2.00 g/100 mL) for the No Ca-No TISAB regression model was determined to be an outlier by applying Dixon's Q-test to the residuals of the regression line obtained when the point was included. This point was thus omitted from the regression model. The 95% confidence intervals for the intercepts and gradients of the four models are given in *Table* 10.

Table 10: Regression data for the response models of the Ca^{2+} studies

Model	Gradient	Gradient CI (95%)	Intercept	Intercept CI (95%)
No Ca-No TISAB	-55.664	1.603	-60.743	0.611
Ca-No TISAB	-56.222	1.219	-60.122	0.452
No Ca-TISAB	-58.329	1.571	-52.265	0.582
Ca-TISAB	-58.094	1.378	-52.723	0.510

When TISAB II is not used, the addition of the Ca^{2+} stock solution resulted in a slight decrease in fluoride concentration at the lower concentrations; however, this is hardly noticeable at the higher concentrations. This is easily explained once again by examining the mole ratios. In the reaction between Ca^{2+} and F^-, for every 1 mole of Ca^{2+}, 2 moles of F^- is required. A 1 mL aliquot of the calibration solution spiked at 5.278 $x10^{-2}$ mg/mL Ca^{2+} contains 1.317 $x10^{-6}$ mol Ca^{2+}. If all the Ca^{2+} complexes with the free fluoride in the sample, twice this many moles of F^- will be complexed – that is, 2.634 10^{-6} mol F^-. Converting this to mass units, 5.00 $x10^{-5}$ g free fluoride will be complexed. At the lowest concentration of 0.250 g/100 mL or 0.250 $x10^{-2}$ g/mL F^-, this is 2.00%, while at the highest concentration (3.00 $x10^{-2}$ g/mL F^-) this is 0.167%. This means that a larger percentage of the total

free fluoride will be complexed at lower concentrations than at higher concentrations, and the effect of the complexation will clearly be more noticeable.

Although the Ca^{2+} does appear to complex free fluoride, it does not significantly impact the effective free fluoride concentration measured, even when no TISAB II is added.

The gradients and intercepts of the response models where TISAB II was added are statistically identical, indicating that any complexation of free fluoride by Ca^{2+} is effectively combated by the addition of TISAB II.

Oxalate Investigation

In addition to a preservative, blood specimen collection tubes routinely contain an anticoagulant, typically potassium oxalate (KOx). Although this is unlikely to interfere with the fluoride concentration reading, a short study was conducted in order to show this. The cut-off concentration of 1.00 g/100 mL fluoride was clearly of greatest interest, and thus samples were prepared at this concentration. The calculated measurement uncertainty was then used to evaluate the statistical significance of any differences in concentrations observed in samples spiked with KOx and in those without.

In *Figure 20* the 99% confidence interval (U) is plotted around the theoretical value 1.00 g/100 mL NaF. The concentrations of all eight samples – those with KOx and those without – lie well within the confidence interval. In addition, the fluoride concentrations of the samples spiked with KOx lie almost directly on top of those for the samples not containing KOx

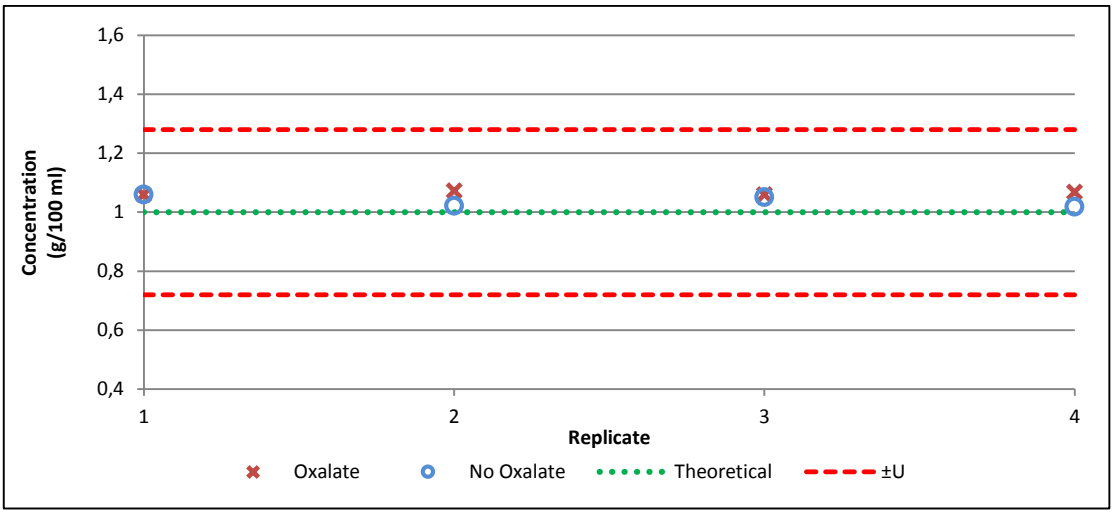

Figure 20: Experimental concentrations obtained for aqueous samples initially prepared at 1.00 g/100 mL NaF, with and without KOx

From the data in *Figure 20*, it can be concluded that there is no significant difference between the fluoride concentrations of those samples with KOx and those without. That is, the addition of KOx as

anticoagulant has no effect on the free fluoride concentration and will thus influence neither its capacity to preserve the blood sample nor the measured fluoride concentration.

3.4. Conclusion

Since for a blood ethanol concentration result to be complete it must be accompanied by a fluoride concentration result, a method for the determination of free fluoride concentration in human whole blood specimens needed to be developed. In addition, due to the challenges involved in calibrating in whole blood matrix, the method needed to allow for aqueous calibration.

It was determined that a two-fold dilution, as recommended in the FISE User Manual,[6] was not sufficient to combat matrix effects, and that specimens should rather be diluted 20-fold in order to satisfactorily suppress the matrix. Furthermore, the addition of a TISAB solution was found to be imperative to decomplex the fluoride, which may have formed complexes with ions such as Fe^{3+}, Mg^{2+} and Ca^{2+} present in the blood. Contrary to initial hypothesis, TISAB II was more effective than TISAB IV in this respect. As such, the final method procedure involved the 20-fold dilution of specimens with dH_2O and TISAB II, where the volume of TISAB II constituted half of the final volume.

This method was then validated by figures of merit, with all calibrations performed in aqueous medium and control specimens prepared in blood matrix. The main purpose of this was to emphasise that the method is fit-for-purpose.

Excellent, repeatable linearity was exhibited by the five aqueous response models, with a 95% confidence interval for the correlation coefficient found to be $r^2 = 0.9975 \pm 0.000084$. Following this, very low limits of detection and quantitation were calculated to be 0.00075 g/100 mL (95% CI: 0.000386 - 0.00145 g/100 mL) and 0.00106 g/100 mL (95% CI: 0.000545 - 0.00205 g/100 mL) respectively. Due to the logarithmic transformation, the confidence intervals are non-symmetrical and must hence be reported as ranges of values.

The method was found to be highly specific for free fluoride in whole blood, with a virtually negligible average blank concentration, and exhibited no significant additive or multiplicative bias. Additionally, the method exhibited small within-batch and between-batch precisions, with overall precisions of 1.11%, 1.10% and 3.37% for the 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL concentrations respectively.

The measurement uncertainty of the method was calculated in the same manner as that of the ethanol method, taking into account contributions of CRM, sample dilution, method bias and method imprecision. The same blood control data as was used in the method validation was used in these calculations. The final expression obtained for this can be used to estimate the MU at any experimental fluoride concentration within the calibration range; however, most concentrations are expected to fall around the cut-off of 1.00 g/100 mL.

At the three internal quality control concentrations of 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL the expanded MU at 99% confidence was found to be 53.0%, 14.5% and 15.1% respectively. That at the lower control concentration is unacceptably high, but specimen fluoride concentrations are not expected to be so low, hence this is not cause for great concern. Additionally, the MU at a concentration of 1.00 g/100 mL, while larger than expected for such an analytical method, is a conservative estimate that allows for absolute certainty that a fluoride concentration is indeed above the required cut-off, while being sufficiently small that the analytical result still has meaning. It would also appear that the relative expanded uncertainty for the method becomes fairly uniform at higher concentrations.

Since the fluoride concentration in blood specimens for ethanol analysis is required to be at least 1.00 g/100 mL, a lower limit was calculated using the MU. As such, it was found that a fluoride concentration determined by this FISE method can only be said to be below the legal cut-off if it is below 0.855 g/100 mL.

Following this, the complexation effects of Fe^{3+}, Mg^{2+} and Ca^{2+} were investigated. Since each of these ions is found in human whole blood, their potential to affect the fluoride concentration of a specimen needed to be evaluated.

The Fe^{3+} ion, being present at the highest concentration of the three, was found to severely affect the free fluoride present in samples. Response models generated by spiking aqueous NaF standards with Fe^{3+} exhibited gradients outside the acceptable range for optimal electrode function and reliable quantitation when TISAB II was not used. However, when TISAB II was added to specimens before analysis, complexation effects were minimised.

In addition, to study the effect of temperature on free fluoride, calibration solutions were chilled and filtered before being analysed at room temperature. For those solutions not spiked with Fe^{3+}, regardless of the presence or absence of TISAB II, this cooling and filtering process had no effect on the fluoride concentration. The response models generated were statistically identical to those for the solutions maintained at room temperature. However, those calibration solutions that were spiked with Fe^{3+} and filtered, even when adding TISAB II, showed a marked decrease in fluoride concentration. The response model generated from the solutions with Fe^{3+} that were cooled and had TISAB II added displayed a discontinuity at 1.00 g/100 mL fluoride. Below this concentration, there is stoichiometrically insufficient fluoride to complex with the Fe^{3+}, and hence little or no FeF_3 is removed upon filtering. Above 1.00 g/100 mL fluoride, FeF_3 is formed, and subsequently removed from the solution by the filtering process.

The corresponding response model generated from the solutions maintained at room temperature exhibited no such decrease in fluoride concentrations. Thus, temperature does impact the extent of complexation, since the complexed fluoride is less soluble at lower temperatures. The enhanced complexation thus observed would seem to imply that the effective free fluoride concentration of the

blood specimen is actually lower than the concentration at which the specimen was initially spiked, and also lower than the decomplexed concentration determined by this FISE method. However, since most blood specimens contain much more than 1.00 g/100 mL fluoride, the effective free fluoride concentration is likely to still be above the cut-off.

The addition of Mg^{2+} did not yield similar results. In fact, it was found to be at too low a concentration to significantly affect the free fluoride concentration. The same is true of the Ca^{2+}, although a slight decrease was seen at the lower fluoride concentrations when TISAB II was not added. Nevertheless, this decrease was not significant.

The addition of potassium oxalate to aqueous samples spiked at 1.00 g/100 mL fluoride, whether TISAB II was added or not, had no statistically significant effect on the fluoride concentration as evaluated against the 99% MU confidence interval for the method.

In conclusion, all calibrators, controls and specimens should be diluted 20-fold to suppress matrix effects, and TISAB II should be added to all solutions in order to combat complexation. Any fluoride concentration determined using this FISE method is deemed below the legally required 1.00 g/100 mL if, and only if, it is below 0.855 g/100 mL.

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Chapter 4

Storage Studies

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4.1. Introduction

Due to claims in literature that ethanol concentrations may increase over time, the reliability of blood alcohol results is being called into question. In fact, neoformation of ethanol as a result of inappropriate storage conditions is often cited as a defence for an elevated blood alcohol result.

Furthermore, certain micro-organisms, such as *Candida albicans*, are capable of fermenting glucose to ethanol.[1-3] Hence, should a blood specimen be contaminated by such a micro-organism, the possibility exists that the ethanol concentration could increase over time. In an effort to prevent this fermentation, a preservative – typically NaF – is added to specimens upon collection.[4, 5]

The ISO/IEC 17025:2005 Guidelines for Testing Laboratories[6] require that the laboratory be able to prove the stability of the analyte in question over the whole storage period. In addition, there are certain optimum conditions under which the fermentation of glucose to ethanol takes place. It thus seems prudent that each laboratory perform their own pre-analytical studies that simulate their specific specimen storage conditions in order to assess the impact of storage on analyte stability, and hence on the final analytical result.

In this set of studies, the impact of time, temperature, NaF content and specimen sterility was evaluated in order to assess the effect of storage conditions on ethanol concentration stability. Subsequently, recommendations on the most appropriate storage conditions for blood alcohol specimens were made. The measurement uncertainty for the GC-MS method as calculated in *Chapter 2* was used to assess the statistical significance of all ethanol concentration results obtained.

4.2. Experimental

4.2.1. General Details

Certified Reference Materials

Aqueous ethanol Certified Reference Material (CRM) (20.909 ± 0.251 g/100 mL) was purchased from the National Metrology Institute of South Africa (NMISA) and is henceforth referred to as ethanol standard.

Stable isotope labelled ethanol-d6 (99%) was obtained from Sigma-Aldrich, Midrand, South Africa.

An aqueous solution of sodium fluoride (2.997 ± 0.075 g/100 mL) was purchased from the National Metrology Institute of South Africa (NMISA) and is hence forth referred to NaF standard solution.

See ***Appendix E*** for Certificates of Analysis.

Reagents and Solvents

Sodium hydrogen carbonate ($NaHCO_3$, 99%) was purchased from Merck, Steinheim, Germany; pentafluorobenzoyl chloride (C_7ClF_5O, 99%) (PFBCl) was obtained from Sigma-Aldrich, Midrand, South Africa; sodium hydroxide pellets (NaOH, 97.0%) were acquired from Merck, Worli, Mumbai.

Phosphate buffered saline (PBS) (pH 7.2) was obtained from Thermo Fischer Scientific, Waltham, MA, United States of America.

All solvents were analytical grade and were used without further preparation.

Dichloromethane (CH_2Cl_2, pesticide grade) was obtained from Sigma-Aldrich, Steinheim, Germany. Acetonitrile (CH_3CN, HPLC grade) was purchased from Sigma-Aldrich, Midrand, South Africa, while deionised water was sourced from Merck, Modderfontein, South Africa.

Equipment

Heparin tubes (**9 mL**), evacuated tubes containing sodium heparin as anticoagulant, were purchased from Lasec, Cape Town, South Africa.

Fluoride tubes (**10 mL**), evacuated tubes containing sodium fluoride and potassium oxalate were obtained from Akasia™ Medical (Pty) Ltd, Johannesburg, South Africa.

Agar plates containing Chloramphenicol ("C-plates") and Nutrient Agar plates were prepared by the Department of Medical Microbiology, Tshwane Academic Division, National Health Laboratory Services.

Schott bottles (**500 mL**) were purchased from Lasec, Cape Town, South Africa.

Syringes (**10 mL, sterile**) and needles (**gauge 21, 2 in.**) were obtained from Sigma-Aldrich, Midrand, South Africa

Colonies and McFarland Standards

A *Candida albicans* ATCC 90028 strain was obtained from the Department of Medical Microbiology, Tshwane Academic Division, National Health Laboratory Service. The 0.5 McFarland Standard was purchased from bioMérieux, France.

Whole Blood

Whole blood will, as before, be referred to as either "pooled blood" or "fresh blood". Pooled blood shall once again refer to blood obtained from the Department of Health, Pretoria, which was prepared by pooling various blood alcohol specimens that were scheduled for destruction. Fresh blood shall refer to blank whole blood collected from healthy volunteers in heparin tubes in accordance with ethical standards (Ethical Clearance Number: **EC150618-013**; See ***Appendix F***). This blood was also pooled prior to use, but contained only endogenous ethanol – that is, naturally occurring ethanol at a very low level, and no fluoride.

Instrumentation

An Agilent 7890A Gas Chromatographic system fitted with an Agilent 7683 Autoinjector and a 5975C Mass Selective Detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis.

The inlet temperature was set at 230 °C and a constant helium carrier gas flow rate of 2.0 mL/minute was used. Sample injection (2 µl) was performed in split mode (20:1) onto a mid-polar fused silica column (ZB5-MSi, 15 m x 0.25 mm, d_f = 0.25 µm, Phenomenex, California, USA). The temperature program had an initial isotherm of 60 °C maintained for 1 minute, followed by a single ramp of 60 °C/min to a temperature 320 °C which was then maintained for 1 minute. This resulted in a total chromatographic time of 6.33 minutes.

The MSD transfer line temperature was set at 280 °C, while the temperatures of the quadrupole and source were 150 °C and 230 °C respectively. A solvent delay time of 1 minute was set before the electron ionisation (EI) source was turned on and all mass spectra were recorded at 70 eV in selected ion monitoring (SIM) mode unless otherwise stated, in which case they were recorded in scan mode.

Processing of chromatographic and mass spectrometric data was performed using Agilent ChemStation software.

A 9609BNWP Fluoride Combination Electrode (ThermoScientific, Chelmsford, MA, USA), filled with Optimum Results A filling solution, was used for analysis of control and specimen solutions. The solution to be analysed was placed into a 20 mL beaker (Lasec, Cape Town, South Africa) with a 3 mm PTFE magnetic stirrer bar (Lasec, Cape Town, South Africa), and agitated vigorously at 500 rpm during analysis. A polystyrene block was placed between the beaker and magnetic stirrer plate to prevent heat transfer to the sample. Additionally, an Orion Automatic Temperature Compensation Probe (927007MD) was placed in a volume of water as reference, and connected to the Orion Star A214 pH/ISE Benchtop Meter throughout to compensate for any fluctuations in temperature during analysis.

Selected ion monitoring mode

When collecting data in SIM mode, the characteristic qualifier ions 212 m/z and 167 m/z were monitored for the ethyl pentafluorobenzoate, while the quantifier ions for the ethyl pentafluorobenzoate and corresponding deuterated internal standard were 240 m/z and 245 m/z, respectively.

Sample preparation conditions

All experimental procedures were performed at room temperature (22 ± 6 °C) unless otherwise stated.

Preparation of calibrators and controls

For the quantitation of ethanol, calibrators and controls were prepared as outlined in *Chapter 2*, while for the quantitation of fluoride the methodology of *Chapter 3* was followed.

Sample Preparation for the Quantitation of Ethanol

To specimen whole blood (**450 µl**) in a reaction tube was added acetonitrile (**50 µl**) as well as internal standard solution (**50 µl**). Tubes were thoroughly vortexed and allowed to equilibrate at room temperature for approximately 30 minutes.

Acetonitrile (**700 µl**) was added and each tube vortexed for 30 seconds to precipitate proteins. After centrifuging for three minutes, the clear upper layer was removed to a new reaction tube. To this was added saturated $NaHCO_3$ (**1000 µl**), dH_2O (**500 µl**) and PFBCl solution (**1000 µl, 5% (v/v) in CH_2Cl_2**).

The tubes were then capped and placed on the multi-shaker for three hours. Following this, the tubes were centrifuged and the organic layer was transferred to a new reaction tube.

The organic layers were washed with saturated $NaHCO_3$ solution (**1000 µl**) followed by dH_2O (**1000 µl**), and then transferred to an amber GC vial.

After drying under compressed air at 35 °C, samples were reconstituted with CH_2Cl_2 (**100 µl**), transferred to glass inserts, and analysed by Gas Chromatography – Mass Spectrometry (GC-MS) on Selected Ion Monitoring (SIM) mode.

Sample Preparation for the Quantitation of Fluoride

Specimen whole blood (**200 µl**) was placed in a beaker with a 3 mm PTFE stirrer bar. TISAB II (**2000 µl**) and dH_2O (**1800 µl**) were added to each beaker, and the resulting solution agitated vigorously for 15 minutes.

The solutions were then analysed by means of the Fluoride Ion Selective Electrode (FISE).

Response Models and Statistical Procedures

Response models were calculated with a non-weighted linear regression on seven calibration points, and for the ethanol a reagent blank was included, comprised of blank whole blood (**450 µl**), acetonitrile (**50 µl**) and internal standard solution (**50 µl**).

Raw concentration results were calculated as the mean of duplicate analyses, after ensuring that the individual concentration values did not differ by more than the precision limits obtained from the expanded measurement uncertainty. These were then corrected for bias by means of the bias correction plots obtained in *Chapter 2* and *Chapter 3*.

The significance of all results obtained was considered in light of the expanded measurement uncertainty of the respective method, as calculated previously.

4.2.2. Effect of Time and Temperature on Ethanol Concentration Stability in Stored Blood Samples

In-house Study

Fresh blood was placed in Schott bottles (**500 mL**) and spiked with the aqueous ethanol standard at the South African legal limit concentrations of 0.02 g/100 mL and 0.05 g/100 mL ethanol, as well as 0.30 g/100 mL ethanol. The bottles were closed securely and placed on the multi-shaker for three hours to mix very gently.

Making use of syringes and needles, two sets of 29 samples at each concentration were prepared by placing the above spiked blood (**approx. 7 mL**) into fluoride tubes (NaF/KOx). Additionally, two sets of 29 blank samples were prepared by placing non-spiked fresh blood (**approx. 7 mL**) in fluoride tubes (NaF/KOx).

One set at each concentration (blank, 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL) was stored at room temperature (22 ± 6 °C) while the remaining four sets were stored under refrigeration (4 ± 3 °C) for 29 weeks. Henceforth *RT* shall denote those samples stored at room temperature, while *Fr* those stored under refrigeration.

One freshly opened tube from each set (eight in total) was analysed in duplicate once a week for ethanol concentration for a period of 29 weeks. The fluoride concentration was also determined in order to establish that it was indeed above 1 g/100 mL.

The obtained Fr and RT ethanol concentration values were then plotted against time, and the MU of the ethanol method was used to evaluate whether any increases or decreases observed were statistically significant.

Departments of Health Parallel Study

Fresh blood was placed in Schott bottles (**500 mL**) and spiked with the aqueous ethanol standard at the South African legal limit concentrations of 0.02 g/100 mL and 0.05 g/100 mL ethanol, as well as 0.30 g/100 mL ethanol. The bottles were closed securely and placed on the multi-shaker for three hours to mix very gently.

Making use of syringes and needles, two sets of 20 samples at each concentration were prepared by placing the above spiked blood (**approx. 7 mL**) into fluoride tubes (NaF/KOx). These tubes were then stored in polystyrene boxes in the refrigerator (2.5 ± 3.5 °C) for eight weeks (Set 1) and 13 weeks (Set 2).

Thereafter, Set 1, including one set at each concentration (0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL), was stored at the Johannesburg Department of Health, while Set 2, including the other three sets, was stored at the Pretoria Department of Health. Henceforth *JHB* shall denote those samples stored at the Johannesburg Department of Health, while *PTA* those stored at the Pretoria

Department of Health. These samples were stored under the same conditions as genuine samples at the Departments of Health.

One freshly opened tube from each set (six in total) was analysed in duplicate once a week for both ethanol concentration, by Gas Chromatography – Flame Ionization Detection (GC-FID) and fluoride concentration (by FISE) for a period of 20 weeks (JHB) and 15 weeks (PTA) by each Department. The temperature at which they were stored was also monitored by the respective Department on a daily basis.

The ethanol concentrations obtained from the two Departments were then plotted against time, and the MU of the GC-MS ethanol method developed in this study was used to evaluate whether any increases or decreases observed were statistically significant. While it should be noted that the GC-FID method would have a different measurement uncertainty compared to the GC-MS method, using the GC-MS measurement uncertainty will allow comparison of the performances of the methods.

4.2.3. Effect of Candida Albicans on Ethanol Concentration Stability in Stored Blood Samples

Preparation of Candida albicans Solutions

A suspension of *Candida albicans* in PBS comparable to the 0.5 McFarland standard ($1.5x10^8$ cells/mL) was prepared, and its optical density confirmed by means of a bioMérieux Densichek 110 V densitometer. This solution will subsequently be referred to as **Stock 1**.

Stock 1 was used to prepare a solution of $1.5x10^6$ cells/mL (**Stock 2**), and **Stock 2** was further diluted to a concentration of $1.5x10^4$ cells/mL (**Stock 3**).

Following this, **Stock 1** was used to prepare working solutions at $3.6x10^7$ cells/mL (**WS 5**) and $1.8x10^7$ cells/mL (**WS 4**), **Stock 2** to prepare working solutions at $3.6x10^5$ cells/mL (**WS 3**) and $1.8x10^5$ cells/mL (**WS 2**), and **Stock 3** to prepare a working solution at $1.8x10^3$ cells/mL (**WS 1**).

Spiking and Inoculation of Specimens

Fresh blood was placed in Schott bottles (**500 mL**) and spiked with the aqueous ethanol standard at the South African legal limit concentrations of 0.02 g/100 mL and 0.05 g/100 mL ethanol. The bottles were closed securely and placed on the multi-shaker for three hours to mix very gently. (***Figure 1A***)

Making use of syringes and needles, two sets of 60 samples at each concentration were prepared by placing the above spiked blood (**approx. 7 mL**) into tubes with and tubes without NaF. (***Figure 1B***)

Finally, 200 µl of WS 1 – WS 5 was added to the four sets of tubes in ***Figure 1B***, yielding the five subsets of specimens detailed in ***Figure 1C***.

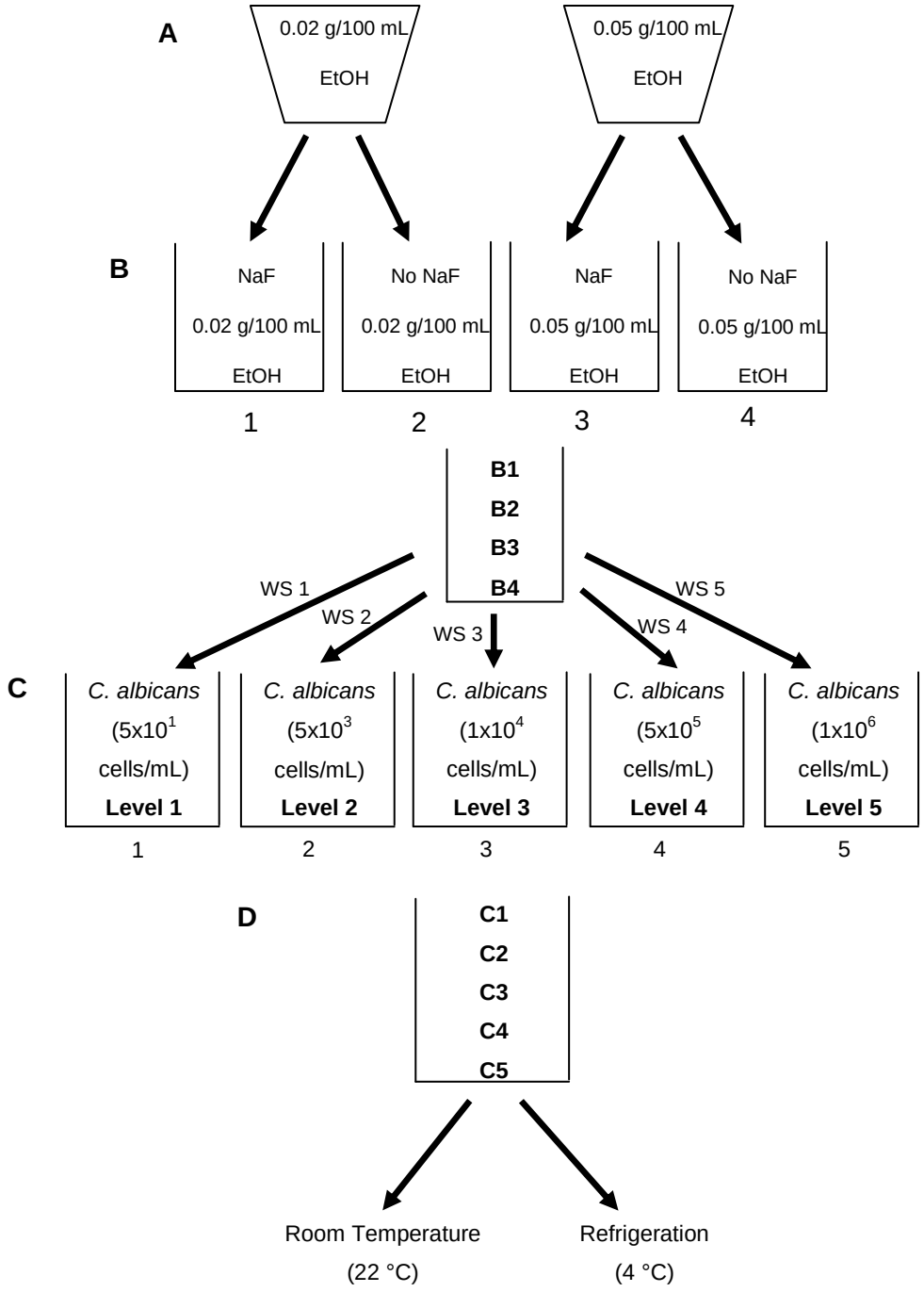

Figure 1: Preparation of inoculate blood specimens

Half of each subset in ***Figure 1C*** was stored at 4 ± 3 °C while the other half was stored at 22 ± 6 °C for up to nine weeks (***Figure 1D***).

A newly opened specimen of each of the sets was analysed in duplicate each day for the first 11 days, and thereafter once a week, for ethanol concentration, fluoride concentration and *C. albicans* concentration.

Those specimens stored at 4 ± 3 °C shall be denoted *Fr*, while those stored at 22 ± 6 °C shall be denoted *RT*.

Colony Quantification

A 100 µl aliquot of each blood specimen was smeared onto two C-plates, and the plates incubated at 30 °C for 24 hours. The resulting colonies on each plate were then counted, and the mean (n = 2) for each specimen obtained.

After week 5, specimens stored at 22 ± 6 °C without NaF (RT_No NaF) were diluted 1000 times before being smeared onto the C-plates.

4.3. Results and Discussion

4.3.1. Effect of Time and Temperature on Ethanol Concentration Stability in Stored Blood Samples

In-house Study

Blood specimens for the determination of ethanol concentration are rarely analysed immediately after collection. In fact, they are often transported to the testing laboratory and then stored there for a period of time before analysis.[7]

Although protocol calls for specimens to be kept cool while being transported to the laboratory and refrigerated thereafter, there is no guarantee that this will indeed be the case. With various literature pieces claiming the possible increase in ethanol concentration over time[1, 2], the reliability of ethanol concentration results is being called in to question.[7]

It would thus seem sensible for each laboratory that analyses for ethanol content to perform a set of storage studies. Samples should be stored under their typical laboratory storage conditions, and the stability of the ethanol concentrations monitored over the anticipated storage time period.

Consequently, samples were prepared at various ethanol concentrations, stored at room temperature and under refrigeration, and the ethanol concentrations monitored on a weekly basis for 29 weeks.

Figure 2, *Figure 3* and *Figure 4* show the ethanol concentrations obtained over the 29 weeks for samples initially spiked at 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL and stored at 4 ± 3 ℃. The 99% confidence interval around the theoretical concentration is shown at each concentration.

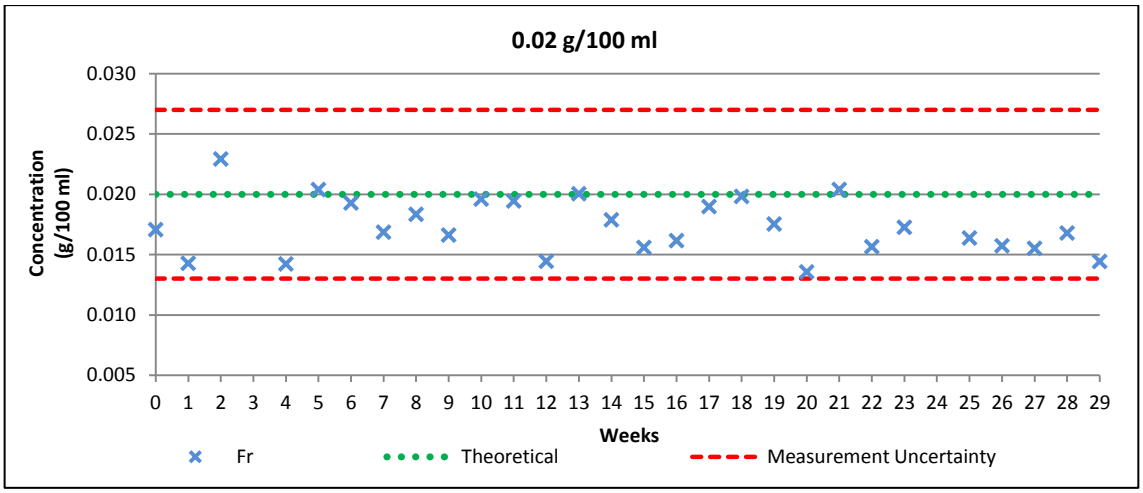

Figure 2: Ethanol concentrations of samples initially spiked at 0.02 g/100 mL and stored at 4 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

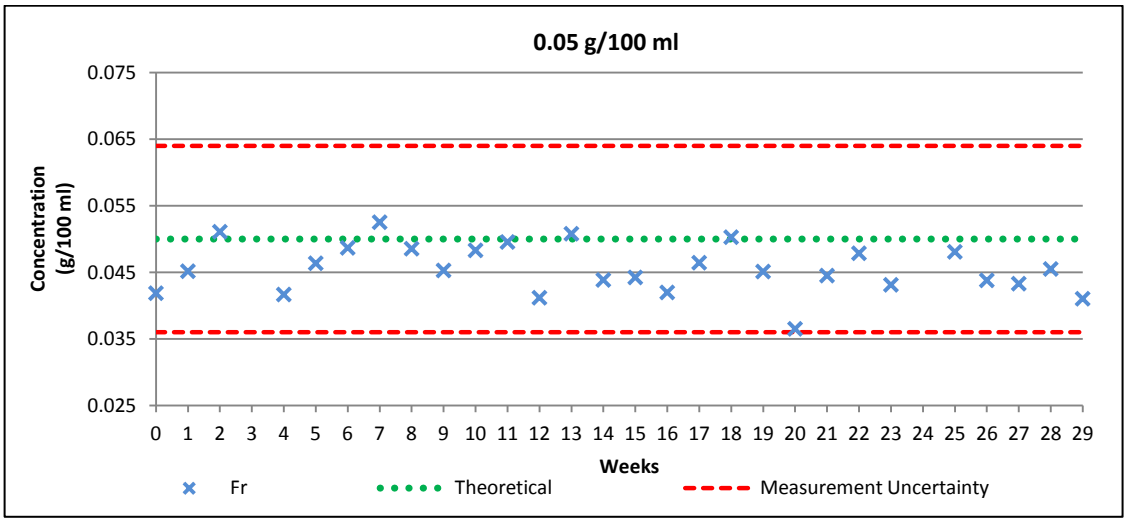

Figure 3: Ethanol concentrations of samples initially spiked at 0.05 g/100 mL and stored at 4 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

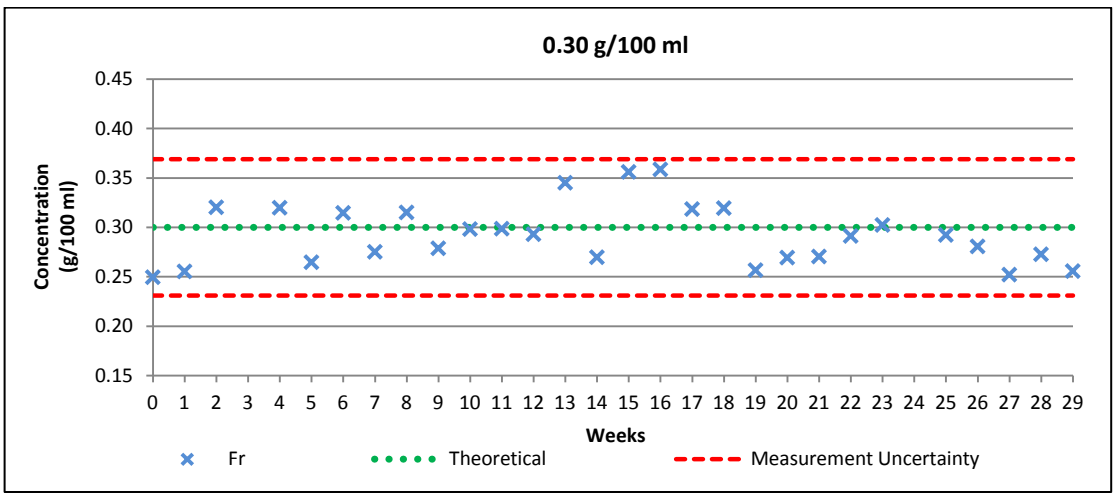

Figure 4: Ethanol concentrations of samples initially spiked at 0.30 g/100 mL and stored at 4 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

Despite showing a slight negative bias, the ethanol concentrations for the Fr specimens remained within the 99% confidence interval around the theoretical value. That is, the ethanol concentrations at all three levels were statistically stable, with no significant increase or decrease for 29 weeks when stored at 4 ± 3 °C.

Figure 5, *Figure 6* and *Figure 7* show the concentrations obtained over the same period of 29 weeks for the corresponding samples stored at 22 ± 6 °C. The 99% confidence interval around the theoretical concentration is shown at each concentration.

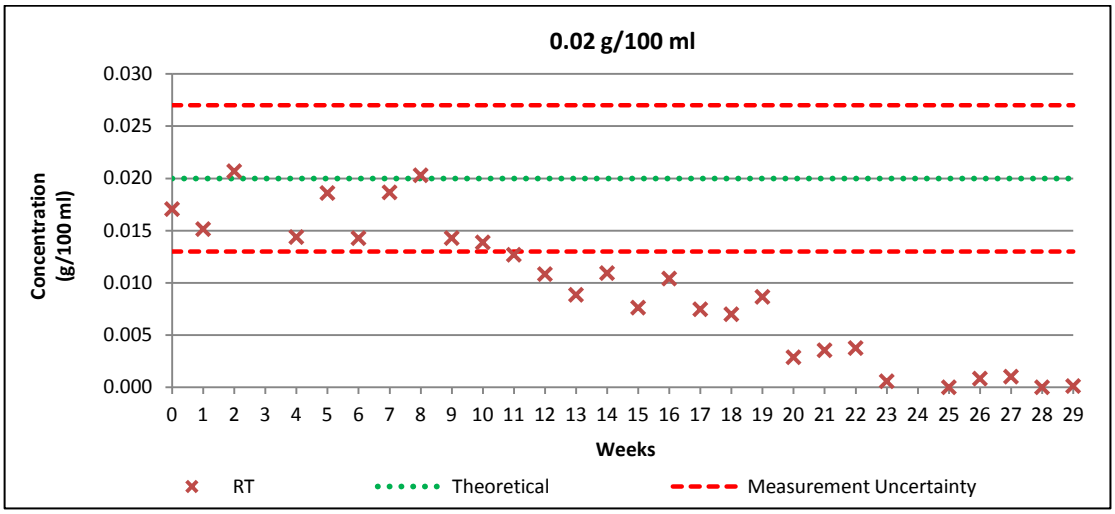

Figure 5: Ethanol concentrations of samples initially spiked at 0.02 g/100 mL and stored at 22 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

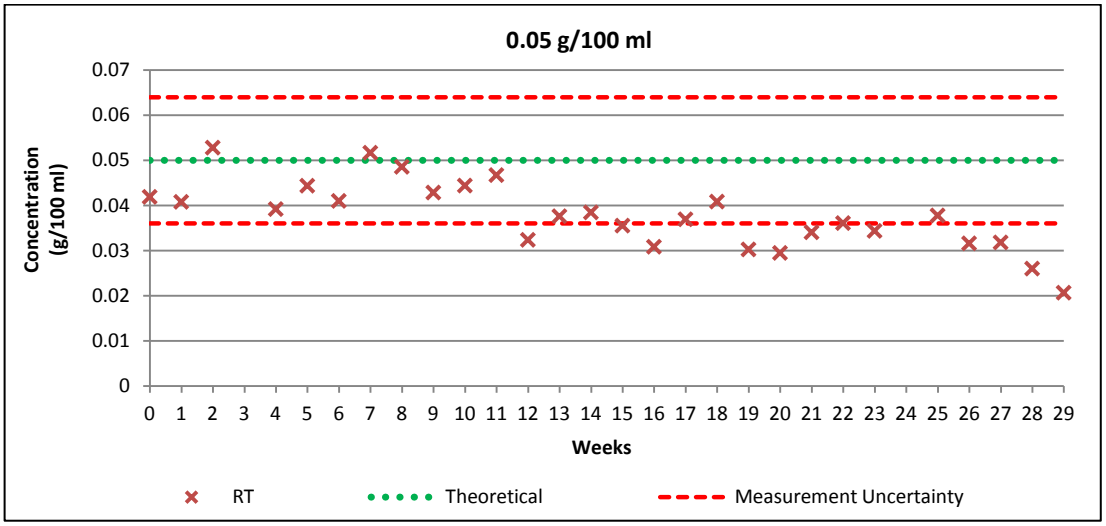

Figure 6: Ethanol concentrations of samples initially spiked at 0.05 g/100 mL and stored at 22 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

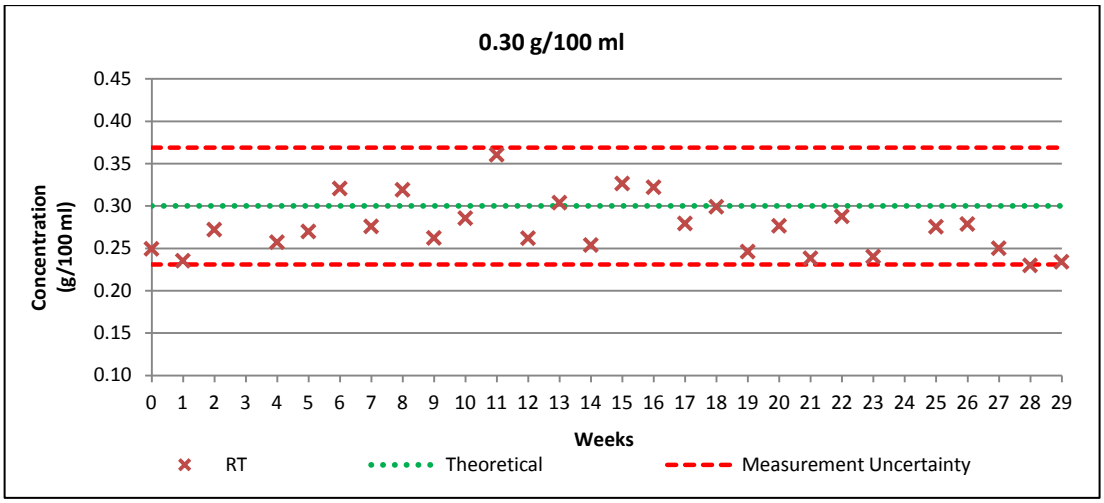

Figure 7: Ethanol concentrations of samples initially spiked at 0.30 g/100 mL and stored at 22 °C for 29 weeks and the corresponding 99% confidence interval around the theoretical concentration

For the first ten weeks, the ethanol concentrations at all three levels were statistically stable. However, from the eleventh week, the concentrations of those samples initially spiked at 0.02 g/100 mL steadily decreased until week 24, when the ethanol content was virtually negligible.

Similarly, those samples initially spiked at 0.05 g/100 mL ethanol exhibited concentrations that were statistically different from the theoretical value after 12 weeks. These concentrations seemed to oscillate around the lower confidence limit until week 25, after which a decidedly decreasing trend was observed. While not completely depleted, the ethanol concentration dropped by 60% and would likely have continued to decrease should the study have continued for a longer period of time.

Although the concentrations of those samples initially spiked at 0.30 g/100 mL were not statistically different from the theoretical value for a period of 29 weeks, there was a visible decrease in concentration values from approximately week 16.

Those samples not spiked with ethanol initially, exhibited no neoformation of ethanol for the 29 week period, with the exception of two RT samples (week 20 and week 29). However, both ethanol concentrations were below the limit of detection of the GC-MS method and were thus taken to be negligible.

From the above results, the ethanol concentration of blood specimens stored with at least 1 g/100 mL fluoride at 4 ± 3 °C can be said to be statistically stable for at least 29 weeks. Similar samples stored at 22 ± 6 °C exhibited ethanol concentrations that were statistically stable for only ten weeks, after which they were significantly decreased, and not increased as is often claimed in court. This decrease can potentially be attributed to the non-enzymatic oxidation of ethanol to acetaldehyde.[8-10]

While the analysis of samples stored at temperatures above 4 °C may not yield accurate estimates of concentration before storage, the ethanol concentration will likely have decreased rather than increased, which would be to the benefit of the defendant. For the accurate quantitation of ethanol in

blood, it is recommended that specimens be stored at or below 4 °C with at least 1 g/100 mL NaF as preservative.

Departments of Health Parallel Study

In South Africa there are four Departments of Health that have a unit that tests for blood alcohol. These are located in Cape Town, Durban, Pretoria and Johannesburg. In order to investigate the stability of ethanol concentration in stored blood specimens, the Pretoria and Johannesburg Departments of Health agreed to store specimens under their individual typical storage conditions, and to monitor ethanol concentration for a period of 15 and 20 weeks respectively. For this study, the initial concentrations of the specimens were unknown to each Department.

Figure 8, *Figure 9*, and *Figure 10* show the ethanol concentrations, determined by HS-GC-FID, for the two Departments for stored specimens initially spiked at 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL ethanol. The 99% confidence interval around the theoretical concentration for the GC-MS method is also shown.

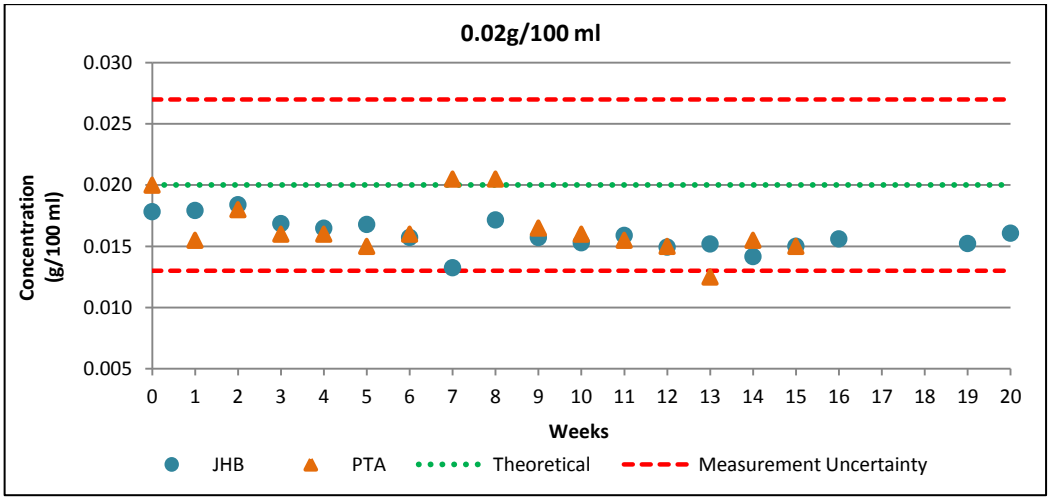

Figure 8: Ethanol concentrations for specimens initially spiked at 0.02 g/100 mL stored at the Johannesburg and Pretoria Departments of Health for a period of 20 and 15 weeks respectively and the 99% confidence interval around the theoretical concentration value as calculated from the GC-MS method

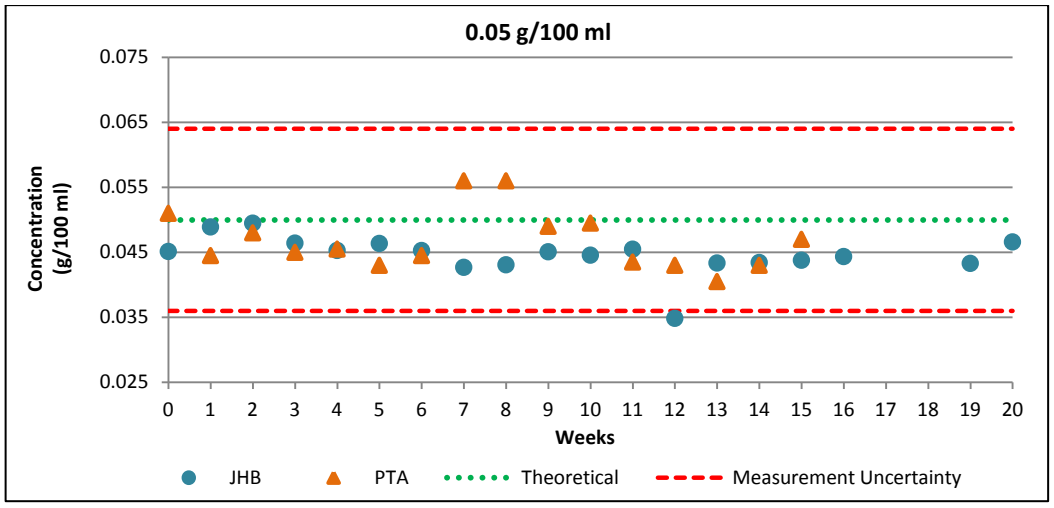

Figure 9: Ethanol concentrations for specimens initially spiked at 0.05 g/100 mL stored at the Johannesburg and Pretoria Departments of Health for a period of 20 and 15 weeks respectively and the 99% confidence interval around the theoretical concentration value as calculated from the GC-MS method

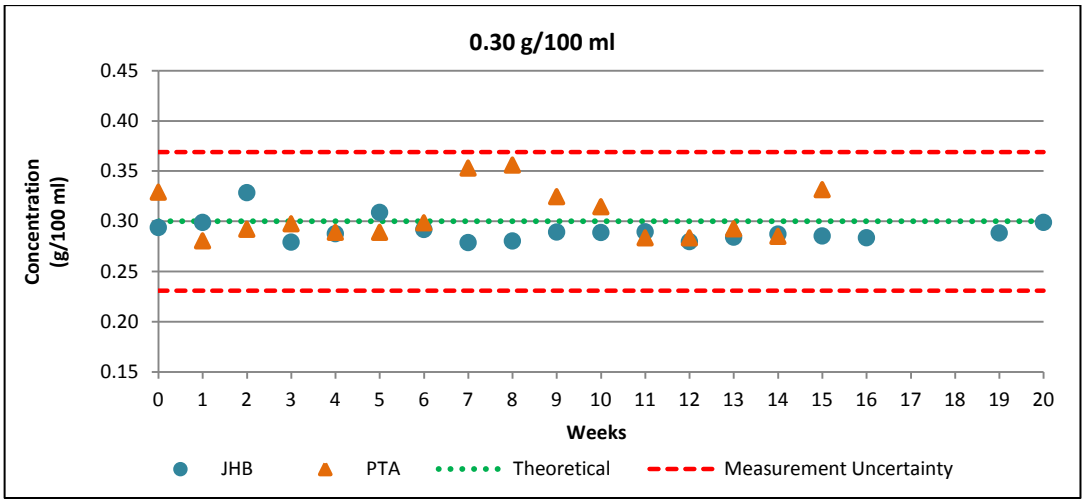

Figure 10: Ethanol concentrations for specimens initially spiked at 0.30 g/100 mL stored at the Johannesburg and Pretoria Departments of Health for a period of 20 and 15 weeks respectively and the 99% confidence interval around the theoretical concentration value as calculated from the GC-MS method

For the PTA specimens, the temperature was found to range between 0 °C and 2 °C, except for week 5 which displayed a larger range of 0 °C to 6 °C. The fluoride concentrations for the specimens were found to be between 1.265 g/100 mL and 1.690 g/100 mL, with the exception of weeks 7 and 8 which ranged between 0.705 g/100 mL and 0.945 g/100 mL.

The temperatures of the JHB specimens ranged between 4.6 °C and 9.2 °C, except in week 14 where a slightly larger range of 2.4 °C to 17.1 °C was observed. The fluoride concentrations for the specimens were found to range between 1.265 g/100 mL and 2.895 g/100 mL. Week 0 exhibited especially low fluoride concentrations of 0.5285-0.6725 g/100mL, while a particularly high concentration of 4.065 g/100 mL was observed for the tube initially spiked at 0.05 g/100 mL and analysed in week 2.

Although a slight decrease in ethanol concentration was seen at each level for both Departments, all three levels lay within the measurement uncertainty of the GC-MS method. A possible explanation for this decrease could be that the entire polystyrene container housing all 20 sample tubes was removed from refrigeration each week, instead of removing one tube at a time.

As expected, the decrease observed in the specimens was much more noticeable in the 0.02 g/100 mL level as compared to the 0.30 g/100 mL where the ethanol concentration was virtually constant.

It is also interesting to note that the ethanol concentrations obtained for the PTA specimens fluctuated vastly more than those of the JHB specimens, and exhibited slightly higher concentrations at week 7 and week 8. These larger concentrations correspond to the lower fluoride concentrations at these weeks.

The fact that these concentration values were obtained by GC-FID and yet still lie within the MU for the GC-MS method speaks to the comparability of the two methods. In *Figure 11* the ethanol concentration values obtained by GC-MS and HS-GC-FID for the refrigerated storage experiments are compared. Both exhibit the same stability, remaining within the 99% confidence interval of the GC-MS method around the theoretical. This shows that the newly developed GC-MS method compares favourably to the well-established HS-GC-FID method that is most commonly used in the analysis of ethanol in blood.

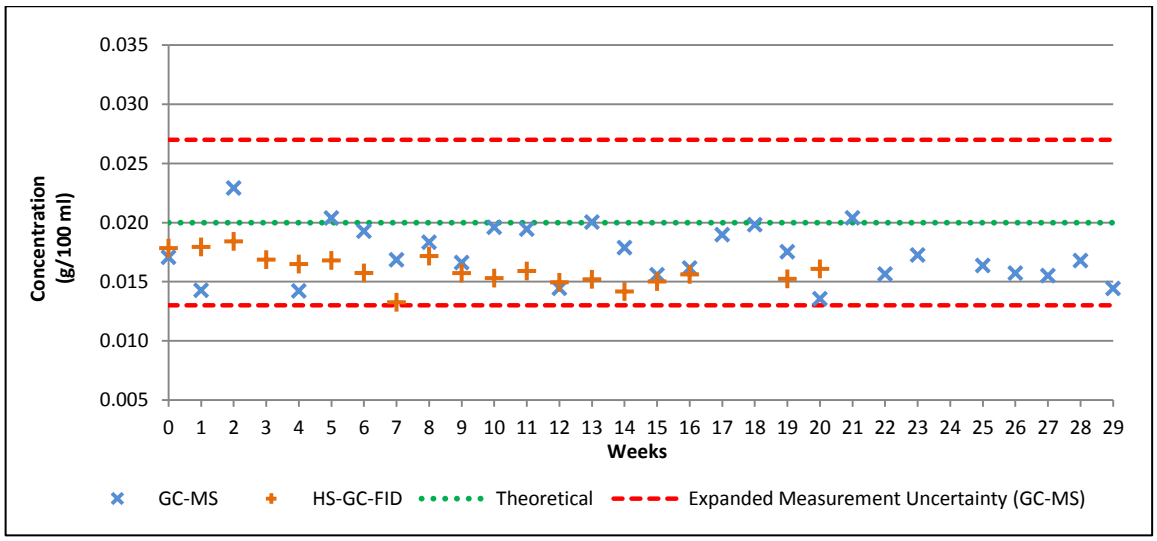

Figure 11: Comparison of ethanol concentration values obtained by GC-MS and HS-GC-FID for specimens stored under similar conditions, and the 99% confidence interval around the theoretical as calculated for the GC-MS method

4.3.2. Effect of Candida Albicans on Ethanol Concentration Stability in Stored Blood Samples

It is common knowledge that yeasts such as *Candida albicans* are capable of fermenting glucose to ethanol.[1, 2, 11] For this reason, possible contamination of the blood specimen with *C. albicans* – or some similar micro-organism – has been cited many times as a defence for an elevated blood ethanol result.[1, 2]

It was thus decided to investigate the effect of *C. albicans* in blood specimens on ethanol concentration, when specimens are stored under ideal conditions (refrigerated with sufficient NaF), non-ideal conditions (room temperature without NaF) and semi-ideal conditions (refrigerated without NaF, and room temperature with NaF).

All that remained was to choose pertinent *C. albicans* inocula cell densities that would result in useful and relevant results. A healthy person should not have *C. albicans* in their blood.[12] However, being an opportunistic pathogen, *C. albicans* bloodstream infections are often found in immunocompromised patients.[12, 13] It proved difficult to find reference ranges for typical levels of *C. albicans* in blood, nevertheless various studies have utilised inocula sizes of 5-25 $x10^1$ cells/mL[14], $1x10^4$ cells/mL[1], and 1-$5x10^5$ cells/mL.[15]

As a result, *C. albicans* solutions were prepared so that inoculated specimen concentrations would range from $5x10^1$ cells/mL to $1x10^6$ cells/mL.

The following figures show plots of the ethanol concentrations over time, with the colony counts overlaid, for the highest and lowest *C. albicans* inoculated concentrations of specimens stored under various conditions. Subsequently, the ethanol concentrations were plotted together with those from the *Time and Temperature* study in order to assess the impact of the presence of *C. albicans*. (See ***Appendix G*** for remaining plots)

Analysis was halted once no colonies were detected for at least three consecutive weeks.

4 °C and NaF

Figure 12 and *Figure 13* show the ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and stored at 4 °C with fluoride, after being inoculated at $1x10^6$ cells/mL and $5x10^1$ cells/mL *C. albicans* respectively. *Figure 14* and *Figure 15* show the same for those specimens initially spiked at 0.05 g/100 mL ethanol. In *Figure 12*, *Figure 13* and *Figure 14*, the colony count for time zero is omitted for clarity.

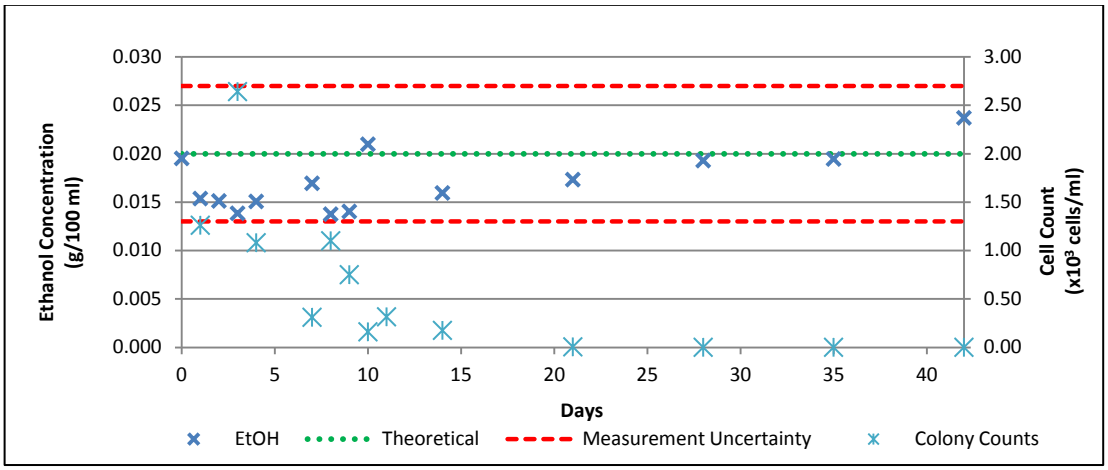

Figure 12: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 4 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

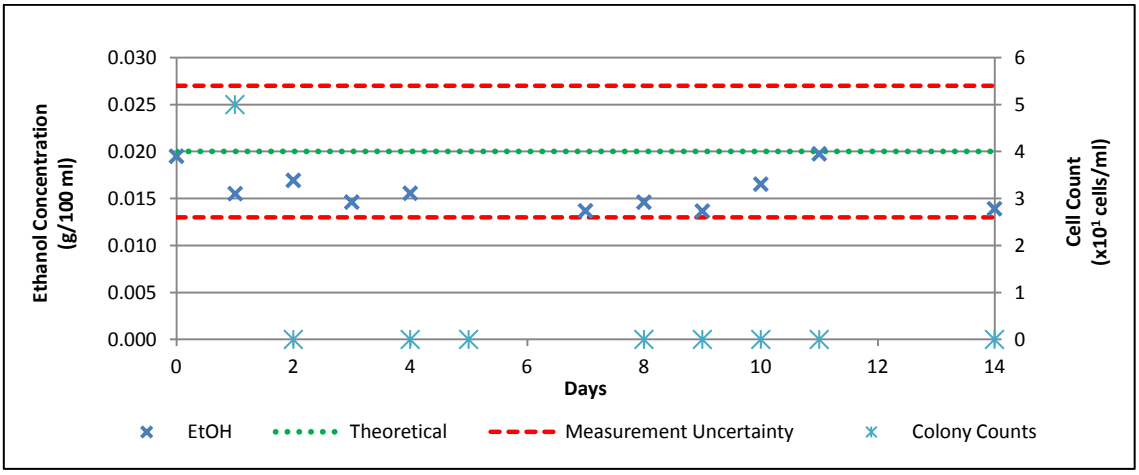

Figure 13: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $5x10^1$ cells/mL C. albicans, and stored at 4 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

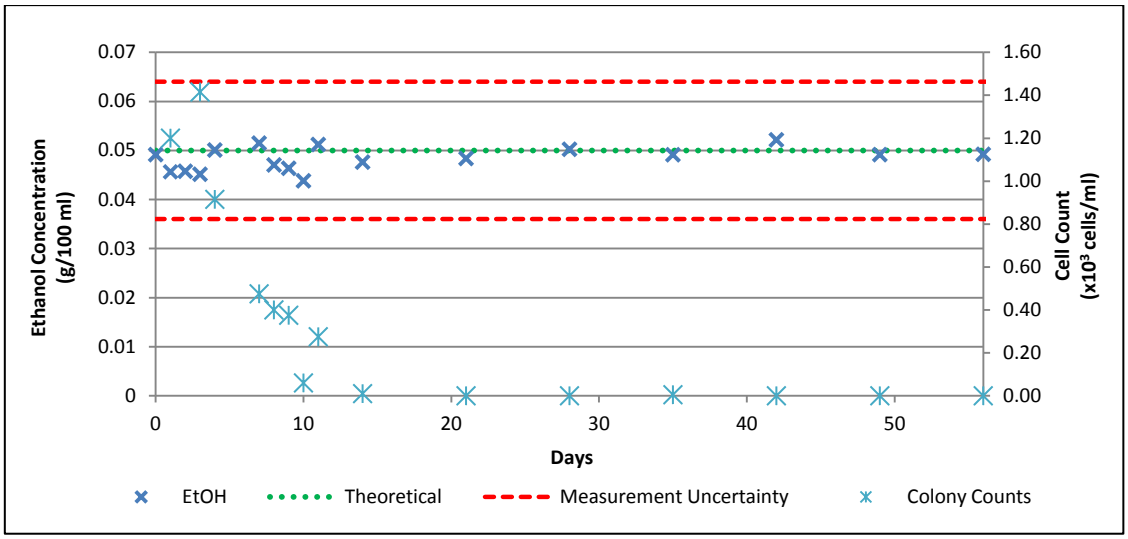

Figure 14: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $1x10^6$ cells/mL C. albicans, and stored at 4 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

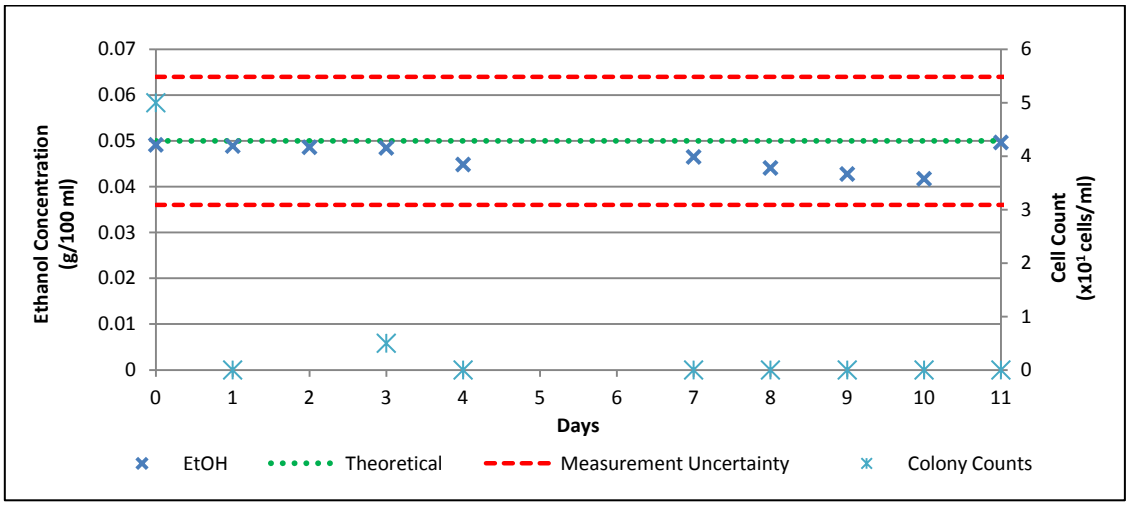

Figure 15: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $5x10^1$ cells/mL C. albicans, and stored at 4 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

In every instance where specimens were stored at 4 °C in the presence of NaF, the ethanol concentration was statistically stable, remaining within the 99% confidence interval around the theoretical concentration. For the specimens initially spiked at 0.02 g/100 mL ethanol, there was, however, a slight negative bias observed.

As was expected, the *C. albicans* died off faster in those specimens that were inoculated at lower concentrations, being completely undetectable after two days, as compared to approximately 14 days for the higher concentration.

Figure 16 shows the overlaid ethanol concentrations for the inoculated specimens (**1x10^6 cells/mL**) and the sterile specimens from the *Time and Temperature* study stored **with NaF** at **4 °C**, initially spiked at 0.02 g/100 mL ethanol. *Figure 17* shows the same for the specimens initially spiked at 0.05 g/100 mL ethanol.

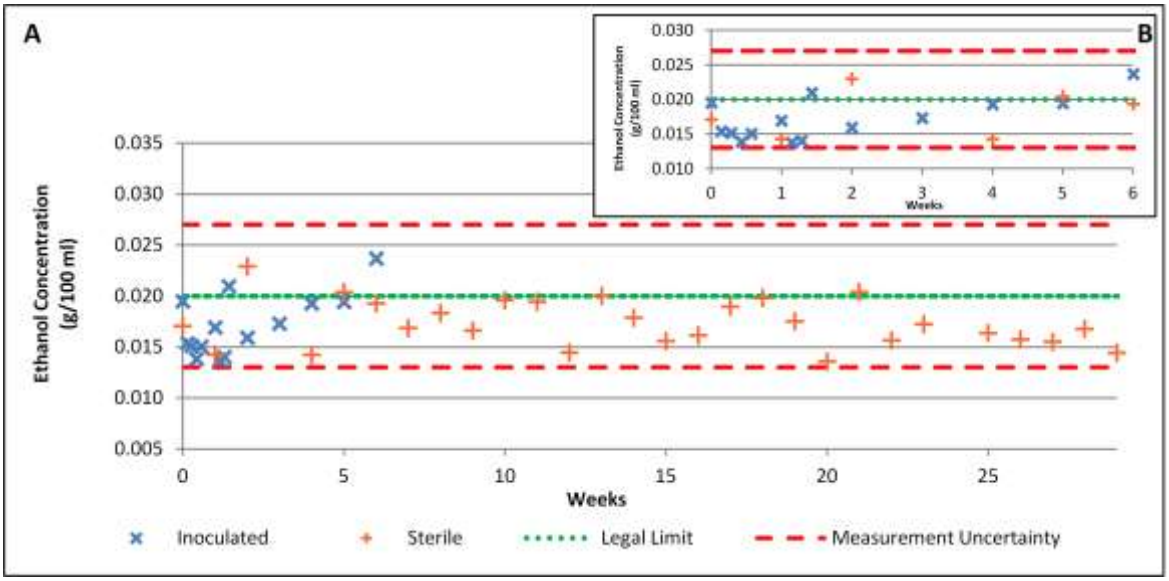

Figure 16A: Ethanol concentrations for sterile specimens and inoculated specimens (1x10^6 cells/mL) with NaF initially spiked at 0.02 g/100 mL ethanol and stored at 4 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 6 weeks

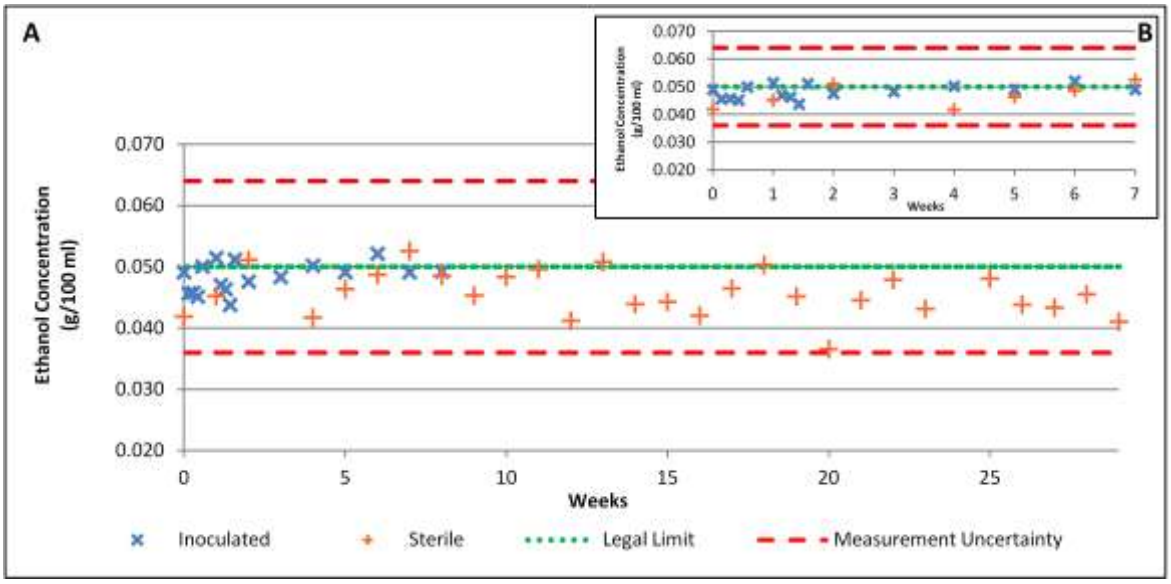

Figure 17A: Ethanol concentrations for sterile specimens and inoculated specimens (1x10^6 cells/mL) with NaF initially spiked at 0.05 g/100 mL ethanol and stored at 4 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 8 weeks

Although the inoculated specimens were only analysed for a period of six and eight weeks for the 0.02 g/100 mL and 0.05 g/100 mL ethanol concentrations respectively, the results compare favourably with those obtained for the sterile specimens. That is, the presence of *C. albicans* had little effect on the stability of the ethanol concentrations of specimens that were refrigerated at 4 °C and contained NaF as preservative.

4 °C and No NaF

Figure 18 and *Figure 19* show the ethanol concentrations and colony counts for the specimens initially spiked at 0.02 g/100 mL ethanol and stored at 4 °C without NaF after being inoculated with $1x10^6$ cells/mL and $5x10^1$ cells/mL *C. albicans* respectively. Similarly, *Figure 20* and *Figure 21* show the ethanol concentrations and colony counts for those specimens initially spiked at 0.05 g/100 mL ethanol. In *Figure 18* and *Figure 20*, the colony count for time zero is omitted for clarity.

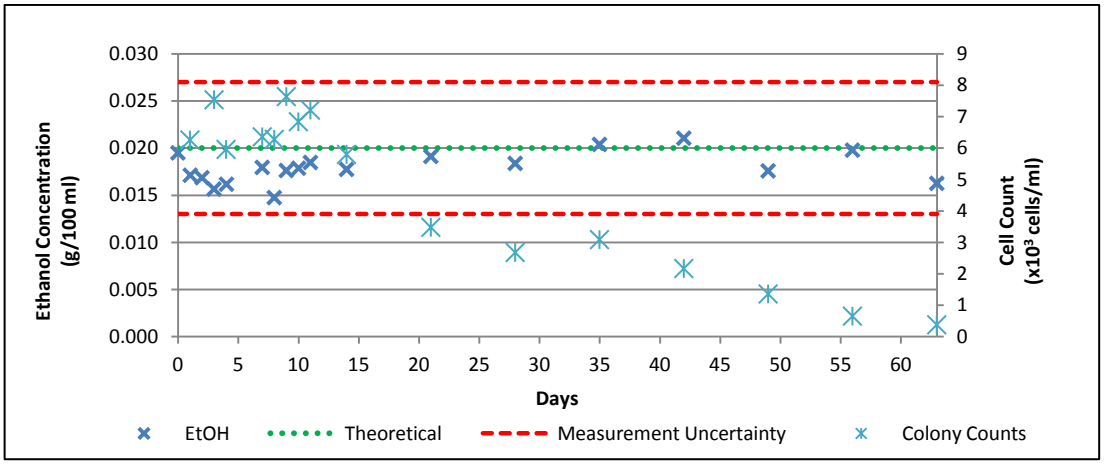

Figure 18: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 4 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

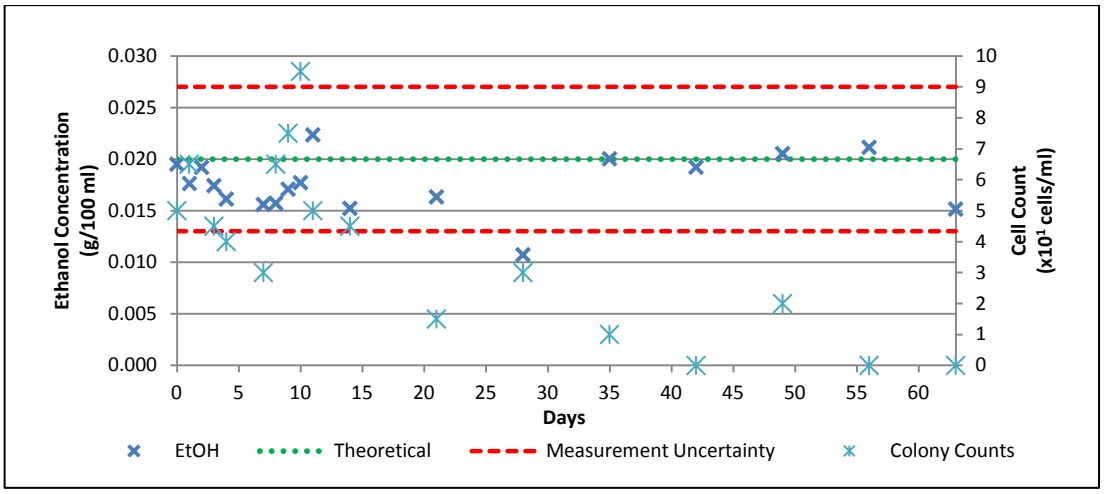

Figure 19: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 4 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

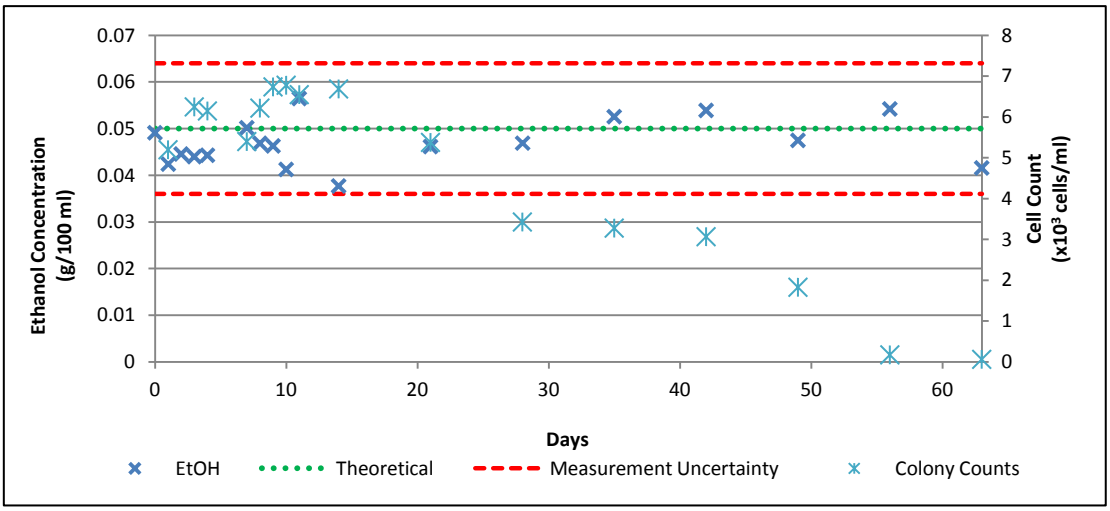

Figure 20: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 4 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

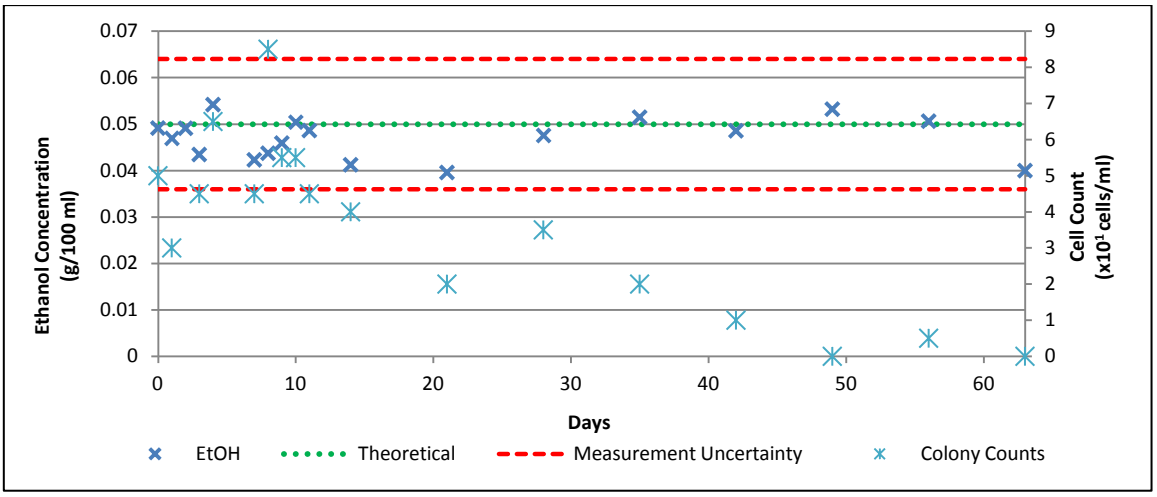

Figure 21: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 4 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

Despite the lack of fluoride and the presence of *C. albicans*, the ethanol concentrations remained statistically stable at 99% confidence, oscillating about the theoretical values for both ethanol levels. In addition, *C. albicans* colonies were still detectable for at least 5 weeks for all inoculation levels, although the colony counts showed a steady decline over time.

For interest sake, *Figure 22* displays images of the agar plates for samples prepared at 0.02 g/100 mL ethanol, inoculated at $1x10^6$ cells/mL and stored at 4 °C in the presence of fluoride in chronological order. It is possible to visually observe the decrease in *C. albicans* concentration, by the decreasing number of colonies on the plates. This correlates with the plot of cell counts against number of days, which is also shown.

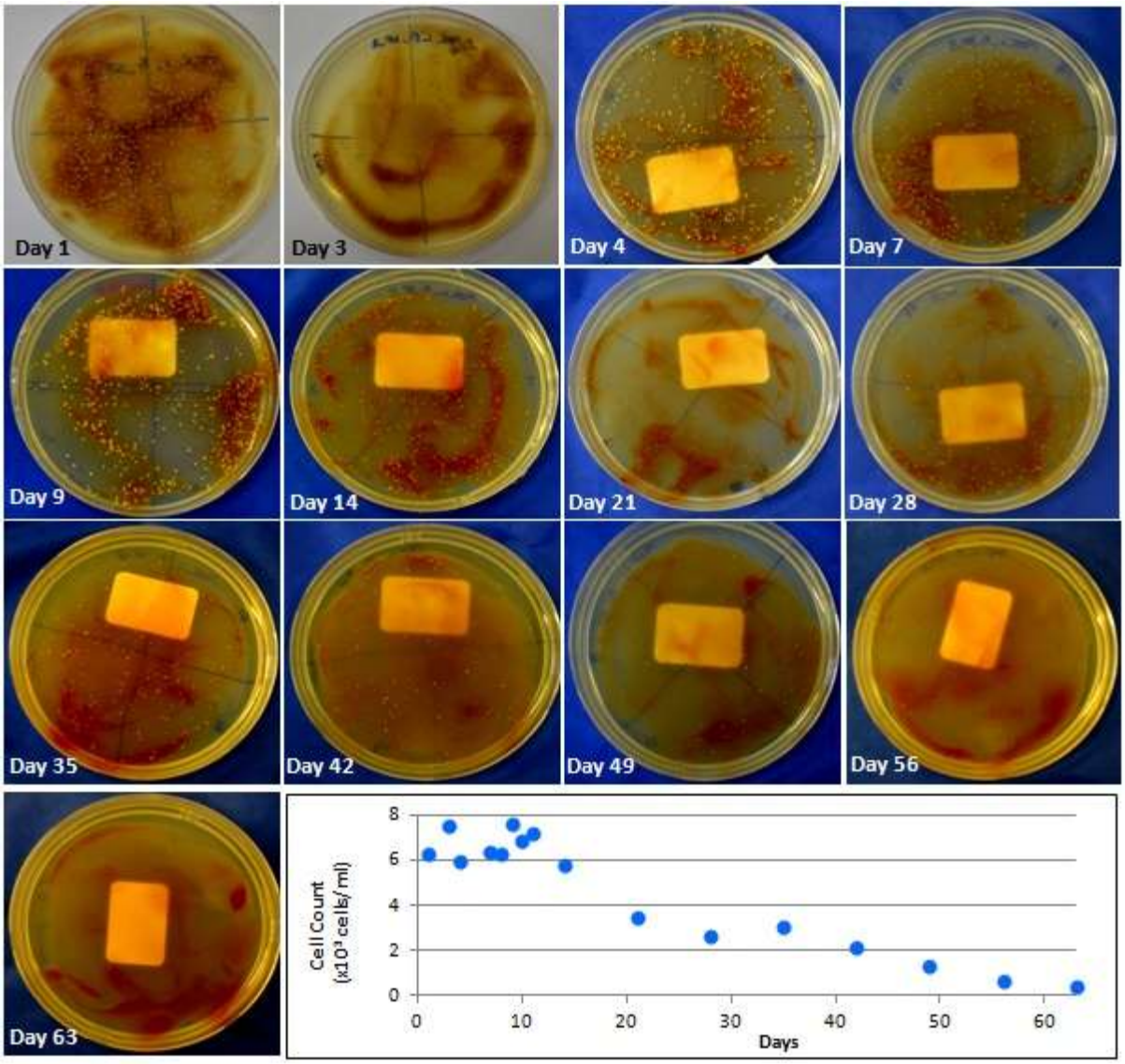

Figure 22: Chronological photos of agar plates of samples prepared at 0.02 g/100 mL ethanol, inoculated at 1x10^6 cells/mL and stored at 4 °C with NaF

Figure 23 shows the overlaid ethanol concentrations for the inoculated specimens (**1x10^6 cells/mL**) stored **without NaF** at **4 °C** and the sterile specimens from the *Time and Temperature* study stored **with NaF** at **4 °C**, initially spiked at 0.02 g/100 mL ethanol. *Figure 24* shows the same for the specimens initially spiked at 0.05 g/100 mL ethanol.

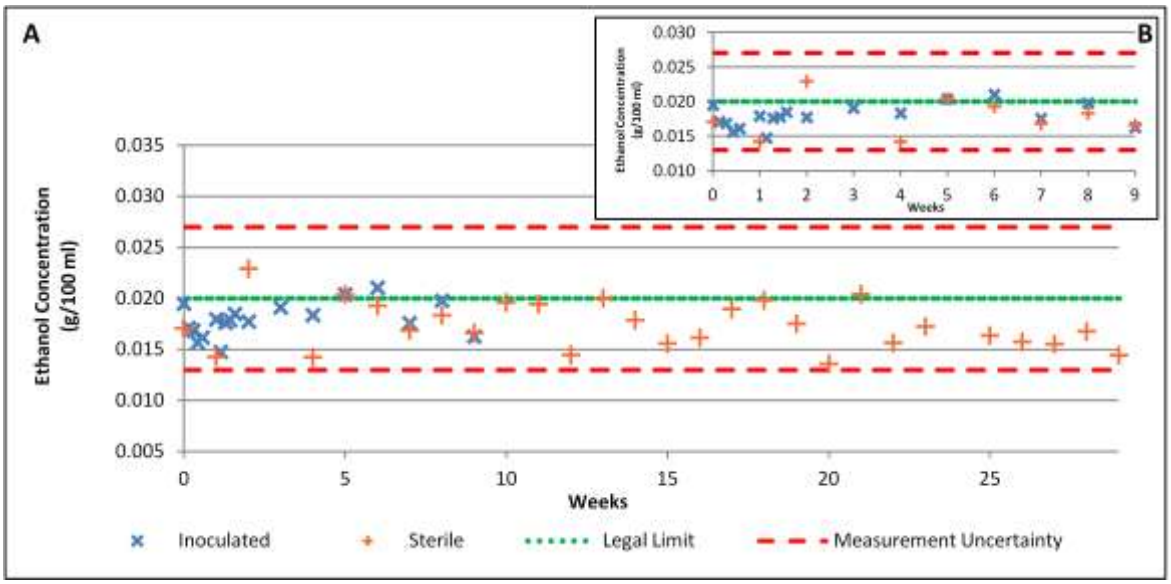

Figure 23A: Ethanol concentrations for sterile specimens with NaF and inoculated specimens ($1x10^6$ cells/mL) without NaF initially spiked at 0.02 g/100 mL ethanol and stored at 4 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 9 weeks

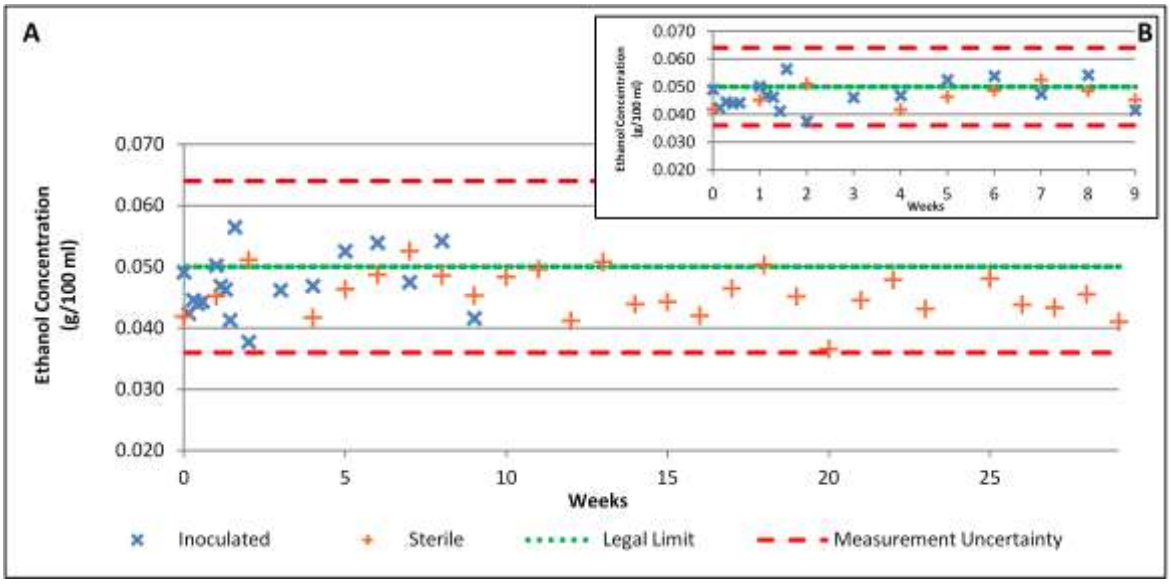

Figure 24A: Ethanol concentrations for sterile specimens with NaF and inoculated specimens ($1x10^6$ cells/mL) without NaF initially spiked at 0.05 g/100 mL ethanol and stored at 4 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 9 weeks

In both instances, the ethanol concentrations for the inoculated specimens were only monitored for 9 weeks. However, they compare favourably with those obtained for the sterile specimens, showing that the presence of *C. albicans* in the specimens combined with the absence of NaF had little effect on the stability of the ethanol concentration, provided that specimens were refrigerated at 4 °C.

22 °C and NaF

Figure 25 and *Figure 26* show the ethanol concentrations and colony counts for specimens initially prepared at 0.02 g/100 mL ethanol and stored at 22 °C in the presence of fluoride after being inoculated at $1x10^6$ cells/mL and $5x10^1$ cells/mL *C. albicans*, while *Figure 27* and *Figure 28* show those for the corresponding specimens initially spiked at 0.05 g/100 mL ethanol. In *Figure 12*, the colony count for time zero is omitted for clarity.

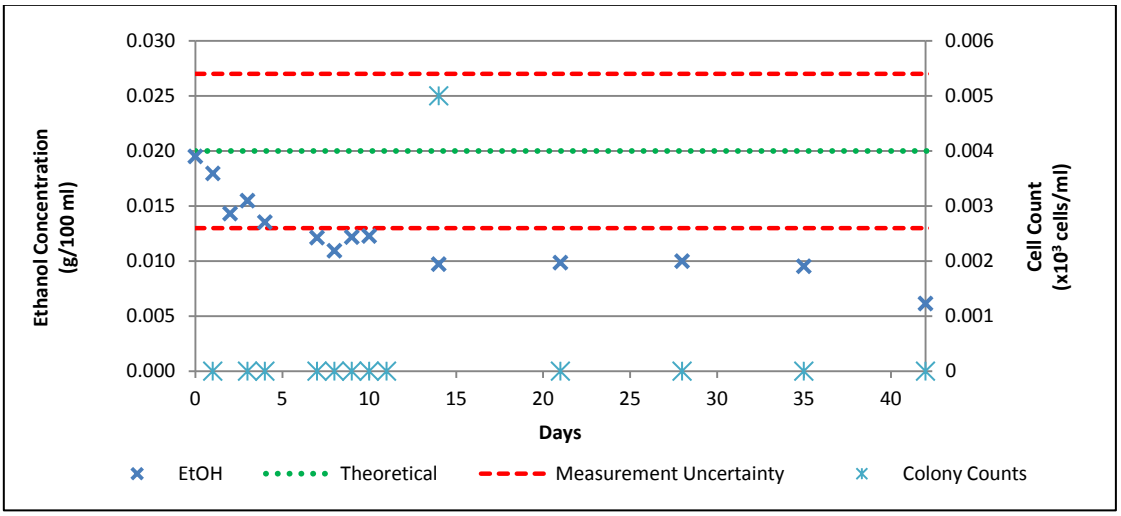

Figure 25: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 22 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

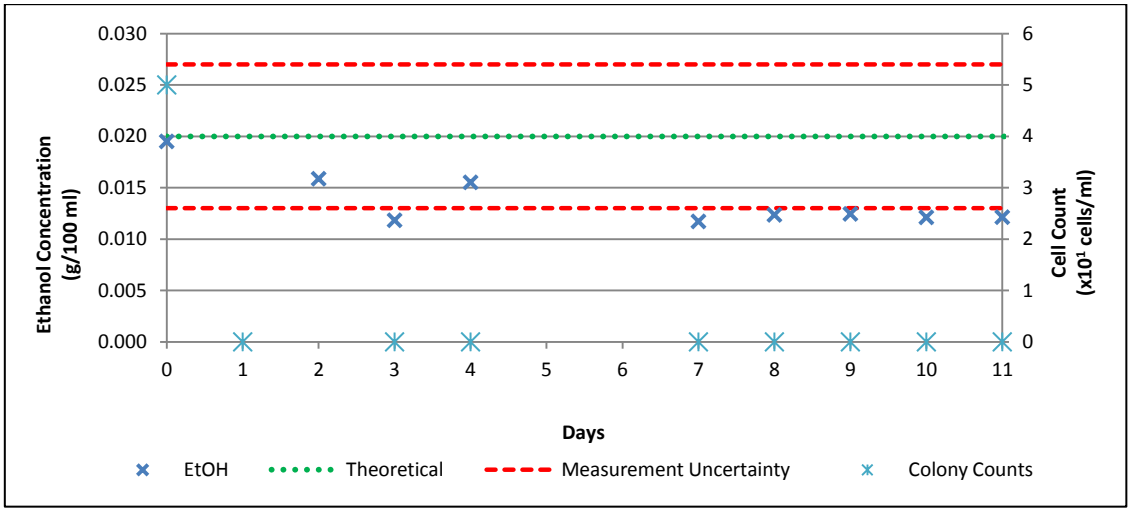

Figure 26: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 22 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

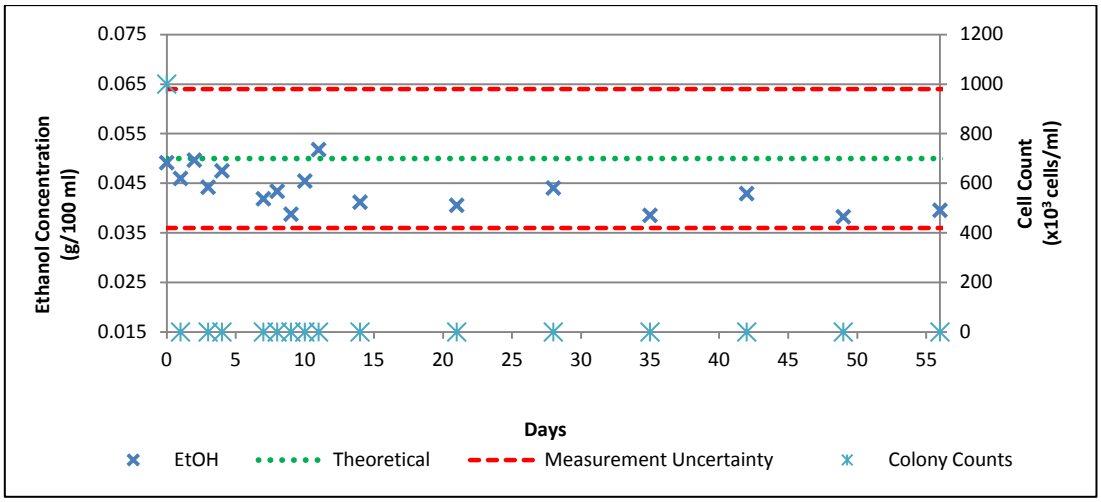

Figure 27: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 22 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

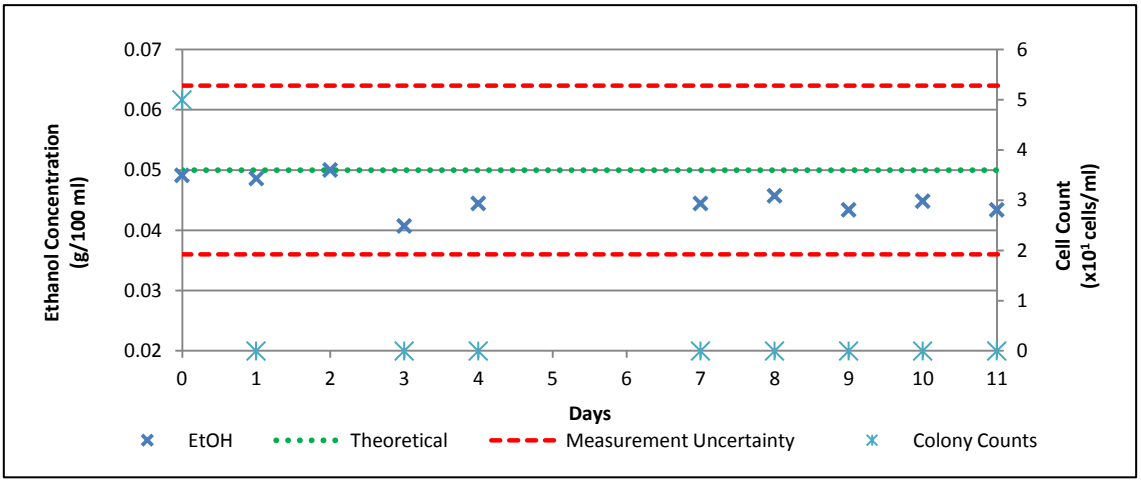

Figure 28: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 22 °C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

For the specimens initially prepared at 0.02 g/100 mL ethanol, the ethanol concentration significantly decreased, irrespective of the *C. albicans* concentration. The higher the *C. albicans* concentration, however, the more substantial the overall decrease in ethanol. In addition, the ethanol concentration decreased rapidly at first within the first seven days, and then seemed to stabilise.

Although the ethanol concentrations of those specimens initially spiked at 0.05 g/100 mL remained statistically stable, there was a noticeable downward trend at all levels of *C. albicans*.

It is interesting to note that while the ethanol concentration did decrease, the *C. albicans* had died off completely within the first 24 hours. This would seem to indicate that the initial presence of the *C. albicans* initiated a sort of cascading effect that continued even after there was no *C. albicans* remaining.

Figure 29 shows the overlaid ethanol concentrations for the inoculated specimens (**1x10^6 cells/mL**) and the sterile specimens from the *Time and Temperature* study stored **with NaF** at **22 °C**, initially spiked at 0.02 g/100 mL ethanol. *Figure 30* shows the same for the specimens initially spiked at 0.05 g/100 mL ethanol.

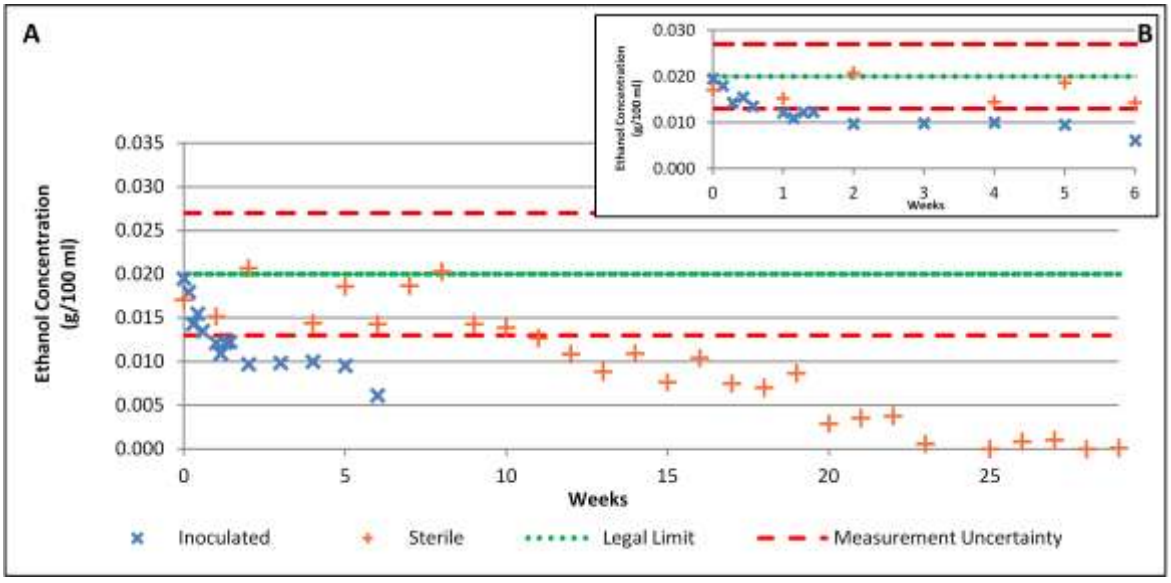

Figure 29A: Ethanol concentrations for sterile specimens and inoculated specimens (1x10^6 cells/mL) with NaF initially spiked at 0.02 g/100 mL ethanol and stored at 22 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 6 weeks

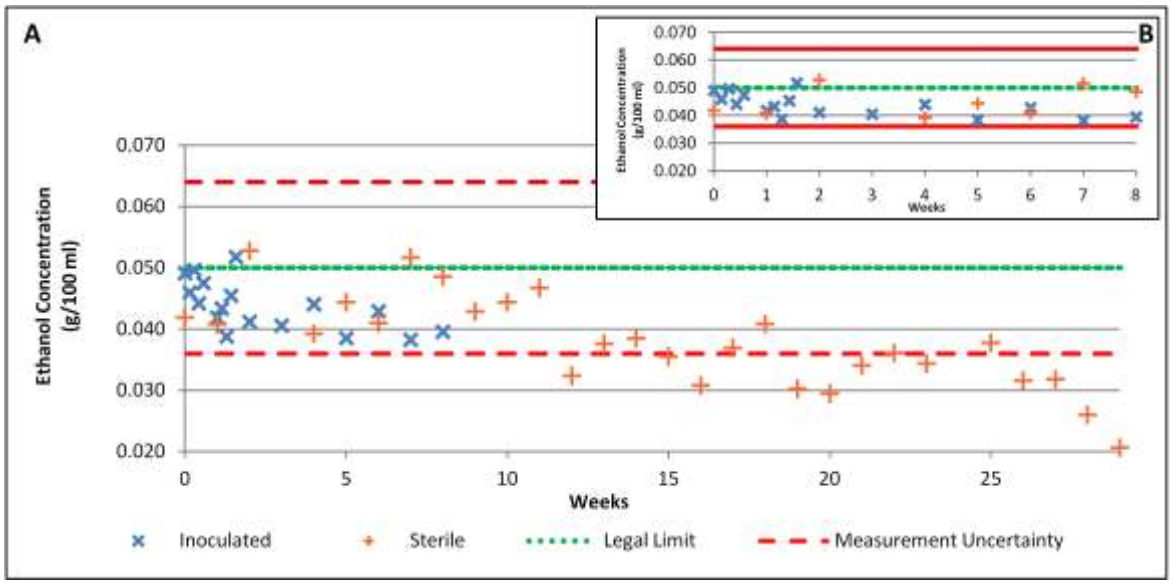

Figure 30A: Ethanol concentrations for sterile specimens and inoculated specimens ($1x10^6$ cells/mL) with NaF initially spiked at 0.05 g/100 mL ethanol and stored at 22 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 8 weeks

The sterile specimens showed a significant decrease in ethanol concentration after 10 and 11 weeks at the 0.02 g/100 mL and 0.05 g/100 mL levels respectively. The steady decline observed in the sterile specimens was mirrored in the inoculated specimens, but at a faster rate. The ethanol concentration of the specimens initially spiked at 0.02 g/100 mL ethanol was significantly lowered by the end of the first week. For those specimens initially spiked at 0.05 g/100 mL ethanol, assuming the observed decreasing trend was to continue, the ethanol concentration would have been significantly lowered by week 9 or week 10.

This just confirms that the presence of *C. albicans* causes a decrease in ethanol concentration, and this decrease, despite the addition of NaF, is exacerbated by long term storage at temperatures around 22 °C.

22 °C and No NaF

Figure 31 and *Figure 32* show the ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol, and stored at 22 °C in the absence of fluoride, after being inoculated with $1x10^6$ cells/mL and $5x10^1$ cells/mL *C. albicans* respectively. Similarly, *Figure 33* and *Figure 34* show the ethanol concentrations and colony counts for specimens spiked at 0.05 g/100 mL. In each instance, the *C. albicans* was not quantifiable for the first 35 days. After this, samples were diluted before being smeared on the agar plates.

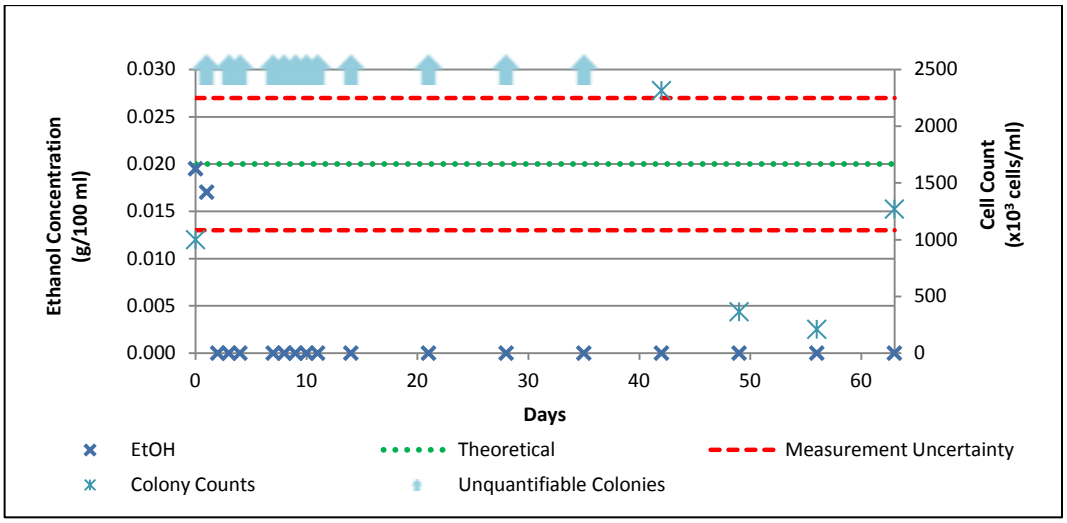

Figure 31: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 22 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

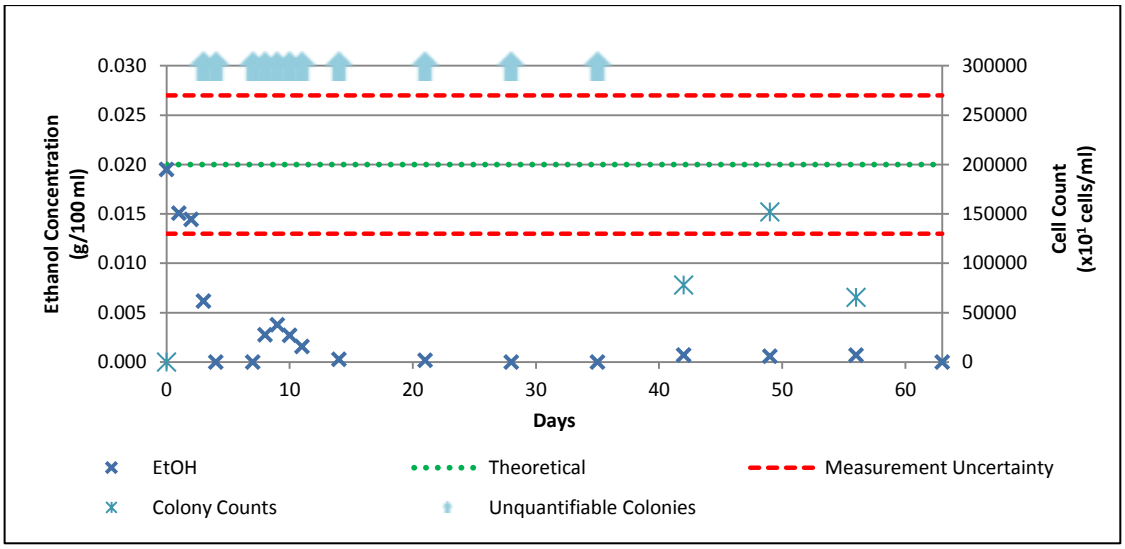

Figure 32: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 22 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

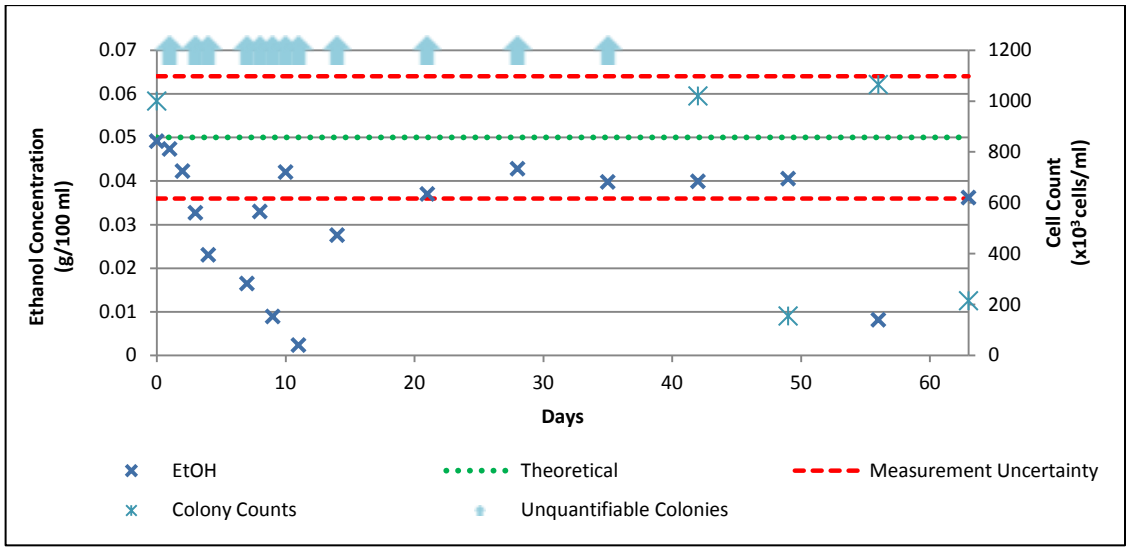

Figure 33: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $1x10^6$ cells/mL *C. albicans*, and stored at 22 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

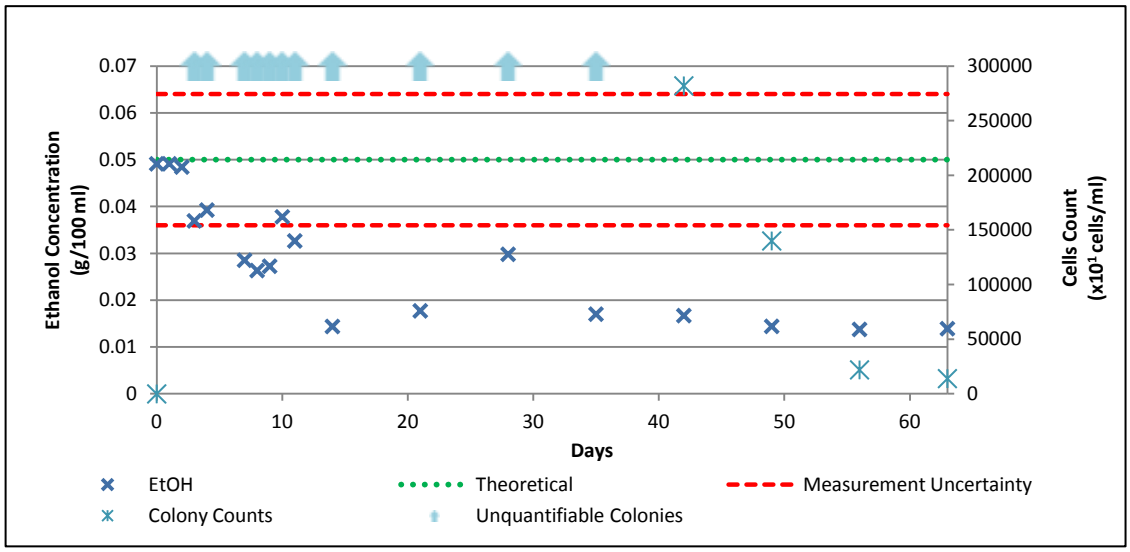

Figure 34: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 mL ethanol and $5x10^1$ cells/mL *C. albicans*, and stored at 22 °C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

The ethanol concentrations of those specimens initially spiked at 0.02 g/100 mL ethanol were completely depleted after only 2 days for the highest concentration of *C. albicans* and 4 days for the lowest. A brief increase in ethanol concentration was observed for the specimens inoculated at the lowest concentration of *C. albicans*; however, the ethanol was quickly depleted once more and did not increase again.

For those specimens initially spiked at 0.05 g/100 mL ethanol, a similar rapid decrease in ethanol concentration was initially observed, with the ethanol concentrations being significantly decreased by three and seven days for the highest and lowest levels of *C. albicans* respectively. At the lowest level of *C. albicans*, the ethanol concentration decreased substantially to less than half the initial

concentration. At the highest level, the ethanol was almost completely depleted after eleven days; however, it increased thereafter to approximately 0.04 g/100 mL.

Interestingly, an increasing-decreasing pattern in ethanol concentration was observed for specimens initially spiked at 0.05 g/100 mL, at all levels of *C. albicans*. This was most noticeable at the higher concentrations of *C. albicans*. It was initially thought that perhaps this could be attributed to the *C. albicans* using and producing ethanol in a cycle. However, in week six, several specimens were plated out onto nutrient agar plates instead of C-plates, and it was subsequently discovered that specimens were possibly contaminated with a *Bacillus* species during spiking. Nutrient agar plates are non-selective, meaning that, unlike the C-plates, they do not select for specific organisms while inhibiting the growth of others.[16]

Figure 35 compares a nutrient agar plate (A) with a C-plate (B) of a specimen initially spiked at 0.05 g/100 mL ethanol and inoculated at $1x10^6$ cells/mL *C. albicans*. Large, "fluffy" colonies are clearly visible in A and are in stark contrast to the small, smooth colonies of *C. albicans* seen in B. These colonies seen in A are likely a *Bacillus* spp.

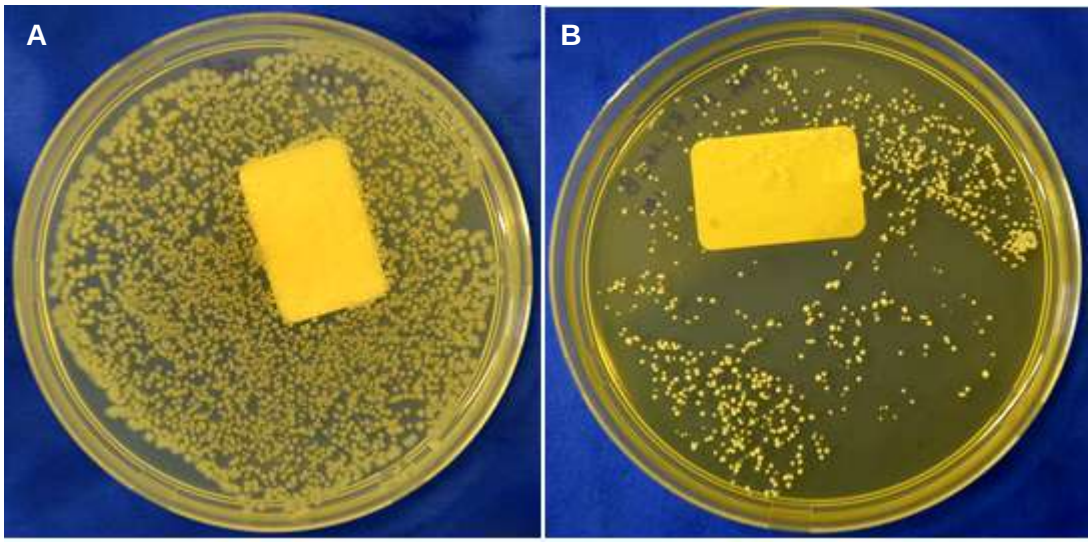

Figure 35 A: Example of nutrient agar plate showing possible *bacillus* spp. as well as the expected *C. albicans.*
B: Corresponding C-plate showing only *C. albicans*

The unexpected increase in ethanol concentrations could potentially be attributed to the presence of the *Bacillus* spp.[17], and it should be noted that while the ethanol concentration did increase, it was never elevated above the prosecution level of 0.064 g/100 mL.

Quantification of *C. albicans* colonies was not possible before week six. However, after week five, specimens were diluted 1000 times before plating out. This process is illustrated in *Figure 36* below. Even after termination of the study at nine weeks, high levels of *C. albicans* were still detected, showing these conditions – room temperature and no preservative – to be optimum conditions for the growth of *C. albicans*.

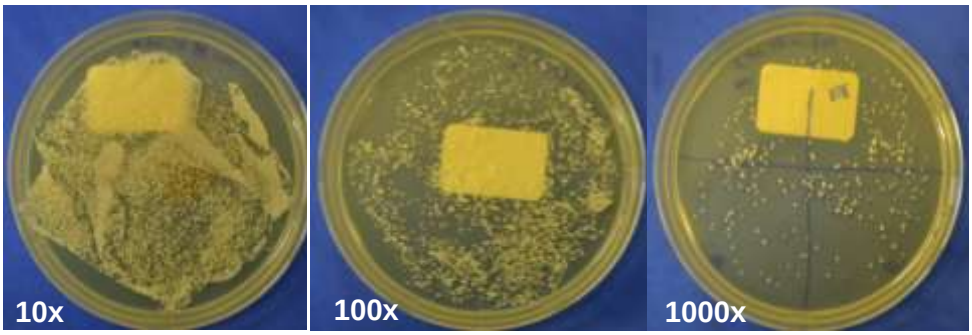

Figure 36: Example of serial dilution of a specimen to allow quantification of *C. albicans* colonies

Figure 37 shows the overlaid ethanol concentrations for the inoculated specimens (**1x10^6 cells/mL**) **without NaF** at **22 °C** and the sterile specimens from the *Time and Temperature* study stored **with NaF** at **22 °C**, initially spiked at 0.02 g/100 mL ethanol. *Figure 38* shows the same for the specimens initially spiked at 0.05 g/100 mL ethanol.

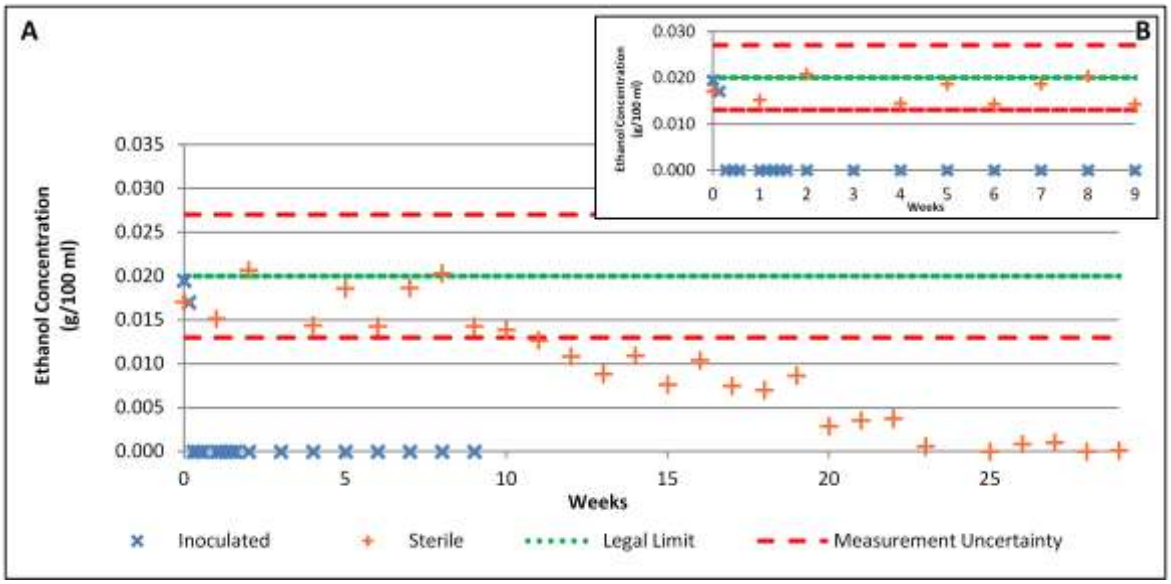

Figure 37A: Ethanol concentrations for sterile specimens with NaF and inoculated specimens (1x10^6 cells/mL) without NaF, initially spiked at 0.02 g/100 mL ethanol and stored at 22 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 9 weeks

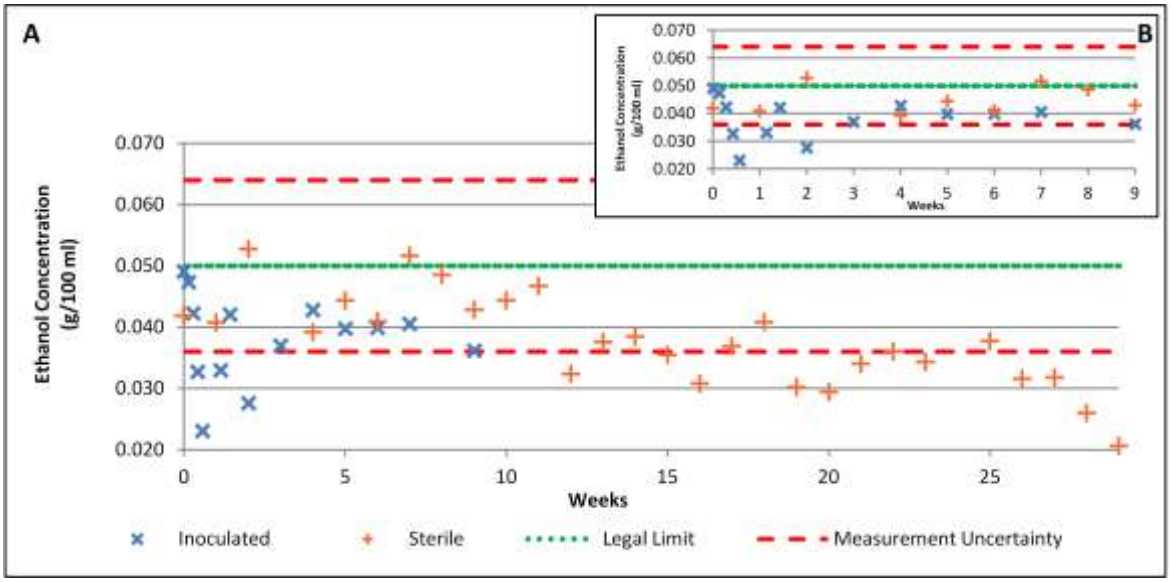

Figure 38A: Ethanol concentrations for sterile specimens with NaF and inoculated specimens ($1x10^6$ cells/mL) without NaF, initially spiked at 0.05 g/100 mL ethanol and stored at 22 °C, with the corresponding 99% confidence interval.

B (inlaid): Expanded scale of **A** from 0 to 9 weeks

Similar to those specimens stored at room temperature with NaF, these inoculated specimens stored without NaF exhibited a statistically decreased ethanol concentration much sooner than did the sterile specimens stored under similar conditions. Additionally, the rate of ~~decline~~ decrease was much faster than that observed for the inoculated specimens stored with NaF.

This highlights the fact that specimens stored at temperatures around 22 °C will be likely to exhibit decreased ethanol concentrations regardless of whether NaF is added or not.

Summary

The storage of blood specimens at room temperature (22 ± 6 °C) resulted in a significant decrease in ethanol concentration at 99% confidence at both legal limits, regardless of whether or not NaF was added to the specimens. This decrease was exacerbated by the presence of *C. albicans*, which had a more significant effect at the lower legal limit of 0.02 g/100 mL.

Blood specimens stored under refrigeration (4 ± 3 °C) exhibited statistically stable ethanol concentrations at 99% confidence at both legal limits. In addition, the presence of *C. albicans* had no significant effect on blood ethanol concentrations of refrigerated specimens, up to the point where no colonies could be detected, after which it was assumed that the *C. albicans* could no longer affect ethanol concentration.

It was also noticed that while the presence of NaF did help to stabilise the ethanol concentration in stored blood specimens, temperature played a much more important role. Regardless of the

presence or absence of NaF in both sterile and inoculated specimens, storage at 4 ± 3 °C was sufficient to stabilise ethanol concentrations over time.

Figure 39 shows agar plates of four specimens stored under each of the storage conditions for comparison of colony growth. Specimens A and B were stored at 4 ± 3 °C while specimens ~~S~~ C and D were stored at 22 ± 6 °C. Additionally, specimens A and C lacked NaF, while specimens B and D contained NaF.

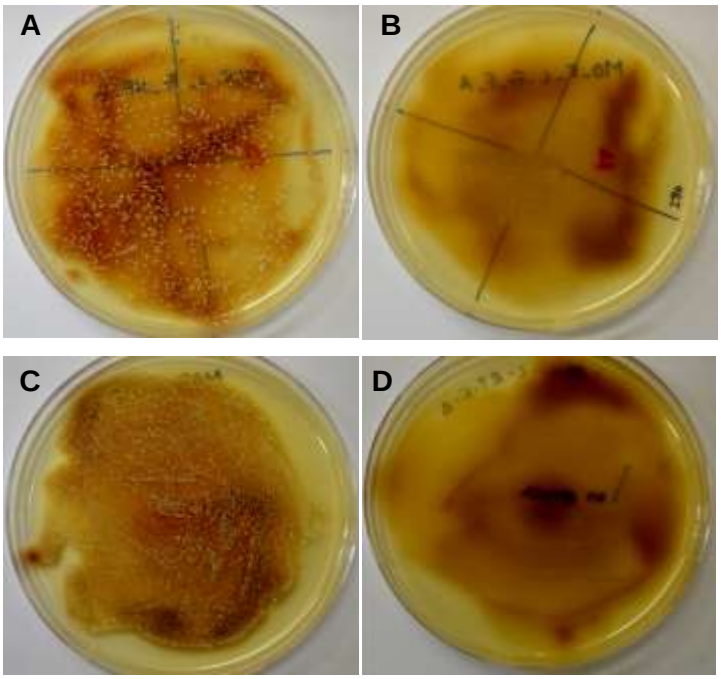

Figure 39: Comparison of the agar plates for the four storage conditions, for specimens initially spiked at 0.05 g/100mL and inoculated at $1x10^6$ cells/mL (**A**: Fr_NF; **B**: Fr_F; **C**: RT_NF; **D**: RT_F) after 24 hours

Interestingly, the *C. albicans* died off faster in those specimens stored at 22 °C with NaF than those stored at 4 °C with NaF. It is hypothesised that those organisms stored at the higher temperature are metabolising faster and thus encountering the fluoride more quickly, while those at the lower temperature are in a sort of “stasis” state where metabolism takes place very slowly. In addition, as was seen in *Chapter 3*, there is less free fluoride available at lower temperatures, and as such the *C. albicans* growth is inhibited to a lesser degree.

4.4. Conclusion

The effect of various storage conditions on the stability of blood ethanol concentration was investigated. It was found that the concentrations of sterile blood specimens spiked at 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL ethanol were statistically stable at 99% confidence for at least 29 weeks provided they were stored under refrigeration (4 ± 3 °C) with the addition of at least 1 g/100 mL NaF.

Identical specimens, spiked at 0.02 g/100 mL, 0.05 g/100 mL, and 0.30 g/100 mL ethanol and containing at least 1 g/100 mL NaF, were stored at room temperature (22 ± 6 °C). The ethanol concentrations of those specimens at 0.02 g/100 mL and 0.05 g/100 mL ethanol were found to be statistically stable at a 99% level of confidence for 11 and 12 weeks respectively, after which a significant decrease in ethanol concentration was observed. The ethanol content at the lowest level was completely depleted by week 24, while that of the 0.05 g/100 mL level was lowered by 60% by termination of the study. The ethanol concentration of the specimens initially spiked at 0.30 g/100 mL did not significantly differ from the theoretical value; however, there was a noticeable decrease from week 16 onwards.

It is often claimed in a court setting that the ethanol concentration of stored blood specimens could have increased before analysis. The results of this study, however, indicated the contrary. While concentrations of specimens stored above 4 °C may not be accurate, it is unlikely that a defendant would be unfairly prosecuted as a result of an artificially raised ethanol concentration. It is more probable that the ethanol concentration of an incorrectly stored specimen will be lower upon analysis than it initially was when the specimen was obtained. This is clearly to the benefit of the defendant. From these results, it is recommended that for the accurate quantitation of ethanol in blood specimens, the specimens be stored at 4 °C with at least 1 g/100 mL NaF.

A similar, parallel study carried out in conjunction with the Pretoria and Johannesburg Departments of Health revealed that the ethanol concentrations at all three levels of interest as determined by HS-GC-FID were statistically stable at 99% confidence according to the measurement uncertainty of the GC-MS method for at least 15 and 20 weeks. This speaks to the comparability of the GC-MS method with the more commonly applied HS-GC-FID method.

Following this, several sets of specimens were prepared at 0.02 g/100 mL and 0.05 g/100 mL ethanol, and inoculated with various concentrations of *C. albicans*. Contamination of blood specimens with this, and similar micro-organisms, is often claimed as a defence for elevated blood alcohol concentrations in a court setting. The results obtained for the inoculated specimens, however, indicated a decrease in ethanol concentration. These results corroborate those obtained for the sterile specimens.

It was seen that storage of blood specimens at room temperature (22 ± 6 °C), whether with or without NaF, resulted in a significant decrease in ethanol concentration at 99% confidence. A greater decrease was observed in the inoculated specimens than in the sterile specimens. The ethanol concentrations of all specimens stored under refrigeration (4 ± 3 °C) were statistically stable at 99% confidence, and even the addition of *C. albicans* had no significant effect.

In addition, it was seen that while the presence of NaF served to assist in stabilising ethanol concentration over time, the temperature at which the specimens were stored had a much greater impact on the ethanol concentrations. Storage at 4 °C was found to be sufficient to stabilise ethanol concentrations over time, regardless of whether NaF was present, or whether specimens were inoculated with *C. albicans*.

It is thus recommended that blood specimens for the analysis of ethanol content be stored at a temperature of 4 °C, and if possible, with at least 1 g/100 mL NaF. Since the temperature at which specimens are transported cannot be guaranteed, the addition of NaF will help to stabilise the ethanol concentration, preventing it from decreasing any further should it have been subjected to higher temperatures.

Furthermore, should these experiments be repeated, greater attention should be paid to ensuring sterility to avoid contamination with undesired species such as the *Bacillus* spp. observed in some of the inoculated specimens. Additionally, blood glucose levels should be monitored throughout, and the effect of varying glucose levels could be investigated.

4.5. References

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Chapter 5

Conclusions and Future Work

Blood ethanol analysis is a very controversial topic in the South African court setting at present. The accurate determination of blood alcohol content is imperative if a fair verdict is to be reached and information on the state of samples at various stages of storage is exceptionally valuable, since blood specimens are not necessarily analysed immediately. It is particularly vital for individual testing laboratories to be able to attest to the stability of ethanol content over time – for at least the intended storage period experienced under their laboratory-specific conditions.

In this study, a Gas Chromatography – Mass Spectrometry (GC-MS) method for the quantitation of ethanol in human whole blood was developed and validated. The method requires no dedicated equipment as it can be carried out on a GC-MS instrument commonly used in routine analysis in the toxicology setting. In developing the most viable method, the supplied GC-MS method was adjusted to minimise the degradation of the GC column due to excess amounts of pentafluorobenzoic acid present in the final sample solution. The adjusted method exhibited excellent linearity and specificity, low limits of detection and quantitation, and sufficiently small overall precisions at the three control levels.

Following this, the measurement uncertainty for the method was determined, taking into account the contributions of the certified reference material, sample dilution, and method bias and imprecision. At the three internal control concentrations of 0.02 g/100 mL, 0.05 g/100 mL and 0.30 g/100 mL, the 99% confidence expanded MU was found to be 35%, 28% and 23% respectively. As such, the prosecution levels at the two South African legal limits were determined to be 0.027 g/100mL for professional drivers and 0.064 g/100mL for public drivers. This novel GC-MS method was thus determined to be fit-for-purpose for the quantitation of ethanol in whole human blood. It is able to distinguish between the different states of drunkenness as laid out in Garriott[1], as well as between the two South African cut-off levels.

Since all blood ethanol concentration results need to be accompanied by the specimen fluoride concentration, a method for the quantitation of free fluoride in human whole blood by means of the Fluoride Ion Selective Electrode (FISE) was developed and validated. It was found that a 20-fold dilution of blood specimens with deionised water and TISAB II with CDTA sufficiently suppresses matrix effects to the extent that calibration may be carried out in aqueous medium, provided that blood specimens and calibrators are prepared in the same manner.

This method for the determination of blood fluoride concentration displayed exceptional linearity, repeatability and precision, with overall precision values of 1.11%, 1.10% and 3.37% for the 0.300 g/100 mL, 1.00 g/100 mL and 2.75 g/100 mL concentrations respectively. Following the same

process for calculating the measurement uncertainty as for the GC-MS method, the expanded MU at 99% confidence was determined to be 14.5% at the 1.00 g/100 mL concentration level. It was interesting to note that at the lower concentrations the expanded MU was unacceptably large; however, it decreased as the fluoride concentration increased, and became more or less uniform at the higher concentrations.

The fluoride method was then used to study the effects of complexation and temperature on free fluoride concentration. It was found that Fe^{3+} at concentrations similar to those seen in human whole blood does severely affect the concentration of free fluoride due to complexation effects. These can, however, be eliminated by sufficient dilution and the addition of TISAB II, which contains a decomplexation reagent.

Considering that most substances are less soluble at lower temperatures, it would stand to reason that sodium fluoride (NaF) is also less soluble in specimens stored at the low temperatures of refrigeration. That is, in specimens stored under refrigeration, the effective free fluoride concentration will be lower. It was found that for solutions not spiked with Fe^{3+} the fluoride concentration was not affected by the lowered temperature. This was not the case for those containing Fe^{3+}, which is more realistic due to the iron content of blood. Those specimens spiked with Fe^{3+}, refrigerated, and filtered to remove any compounds not in solution were found to have a severely lowered fluoride concentration, especially below 1.00 g/100 mL, irrespective of whether TISAB II was added or not. Since similarly prepared solutions kept at room temperature did not show a decrease in fluoride concentration, it can be concluded that temperature does indeed impact the extent of fluoride complexation.

Replacing the Fe^{3+} with Mg^{2+} or Ca^{2+}, which are the ions found at the next highest concentrations in whole blood, had no noticeable effect on the free fluoride concentration. This is likely to be due to the low concentrations of these ions.

Since a typical blood specimen will contain Fe^{3+}, and is likely to be stored under refrigeration, it is probable that the Fe^{3+} will complex with the fluoride, lowering the effective free fluoride concentration. Although the addition of TISAB II does minimise this effect by decomplexing the fluoride before analysis, this fluoride concentration measured by the FISE may not be a true reflection of the effective fluoride concentration – that is, the fluoride available to preserve the specimen. The concentration of this fluoride is required to be 1.00 g/100 mL, but if the fluoride is complexed by iron and other free ions present in the blood matrix, the possibility exists that the effective free fluoride concentration is in actual fact *lower* than the specimen is initially spiked at, and hence lower than the decomplexed concentration determined by this method. There is some comfort in the fact that since blood specimen collection tubes are rarely filled to capacity, the initial fluoride concentration of most blood specimens is usually much greater than the required 1.00 g/100 mL.

Having developed the necessary tools, it was then possible to investigate the effect of various storage conditions on the stability of ethanol concentration in blood specimens. It was found that in sterile specimens stored at 4 ± 3 °C the ethanol concentration was statistically stable at 99% confidence for at least 29 weeks, while those specimens initially spiked at 0.02 g/100 mL and 0.05 g/100 mL, and stored at 22 ± 6 °C, exhibited statistically decreased ethanol concentrations after 11 and 12 weeks respectively. The results from the specimens stored at 4 ± 3 °C compared favourably with those obtained from the Johannesburg Department of Health, showing the comparability of the novel GC-MS method and the commonly used Head Space-Gas Chromatography-Flame Ionisation Detection (HS-GC-FID) method. These results allowed the recommendation that for the accurate quantitation of ethanol in whole blood specimens, the specimens should be stored at 4 °C with at least 1.00 g/100 mL NaF. In addition, the defence of "incorrect storage temperature" causing neoformation of ethanol and thereby resulting in an elevated BAC result can no longer be claimed. While storage of blood specimens may result in the determined ethanol content being found to be different to that when the specimen was taken, the ethanol concentration will be lowered, which is to the benefit of the defendant.

Another claim often made is that the contamination of blood specimens with some yeast or other such micro-organism, such as *C. albicans*, may artificially raise the ethanol content. Specimens were prepared at 0.02 g/100 mL and 0.05 g/100 mL ethanol and inoculated with various levels of *C. albicans*. Those specimens stored at 4 ± 3 °C, both with and without NaF, exhibited statistically stable ethanol concentrations at 99% confidence. The specimens stored at 22 ± 6 °C, however, mimicked the results seen for the sterile specimens, albeit at a greater rate. That is, the ethanol concentrations of specimens "contaminated" with *C. albicans* and stored at room temperature were significantly decreased in a shorter period of time than that of the sterile specimens. It was noted that although the NaF does play a role in stabilising the ethanol concentration, the temperature at which specimens are stored has a greater impact.

From the above results, it is strongly recommended that, for the accurate quantitation of ethanol content, blood specimens be stored at approximately 4 °C and, if at all possible, with 1 g/100 mL NaF. In addition, the above results suggest that the neoformation of ethanol in specimens stored at higher temperatures, or in specimens contaminated by *C. albicans*, is highly unlikely. As such, the related court defence for elevated BAC results is rendered moot and unrealistic.

There is clearly still much work that needs to be done on this topic.

The decrease in ethanol concentration observed in the sterile specimens needs to be investigated fully and its cause determined, whether it be due to enzymatic oxidation of ethanol to acetaldehyde, or due to some other cause. Additionally, it could be useful to obtain information on the stability of ethanol concentrations in specimens subjected to other temperature ranges, for example one or more

freeze-thaw cycles, or substantially raised temperatures such as would be achieved in a motor vehicle.

There are also various other micro-organisms that have been known to produce ethanol from glucose. These include *Proteus mirabilis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*.[2-7] A similar inoculation storage study as was performed with the *Candida albicans* could be carried out using each of these species. Should this be done, specific attention should be paid to working aseptically to avoid specimen contamination with other species. Additionally, the glucose concentration of all specimens should be monitored throughout the study, and the effect of varying glucose levels could be investigated.

Of course, each study could also be repeated for longer periods of time to gain information on ethanol stability during even longer term storage.

Finally, a full ruggedness study should be performed for the fluoride method, and it could be useful to repeat the temperature studies making used of Ca^{2+} and Mg^{2+} to determine the extent of complexation of fluoride with these ions at lower temperatures.

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Appendix A

Chromatograms and Mass Spectra

Throughout the study, Total Ion Chromatograms (TICs) with peaks for derivatised ethanol and derivatised ethanol-d6 were obtained. The extracted ion chromatograms (XICs) and mass spectra in each case were nearly identical, only varying slightly in retention times and chromatographic quality. The TIC shown in Figure 3 of Chapter 2 and the XIC and mass spectrum given in Figure 2 or Chapter 2 are representative of the general data obtained. Hence, what follows are only some pertinent chromatograms and mass spectra.

Stability of Derivatised Analyte

The bias-corrected ethanol concentrations obtained for the repeated injection of a medium level internal quality control sample over a period of 16 hours, during which the sample stood on the autosampler tray, were found not to differ statistically. The only variation observed over the 16 hour period was a deterioration in chromatographic quality. This is seen in *Figure 1*, *Figure 2* and *Figure 3*.

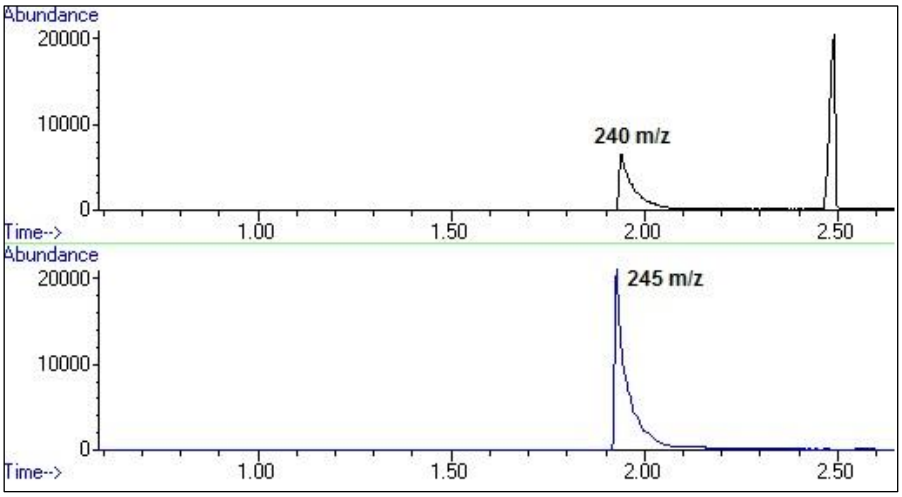


Figure 1: XICs for derivatised ethanol and ethanol-d6 for the medium level internal quality control sample immediately after being placed on the autosampler tray.

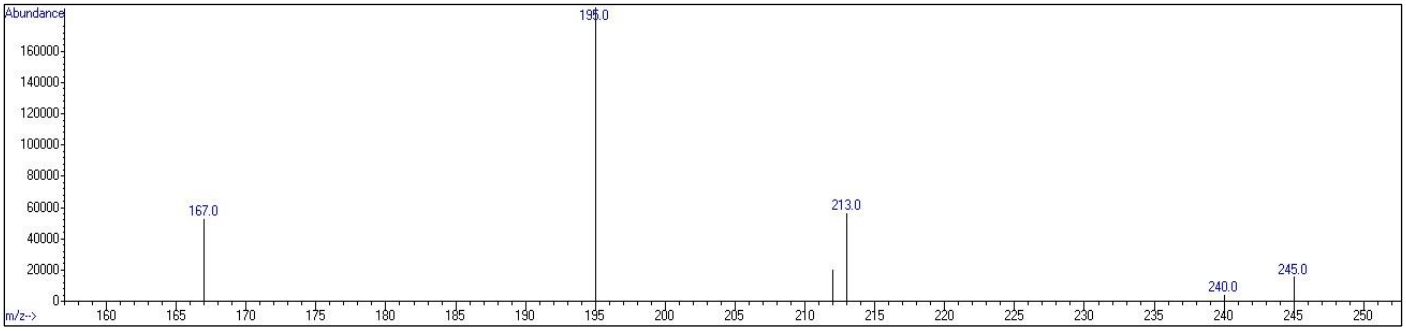


Figure 2: Mass spectrum for derivatised ethanol and ethanol-d6 for the medium level internal quality control sample immediately after being placed on the autosampler tray

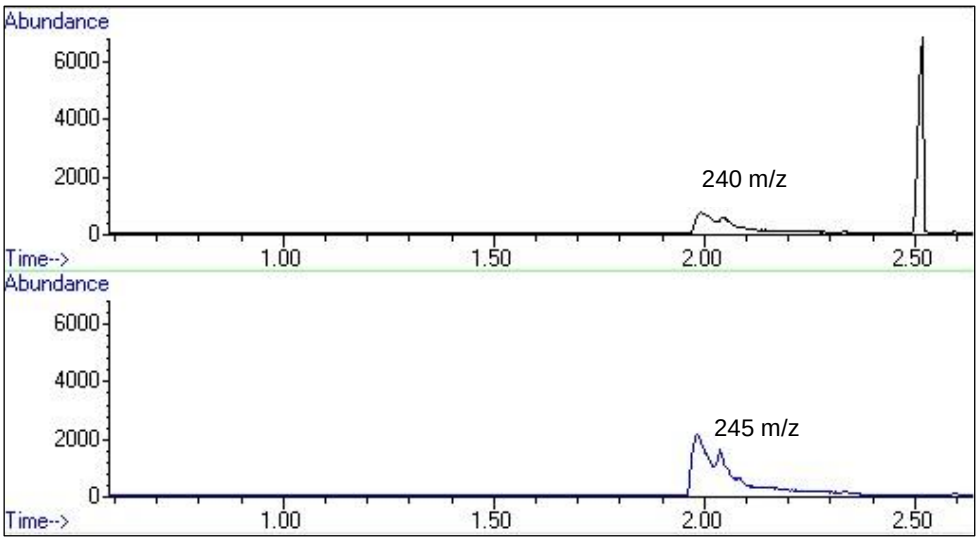
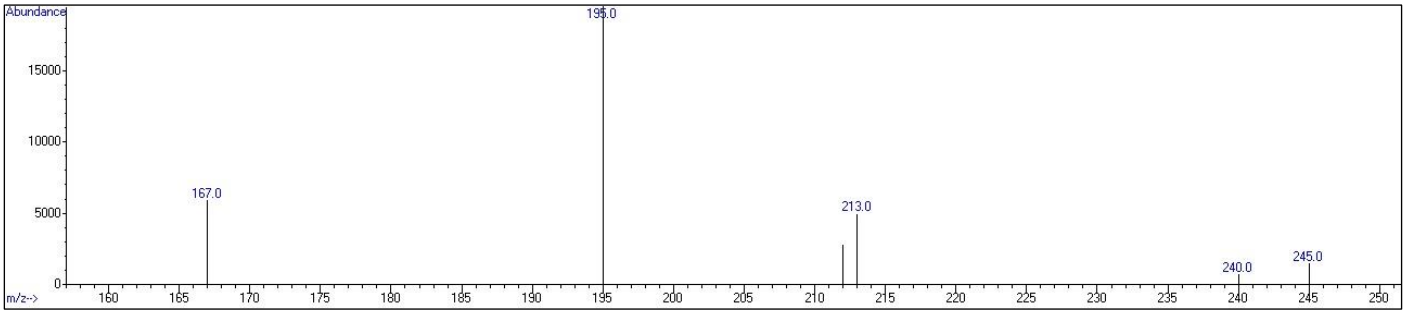


Figure 3: XICs for derivatised ethanol and ethanol-d6 for the medium level internal quality control sample after 8 hours on the autosampler tray



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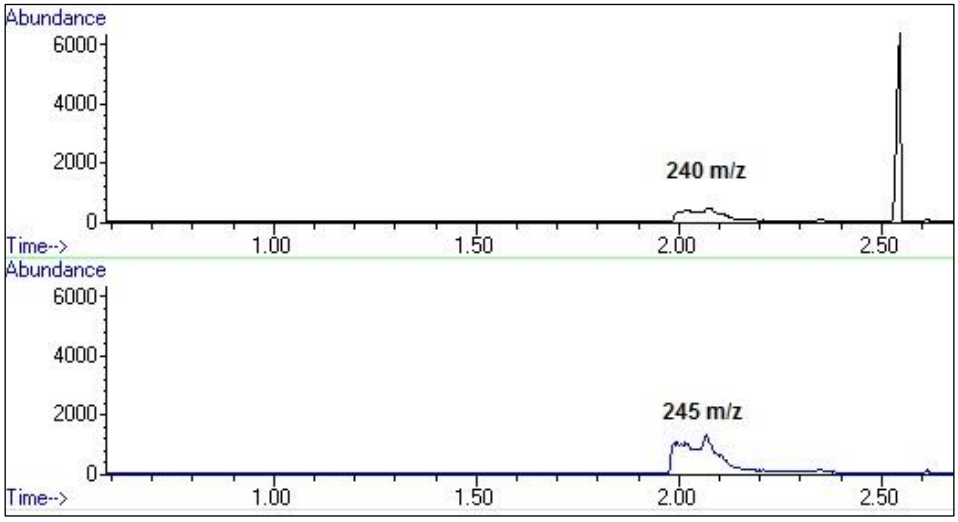


Figure 5: XICs for derivatised ethanol and ethanol-d6 for the medium level internal quality control sample after 16 hours on the autosampler tray.

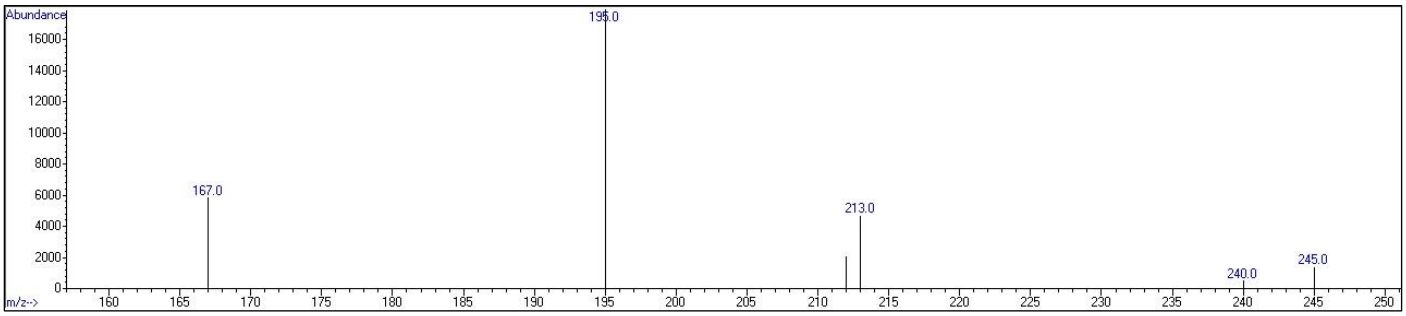


Figure 6: Mass spectrum for derivatised ethanol and ethanol-d6 for the medium level internal quality control sample after 16 hours on the autosampler tray

Storage Studies

Once again, in all the storage studies performed, the TICs, XICs and mass spectra all exhibited the same type of data, just with varying quantities of ethanol. An example of this is given below in *Figure 6*, *Figure 7* and *Figure 8.* The XICs of the specimens initially spiked at 0.02 g/100 ml ethanol and stored at 22 ±6°C for one, 20 and 29 weeks are shown. There is a noticeable decrease in abundance in the 240 m/z peak relative to the 245 m/z peak. This was observed in each study where the ethanol concentration decreased.

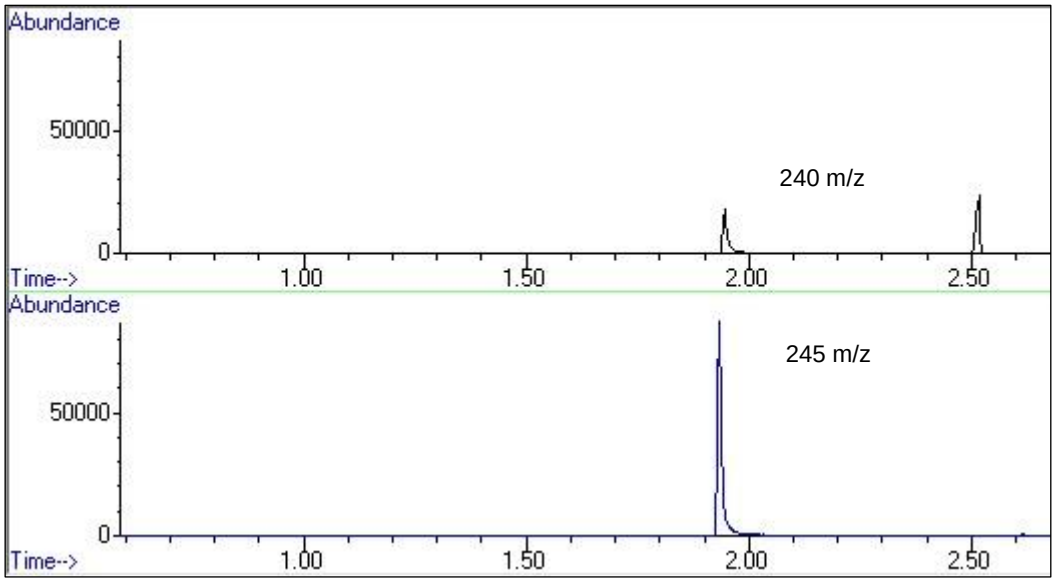


Figure 7: XICs for a specimen initially spiked with 0.02 g/100 ml ethanol and stored for 1 week at 22 ± 6°C

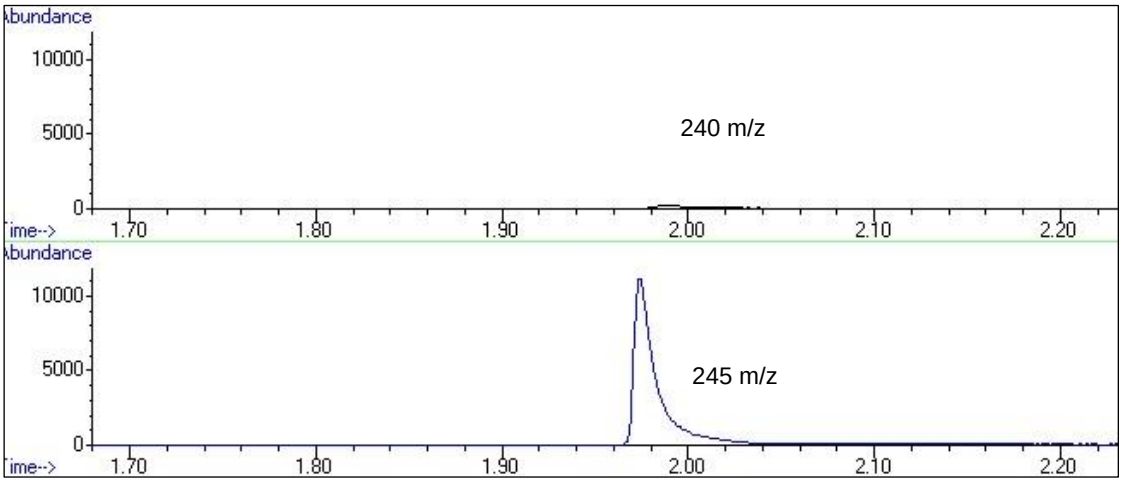
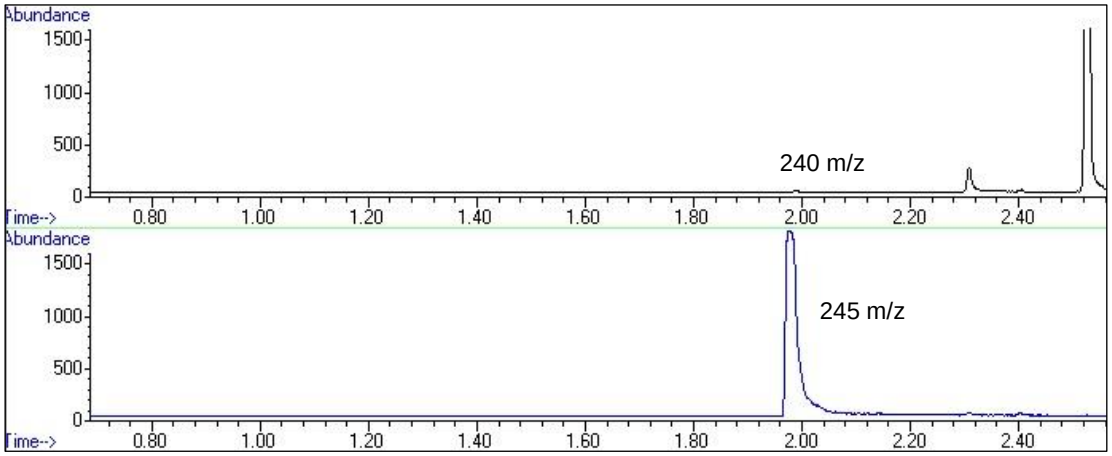


Figure 8: XICs for a specimen initially spiked with 0.02 g/100 ml ethanol and stored for 20 weeks at 22 ± 6°C



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Appendix B

Pipette Calibration Uncertainty

Chapter 2:

Ethanol

Table 1: Pipette internal volume and percentage error for the 20 – 200 µl pipette

Volume (µl)	CV (%)
20	0.26
100	0.03
200	0.06

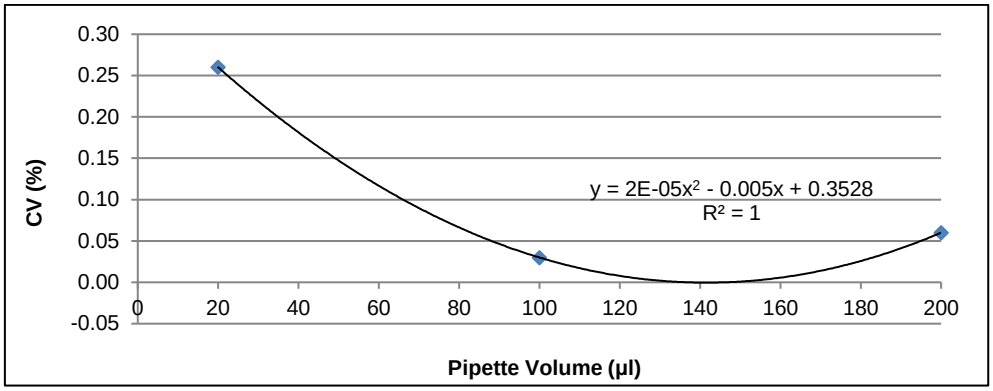


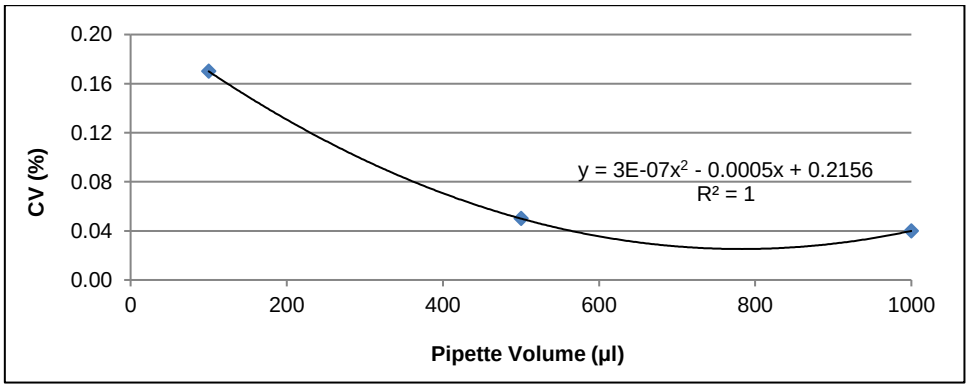
Figure 1: Pipette internal volume versus percentage error for the 20 – 200 µl pipette

Chapter 3:

Fluoride

Table 2: Pipette internal volume and percentage error for the 100 – 1000 µl pipette

Volume (µl)	CV (%)
100	0.17
500	0.05
1000	0.04



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C.1. Chapter 2: Ethanol

C.3.1. Method Validation

Bias and Precision

Table 1: Experimental ethanol concentrations obtained over five days and used in calculations of bias and precision

	LC	MC	HC
Day 1	0.023474	0.052443	0.311924
	0.024247	0.052817	0.30374
	0.021975	0.055826	0.34769
	0.023186	0.056755	0.30236
	0.022476	0.054594	0.34310
Day 2	0.023775	0.055795	0.33084
	0.022180	0.059281	0.33826
	0.023950	0.052892	0.31578
	0.023873	0.055929	0.31304
	0.021938	0.052728	0.27968
Day 3	0.018386	0.045927	0.26115
	0.021421	0.044700	0.24960
	0.021524	0.048511	0.26998
	0.019256	0.044811	0.26400
	0.019756	0.046071	0.26018
Day 4	0.018257	0.054754	0.27328
	0.019495	0.056003	0.27544
	0.018023	0.054235	0.28875
	0.013367*	0.054084	0.28919
	0.019497	0.083435*	0.28272
Day 5	0.021579	0.047310	0.29598
	0.021582	0.045956	0.28483
	0.022288	0.044338	0.29744
	0.022517	0.045475	0.30230
	0.038250	0.045973	0.41583*

* Outlier according to Grubbs' Test for outliers.

Table 2: ANOVA calculation results for the low level ethanol internal quality controls (0.02 g/100ml) used in the pre-validation

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.58277E-05	4	1.64569E-05	17.2367	5.68362E-06	2.92774
Within Groups	1.71857E-05	18	9.54762E-07			

Table 3: ANOVA calculation results for the medium level ethanol internal controls (0.05 g/100ml) used in the pre-validation

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000470165	4	0.000117541	37.80200789	8.48788E-09	2.895107308
Within Groups	5.90785E-05	19	3.10939E-06			

Table 4: ANOVA calculation results for the high level ethanol internal controls (0.30 g/100ml) used in the pre-validation

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.012332266	3	0.004110755	14.93124436	6.72521E-05	3.238871517
Within Groups	0.004404997	16	0.000275312			

Ruggedness

Table 5: Ethanol concentrations obtained for two sets of medium internal quality control samples, where one set was dried completely and one set was not

	Theoretical Concentration (g/100ml)	**Replicate 1**	**Replicate 2**	**Replicate 3**
Dry	0.05	0.052260508	0.051317381	0.052664826
Not Dry	0.05	0.049756121	0.05145655	0.049613249

Selectivity

Table 6: Abundance ratios for the identification of ethanol

240/212	**240/195**	**240/167**	**212/195**	**212/167**	**195/167**
0.207048	0.051259	0.197407	0.247569	0.953434	3.851179
0.215947	0.077311	0.183747	0.358007	0.850888	2.376736
0.220089	0.064886	0.205442	0.294816	0.933451	3.166220
0.212582	0.067434	0.186654	0.317215	0.878030	2.767936
0.216429	0.061169	0.170669	0.282628	0.788568	2.790132
0.216408	0.059452	0.196407	0.274723	0.907578	3.303610
0.215753	0.061334	0.185266	0.284278	0.858695	3.020619
0.207958	0.065674	0.177905	0.315803	0.855484	2.708918
0.190608	0.055995	0.167945	0.293770	0.881100	2.999286
0.222509	0.066035	0.192860	0.296775	0.866749	2.920562
0.216214	0.069540	0.203887	0.321624	0.942987	2.931952
0.210846	0.068692	0.199481	0.325790	0.946099	2.904017
0.198449	0.065589	0.178051	0.330510	0.897212	2.714629
0.197099	0.061303	0.176952	0.311029	0.897783	2.886496
0.245606	0.062148	0.192337	0.253039	0.783110	3.094822
0.203523	0.049511	0.152617	0.243271	0.749875	3.082475
0.227443	0.079853	0.241909	0.351091	1.063602	3.029415
0.240807	0.081340	0.248093	0.337779	1.030259	3.050095
0.250479	0.085048	0.251774	0.339541	1.005168	2.960375
0.226413	0.079711	0.250544	0.352061	1.106579	3.143146
0.232891	0.082862	0.252255	0.355797	1.083145	3.044273
0.234687	0.084594	0.260158	0.360457	1.108533	3.075357
0.236269	0.083445	0.247220	0.353178	1.046349	2.962668
0.232698	0.084968	0.262394	0.365144	1.127615	3.088139
0.219371	0.076656	0.232846	0.349436	1.061427	3.037546
0.229043	0.067594	0.234084	0.295114	1.022010	3.463102
0.237602	0.082976	0.258132	0.349223	1.086406	3.110925

0.224399	0.081315	0.240299	0.362368	1.070855	2.955159
0.227557	0.076700	0.217314	0.337060	0.954989	2.833294
0.203191	0.057211	0.160111	0.281564	0.787983	2.798589
0.232653	0.085244	0.260887	0.366401	1.121359	3.060470
0.234156	0.060531	0.173899	0.258506	0.742665	2.872908
0.212829	0.068234	0.210161	0.320606	0.987466	3.080003
0.235663	0.073602	0.228490	0.312320	0.969560	3.104383
0.242779	0.079204	0.238795	0.326239	0.983591	3.014938
0.069508*	0.022643*	0.069977*	0.325758*	1.006744*	3.090469*
0.224204	0.074573	0.229967	0.332610	1.025702	3.083795
0.231207	0.074242	0.243371	0.321107	1.052613	3.278076
0.236448	0.080920	0.240331	0.342232	1.016425	2.969991
0.226324	0.073751	0.219168	0.325863	0.968379	2.971731
0.220731	0.074904	0.218687	0.339347	0.990739	2.919549
0.221141	0.074091	0.240605	0.335039	1.088013	3.247419
0.212288	0.046099	0.159551	0.217154	0.751581	3.461059
0.190852	0.038406	0.137086	0.201233	0.718282	3.569399
0.179257	0.055420	0.166918	0.309165	0.931164	3.011872
0.197633	0.047232	0.161741	0.238991	0.818392	3.424363
0.197164	0.069375	0.234170	0.351866	1.187690	3.375403
0.206486	0.072921	0.252017	0.353154	1.220506	3.456018
0.205574	0.073253	0.236959	0.356337	1.152671	3.234782
0.196080	0.068812	0.225823	0.350941	1.151687	3.281712
0.197336	0.072011	0.240207	0.364918	1.217253	3.335686
0.202613	0.073320	0.237276	0.361870	1.171078	3.236185
0.204719	0.067121	0.241536	0.327870	1.179842	3.598504
0.202909	0.072920	0.250555	0.359374	1.234813	3.436010
0.207604	0.077934	0.255727	0.375398	1.231800	3.281321
0.213475	0.068885	0.231537	0.322681	1.084605	3.361227
0.196509	0.037968	0.129563	0.193211	0.659326	3.412466
0.226529	0.039598	0.161793	0.174801	0.714226	4.085942
0.240270	0.056670	0.189782	0.235860	0.789869	3.348890
0.245341	0.057695	0.196118	0.235163	0.799371	3.399222
0.261366	0.079559	0.315845	0.304395	1.208439	3.969969
0.250606	0.079178	0.263741	0.315945	1.052413	3.331000
0.248762	0.074201	0.252648	0.298280	1.015622	3.404929
0.259861	0.079164	0.277847	0.304642	1.069216	3.509747
0.261134	0.083926	0.287768	0.321390	1.101996	3.428849
0.256220	0.082882	0.291154	0.323481	1.136343	3.512862
0.242109	0.080707	0.273300	0.333349	1.128832	3.386341
0.254194	0.082818	0.280170	0.325808	1.102190	3.382948
0.251610	0.079168	0.295832	0.314645	1.175758	3.736780
0.260113	0.088231	0.303561	0.339203	1.167037	3.440524

* Outlier according to Grubbs' Test for outliers.

C.3.2. Measurement Uncertainty

Sample Dilution Uncertainty Contribution

Table 7: Masses for ten volumes of 50 µl and 450 µl, with the standard deviation of each set

Replicate	Mass for 50 µl (mg)	Mass for 450 µl (mg)
1	49.98	449.95
2	49.78	450.22
3	49.99	448.52
4	50.04	447.90
5	49.78	449.62
6	49.99	448.20
7	49.92	448.61
8	49.96	447.98
9	49.92	449.55
10	49.52	449.18
Standard Deviation	**0.16**	**0.84**

Table 8: Seventy-seven experimentally obtained ethanol concentrations at the three internal quality control levels, recorded over a period of 13 months used in the measurement uncertainty calculations

	Low Control	Medium Control	High Control
Theoretical Concentration (g/100ml)	**0.02**	**0.05**	**0.30**
1	0.02311	0.05413	0.32571
2	0.02333	0.05371	0.32273
3	0.02298	0.05434	0.32331
4	0.02391	0.05433	0.32565
5	0.02034	0.04454	0.26258
6	0.02039	0.04661	0.26508
7	0.01888	0.04600	0.28232
8	0.01876	0.05857	0.28574
9	0.02559	0.05966	0.35312
10	0.02613	0.05424	0.35663
11	0.02522	0.04476	0.35892
12	0.01999	0.04733	0.26645
13	0.02653	0.05538	0.28514
14	0.02158	0.05416	0.29041
15	0.02240	0.04294	0.29987
16	0.01536	0.04978	0.31768
17	0.01508	0.04515	0.26621
18	0.01560	0.04572	0.28606
19	0.02075	0.05611	0.33546
20	0.02201	0.04720	0.35555
21	0.02298	0.05532	0.26073

22	0.02482	0.05540	0.29021
23	0.02538	0.05653	0.29960
24	0.02002	0.05763	0.32715
25	0.01938	0.04226	0.32720
26	0.02342	0.04175	0.29349
27	0.02515	0.05375	0.28481
28	0.02134	0.05117	0.30162
29	0.01857	0.05078	0.30333
30	0.01874	0.04775	0.33403
31	0.01871	0.05197	0.34233
32	0.01981	0.05291	0.31360
33	0.01907	0.04876	0.27573
34	0.01713	0.04751	0.30892
35	0.01768	0.05122	0.31874
36	0.01951	0.05058	0.29589
37	0.02224	0.05049	0.28825
38	0.02086	0.06132	0.33225
39	0.02264	0.05859	0.36368
40	0.02300	0.05107	0.34925
41	0.02033	0.04783	0.30628
42	0.02020	0.04841	0.30921
43	0.02368	0.04860	0.32088
44	0.02304	0.05775	0.31330
45	0.01995	0.05504	0.31293
46	0.02011	0.05333	0.33747
47	0.01977	0.04804	0.30095
48	0.02502	0.04266	0.30481
49	0.02014	0.06435	0.29228
50	0.01902	0.04398	0.31292
51	0.02117	0.04307	0.31374
52	0.02104	0.05331	0.33335
53	0.02493	0.05293	0.32408
54	0.02140	0.04925	0.33792
55	0.02148	0.05000	0.34683
56	0.02404	0.05659	0.30996
57	0.02354	0.06323	0.35342
58	0.01952	0.06185	0.37138
59	0.02029	0.05861	0.32926
60	0.02020	0.05467	0.29517
61	0.02284	0.05078	0.32194
62	0.02541	0.05187	0.32550
63	0.02673	0.04325	0.33977
64	0.02051	0.05780	0.27477
65	0.02245	0.06161	0.30718

66	0.02307	0.06167	0.33203
67	0.02189	0.05250	0.31136
68	0.01674	0.05377	0.29988
69	0.01887	0.05724	0.31170
70	0.02488	0.05577	0.30090
71	0.01915	0.05015	0.27691
72	0.01945	0.05239	0.26834
73	0.02041	0.05301	0.28773
74	0.01836	0.04982	0.34000
75	0.02075	0.05003	0.32857
76	0.02065	0.04958	0.29182
77	0.02359	0.05369	0.26767
Average	**0.02134**	**0.05208**	**0.31153**
Standard Deviation	**0.00264**	**0.00531**	**0.02657**

Procedural Uncertainty Contribution: Imprecision

Method 1

Table 9: Differences between replicate concentration values for the 77 ethanol internal quality control concentrations used in the calculation of u_{imp} by Gullberg's method

	Δ Low Control	**Δ Medium Control**	**Δ High Control**
1	0.002272	-0.003383	-0.043948
2	0.000288	-0.001777	-0.040743
3	0.001596	0.002902	0.015058
4	0.000078	0.003200	0.025223
5	0.002166	0.008211	-0.002852
6	-0.002268	-0.003811	0.009800
7	-0.001238	0.000144	0.013747
8	-0.001474	0.000949	0.006033
9	-0.006180	0.005315	-0.014286
10	-0.003946	-0.004113	0.002149
11	-0.005419	0.000208	0.061109
12	0.000853	-0.000024	-0.009849
13	0.012412	-0.001249	0.012588
14	-0.000003	0.000151	0.011150
15	-0.000229	0.054046	-0.004859
16	0.000741	-0.004932	0.122495
17	0.000695	0.001618	0.016910
18	-0.000856	-0.000498	-0.038721
19	0.001088	-0.000951	-0.019114
20	0.002711	-0.000462	-0.042895
21	0.000228	0.002859	-0.016979
22	0.001793	0.003723	-0.032167
23	0.001318	-0.000208	-0.022710

24	0.000913	-0.004290	0.036399
25	0.001250	0.000922	0.022119
26	0.000144	0.000363	-0.065284
27	0.001463	0.002811	-0.001780
28	0.003466	-0.002490	-0.012664
29	0.003514	0.003866	-0.014578
30	-0.000003	0.000759	-0.058765
31	0.001288	0.001997	-0.021511
32	-0.000199	-0.004945	0.014734
33	-0.000719	-0.003458	0.002930
34	-0.002958	-0.004958	-0.003337
35	0.003242	0.000398	-0.002097
36	-0.002865	-0.000260	-0.002073
37	0.000718	0.001146	-0.000167
38	0.000581	0.001171	0.005682
39	-0.002158	0.000192	-0.001537
40	-0.001122	-0.000694	-0.004195
41	0.000310	-0.006430	-0.032685
42	0.000256	0.000490	-0.033675
43	0.000756	-0.001337	-0.015572
44	-0.001369	-0.000373	0.006583
45	-0.001041	0.003909	-0.023006
46	-0.002862	-0.004314	-0.032597
47	-0.003666	0.000095	-0.003895
48	-0.003246	0.002798	0.007670
49	-0.001560	-0.014348	-0.034969
50	-0.003355	0.000180	-0.040188
51	-0.000965	0.000277	-0.004959
52	0.001656	0.001314	-0.001914
53	-0.003614	0.000480	-0.030998
54	0.006678	0.006329	0.001307
55	-0.003953	0.002528	0.018648
56	-0.006416	-0.000205	-0.105665
57	0.000069	-0.002001	0.021953
58	-0.002050	-0.002347	-0.029422
59	0.000180	-0.000087	-0.040760
60	0.001509	-0.005373	-0.005439
61	0.000459	-0.000615	-0.002612
62	-0.001494	-0.000039	-0.052767
63	-0.000620	-0.002516	0.012562
64	0.001237	-0.000733	-0.091437
65	-0.002311	-0.003274	-0.001523
66	-0.001041	-0.003595	0.010033
67	-0.003250	-0.003963	0.005572

68	0.003150	0.001930	-0.015358
69	-0.002863	-0.001156	-0.006166
70	0.001609	-0.001160	-0.045251
71	0.000662	-0.002380	0.007898
72	0.001696	-0.004358	-0.003059
73	-0.002284	-0.002019	-0.006034
74	-0.002532	-0.000771	-0.008818
75	0.000497	-0.002206	-0.018038
76	0.000737	-0.000625	-0.000767
77	-0.000023	-0.006822	-0.023223
Sum of squares	**0.000572**	**0.003764**	**0.077981**
SD_G	**0.001927**	**0.004944**	**0.022503**

Method 2

Table 10: Relative concentration values for the three sets of 77 experimentally obtained ethanol quality control concentrations

	Low Control Relative Concentration	Medium Control Relative Concentration	High Control Relative Concentration
Average Concentration	**0.02134**	**0.05208**	**0.31153**
1	1.08310	1.03954	1.04553
2	1.09336	1.03131	1.03596
3	1.07686	1.04355	1.03782
4	1.12064	1.04327	1.04533
5	0.95318	0.85531	0.84287
6	0.95559	0.89496	0.85091
7	0.88465	0.88332	0.90623
8	0.87920	1.12479	0.91721
9	1.19920	1.14561	1.13350
10	1.22457	1.04165	1.14476
11	1.18208	0.85953	1.15212
12	0.93681	0.90884	0.85530
13	1.24312	1.06343	0.91530
14	1.01140	1.04002	0.93221
15	1.04992	0.82450	0.96258
16	0.72005	0.95584	1.01975
17	0.70659	0.86696	0.85454
18	0.73089	0.87804	0.91825
19	0.97255	1.07738	1.07683
20	1.03148	0.90630	1.14131
21	1.07715	1.06230	0.83692
22	1.16306	1.06387	0.93156
23	1.18948	1.08554	0.96170
24	0.93804	1.10669	1.05015
25	0.90837	0.81153	1.05031

26	1.09756	0.80170	0.94211
27	1.17848	1.03212	0.91423
28	1.00014	0.98256	0.96820
29	0.87017	0.97512	0.97369
30	0.87821	0.91697	1.07225
31	0.87688	0.99797	1.09888
32	0.92833	1.01597	1.00665
33	0.89367	0.93630	0.88510
34	0.80291	0.91233	0.99163
35	0.82841	0.98360	1.02314
36	0.91455	0.97124	0.94982
37	1.04239	0.96957	0.92529
38	0.97754	1.17747	1.06650
39	1.06085	1.12516	1.16740
40	1.07777	0.98079	1.12108
41	0.95270	0.91842	0.98317
42	0.94664	0.92957	0.99257
43	1.10961	0.93317	1.03002
44	1.07985	1.10897	1.00569
45	0.93476	1.05697	1.00449
46	0.94228	1.02405	1.08326
47	0.92657	0.92258	0.96604
48	1.17280	0.81913	0.97844
49	0.94395	1.23569	0.93822
50	0.89116	0.84447	1.00448
51	0.99230	0.82706	1.00711
52	0.98620	1.02377	1.07006
53	1.16847	1.01638	1.04029
54	1.00293	0.94582	1.08473
55	1.00687	0.96020	1.11331
56	1.12667	1.08663	0.99498
57	1.10341	1.21429	1.13446
58	0.91501	1.18764	1.19211
59	0.95082	1.12547	1.05693
60	0.94652	1.04985	0.94748
61	1.07027	0.97505	1.03341
62	1.19109	0.99603	1.04486
63	1.25260	0.83045	1.09066
64	0.96101	1.10998	0.88201
65	1.05233	1.18309	0.98604
66	1.08112	1.18416	1.06581
67	1.02571	1.00824	0.99947
68	0.78462	1.03251	0.96260
69	0.88417	1.09913	1.00055

70	1.16616	1.07092	0.96588
71	0.89768	0.96296	0.88888
72	0.91152	1.00595	0.86136
73	0.95642	1.01787	0.92361
74	0.86059	0.95677	1.09138
75	0.97233	0.96074	1.05471
76	0.96796	0.95214	0.93675
77	1.10572	1.03099	0.85921
Standard Deviation	**0.12352**	**0.10204**	**0.08528**

C.2. Chapter 3: Fluoride

C.3.1. Method Validation

Limits of Detection and Quantitation

Table 11: Average potential values (mV) obtained by FISE for ten duplicate blank samples

Duplicate	Potential (mV)
1	152.9
2	142.9
3	139.3
4	122.4
5	145.1
6	146.2
7	133.1
8	131.7
9	138.2
10	135.8
Average	**138.7**
Standard Deviation	**8.6**

Bias and Precision

Table 12: Experimental fluoride concentrations obtained over five days and used in calculations of bias and precision

	LC	MC	HC
Day 1	0.277772	1.001392	2.372608
	0.273526	0.993709	2.372608
	0.282084	1.013028	2.552713
	0.281000	0.997543	2.465756
	0.277772	0.997543	2.446839
Day 2	0.322968	0.950369	2.463643
	0.326711	0.939481	2.379934
	0.322968	0.918078	2.370810
	0.322968	0.914558	2.370810
	0.319268	0.914558	2.299070

	0.298082	0.960880	2.428343
	0.296932	0.975843	2.336325
Day 3	0.294647	0.983411	2.437742
	0.295788	0.994874	2.345368
	0.302723	0.991039	2.428343
	0.305845	0.983237	3.185310*
	0.300027	0.983237	2.659148
Day 4	0.303505	0.990820	2.689970
	0.302341	0.987022	2.608562
	0.302341	0.987022	2.628680
	0.310488	1.018944	2.233119
	0.308132	1.018944	2.207747
Day 5	0.308132	1.015070	2.190993
	0.308132	0.977134	2.284741
	0.301170	0.973419	2.216172

* Outlier according to Grubbs' Test for outliers.

Table 13: Absolute differences in fluoride concentration at the 0.300 g/100ml control level for the five days of validation

LC	Theoretical Concentration (g/100ml)	Δ Concentration 1 (g/100ml)	Δ Concentration 2 (g/100ml)	Δ Concentration 3 (g/100ml)	Δ Concentration 4 (g/100ml)	Δ Concentration 5 (g/100ml)
Day 1	0.302	0.024	0.028	0.020	0.021	0.024
Day 2	0.303	0.020	0.024	0.020	0.020	0.029
Day 3	0.305	0.007	0.008	0.010	0.009	0.002
Day 4	0.298	0.008	0.002	0.005	0.004	0.004
Day 5	0.298	0.013	0.010	0.010	0.010	0.003

Table 14: Absolute differences in fluoride concentration at the 1.00 g/100ml control level for the five days of validation

MC	Theoretical Concentration (g/100ml)	Δ Concentration 1 (g/100ml)	Δ Concentration 2 (g/100ml)	Δ Concentration 3 (g/100ml)	Δ Concentration 4 (g/100ml)	Δ Concentration 5 (g/100ml)
Day 1	0.999	0.002	0.005	0.014	0.001	0.001
Day 2	1.00	0.036	0.047	0.069	0.072	0.072
Day 3	1.01	0.044	0.029	0.022	0.010	0.014
Day 4	0.997	0.014	0.014	0.006	0.010	0.010
Day 5	1.00	0.019	0.019	0.015	0.023	0.026

Table 15: Absolute differences in fluoride concentration at the 2.75 g/100ml control level for the five days of validation

HC	Theoretical Concentration (g/100ml)	Δ Concentration 1 (g/100ml)	Δ Concentration 2 (g/100ml)	Δ Concentration 3 (g/100ml)	Δ Concentration 4 (g/100ml)	Δ Concentration 5 (g/100ml)
Day 1	2.751	0.378	0.378	0.198	0.285	0.304
Day 2	2.749	0.219	0.306	0.315	0.315	0.390
Day 3	2.759	0.331	0.423	0.321	0.321	0.331

Day 4	2.755		0.096	0.065	0.146	0.126
Day 5	2.753	0.519	0.545	0.561	0.468	0.536

Table 16: ANOVA calculation results for the low level fluoride internal quality controls (0.300 g/100ml)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001591	4	0.0003977	36.9610	5.6604E-09	2.86608
Within Groups	0.000215	20	0.0000108			

Table 17: ANOVA calculation results for the low level fluoride internal quality controls (1.00 g/100ml)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009012	4	0.0022529	21.7381	4.7738E-07	2.86608
Within Groups	0.002073	20	0.0001036			

Table 18: ANOVA calculation results for the low level fluoride internal quality controls (2.75 g/100ml)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.393984	4	0.0984960	34.8678	1.6594E-08	2.89511
Within Groups	0.053672	19	0.0028248			

C.3.2. Measurement Uncertainty

Sample Dilution Uncertainty Contribution

Table 19: Masses for ten volumes of 200 µl, 800 µl and 1000 µl, with the standard deviation of each set

Replicate	Mass for 200 µl (mg)	Mass for 800 µl (mg)	Mass for 1000 µl (mg)
1	200.19	799.74	1000.73
2	199.92	799.05	1000.50
3	199.46	799.64	1001.04
4	199.68	801.63	999.42
5	199.85	800.86	998.66
6	200.29	799.73	999.81
7	199.47	800.46	1001.62
8	200.27	799.62	1001.35
9	200.28	800.57	998.82
10	199.97	801.32	999.42
Standard Deviation	0.3227934	0.8370557	1.0551782

C.3.3. Method Applications

Complexation Studies

Iron

Table 20: Potential values for calibration solutions prepared without Fe^{3+}

	Concentration (g/100ml)	log[F]	Fr-No Fe TISAB Potential (mV)	Fr-No Fe No TISAB Potential (mV)	RT-No Fe TISAB Potential (mV)	RT-No Fe No TISAB Potential (mV)
Standard 1	0.250	-0.602	-19.2	-30.0	-18.6	-29.9
Standard 2	0.500	-0.301	-36.5	-46.6	-36.2	-46.6
Standard 3	1.00	0.000	-54.3	-63.5	-53.9	-63.8
Standard 4	1.50	0.176	-64.4	-73.1	-63.9	-73.0
Standard 5	2.00	0.301	-71.9	-80.1	-71.6	-80.1
Standard 6	2.50	0.398	-77.5	-85.3	-77.2	-85.4
Standard 7	3.00	0.477	-81.9	-89.5	-81.9	-89.4

Table 21: Potential values for calibration solutions prepared with Fe^{3+}

	Concentration (g/100ml)	log[F]	Fr-Fe TISAB Potential (mV)	Fr-Fe No TISAB Potential (mV)	RT-Fe TISAB Potential (mV)	RT-Fe No TISAB Potential (mV)
Standard 1	0.250	-0.602	-17.7	13.7	-18.2	11.8
Standard 2	0.500	-0.301	-33.3	-24.7	-35.6	-28.2
Standard 3	1.00	0.000	-35.0	-45.3	-53.3	-53.4
Standard 4	1.50	0.176	-52.5	-62.2	-60.7	-66.3
Standard 5	2.00	0.301	-63.4	-72.5	-69.1	-76.1
Standard 6	2.50	0.398	-71.1	-79.5	-75.0	-82.2
Standard 7	3.00	0.477	-76.6	-84.7	-80.4	-86.5

Magnesium

Table 22: Potential values for calibration solutions in the Mg^{2+} studies

	Concentration (g/100ml)	log[F]	No Mg-No TISAB	Mg-No TISAB	No Mg-TISAB	Mg-TISAB
Standard 1	0.250	-0.602	-27.3	-26.8	-17.3	-18.1
Standard 2	0.500	-0.301	-43.4	-38.6	-34.0	-34.6
Standard 3	1.00	0.000	-61.6	-61.3	-53.2	-53.5
Standard 4	1.50	0.176	-70.5	-70.3	-62.2	-62.4
Standard 5	2.00	0.301	-73.1*	-77.3	-70.2	-69.7
Standard 6	2.50	0.398	-82.9	-82.2	-75.5	-75.0
Standard 7	3.00	0.477	-87.1	-87.0	-79.8	-79.5

* Outlier according to Grubbs' Test for outliers.

Calcium

Table 23: Potential values for calibration solutions in the Ca^{2+} studies

	Concentration (g/100ml)	log[F]	No Ca-No TISAB	Ca-No TISAB	No Ca-TISAB	Ca-TISAB
Standard 1	0.250	-0.602	-27.3	-26.4	-17.3	-17.9
Standard 2	0.500	-0.301	-43.4	-42.6	-34.0	-34.6

Standard 3	1.00	0.000	-61.6	-60.9	-53.2	-53.6
Standard 4	1.50	0.176	-70.5	-69.9	-62.2	-62.8
Standard 5	2.00	0.301	-73.1*	-77.2	-70.2	-70.4
Standard 6	2.50	0.398	-82.9	-82.6	-75.5	-75.9
Standard 7	3.00	0.477	-87.1	-86.7	-79.8	-80.2

* Outlier according to Grubbs' Test for outliers.

Oxalate Investigation

Table 24: Potential and concentration values obtained for samples with and without KOx

Sample	Potential without Kox (mV)	Concentration without KOx (g/100 ml)	Potential with Kox (mV)	Concentration with KOx (g/100 ml)
1	-52.6	1.06	-52.6	1.06
2	-51.7	1.02	-52.9	1.07
3	-52.4	1.05	-52.6	1.06
4	-51.6	1.02	-52.8	1.07

C.3. Chapter 4: Storage Studies

C.3.1. Time and Temperature

In-house Study

Table 25: Experimental ethanol concentrations and bias corrected ethanol concentrations for the samples initially spiked at 0.02 g/100 ml (low), 0.05 g/100 ml (medium), and 0.30 g/100 ml (high) and stored at 4°C for 29 weeks

Week	Low Experimental Concentration (g/100 ml)	Low Corrected Experimental Concentration (g/100 ml)	Medium Experimental Concentration (g/100 ml)	Medium Corrected Experimental Concentration (g/100 ml)	High Experimental Concentration (g/100 ml)	High Corrected Experimental Concentration (g/100 ml)
Theoretical		**0.02**		**0.05**		**0.30**
0	0.0181	0.0171	0.0439	0.0419	0.259	0.249
1	0.0152	0.0143	0.0473	0.0452	0.265	0.255
2	0.0242	0.0229	0.0534	0.0511	0.332	0.320
3	ND	ND	ND	ND	ND	ND
4	0.0152	0.0142	0.0436	0.0417	0.332	0.320
5	0.0216	0.0204	0.0485	0.0464	0.275	0.265
6	0.0204	0.0193	0.0509	0.0487	0.327	0.315
7	0.0179	0.0169	0.0549	0.0525	0.286	0.275
8	0.0194	0.0183	0.0508	0.0486	0.327	0.315
9	0.0177	0.0166	0.0474	0.0453	0.289	0.279
10	0.0207	0.0196	0.0505	0.0483	0.310	0.298
11	0.0206	0.0194	0.0518	0.0496	0.310	0.299
12	0.0154	0.0144	0.0431	0.0412	0.304	0.293
13	0.0212	0.0200	0.0531	0.0508	0.358	0.345
14	0.0190	0.0179	0.0459	0.0439	0.280	0.270
15	0.0166	0.0156	0.0463	0.0442	0.370	0.356
16	0.0172	0.0162	0.0440	0.0420	0.372	0.358

17	0.0201	0.0190	0.0486	0.0465	0.331	0.318
18	0.0210	0.0198	0.0526	0.0503	0.332	0.319
19	0.0186	0.0175	0.0472	0.0451	0.266	0.257
20	0.0145	0.0136	0.0383	0.0365	0.280	0.269
21	0.0216	0.0204	0.0466	0.0445	0.281	0.270
22	0.0166	0.0156	0.0501	0.0479	0.302	0.291
23	0.0183	0.0173	0.0452	0.0431	0.314	0.302
24	ND	ND	ND	ND	ND	ND
25	0.0174	0.0164	0.0503	0.0481	0.303	0.292
26	0.0167	0.0157	0.0459	0.0438	0.291	0.281
27	0.0165	0.0155	0.0453	0.0433	0.262	0.252
28	0.0178	0.0168	0.0476	0.0455	0.283	0.273
29	0.0154	0.0144	0.0430	0.0411	0.265	0.256

ND: No Data

Table 26: Experimental ethanol concentrations and bias corrected ethanol concentrations for the samples initially spiked at 0.02 g/100 ml (low), 0.05 g/100 ml (medium), and 0.30 g/100 ml (high) and stored at 22°C for 29 weeks

Week	Low Experimental Concentration (g/100 ml)	Low Corrected Experimental Concentration (g/100 ml)	Medium Experimental Concentration (g/100 ml)	Medium Corrected Experimental Concentration (g/100 ml)	High Experimental Concentration (g/100 ml)	High Corrected Experimental Concentration (g/100 ml)
Theoretical		**0.02**		**0.05**		**0.30**
0	0.01812	0.01706	0.04386	0.04188	0.25908	0.24944
1	0.01613	0.01515	0.04271	0.04078	0.24453	0.23541
2	0.02188	0.02069	0.05517	0.05279	0.28259	0.27211
3	ND	ND	ND	ND	ND	ND
4	0.01534	0.01439	0.04105	0.03918	0.26718	0.25725
5	0.01971	0.01860	0.04642	0.04436	0.28031	0.26992
6	0.01523	0.01428	0.04293	0.04099	0.33294	0.32067
7	0.01978	0.01867	0.05400	0.05167	0.28650	0.27589
8	0.02148	0.02030	0.05076	0.04854	0.33132	0.31911
9	0.01524	0.01429	0.04485	0.04284	0.27243	0.26231
10	0.01481	0.01387	0.04645	0.04439	0.29659	0.28562
11	0.01358	0.01269	0.04886	0.04671	0.37419	0.36045
12	0.01166	0.01083	0.03398	0.03236	0.27210	0.26200
13	0.00961	0.00885	0.03940	0.03759	0.31556	0.30391
14	0.01176	0.01093	0.04029	0.03845	0.26372	0.25392
15	0.00833	0.00763	0.03724	0.03551	0.33900	0.32651
16	0.01122	0.01041	0.03239	0.03082	0.33443	0.32210
17	0.00817	0.00747	0.03870	0.03691	0.29012	0.27937
18	0.00768	0.00699	0.04277	0.04084	0.31054	0.29907
19	0.00940	0.00866	0.03178	0.03024	0.25569	0.24617
20	0.00340	0.00287	0.03097	0.02946	0.28722	0.27658
21	0.00410	0.00355	0.03572	0.03403	0.24752	0.23830
22	0.00432	0.00376	0.03783	0.03607	0.29881	0.28776

23	0.00102	0.00058	0.03604	0.03434	0.24959	0.24029
24	ND	ND	ND	ND	ND	ND
25	0.00000	0.00000	0.03958	0.03776	0.28607	0.27547
26	0.00129	0.00083	0.03317	0.03158	0.28944	0.27872
27	0.00148	0.00102	0.03338	0.03178	0.25980	0.25014
28	0.00000	0.00000	0.02737	0.02598	0.23883	0.22991
29	0.00055	0.00012	0.02183	0.02064	0.24322	0.23415

ND: No Data

Departments of Health Parallel Study

Table 27: Ethanol concentrations for specimens stored at and analysed by the Pretoria Department of Health (HS-GC-MS)

Week	L1 (g/100ml)	L2 (g/100ml)	L3 (g/100ml)
Theoretical	**0.02**	**0.05**	**0.30**
0	0.0200	0.0510	0.3290
1	0.0155	0.0445	0.2805
2	0.0180	0.0480	0.2920
3	0.0160	0.0450	0.2975
4	0.0160	0.0455	0.2890
5	0.0150	0.0430	0.2890
6	0.0160	0.0445	0.2985
7	0.0205	0.0560	0.3530
8	0.0205	0.0560	0.3560
9	0.0165	0.0490	0.3245
10	0.0160	0.0495	0.3145
11	0.0155	0.0435	0.2835
12	0.0150	0.0430	0.2835
13	0.0125	0.0405	0.2925
14	0.0155	0.0430	0.2850
15	0.0150	0.0470	0.3315

Table 28: Fluoride concentrations for specimens stored at and analysed by the Pretoria Department of Health

Week	L1 (g/100ml)	L2 (g/100ml)	L3 (g/100ml)
0	1.52	1.53	1.73
1	1.46	1.25	1.52
2	0.720	0.960	0.840
3	2.29	2.23	1.73
4	1.80	2.13	1.77
5	1.82	1.78	1.81
6	2.15	1.99	1.55
7	0.870	0.780	0.820
8	0.705	0.890	0.765
9	2.10	1.96	2.03
10	2.10	1.96	2.03
11	2.07	1.94	2.04
12	2.05	2.13	1.96
13	1.99	2.11	2.09
14	1.89	2.15	2.04
15	1.89	1.84	2.18

Table 29: Ethanol concentrations for specimens stored at and analysed by the Johannesburg Department of Health (HS-GC-MS)

Week	L1 (g/100ml)	L2 (g/100ml)	L3 (g/100ml)
Theoretical	0.02	0.05	0.3
0	0.0178	0.0451	0.2938
1	0.0179	0.0489	0.2989
2	0.0184	0.0495	0.3285
3	0.0169	0.0464	0.2793
4	0.0165	0.0453	0.2877
5	0.0168	0.0464	0.3089
6	0.0157	0.0453	0.2919
7	0.0133	0.0427	0.2788
8	0.0172	0.0431	0.2804
9	0.0157	0.0451	0.2893
10	0.0153	0.0446	0.2889
11	0.0159	0.0455	0.2896
12	0.0149	0.0348	0.2798
13	0.0152	0.0434	0.2842
14	0.0142	0.0434	0.2873
15	0.0150	0.0438	0.2854
16	0.0156	0.0443	0.2836
17	ND	ND	ND

18	ND	ND	ND
19	0.0152	0.0433	0.2884
20	0.0161	0.0466	0.2989

ND: No Data

Table 30: Fluoride concentrations for specimens stored at and analysed by the Johannesburg Department of Health

Week	L1 (g/100ml)	L2 (g/100ml)	L3 (g/100ml)
0	0.581	0.644	0.673
1	1.67	1.62	1.92
2	4.07	1.42	1.70
3	1.75	2.40	2.40
4	2.29	2.03	2.69
5	2.37	1.48	1.27
6	1.55	2.07	2.06
7	2.84	2.09	2.15
8	1.85	1.74	1.55
9	2.83	2.81	2.93
10	2.53	2.56	2.64
11	2.03	2.07	1.67
12	1.65	1.60	1.62
13	2.45	2.44	1.87
14	1.73	1.97	1.99
15	2.50	2.63	2.44
16	2.41	3.04	2.78
17	ND	ND	ND
18	ND	ND	ND
19	1.84	2.23	1.74
20	2.22	1.39	2.62

ND: No Data

C.3.2. Candida albicans Study

Table 31: Ethanol concentrations for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of *C. albicans* and stored at 4°C in the presence of NaF for up to 7 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
	***C. albicans* Concentration (cells/ml)**	**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.02	0.01950	0.01950	0.01950	0.01950	0.01950
1	0.02	0.01552		0.01560	0.01594	0.01536
2	0.02	0.01693	0.01754	0.01573	0.01489	0.01511
3	0.02	0.01462	0.01477	0.01653	0.01350	0.01383
4	0.02	0.01556	0.01465	0.01556	0.01557	0.01506
7	0.02	0.01370	0.01389	0.01372	0.01475	0.01693
8	0.02	0.01461	0.01462	0.01497	0.01437	0.01375
9	0.02	0.01366	0.01378	0.01561	0.01463	0.01403

10	0.02	0.01653	0.01629	0.01923	0.01620	0.02096
11	0.02	0.01976	0.01855	ND	ND	ND
14	0.02	0.01392	0.01681	0.01465	0.01589	0.01594
21	0.02	0.01118	0.01577	0.01455	0.01649	0.01731
28	0.02	0.00281	0.01513	0.01926	0.00354	0.01928
35	0.02	ND	ND	ND	0.02081	0.01945
42	0.02	ND	ND	ND	0.01682	0.02369
49	0.02	ND	ND	ND	0.01684	ND

ND: No Data

Table 32: Ethanol concentrations for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the presence of NaF for up to 8 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.05	0.04914	0.04914	0.04914	0.04914	0.04914
1	0.05	0.04886	0.04724	0.04606	0.04439	0.04566
2	0.05	0.04862	0.04607	0.04235	0.04401	0.04576
3	0.05	0.04848	0.04455	0.04164	0.04324	0.04515
4	0.05	0.04485	0.04724	0.04599	0.04640	0.05008
7	0.05	0.04651	0.04527	0.04758	0.04725	0.05149
8	0.05	0.04410	0.04482	0.04105	0.04804	0.04704
9	0.05	0.04278	0.04266	0.04352	0.04495	0.04636
10	0.05	0.04173	0.04348	0.04845	0.04281	0.04381
11	0.05	0.04967	0.05102	0.05444	0.05082	0.05121
14	0.05	0.03594	0.04474	0.05092	0.04553	0.04762
21	0.05	0.04143	0.04328	0.04531	0.04775	0.04836
28	0.05	0.01089	0.01603	0.04566	0.02183	0.05024
35	0.05	ND	ND	ND	ND	0.04912
42	0.05	ND	ND	ND	ND	0.05216
49	0.05	ND	ND	ND	ND	0.04914
56	0.05	ND	ND	ND	ND	0.04921

ND: No Data

Table 33: Ethanol concentrations for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the absence of NaF for up to 9 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.02	0.01950	0.01950	0.01950	0.01950	0.01950
1	0.02	0.01765	0.01860	ND	0.01704	0.01712
2	0.02	0.01920	0.01702	0.01747	0.01590	0.01687
3	0.02	0.01743	0.01627	0.01335	0.01478	0.01566
4	0.02	0.01613	0.01644	0.01614	0.01539	0.01617
7	0.02	0.01560	0.01722	0.01803	0.01626	0.01796

8	0.02	0.01572	0.01502	0.01640	0.01616	0.01476
9	0.02	0.01708	0.01665	0.01710	0.01695	0.01763
10	0.02	0.01772	0.01555	0.01922	0.02248	0.01788
11	0.02	0.02235	0.01839	0.01851	0.01851	0.01848
14	0.02	0.01522	0.01549	0.01620	0.01693	0.01775
21	0.02	0.01634	0.01883	0.01920	0.01794	0.01912
28	0.02	0.01073	0.00943	0.01668	0.01141	0.01838
35	0.02	0.02003	0.02236	0.01964	0.02014	0.02039
42	0.02	0.01922	0.01978	0.02137	0.02137	0.02106
49	0.02	0.02053	0.02102	0.01842	0.01794	0.01759
56	0.02	0.02114	0.01987	0.01847	0.02033	0.01979
63	0.02	0.01515	0.01505	0.01582	0.01530	0.01627

ND: No Data

Table 34: Ethanol concentrations for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the absence of NaF for up to 9 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.05	0.04914	0.04914	0.04914	0.04914	0.04914
1	0.05	0.04692	0.04347	0.04666	0.04438	0.04241
2	0.05	0.04910	0.04549	0.04222	0.04319	0.04459
3	0.05	0.04345	0.03951	0.03679	0.03992	0.04402
4	0.05	0.05419	0.04205	0.04596	0.04333	0.04432
7	0.05	0.04230	0.04107	0.04751	0.04642	0.05021
8	0.05	0.04373	0.04536	0.04468	0.04369	0.04689
9	0.05	0.04591	0.04754	0.04554	0.04519	0.04632
10	0.05	0.05040	0.04186	0.04419	0.04652	0.04128
11	0.05	0.04862	0.05178	0.04463	0.04363	0.05648
14	0.05	0.04124	0.04306	0.03616	0.02806	0.03773
21	0.05	0.03958	0.03996	0.04703	0.04440	0.04618
28	0.05	0.04757	0.02207	0.04514	0.03768	0.04688
35	0.05	0.05147	0.05091	0.04858	0.05318	0.05259
42	0.05	0.04855	0.04938	0.04937	0.05475	0.05393
49	0.05	0.05321	0.05389	0.05317	0.04869	0.04751
56	0.05	0.05063	0.05032	0.04888	0.05435	0.05425
63	0.05	0.03998	0.03997	0.03986	0.04209	0.04160

Table 35: Ethanol concentrations for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the presence of NaF for up to 7 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.02	0.01950	0.01950	0.01950	0.01950	0.01950
1	0.02	ND	0.01518	0.01616	ND	0.01797

2	0.02	0.01587	0.01506	0.01529	0.01841	0.01431
3	0.02	0.01183	0.01277	0.01201	0.01424	0.01548
4	0.02	0.01552	0.01270	0.01308	0.01337	0.01353
7	0.02	0.01172	0.01359	0.01236	0.01193	0.01212
8	0.02	0.01235	0.01279	0.01252	0.01229	0.01093
9	0.02	0.01245	0.01282	0.01137	0.01300	0.01217
10	0.02	0.01211	0.01264	0.01301	0.01410	0.01227
11	0.02	0.01213	0.01287	ND	ND	ND
14	0.02	0.00996	0.01028	0.01026	0.00883	0.00972
21	0.02	0.00855	0.00860	0.00983	0.01649	0.00986
28	0.02	0.00517	0.00923	0.01207	0.01026	0.00999
35	0.02	ND	ND	ND	0.00622	0.00952
42	0.02	ND	ND	ND	0.00440	0.00614
49	0.02	ND	ND	ND	0.00371	ND

ND: No Data

Table 36: Ethanol concentrations for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the presence of NaF for up to 8 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.05	0.04914	0.04914	0.04914	0.04914	0.04914
1	0.05	0.04858	0.04764	0.04780	0.06483	0.04598
2	0.05	0.05004	0.04843	0.04713	0.04804	0.04967
3	0.05	0.04075	0.04005	0.04116	0.04269	0.04420
4	0.05	0.04448	0.04249	0.04813	0.04595	0.04751
7	0.05	0.04448	0.04539	0.04225	0.04443	0.04187
8	0.05	0.04575	0.04353	ND	0.04631	0.04339
9	0.05	0.04342	0.04453	0.04405	0.04385	0.03876
10	0.05	0.04486	0.04528	0.04819	0.04385	0.04544
11	0.05	0.04342	0.04934	0.05021	0.04788	0.05179
14	0.05	0.02871	0.03448	0.03981	0.03685	0.04118
21	0.05	0.04218	0.04196	0.03939	0.04153	0.04055
28	0.05	0.04314	0.03895	0.04672	0.04310	0.04404
35	0.05	ND	ND	ND	ND	0.03853
42	0.05	ND	ND	ND	ND	0.04291
49	0.05	ND	ND	ND	ND	0.03824
56	0.05	ND	ND	ND	ND	0.03955

ND: No Data

Table 37: Ethanol concentrations for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the absence of NaF for up to 9 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.02	0.01950	0.01950	0.01950	0.01950	0.01950
1	0.02	0.01508	0.01504	0.01594	0.01524	0.01703
2	0.02	0.01445	0.01556	0.01373	0.00204	0.00000
3	0.02	0.00617	0.00715	0.00344	0.00000	0.00000
4	0.02	0.00000	0.00144	0.00000	0.00000	0.00000
7	0.02	0.00000	0.00000	0.00000	0.00000	0.00000
8	0.02	0.00276	0.00332	0.00100	0.00000	0.00000
9	0.02	0.00378	0.00354	0.00053	0.00000	0.00000
10	0.02	0.00271	0.00498	0.00000	0.00000	0.00000
11	0.02	0.00158	0.00085	0.00036	0.00000	0.00000
14	0.02	0.00028	0.00035	0.00022	0.00000	0.00000
21	0.02	0.00018	0.00035	0.00000	0.00000	0.00000
28	0.02	0.00000	0.00000	0.00000	0.00000	0.00000
35	0.02	0.00000	0.00000	0.00000	0.00000	0.00000
42	0.02	0.00072	0.00063	0.00040	0.00000	0.00000
49	0.02	0.00059	0.00080	0.00039	0.00000	0.00000
56	0.02	0.00070	0.00044	0.00060	0.00000	0.00000
63	0.02	0.00000	0.00026	0.00050	0.00000	0.00000

Table 38: Ethanol concentrations for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the absence of NaF for up to 9 weeks

Days	Theoretical	Level 1	Level 2	Level 3	Level 4	Level 5
***C. albicans* Concentration (cells/ml)**		**$5x10^1$**	**$5x10^3$**	**$1x10^4$**	**$5x10^5$**	**$1x10^6$**
0	0.02	0.04914	0.04914	0.04914	0.04914	0.04914
1	0.02	0.04913	0.04897	0.04891	0.04814	0.04734
2	0.02	0.04845	0.04475	0.04534	0.04204	0.04227
3	0.02	0.03695	0.03978	0.03818	0.03517	0.03272
4	0.02	0.03927	0.03946	0.03675	0.01811	0.02307
7	0.02	0.02855	0.02850	0.02258	0.02280	0.01650
8	0.02	0.02635	0.04155	0.02196	0.03156	0.03301
9	0.02	0.02727	0.02842	0.04054	0.02908	0.00893
10	0.02	0.03780	0.02662	0.02451	0.02043	0.04205
11	0.02	0.03268	0.02312	0.04389	0.01129	0.00238
14	0.02	0.01440	0.01228	0.02982	0.00299	0.02759
21	0.02	0.01775	0.01465	0.03725	0.02387	0.03703
28	0.02	0.02982	0.02248	0.04398	0.01621	0.04282
35	0.02	0.01704	0.01975	0.05154	0.00608	0.03979
42	0.02	0.01672	0.01535	0.03759	0.03651	0.03992

49	0.02	0.01443	0.01772	0.04795	0.00489	0.04054
56	0.02	0.01378	0.01376	0.00861	0.02805	0.00818
63	0.02	0.01394	0.01033	0.00663	0.02641	0.03620

Table 39: Colony counts for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the presence of NaF for up to 7 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.02	0.02	0.02	0.02	0.02
0	5	500	1000	5000	1000
1	0.00	0.00	0.00	3.85	1.26
3	0.00	0.00	1.50	2.70	2.64
4	0.00	0.00	0.00	1.35	1.08
7	0.00	0.00	0.00	0.40	0.31
8	0.00	0.00	0.00	0.55	1.10
9	0.00	0.00	0.00	1.10	0.75
10	0.00	0.00	0.00	0.15	0.16
11	0.00	0.00	0.00	0.00	0.32
14	ND	ND	ND	0.00	0.18
21	ND	ND	ND	0.25	0.01
28	ND	ND	ND	0.05	0.00
35	ND	ND	ND	0.00	0.00
42	ND	ND	ND	0.00	0.00
49	ND	ND	ND	0.00	ND

ND: No Data

Table 40: Colony counts for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the presence of NaF for up to 8 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.05	0.05	0.05	0.05	0.05
0	5	500	1000	5000	1000
1	0.00	0.00	0.50	6.15	1.20
3	0.50	0.00	0.50	4.75	1.42
4	0.00	0.00	0.00	4.65	0.92
7	0.00	0.00	0.00	0.35	0.48
8	0.00	0.00	0.00	0.35	0.40
9	0.00	0.00	0.00	0.80	0.38
10	0.00	0.00	0.00	0.20	0.06
11	0.00	0.00	0.00	0.25	0.28
14	ND	ND	ND	0.00	0.01
21	ND	ND	ND	0.00	0.00
28	ND	ND	ND	0.00	0.00
35	ND	ND	ND	ND	0.01
42	ND	ND	ND	ND	0.00
49	ND	ND	ND	ND	0.00
56	ND	ND	ND	ND	0.00

ND: No Data

Table 41: Colony counts for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the absence of NaF for up to 9 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.02	0.02	0.02	0.02	0.02
0	5	500	1000	5000	1000
1	6.50	7.50	11.50	54.40	6.26
3	4.50	4.50	10.50	49.10	7.54
4	4.00	3.50	7.00	49.50	5.96
7	3.00	7.00	9.50	44.90	6.36
8	6.50	5.00	5.50	36.80	6.28
9	7.50	4.00	9.50	42.30	7.64
10	9.50	7.00	3.50	37.20	6.84
11	5.00	9.50	13.00	53.40	7.21
14	4.50	5.00	4.50	31.60	5.80
21	1.50	2.50	4.50	26.30	3.48
28	3.00	1.00	2.00	20.80	2.68
35	1.00	2.00	2.50	21.10	3.09
42	0.00	0.00	2.50	13.70	2.17
49	2.00	1.00	1.50	7.00	1.36
56	0.00	0.00	2.00	3.00	0.66

63	0.00	0.00	1.00	1.80	0.38

Table 42: Colony counts for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 4°C in the absence of NaF for up to 9 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.05	0.05	0.05	0.05	0.05
0	5	500	1000	5000	1000
1	3.00	7.00	11.00	40.00	5.20
3	4.50	6.50	9.00	50.50	6.25
4	6.50	7.00	13.00	61.20	6.15
7	4.50	3.00	9.00	41.00	5.40
8	8.50	4.00	8.00	39.40	6.22
9	5.50	4.00	11.00	35.00	6.74
10	5.50	7.00	8.50	33.40	6.78
11	4.50	5.00	5.00	52.10	6.55
14	4.00	5.00	7.00	34.00	6.69
21	2.00	4.50	12.50	26.50	5.38
28	3.50	2.50	2.00	26.60	3.43
35	2.00	0.00	2.00	24.40	3.28
42	1.00	0.00	2.50	9.50	3.07
49	0.00	0.00	0.00	6.65	1.83
56	0.50	0.50	0.50	2.35	0.18
63	0.00	0.00	0.00	1.45	0.07

Table 43: Colony counts for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the presence of NaF for up to 7 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.02	0.02	0.02	0.02	0.02
0	5	500	1000	5000	1000
1	0.00	0.00	0.00	0.05	0.00
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
11	0.00	0.00	0.00	0.00	0.00
14	ND	ND	ND	0.00	0.01
21	ND	ND	ND	0.00	0.00
28	ND	ND	ND	0.00	0.00
35	ND	ND	ND	0.00	0.00
42	ND	ND	ND	0.00	0.00

49	ND	ND	ND	0.00	ND

ND: No Data

Table 44: Colony counts for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the presence of NaF for up to 8 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.05	0.05	0.05	0.05	0.05
0	5	500	1000	5000	1000
1	0.00	0.00	3.00	0.05	0.00
3	0.00	0.50	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
11	0.00	0.00	0.00	0.00	0.00
14	ND	ND	ND	0.00	0.00
21	ND	ND	ND	0.00	0.00
28	ND	ND	ND	0.00	0.00
35	ND	ND	ND	ND	0.00
42	ND	ND	ND	ND	0.00
49	ND	ND	ND	ND	0.00
56	ND	ND	ND	ND	0.00

ND: No Data

Table 45: Colony counts for specimens initially spiked at 0.02 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the absence of NaF for up to 9 weeks

Days	Level 1 ($x10^1$)	Level 2 ($x10^1$)	Level 3 ($x10^1$)	Level 4 ($x10^2$)	Level 5 ($x10^3$)
Ethanol Concentration (g/100 ml)	0.02	0.02	0.02	0.02	0.02
0	5	500	1000	5000	1000
1	440	370	545	UQ	UQ
3	UQ	UQ	UQ	UQ	UQ
4	UQ	UQ	UQ	UQ	UQ
7	UQ	UQ	UQ	UQ	UQ
8	UQ	UQ	UQ	UQ	UQ
9	UQ	UQ	UQ	UQ	UQ
10	UQ	UQ	UQ	UQ	UQ
11	UQ	UQ	UQ	UQ	UQ
14	UQ	UQ	UQ	UQ	UQ
21	UQ	UQ	UQ	UQ	UQ
28	UQ	UQ	UQ	UQ	UQ
35	UQ	UQ	UQ	UQ	UQ
42	78000	334000	261000	11400	2315

49	152000	74000	92500	7550	365
56	65500	102500	87000	7600	210
63	255000	680000	85500	19900	1270

UQ: Unquantifiable

Table 46: Colony counts for specimens initially spiked at 0.05 g/100 ml ethanol, inoculated at five levels of C. albicans and stored at 22°C in the absence of NaF for up to 9 weeks

Days	**Level 1 ($x10^1$)**	**Level 2 ($x10^1$)**	**Level 3 ($x10^1$)**	**Level 4 ($x10^2$)**	**Level 5 ($x10^3$)**
Ethanol Concentration (g/100 ml)	0.05	0.05	0.05	0.05	0.05
0	5	500	1000	5000	1000
1	110	54	226	UQ	UQ
3	UQ	UQ	250	UQ	UQ
4	UQ	UQ	UQ	UQ	UQ
7	UQ	UQ	UQ	UQ	UQ
8	UQ	UQ	UQ	UQ	UQ
9	UQ	UQ	UQ	UQ	UQ
10	UQ	UQ	UQ	UQ	UQ
11	UQ	UQ	UQ	UQ	UQ
14	UQ	UQ	UQ	UQ	UQ
21	UQ	UQ	UQ	UQ	UQ
28	UQ	UQ	UQ	UQ	UQ
35	UQ	UQ	UQ	UQ	UQ
42	282000	381000	59000	11550	1020
49	140000	177000	20000	2100	155
56	22000	121000	245500	7850	1065
63	14000	62000	92000	3750	215

UQ: Unquantifiable

Appendix D

Normality

D.1. Ethanol

D.1.1. Step 1: Order Data in Ascending Order

Table 1: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) ethanol internal quality controls ordered in ascending order

i	LC	MC	HC
Theoretical Concentration (g/100 ml)	**0.02**	**0.05**	**0.30**
1	0.01508	0.04175	0.26073
2	0.01536	0.04226	0.26258
3	0.01560	0.04263	0.26508
4	0.01603	0.04266	0.26621
5	0.01624	0.04294	0.26645
6	0.01674	0.04307	0.26834
7	0.01683	0.04325	0.27477
8	0.01713	0.04398	0.27573
9	0.01768	0.04454	0.27691
10	0.01774	0.04476	0.28232
11	0.01836	0.04515	0.28481
12	0.01847	0.04522	0.28514
13	0.01857	0.04572	0.28574
14	0.01871	0.04600	0.28606
15	0.01874	0.04623	0.28621
16	0.01876	0.04661	0.28773
17	0.01876	0.04720	0.28825
18	0.01887	0.04733	0.29021
19	0.01888	0.04751	0.29041
20	0.01902	0.04775	0.29228
21	0.01903	0.04775	0.29349
22	0.01907	0.04783	0.29517
23	0.01912	0.04804	0.29589
24	0.01915	0.04841	0.29960
25	0.01938	0.04860	0.29987
26	0.01945	0.04861	0.29988
27	0.01951	0.04867	0.30090
28	0.01952	0.04876	0.30095
29	0.01952	0.04890	0.30162
30	0.01958	0.04925	0.30333
31	0.01975	0.04958	0.30481

32	0.01977	0.04958	0.30628
33	0.01981	0.04978	0.30718
34	0.01990	0.04982	0.30892
35	0.01995	0.05000	0.30921
36	0.01995	0.05003	0.30996
37	0.01999	0.05005	0.31136
38	0.02000	0.05015	0.31170
39	0.02000	0.05049	0.31292
40	0.02002	0.05058	0.31293
41	0.02011	0.05078	0.31330
42	0.02014	0.05078	0.31360
43	0.02020	0.05094	0.31374
44	0.02020	0.05099	0.31768
45	0.02029	0.05107	0.31874
46	0.02033	0.05117	0.32088
47	0.02034	0.05122	0.32194
48	0.02039	0.05187	0.32273
49	0.02041	0.05193	0.32331
50	0.02049	0.05197	0.32408
51	0.02051	0.05239	0.32550
52	0.02065	0.05250	0.32565
53	0.02075	0.05280	0.32571
54	0.02075	0.05291	0.32715
55	0.02086	0.05293	0.32720
56	0.02104	0.05301	0.32857
57	0.02117	0.05308	0.32926
58	0.02131	0.05325	0.33203
59	0.02134	0.05331	0.33225
60	0.02140	0.05333	0.33335
61	0.02148	0.05337	0.33403
62	0.02158	0.05357	0.33546
63	0.02166	0.05369	0.33747
64	0.02189	0.05371	0.33792
65	0.02194	0.05375	0.33977
66	0.02201	0.05377	0.34000
67	0.02202	0.05388	0.34233
68	0.02224	0.05413	0.34683
69	0.02230	0.05416	0.34925
70	0.02240	0.05424	0.35312
71	0.02245	0.05433	0.35342
72	0.02250	0.05434	0.35555
73	0.02264	0.05467	0.35663
74	0.02280	0.05491	0.35811
75	0.02284	0.05504	0.35892

76	0.02298	0.05532	0.36368
77	0.02298	0.05538	0.37138
78	0.02300	0.05540	
79	0.02300	0.05564	
80	0.02304	0.05577	
81	0.02307	0.05594	
82	0.02311	0.05607	
83	0.02333	0.05611	
84	0.02342	0.05653	
85	0.02354	0.05659	
86	0.02359	0.05679	
87	0.02368	0.05705	
88	0.02391	0.05705	
89	0.02401	0.05724	
90	0.02404	0.05734	
91	0.02457	0.05758	
92	0.02482	0.05763	
93	0.02488	0.05775	
94	0.02488	0.05780	
95	0.02493	0.05780	
96	0.02502	0.05811	
97	0.02515	0.05821	
98	0.02522	0.05827	
99	0.02538	0.05857	
100	0.02541	0.05859	
101	0.02559	0.05861	
102	0.02613	0.05949	
103	0.02653	0.05966	
104	0.02673	0.06051	
105		0.06132	
106		0.06161	
107		0.06167	
108		0.06185	
109		0.06323	
110		0.06435	
μ	**0.02111**	**0.05247**	**0.31263**
σ	**0.00260**	**0.00512**	**0.02684**

D.1.2. Step 2: Calculate Standardized Normal Variable, z

$$z = \frac{(x - \mu)}{\sigma}$$ Equation 1

Table 2: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) ethanol internal quality controls ordered in ascending order, and the corresponding z-values

i	LC	z (LC)	MC	z (MC)	HC	z (HC)
Theoretical Concentration (g/100 ml)	**0.02**		**0.05**		**0.30**	
1	0.01508	-2.31825	0.04175	-2.09547	0.26073	-1.93375
2	0.01536	-2.20790	0.04226	-1.99545	0.26258	-1.86470
3	0.01560	-2.11900	0.04263	-1.92304	0.26508	-1.77138
4	0.01603	-1.95373	0.04266	-1.91807	0.26621	-1.72927
5	0.01624	-1.87141	0.04294	-1.86344	0.26645	-1.72043
6	0.01674	-1.67843	0.04307	-1.83744	0.26834	-1.65009
7	0.01683	-1.64575	0.04325	-1.80296	0.27477	-1.41043
8	0.01713	-1.52842	0.04398	-1.66021	0.27573	-1.37459
9	0.01768	-1.31936	0.04454	-1.54991	0.27691	-1.33073
10	0.01774	-1.29557	0.04476	-1.50704	0.28232	-1.12931
11	0.01836	-1.05553	0.04515	-1.43141	0.28481	-1.03650
12	0.01847	-1.01244	0.04522	-1.41640	0.28514	-1.02409
13	0.01857	-0.97696	0.04572	-1.31870	0.28574	-1.00190
14	0.01871	-0.92195	0.04600	-1.26496	0.28606	-0.98987
15	0.01874	-0.91104	0.04623	-1.21926	0.28621	-0.98430
16	0.01876	-0.90288	0.04661	-1.14653	0.28773	-0.92768
17	0.01876	-0.90189	0.04720	-1.03114	0.28825	-0.90811
18	0.01887	-0.86216	0.04733	-1.00525	0.29021	-0.83543
19	0.01888	-0.85822	0.04751	-0.96979	0.29041	-0.82788
20	0.01902	-0.80486	0.04775	-0.92254	0.29228	-0.75803
21	0.01903	-0.79953	0.04775	-0.92213	0.29349	-0.71294
22	0.01907	-0.78426	0.04783	-0.90780	0.29517	-0.65055
23	0.01912	-0.76481	0.04804	-0.86550	0.29589	-0.62349
24	0.01915	-0.75138	0.04841	-0.79432	0.29960	-0.48559
25	0.01938	-0.66370	0.04860	-0.75772	0.29987	-0.47535
26	0.01945	-0.63790	0.04861	-0.75485	0.29988	-0.47506
27	0.01951	-0.61302	0.04867	-0.74223	0.30090	-0.43707
28	0.01952	-0.60978	0.04876	-0.72587	0.30095	-0.43515
29	0.01952	-0.60925	0.04890	-0.69896	0.30162	-0.41010
30	0.01958	-0.58803	0.04925	-0.62902	0.30333	-0.34639
31	0.01975	-0.52083	0.04958	-0.56471	0.30481	-0.29126
32	0.01977	-0.51447	0.04958	-0.56451	0.30628	-0.23641
33	0.01981	-0.50002	0.04978	-0.52705	0.30718	-0.20301
34	0.01990	-0.46537	0.04982	-0.51762	0.30892	-0.13816
35	0.01995	-0.44733	0.05000	-0.48269	0.30921	-0.12729
36	0.01995	-0.44647	0.05003	-0.47719	0.30996	-0.09931
37	0.01999	-0.43052	0.05005	-0.47345	0.31136	-0.04724
38	0.02000	-0.42738	0.05015	-0.45463	0.31170	-0.03468

39	0.02000	-0.42539	0.05049	-0.38729	0.31292	0.01099
40	0.02002	-0.42042	0.05058	-0.37037	0.31293	0.01109
41	0.02011	-0.38569	0.05078	-0.33154	0.31330	0.02496
42	0.02014	-0.37194	0.05078	-0.33090	0.31360	0.03613
43	0.02020	-0.35085	0.05094	-0.29873	0.31374	0.04147
44	0.02020	-0.34989	0.05099	-0.29021	0.31768	0.18817
45	0.02029	-0.31562	0.05107	-0.27315	0.31874	0.22749
46	0.02033	-0.30023	0.05117	-0.25514	0.32088	0.30741
47	0.02034	-0.29624	0.05122	-0.24460	0.32194	0.34676
48	0.02039	-0.27649	0.05187	-0.11809	0.32273	0.37637
49	0.02041	-0.26975	0.05193	-0.10678	0.32331	0.39791
50	0.02049	-0.23629	0.05197	-0.09837	0.32408	0.42652
51	0.02051	-0.23209	0.05239	-0.01715	0.32550	0.47963
52	0.02065	-0.17508	0.05250	0.00611	0.32565	0.48507
53	0.02075	-0.13927	0.05280	0.06426	0.32571	0.48736
54	0.02075	-0.13743	0.05291	0.08477	0.32715	0.54105
55	0.02086	-0.09655	0.05293	0.08898	0.32720	0.54286
56	0.02104	-0.02551	0.05301	0.10410	0.32857	0.59389
57	0.02117	0.02449	0.05308	0.11766	0.32926	0.61966
58	0.02131	0.07644	0.05325	0.15171	0.33203	0.72282
59	0.02134	0.08877	0.05331	0.16418	0.33225	0.73081
60	0.02140	0.11164	0.05333	0.16701	0.33335	0.77212
61	0.02148	0.14402	0.05337	0.17433	0.33403	0.79747
62	0.02158	0.18111	0.05357	0.21507	0.33546	0.85065
63	0.02166	0.21034	0.05369	0.23765	0.33747	0.92531
64	0.02189	0.29843	0.05371	0.24087	0.33792	0.94237
65	0.02194	0.31994	0.05375	0.24910	0.33977	1.01116
66	0.02201	0.34574	0.05377	0.25307	0.34000	1.01959
67	0.02202	0.34995	0.05388	0.27482	0.34233	1.10654
68	0.02224	0.43524	0.05413	0.32462	0.34683	1.27405
69	0.02230	0.45563	0.05416	0.32954	0.34925	1.36426
70	0.02240	0.49700	0.05424	0.34614	0.35312	1.50835
71	0.02245	0.51675	0.05433	0.36258	0.35342	1.51956
72	0.02250	0.53428	0.05434	0.36549	0.35555	1.59909
73	0.02264	0.58660	0.05467	0.42952	0.35663	1.63910
74	0.02280	0.64845	0.05491	0.47680	0.35811	1.69440
75	0.02284	0.66388	0.05504	0.50200	0.35892	1.72446
76	0.02298	0.71791	0.05532	0.55627	0.36368	1.90188
77	0.02298	0.72026	0.05538	0.56771	0.37138	2.18867
78	0.02300	0.72532	0.05540	0.57221		
79	0.02300	0.72689	0.05564	0.61890		
80	0.02304	0.74241	0.05577	0.64396		
81	0.02307	0.75279	0.05594	0.67807		
82	0.02311	0.76908	0.05607	0.70362		

83	0.02333	0.85321	0.05611	0.70971
84	0.02342	0.88763	0.05653	0.79266
85	0.02354	0.93561	0.05659	0.80376
86	0.02359	0.95449	0.05679	0.84390
87	0.02368	0.98644	0.05705	0.89356
88	0.02391	1.07686	0.05705	0.89407
89	0.02401	1.11643	0.05724	0.93097
90	0.02404	1.12634	0.05734	0.95126
91	0.02457	1.32909	0.05758	0.99795
92	0.02482	1.42474	0.05763	1.00788
93	0.02488	1.44874	0.05775	1.03107
94	0.02488	1.45010	0.05780	1.03994
95	0.02493	1.46905	0.05780	1.04143
96	0.02502	1.50454	0.05811	1.10111
97	0.02515	1.55116	0.05821	1.12077
98	0.02522	1.58066	0.05827	1.13324
99	0.02538	1.64131	0.05857	1.19208
100	0.02541	1.65455	0.05859	1.19590
101	0.02559	1.72105	0.05861	1.19901
102	0.02613	1.92905	0.05949	1.37086
103	0.02653	2.08120	0.05966	1.40397
104	0.02673	2.15894	0.06051	1.57089
105			0.06132	1.72807
106			0.06161	1.78526
107			0.06167	1.79620
108			0.06185	1.83161
109			0.06323	2.10281
110			0.06435	2.32053

D.1.3. Step 3: Calculate Theoretical Normal Cumulative Distribution Values, F(x)

$$F(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$ Equation 2

Table 3: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) ethanol internal quality controls ordered in ascending order, corresponding z-values and F(x) values

i	LC	z (LC)	$F(x)_{LC}$	MC	z (MC)	$F(x)_{MC}$	HC	z (HC)	$F(x)_{HC}$
Theoretical Concentration (g/100 ml)	**0.02**			**0.05**			**0.30**		
1	0.01508	-2.31825	0.01022	0.04175	-2.09547	0.01806	0.26073	-1.93375	0.02657
2	0.01536	-2.20790	0.01363	0.04226	-1.99545	0.02300	0.26258	-1.86470	0.03111
3	0.01560	-2.11900	0.01705	0.04263	-1.92304	0.02724	0.26508	-1.77138	0.03825

4	0.01603	-1.95373	0.02537	0.04266	-1.91807	0.02755	0.26621	-1.72927	0.04188
5	0.01624	-1.87141	0.03064	0.04294	-1.86344	0.03120	0.26645	-1.72043	0.04268
6	0.01674	-1.67843	0.04663	0.04307	-1.83744	0.03307	0.26834	-1.65009	0.04946
7	0.01683	-1.64575	0.04991	0.04325	-1.80296	0.03570	0.27477	-1.41043	0.07921
8	0.01713	-1.52842	0.06320	0.04398	-1.66021	0.04844	0.27573	-1.37459	0.08463
9	0.01768	-1.31936	0.09352	0.04454	-1.54991	0.06058	0.27691	-1.33073	0.09164
10	0.01774	-1.29557	0.09756	0.04476	-1.50704	0.06590	0.28232	-1.12931	0.12938
11	0.01836	-1.05553	0.14559	0.04515	-1.43141	0.07616	0.28481	-1.03650	0.14998
12	0.01847	-1.01244	0.15566	0.04522	-1.41640	0.07833	0.28514	-1.02409	0.15290
13	0.01857	-0.97696	0.16429	0.04572	-1.31870	0.09363	0.28574	-1.00190	0.15820
14	0.01871	-0.92195	0.17828	0.04600	-1.26496	0.10294	0.28606	-0.98987	0.16112
15	0.01874	-0.91104	0.18114	0.04623	-1.21926	0.11137	0.28621	-0.98430	0.16248
16	0.01876	-0.90288	0.18329	0.04661	-1.14653	0.12579	0.28773	-0.92768	0.17679
17	0.01876	-0.90189	0.18356	0.04720	-1.03114	0.15124	0.28825	-0.90811	0.18191
18	0.01887	-0.86216	0.19430	0.04733	-1.00525	0.15739	0.29021	-0.83543	0.20174
19	0.01888	-0.85822	0.19539	0.04751	-0.96979	0.16608	0.29041	-0.82788	0.20387
20	0.01902	-0.80486	0.21045	0.04775	-0.92254	0.17812	0.29228	-0.75803	0.22422
21	0.01903	-0.79953	0.21199	0.04775	-0.92213	0.17823	0.29349	-0.71294	0.23794
22	0.01907	-0.78426	0.21644	0.04783	-0.90780	0.18199	0.29517	-0.65055	0.25767
23	0.01912	-0.76481	0.22219	0.04804	-0.86550	0.19338	0.29589	-0.62349	0.26648
24	0.01915	-0.75138	0.22621	0.04841	-0.79432	0.21350	0.29960	-0.48559	0.31363
25	0.01938	-0.66370	0.25344	0.04860	-0.75772	0.22431	0.29987	-0.47535	0.31727
26	0.01945	-0.63790	0.26177	0.04861	-0.75485	0.22517	0.29988	-0.47506	0.31737
27	0.01951	-0.61302	0.26993	0.04867	-0.74223	0.22897	0.30090	-0.43707	0.33103
28	0.01952	-0.60978	0.27100	0.04876	-0.72587	0.23396	0.30095	-0.43515	0.33173
29	0.01952	-0.60925	0.27118	0.04890	-0.69896	0.24229	0.30162	-0.41010	0.34087
30	0.01958	-0.58803	0.27826	0.04925	-0.62902	0.26467	0.30333	-0.34639	0.36452
31	0.01975	-0.52083	0.30124	0.04958	-0.56471	0.28613	0.30481	-0.29126	0.38543
32	0.01977	-0.51447	0.30346	0.04958	-0.56451	0.28620	0.30628	-0.23641	0.40656
33	0.01981	-0.50002	0.30853	0.04978	-0.52705	0.29908	0.30718	-0.20301	0.41956
34	0.01990	-0.46537	0.32083	0.04982	-0.51762	0.30236	0.30892	-0.13816	0.44506
35	0.01995	-0.44733	0.32732	0.05000	-0.48269	0.31466	0.30921	-0.12729	0.44936
36	0.01995	-0.44647	0.32763	0.05003	-0.47719	0.31662	0.30996	-0.09931	0.46045
37	0.01999	-0.43052	0.33341	0.05005	-0.47345	0.31795	0.31136	-0.04724	0.48116
38	0.02000	-0.42738	0.33455	0.05015	-0.45463	0.32469	0.31170	-0.03468	0.48617
39	0.02000	-0.42539	0.33528	0.05049	-0.38729	0.34927	0.31292	0.01099	0.50438
40	0.02002	-0.42042	0.33709	0.05058	-0.37037	0.35555	0.31293	0.01109	0.50443
41	0.02011	-0.38569	0.34986	0.05078	-0.33154	0.37012	0.31330	0.02496	0.50996
42	0.02014	-0.37194	0.35497	0.05078	-0.33090	0.37036	0.31360	0.03613	0.51441
43	0.02020	-0.35085	0.36285	0.05094	-0.29873	0.38257	0.31374	0.04147	0.51654
44	0.02020	-0.34989	0.36321	0.05099	-0.29021	0.38583	0.31768	0.18817	0.57463
45	0.02029	-0.31562	0.37615	0.05107	-0.27315	0.39237	0.31874	0.22749	0.58998
46	0.02033	-0.30023	0.38200	0.05117	-0.25514	0.39931	0.32088	0.30741	0.62074
47	0.02034	-0.29624	0.38352	0.05122	-0.24460	0.40338	0.32194	0.34676	0.63561

48	0.02039	-0.27649	0.39109	0.05187	-0.11809	0.45300	0.32273	0.37637	0.64668
49	0.02041	-0.26975	0.39368	0.05193	-0.10678	0.45748	0.32331	0.39791	0.65465
50	0.02049	-0.23629	0.40660	0.05197	-0.09837	0.46082	0.32408	0.42652	0.66514
51	0.02051	-0.23209	0.40823	0.05239	-0.01715	0.49316	0.32550	0.47963	0.68426
52	0.02065	-0.17508	0.43051	0.05250	0.00611	0.50244	0.32565	0.48507	0.68619
53	0.02075	-0.13927	0.44462	0.05280	0.06426	0.52562	0.32571	0.48736	0.68700
54	0.02075	-0.13743	0.44534	0.05291	0.08477	0.53378	0.32715	0.54105	0.70576
55	0.02086	-0.09655	0.46154	0.05293	0.08898	0.53545	0.32720	0.54286	0.70639
56	0.02104	-0.02551	0.48982	0.05301	0.10410	0.54146	0.32857	0.59389	0.72371
57	0.02117	0.02449	0.50977	0.05308	0.11766	0.54683	0.32926	0.61966	0.73226
58	0.02131	0.07644	0.53046	0.05325	0.15171	0.56029	0.33203	0.72282	0.76510
59	0.02134	0.08877	0.53537	0.05331	0.16418	0.56521	0.33225	0.73081	0.76755
60	0.02140	0.11164	0.54444	0.05333	0.16701	0.56632	0.33335	0.77212	0.77998
61	0.02148	0.14402	0.55726	0.05337	0.17433	0.56920	0.33403	0.79747	0.78741
62	0.02158	0.18111	0.57186	0.05357	0.21507	0.58514	0.33546	0.85065	0.80252
63	0.02166	0.21034	0.58330	0.05369	0.23765	0.59392	0.33747	0.92531	0.82260
64	0.02189	0.29843	0.61731	0.05371	0.24087	0.59517	0.33792	0.94237	0.82700
65	0.02194	0.31994	0.62549	0.05375	0.24910	0.59836	0.33977	1.01116	0.84403
66	0.02201	0.34574	0.63523	0.05377	0.25307	0.59989	0.34000	1.01959	0.84604
67	0.02202	0.34995	0.63681	0.05388	0.27482	0.60827	0.34233	1.10654	0.86575
68	0.02224	0.43524	0.66831	0.05413	0.32462	0.62726	0.34683	1.27405	0.89868
69	0.02230	0.45563	0.67567	0.05416	0.32954	0.62913	0.34925	1.36426	0.91376
70	0.02240	0.49700	0.69040	0.05424	0.34614	0.63538	0.35312	1.50835	0.93427
71	0.02245	0.51675	0.69733	0.05433	0.36258	0.64154	0.35342	1.51956	0.93569
72	0.02250	0.53428	0.70343	0.05434	0.36549	0.64263	0.35555	1.59909	0.94510
73	0.02264	0.58660	0.72126	0.05467	0.42952	0.66623	0.35663	1.63910	0.94940
74	0.02280	0.64845	0.74165	0.05491	0.47680	0.68325	0.35811	1.69440	0.95491
75	0.02284	0.66388	0.74661	0.05504	0.50200	0.69217	0.35892	1.72446	0.95769
76	0.02298	0.71791	0.76359	0.05532	0.55627	0.71099	0.36368	1.90188	0.97141
77	0.02298	0.72026	0.76432	0.05538	0.56771	0.71488	0.37138	2.18867	0.98569
78	0.02300	0.72532	0.76587	0.05540	0.57221	0.71641			
79	0.02300	0.72689	0.76635	0.05564	0.61890	0.73201			
80	0.02304	0.74241	0.77108	0.05577	0.64396	0.74020			
81	0.02307	0.75279	0.77421	0.05594	0.67807	0.75114			
82	0.02311	0.76908	0.77908	0.05607	0.70362	0.75916			
83	0.02333	0.85321	0.80323	0.05611	0.70971	0.76106			
84	0.02342	0.88763	0.81263	0.05653	0.79266	0.78601			
85	0.02354	0.93561	0.82526	0.05659	0.80376	0.78923			
86	0.02359	0.95449	0.83008	0.05679	0.84390	0.80064			
87	0.02368	0.98644	0.83804	0.05705	0.89356	0.81422			
88	0.02391	1.07686	0.85923	0.05705	0.89407	0.81436			
89	0.02401	1.11643	0.86788	0.05724	0.93097	0.82407			
90	0.02404	1.12634	0.86999	0.05734	0.95126	0.82926			
91	0.02457	1.32909	0.90809	0.05758	0.99795	0.84085			

92	0.02482	1.42474	0.92288	0.05763	1.00788	0.84324
93	0.02488	1.44874	0.92630	0.05775	1.03107	0.84875
94	0.02488	1.45010	0.92648	0.05780	1.03994	0.85082
95	0.02493	1.46905	0.92909	0.05780	1.04143	0.85116
96	0.02502	1.50454	0.93378	0.05811	1.10111	0.86458
97	0.02515	1.55116	0.93957	0.05821	1.12077	0.86881
98	0.02522	1.58066	0.94302	0.05827	1.13324	0.87144
99	0.02538	1.64131	0.94963	0.05857	1.19208	0.88338
100	0.02541	1.65455	0.95099	0.05859	1.19590	0.88413
101	0.02559	1.72105	0.95738	0.05861	1.19901	0.88474
102	0.02613	1.92905	0.97314	0.05949	1.37086	0.91479
103	0.02653	2.08120	0.98129	0.05966	1.40397	0.91984
104	0.02673	2.15894	0.98457	0.06051	1.57089	0.94190
105				0.06132	1.72807	0.95801
106				0.06161	1.78526	0.96289
107				0.06167	1.79620	0.96377
108				0.06185	1.83161	0.96650
109				0.06323	2.10281	0.98226
110				0.06435	2.32053	0.98984

D.1.4. Step 4: Calculate Experimental CDF Values, CDF(i)

$$CDF(i) = \frac{i}{n} \quad \text{for } 1 < i < n$$

Equation 3

Table 4: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) ethanol internal quality controls ordered in ascending order, corresponding F(x) values, and CDF values

i	LC	$F(x)_{LC}$	CDF_{LC}	MC	$F(x)_{MC}$	CDF_{MC}	HC	$F(x)_{HC}$	CDF_{HC}
Theoretical Concentration (g/100 ml)	**0.02**			**0.05**			**0.3**		
1	0.01508	0.01022	0.00962	0.04175	0.01806	0.00909	0.26073	0.02657	0.012987
2	0.01536	0.01363	0.01923	0.04226	0.02300	0.01818	0.26258	0.03111	0.025974
3	0.01560	0.01705	0.02885	0.04263	0.02724	0.02727	0.26508	0.03825	0.038961
4	0.01603	0.02537	0.03846	0.04266	0.02755	0.03636	0.26621	0.04188	0.051948
5	0.01624	0.03064	0.04808	0.04294	0.03120	0.04545	0.26645	0.04268	0.064935
6	0.01674	0.04663	0.05769	0.04307	0.03307	0.05455	0.26834	0.04946	0.077922
7	0.01683	0.04991	0.06731	0.04325	0.03570	0.06364	0.27477	0.07921	0.090909
8	0.01713	0.06320	0.07692	0.04398	0.04844	0.07273	0.27573	0.08463	0.103896
9	0.01768	0.09352	0.08654	0.04454	0.06058	0.08182	0.27691	0.09164	0.116883
10	0.01774	0.09756	0.09615	0.04476	0.06590	0.09091	0.28232	0.12938	0.12987
11	0.01836	0.14559	0.10577	0.04515	0.07616	0.10000	0.28481	0.14998	0.142857
12	0.01847	0.15566	0.11538	0.04522	0.07833	0.10909	0.28514	0.15290	0.155844

13	0.01857	0.16429	0.12500	0.04572	0.09363	0.11818	0.28574	0.15820	0.168831
14	0.01871	0.17828	0.13462	0.04600	0.10294	0.12727	0.28606	0.16112	0.181818
15	0.01874	0.18114	0.14423	0.04623	0.11137	0.13636	0.28621	0.16248	0.194805
16	0.01876	0.18329	0.15385	0.04661	0.12579	0.14545	0.28773	0.17679	0.207792
17	0.01876	0.18356	0.16346	0.04720	0.15124	0.15455	0.28825	0.18191	0.220779
18	0.01887	0.19430	0.17308	0.04733	0.15739	0.16364	0.29021	0.20174	0.233766
19	0.01888	0.19539	0.18269	0.04751	0.16608	0.17273	0.29041	0.20387	0.246753
20	0.01902	0.21045	0.19231	0.04775	0.17812	0.18182	0.29228	0.22422	0.25974
21	0.01903	0.21199	0.20192	0.04775	0.17823	0.19091	0.29349	0.23794	0.272727
22	0.01907	0.21644	0.21154	0.04783	0.18199	0.20000	0.29517	0.25767	0.285714
23	0.01912	0.22219	0.22115	0.04804	0.19338	0.20909	0.29589	0.26648	0.298701
24	0.01915	0.22621	0.23077	0.04841	0.21350	0.21818	0.29960	0.31363	0.311688
25	0.01938	0.25344	0.24038	0.04860	0.22431	0.22727	0.29987	0.31727	0.324675
26	0.01945	0.26177	0.25000	0.04861	0.22517	0.23636	0.29988	0.31737	0.337662
27	0.01951	0.26993	0.25962	0.04867	0.22897	0.24545	0.30090	0.33103	0.350649
28	0.01952	0.27100	0.26923	0.04876	0.23396	0.25455	0.30095	0.33173	0.363636
29	0.01952	0.27118	0.27885	0.04890	0.24229	0.26364	0.30162	0.34087	0.376623
30	0.01958	0.27826	0.28846	0.04925	0.26467	0.27273	0.30333	0.36452	0.38961
31	0.01975	0.30124	0.29808	0.04958	0.28613	0.28182	0.30481	0.38543	0.402597
32	0.01977	0.30346	0.30769	0.04958	0.28620	0.29091	0.30628	0.40656	0.415584
33	0.01981	0.30853	0.31731	0.04978	0.29908	0.30000	0.30718	0.41956	0.428571
34	0.01990	0.32083	0.32692	0.04982	0.30236	0.30909	0.30892	0.44506	0.441558
35	0.01995	0.32732	0.33654	0.05000	0.31466	0.31818	0.30921	0.44936	0.454545
36	0.01995	0.32763	0.34615	0.05003	0.31662	0.32727	0.30996	0.46045	0.467532
37	0.01999	0.33341	0.35577	0.05005	0.31795	0.33636	0.31136	0.48116	0.480519
38	0.02000	0.33455	0.36538	0.05015	0.32469	0.34545	0.31170	0.48617	0.493506
39	0.02000	0.33528	0.37500	0.05049	0.34927	0.35455	0.31292	0.50438	0.506494
40	0.02002	0.33709	0.38462	0.05058	0.35555	0.36364	0.31293	0.50443	0.519481
41	0.02011	0.34986	0.39423	0.05078	0.37012	0.37273	0.31330	0.50996	0.532468
42	0.02014	0.35497	0.40385	0.05078	0.37036	0.38182	0.31360	0.51441	0.545455
43	0.02020	0.36285	0.41346	0.05094	0.38257	0.39091	0.31374	0.51654	0.558442
44	0.02020	0.36321	0.42308	0.05099	0.38583	0.40000	0.31768	0.57463	0.571429
45	0.02029	0.37615	0.43269	0.05107	0.39237	0.40909	0.31874	0.58998	0.584416
46	0.02033	0.38200	0.44231	0.05117	0.39931	0.41818	0.32088	0.62074	0.597403
47	0.02034	0.38352	0.45192	0.05122	0.40338	0.42727	0.32194	0.63561	0.61039
48	0.02039	0.39109	0.46154	0.05187	0.45300	0.43636	0.32273	0.64668	0.623377
49	0.02041	0.39368	0.47115	0.05193	0.45748	0.44545	0.32331	0.65465	0.636364
50	0.02049	0.40660	0.48077	0.05197	0.46082	0.45455	0.32408	0.66514	0.649351
51	0.02051	0.40823	0.49038	0.05239	0.49316	0.46364	0.32550	0.68426	0.662338
52	0.02065	0.43051	0.50000	0.05250	0.50244	0.47273	0.32565	0.68619	0.675325
53	0.02075	0.44462	0.50962	0.05280	0.52562	0.48182	0.32571	0.68700	0.688312
54	0.02075	0.44534	0.51923	0.05291	0.53378	0.49091	0.32715	0.70576	0.701299
55	0.02086	0.46154	0.52885	0.05293	0.53545	0.50000	0.32720	0.70639	0.714286
56	0.02104	0.48982	0.53846	0.05301	0.54146	0.50909	0.32857	0.72371	0.727273

57	0.02117	0.50977	0.54808	0.05308	0.54683	0.51818	0.32926	0.73226	0.74026
58	0.02131	0.53046	0.55769	0.05325	0.56029	0.52727	0.33203	0.76510	0.753247
59	0.02134	0.53537	0.56731	0.05331	0.56521	0.53636	0.33225	0.76755	0.766234
60	0.02140	0.54444	0.57692	0.05333	0.56632	0.54545	0.33335	0.77998	0.779221
61	0.02148	0.55726	0.58654	0.05337	0.56920	0.55455	0.33403	0.78741	0.792208
62	0.02158	0.57186	0.59615	0.05357	0.58514	0.56364	0.33546	0.80252	0.805195
63	0.02166	0.58330	0.60577	0.05369	0.59392	0.57273	0.33747	0.82260	0.818182
64	0.02189	0.61731	0.61538	0.05371	0.59517	0.58182	0.33792	0.82700	0.831169
65	0.02194	0.62549	0.62500	0.05375	0.59836	0.59091	0.33977	0.84403	0.844156
66	0.02201	0.63523	0.63462	0.05377	0.59989	0.60000	0.34000	0.84604	0.857143
67	0.02202	0.63681	0.64423	0.05388	0.60827	0.60909	0.34233	0.86575	0.87013
68	0.02224	0.66831	0.65385	0.05413	0.62726	0.61818	0.34683	0.89868	0.883117
69	0.02230	0.67567	0.66346	0.05416	0.62913	0.62727	0.34925	0.91376	0.896104
70	0.02240	0.69040	0.67308	0.05424	0.63538	0.63636	0.35312	0.93427	0.909091
71	0.02245	0.69733	0.68269	0.05433	0.64154	0.64545	0.35342	0.93569	0.922078
72	0.02250	0.70343	0.69231	0.05434	0.64263	0.65455	0.35555	0.94510	0.935065
73	0.02264	0.72126	0.70192	0.05467	0.66623	0.66364	0.35663	0.94940	0.948052
74	0.02280	0.74165	0.71154	0.05491	0.68325	0.67273	0.35811	0.95491	0.961039
75	0.02284	0.74661	0.72115	0.05504	0.69217	0.68182	0.35892	0.95769	0.974026
76	0.02298	0.76359	0.73077	0.05532	0.71099	0.69091	0.36368	0.97141	0.987013
77	0.02298	0.76432	0.74038	0.05538	0.71488	0.70000	0.37138	0.98569	1
78	0.02300	0.76587	0.75000	0.05540	0.71641	0.70909			
79	0.02300	0.76635	0.75962	0.05564	0.73201	0.71818			
80	0.02304	0.77108	0.76923	0.05577	0.74020	0.72727			
81	0.02307	0.77421	0.77885	0.05594	0.75114	0.73636			
82	0.02311	0.77908	0.78846	0.05607	0.75916	0.74545			
83	0.02333	0.80323	0.79808	0.05611	0.76106	0.75455			
84	0.02342	0.81263	0.80769	0.05653	0.78601	0.76364			
85	0.02354	0.82526	0.81731	0.05659	0.78923	0.77273			
86	0.02359	0.83008	0.82692	0.05679	0.80064	0.78182			
87	0.02368	0.83804	0.83654	0.05705	0.81422	0.79091			
88	0.02391	0.85923	0.84615	0.05705	0.81436	0.80000			
89	0.02401	0.86788	0.85577	0.05724	0.82407	0.80909			
90	0.02404	0.86999	0.86538	0.05734	0.82926	0.81818			
91	0.02457	0.90809	0.87500	0.05758	0.84085	0.82727			
92	0.02482	0.92288	0.88462	0.05763	0.84324	0.83636			
93	0.02488	0.92630	0.89423	0.05775	0.84875	0.84545			
94	0.02488	0.92648	0.90385	0.05780	0.85082	0.85455			
95	0.02493	0.92909	0.91346	0.05780	0.85116	0.86364			
96	0.02502	0.93378	0.92308	0.05811	0.86458	0.87273			
97	0.02515	0.93957	0.93269	0.05821	0.86881	0.88182			
98	0.02522	0.94302	0.94231	0.05827	0.87144	0.89091			
99	0.02538	0.94963	0.95192	0.05857	0.88338	0.90000			
100	0.02541	0.95099	0.96154	0.05859	0.88413	0.90909			

101	0.02559	0.95738	0.97115	0.05861	0.88474	0.91818
102	0.02613	0.97314	0.98077	0.05949	0.91479	0.92727
103	0.02653	0.98129	0.99038	0.05966	0.91984	0.93636
104	0.02673	0.98457	1.00000	0.06051	0.94190	0.94545
105				0.06132	0.95801	0.95455
106				0.06161	0.96289	0.96364
107				0.06167	0.96377	0.97273
108				0.06185	0.96650	0.98182
109				0.06323	0.98226	0.99091
110				0.06435	0.98984	1.00000

D.1.5. Step 5: Plot Sample Data versus CDF and F values

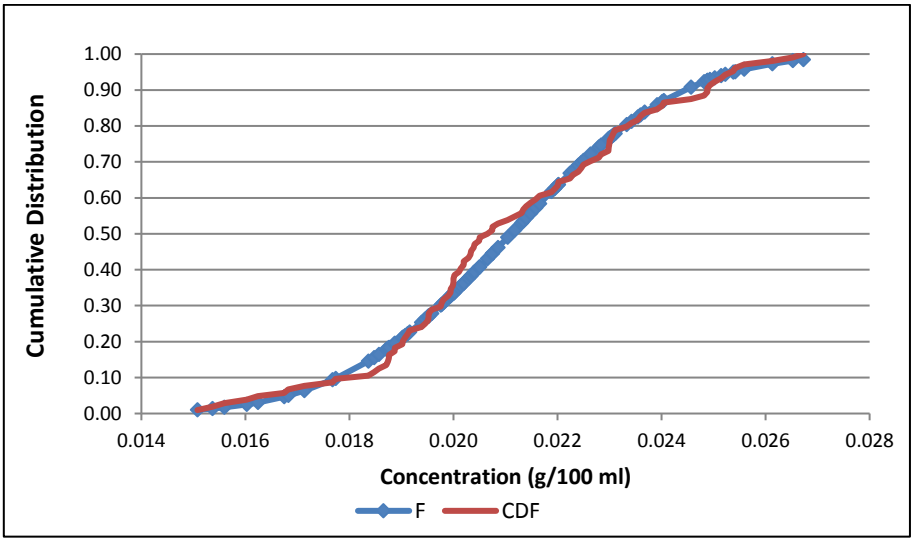

Figure 1: Plot of ethanol concentration (g/100 ml) versus F and CDF for the LC data

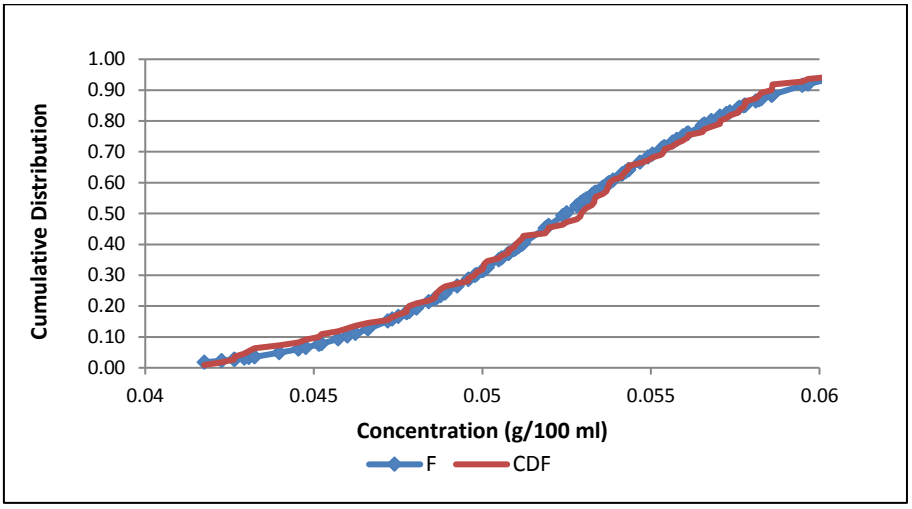

Figure 2: Plot of ethanol concentration (g/100 ml) versus F and CDF for the MC data

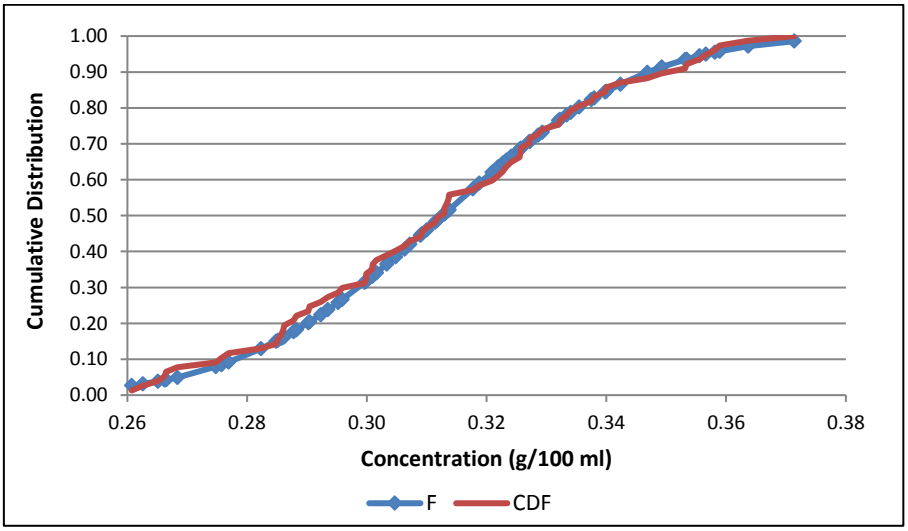

Figure 3: Plot of ethanol concentration (g/100 ml) versus F and CDF for the HC data

D.1.6. Step 6: Calculate Differences Between CDF and F values, D

$$D_i = |CDF_i - F(x_i)| \quad \text{for } 1 < i < n \qquad \text{Equation 4}$$

Table 5: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) ethanol internal quality controls ordered in ascending order, and corresponding differences between CDF and F values, D

i	LC	D_{LC}	MC	D_{MC}	HC	D_{HC}
Theoretical Concentration (g/100 ml)	**0.02**		**0.05**		**0.30**	
1	0.01508	0.000602	0.041749	0.008974	0.260725	0.013585
2	0.01536	0.005605	0.042261	0.004815	0.262579	0.005138
3	0.01560	0.011801	0.042631	3.51E-05	0.265083	0.000712
4	0.01603	0.013095	0.042657	0.008812	0.266214	0.010068
5	0.01624	0.017433	0.042936	0.014254	0.266451	0.022258
6	0.01674	0.011061	0.043069	0.021473	0.268339	0.028459
7	0.01683	0.0174	0.043246	0.02794	0.274772	0.011703
8	0.01713	0.013719	0.043976	0.024291	0.275734	0.019266
9	0.01768	0.006986	0.044541	0.021237	0.276911	0.025245
10	0.01774	0.001408	0.04476	0.025009	0.282317	0.000487
11	0.01836	0.039822	0.045147	0.023843	0.284809	0.007128
12	0.01847	0.040279	0.045224	0.030762	0.285142	0.002947
13	0.01857	0.039294	0.045724	0.024547	0.285737	0.010635
14	0.01871	0.043661	0.045999	0.02433	0.28606	0.020699
15	0.01874	0.036907	0.046233	0.024991	0.28621	0.032321
16	0.01876	0.029449	0.046605	0.019666	0.287729	0.031007
17	0.01876	0.020096	0.047196	0.003308	0.288255	0.038868
18	0.01887	0.021224	0.047328	0.006249	0.290206	0.032028
19	0.01888	0.012693	0.04751	0.006651	0.290408	0.042884
20	0.01902	0.018142	0.047752	0.003694	0.292283	0.035524
21	0.01903	0.01007	0.047754	0.01268	0.293493	0.034786
22	0.01907	0.004905	0.047827	0.018008	0.295168	0.028045
23	0.01912	0.001039	0.048043	0.015709	0.295894	0.03222
24	0.01915	0.004558	0.048408	0.004677	0.299596	0.001941
25	0.01938	0.013056	0.048595	0.002963	0.299871	0.007405
26	0.01945	0.011769	0.04861	0.011195	0.299878	0.020291
27	0.01951	0.010316	0.048674	0.01648	0.300898	0.01962
28	0.01952	0.001772	0.048758	0.020586	0.30095	0.031909
29	0.01952	0.007666	0.048896	0.021347	0.301622	0.035756
30	0.01958	0.010206	0.049254	0.008061	0.303332	0.025087
31	0.01975	0.003164	0.049583	0.004317	0.304812	0.017172
32	0.01977	0.00423	0.049584	0.004707	0.306284	0.009027
33	0.01981	0.008777	0.049776	0.000921	0.30718	0.009009

34	0.01990	0.006091	0.049824	0.006728	0.308921	0.003499
35	0.01995	0.00922	0.050003	0.003523	0.309213	0.005188
36	0.01995	0.018524	0.050031	0.010658	0.309964	0.007087
37	0.01999	0.022362	0.05005	0.018416	0.311362	0.000643
38	0.02000	0.030832	0.050146	0.020766	0.311699	0.007341
39	0.02000	0.039724	0.050491	0.005273	0.312925	0.00211
40	0.02002	0.047525	0.050577	0.008082	0.312927	0.015054
41	0.02011	0.044366	0.050776	0.002608	0.3133	0.02251
42	0.02014	0.048876	0.050779	0.011458	0.313599	0.031044
43	0.02020	0.050611	0.050944	0.008337	0.313743	0.0419
44	0.02020	0.059866	0.050988	0.014173	0.31768	0.003202
45	0.02029	0.056547	0.051075	0.016723	0.318736	0.005564
46	0.02033	0.060307	0.051167	0.018876	0.320881	0.023333
47	0.02034	0.0684	0.051221	0.023891	0.321937	0.025224
48	0.02039	0.070453	0.051869	0.016635	0.322732	0.023303
49	0.02041	0.077476	0.051926	0.012029	0.32331	0.018289
50	0.02049	0.074165	0.051969	0.006274	0.324078	0.015785
51	0.02051	0.082151	0.052385	0.029524	0.325504	0.021918
52	0.02065	0.069492	0.052504	0.029708	0.32565	0.010864
53	0.02075	0.064996	0.052802	0.043802	0.325711	0.001312
54	0.02075	0.073887	0.052907	0.042869	0.327152	0.004466
55	0.02086	0.067306	0.052928	0.03545	0.327201	0.007898
56	0.02104	0.048639	0.053006	0.032366	0.32857	0.003565
57	0.02117	0.038307	0.053075	0.028649	0.329262	0.007999
58	0.02131	0.027229	0.053249	0.033021	0.332031	0.011858
59	0.02134	0.03194	0.053313	0.028842	0.332246	0.001319
60	0.02140	0.032479	0.053328	0.020862	0.333354	0.000757
61	0.02148	0.029283	0.053365	0.01465	0.334035	0.004797
62	0.02158	0.024294	0.053574	0.021506	0.335462	0.002677
63	0.02166	0.022469	0.053689	0.021196	0.337466	0.004415
64	0.02189	0.001926	0.053706	0.013354	0.337924	0.00417
65	0.02194	0.000493	0.053748	0.00745	0.33977	0.000126
66	0.02201	0.000617	0.053768	0.000106	0.339997	0.011103
67	0.02202	0.007417	0.053879	0.000818	0.342331	0.004375
68	0.02224	0.014461	0.054134	0.009082	0.346827	0.015561
69	0.02230	0.012209	0.054159	0.001855	0.349248	0.017652
70	0.02240	0.017328	0.054244	0.000982	0.353116	0.025177
71	0.02245	0.014641	0.054329	0.003915	0.353417	0.013612
72	0.02250	0.01112	0.054343	0.01192	0.355551	0.010035
73	0.02264	0.01934	0.054671	0.002591	0.356625	0.001352
74	0.02280	0.030116	0.054913	0.010522	0.358109	0.006134
75	0.02284	0.025461	0.055042	0.010347	0.358916	0.016338
76	0.02298	0.032825	0.05532	0.020076	0.363679	0.015606
77	0.02298	0.023933	0.055378	0.014884	0.371376	0.014311

78	0.02300	0.015872	0.055401	0.007319
79	0.02300	0.006737	0.05564	0.013826
80	0.02304	0.001849	0.055769	0.012927
81	0.02307	0.004634	0.055943	0.014774
82	0.02311	0.009384	0.056074	0.01371
83	0.02333	0.00515	0.056105	0.006514
84	0.02342	0.004937	0.05653	0.022376
85	0.02354	0.007955	0.056586	0.016506
86	0.02359	0.00316	0.056792	0.018819
87	0.02368	0.001503	0.057046	0.023312
88	0.02391	0.013075	0.057049	0.014358
89	0.02401	0.012111	0.057237	0.014975
90	0.02404	0.004605	0.057341	0.011081
91	0.02457	0.033091	0.05758	0.013575
92	0.02482	0.038268	0.057631	0.006881
93	0.02488	0.032065	0.05775	0.003292
94	0.02488	0.022638	0.057795	0.003729
95	0.02493	0.015628	0.057803	0.012473
96	0.02502	0.010702	0.058108	0.008151
97	0.02515	0.006876	0.058209	0.013011
98	0.02522	0.000714	0.058273	0.019466
99	0.02538	0.002289	0.058574	0.016616
100	0.02541	0.010546	0.058593	0.024959
101	0.02559	0.013775	0.058609	0.033445
102	0.02613	0.007631	0.059489	0.012482
103	0.02653	0.009093	0.059658	0.016527
104	0.02673	0.015427	0.060512	0.003559
105			0.061317	0.003467
106			0.06161	0.000746
107			0.061665	0.008959
108			0.061847	0.015323
109			0.063235	0.00865
110			0.064349	0.010156

D.1.7. Step 7: Find Largest Difference

Table 6: Largest Differences for the LC, MC and HC ethanol internal quality controls

	LC	MC	HC
Theoretical Concentration (g/100 ml)	0.02	0.05	0.3
D_T	0.08215	0.04380	0.04288

D.1.8. Step 8: Compare Largest Difference With Critical Difference Value

For data sets of over 50, the critical difference value, D_C, at 95% confidence is calculated by

$$D_C = \frac{1.35810}{\sqrt{n}} \qquad \text{Equation 5}$$

When D_T is less than D_C the sample set is considered to be normally distributed.

Table 7: Largest Differences and Critical Differences for the LC, MC, and HC ethanol internal quality control values

	LC	MC	HC
Theoretical Concentration (g/100 ml)	**0.02**	**0.05**	**0.3**
D_T	0.08215	0.04380	0.04288
D_C	0.13336	0.12967	0.15499
Distribution	**Normal**	**Normal**	**Normal**

D.2. Fluoride

D.2.1. Step 1: Order Data in Ascending Order

Table 8: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) fluoride internal quality controls ordered in ascending order

	LC	MC	HC
Theoretical Concentration (g/100 ml)	**0.300**	**1.00**	**2.75**
1	0.27353	0.91456	2.19099
2	0.27777	0.91456	2.20775
3	0.27777	0.91808	2.21617
4	0.28100	0.93948	2.23312
5	0.28208	0.95037	2.28474
6	0.29465	0.96088	2.29907
7	0.29579	0.97342	2.33633
8	0.29693	0.97584	2.34537
9	0.29808	0.97713	2.37081
10	0.30003	0.98324	2.37081
11	0.30117	0.98324	2.37261
12	0.30234	0.98341	2.37261
13	0.30234	0.98702	2.37993
14	0.30272	0.98702	2.42834
15	0.30350	0.99082	2.42834
16	0.30585	0.99104	2.43774
17	0.30813	0.99371	2.44684

18	0.30813	0.99487	2.46364
19	0.30813	0.99754	2.46576
20	0.31049	0.99754	2.55271
21	0.31927	1.00139	2.60856
22	0.32297	1.01303	2.62868
23	0.32297	1.01507	2.65915
24	0.32297	1.01894	2.68997
25	0.32671	1.01894	
μ	0.30181	0.97925	2.40792
σ	0.01499	0.03065	0.14125

D.2.2. Step 2: Calculate Standardized Normal Variable, z

Table 9: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) fluoride internal quality controls ordered in ascending order, and the corresponding z-values

	LC	**z (LC)**	**MC**	**z (MC)**	**HC**	**z (HC)**
Theoretical Concentration (g/100 ml)	**0.300**		**1.00**		**2.75**	
1	0.27353	-1.88748	0.91456	-2.11055	2.19099	-1.53581
2	0.27777	-1.60416	0.91456	-2.11055	2.20775	-1.41719
3	0.27777	-1.60416	0.91808	-1.99572	2.21617	-1.35754
4	0.28100	-1.38880	0.93948	-1.29741	2.23312	-1.23756
5	0.28208	-1.31645	0.95037	-0.94217	2.28474	-0.87208
6	0.29465	-0.47815	0.96088	-0.59922	2.29907	-0.77064
7	0.29579	-0.40205	0.97342	-0.19011	2.33633	-0.50687
8	0.29693	-0.32566	0.97584	-0.11104	2.34537	-0.44285
9	0.29808	-0.24897	0.97713	-0.06891	2.37081	-0.26272
10	0.30003	-0.11917	0.98324	0.13022	2.37081	-0.26272
11	0.30117	-0.04291	0.98324	0.13022	2.37261	-0.25000
12	0.30234	0.03523	0.98341	0.13590	2.37261	-0.25000
13	0.30234	0.03523	0.98702	0.25369	2.37993	-0.19813
14	0.30272	0.06075	0.98702	0.25369	2.42834	0.14460
15	0.30350	0.11288	0.99082	0.37763	2.42834	0.14460
16	0.30585	0.26907	0.99104	0.38475	2.43774	0.21114
17	0.30813	0.42165	0.99371	0.47188	2.44684	0.27555
18	0.30813	0.42165	0.99487	0.50989	2.46364	0.39452
19	0.30813	0.42165	0.99754	0.59697	2.46576	0.40949
20	0.31049	0.57887	0.99754	0.59697	2.55271	1.02513
21	0.31927	1.16473	1.00139	0.72254	2.60856	1.42054
22	0.32297	1.41162	1.01303	1.10218	2.62868	1.56297
23	0.32297	1.41162	1.01507	1.16880	2.65915	1.77868
24	0.32297	1.41162	1.01894	1.29518	2.68997	1.99689
25	0.32671	1.66138	1.01894	1.29518		

D.2.3. Step 3: Calculate Theoretical Normal Cumulative Distribution Values, F(x)

Table 10: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) fluoride internal quality controls ordered in ascending order, corresponding z-values and F(x) values

	LC	z (LC)	$F(x)_{LC}$	MC	$F(x)_{MC}$	z (MC)	HC	z (HC)	$F(x)_{HC}$
Theoretical Concentration (g/100 ml)	**0.300**			**1.00**			**2.75**		
1	0.27353	-1.88748	0.02955	0.91456	0.01741	-2.11055	2.19099	-1.53581	0.062292
2	0.27777	-1.60416	0.05434	0.91456	0.01741	-2.11055	2.20775	-1.41719	0.078213
3	0.27777	-1.60416	0.05434	0.91808	0.02298	-1.99572	2.21617	-1.35754	0.087304
4	0.28100	-1.38880	0.08245	0.93948	0.09725	-1.29741	2.23312	-1.23756	0.107939
5	0.28208	-1.31645	0.09401	0.95037	0.17305	-0.94217	2.28474	-0.87208	0.191581
6	0.29465	-0.47815	0.31627	0.96088	0.27451	-0.59922	2.29907	-0.77064	0.220461
7	0.29579	-0.40205	0.34382	0.97342	0.42461	-0.19011	2.33633	-0.50687	0.306122
8	0.29693	-0.32566	0.37234	0.97584	0.45579	-0.11104	2.34537	-0.44285	0.328936
9	0.29808	-0.24897	0.40169	0.97713	0.47253	-0.06891	2.37081	-0.26272	0.396383
10	0.30003	-0.11917	0.45257	0.98324	0.55180	0.13022	2.37081	-0.26272	0.396383
11	0.30117	-0.04291	0.48289	0.98324	0.55180	0.13022	2.37261	-0.25000	0.401296
12	0.30234	0.03523	0.51405	0.98341	0.55405	0.13590	2.37261	-0.25000	0.401296
13	0.30234	0.03523	0.51405	0.98702	0.60013	0.25369	2.37993	-0.19813	0.421473
14	0.30272	0.06075	0.52422	0.98702	0.60013	0.25369	2.42834	0.14460	0.557488
15	0.30350	0.11288	0.54494	0.99082	0.64715	0.37763	2.42834	0.14460	0.557488
16	0.30585	0.26907	0.60606	0.99104	0.64979	0.38475	2.43774	0.21114	0.583613
17	0.30813	0.42165	0.66336	0.99371	0.68149	0.47188	2.44684	0.27555	0.608553
18	0.30813	0.42165	0.66336	0.99487	0.69494	0.50989	2.46364	0.39452	0.653402
19	0.30813	0.42165	0.66336	0.99754	0.72474	0.59697	2.46576	0.40949	0.658909
20	0.31049	0.57887	0.71866	0.99754	0.72474	0.59697	2.55271	1.02513	0.847349
21	0.31927	1.16473	0.87794	1.00139	0.76502	0.72254	2.60856	1.42054	0.922274
22	0.32297	1.41162	0.92097	1.01303	0.86481	1.10218	2.62868	1.56297	0.94097
23	0.32297	1.41162	0.92097	1.01507	0.87876	1.16880	2.65915	1.77868	0.962354
24	0.32297	1.41162	0.92097	1.01894	0.90237	1.29518	2.68997	1.99689	0.977082
25	0.32671	1.66138	0.95168	1.01894	0.90237	1.29518			

D.2.4. Step 4: Calculate Experimental CDF Values, CDF(i)

Table 11: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) fluoride internal quality controls ordered in ascending order, corresponding F(x) values, and CDF values

	LC	$F(x)_{LC}$	CDF_{LC}	MC	$F(x)_{MC}$	CDF_{MC}	HC	$F(x)_{HC}$	CDF_{HC}
Theoretical Concentration (g/100 ml)	**0.300**			**1.00**			**2.75**		
1	0.27353	0.02955	0.040	0.91456	0.01741	0.04	2.19099	0.06229	0.042
2	0.27777	0.05434	0.080	0.91456	0.01741	0.08	2.20775	0.07821	0.083
3	0.27777	0.05434	0.120	0.91808	0.02298	0.12	2.21617	0.08730	0.125

4	0.28100	0.08245	0.160	0.93948	0.09725	0.16	2.23312	0.10794	0.167
5	0.28208	0.09401	0.200	0.95037	0.17305	0.20	2.28474	0.19158	0.208
6	0.29465	0.31627	0.240	0.96088	0.27451	0.24	2.29907	0.22046	0.250
7	0.29579	0.34382	0.280	0.97342	0.42461	0.28	2.33633	0.30612	0.292
8	0.29693	0.37234	0.320	0.97584	0.45579	0.32	2.34537	0.32894	0.333
9	0.29808	0.40169	0.360	0.97713	0.47253	0.36	2.37081	0.39638	0.375
10	0.30003	0.45257	0.400	0.98324	0.55180	0.40	2.37081	0.39638	0.417
11	0.30117	0.48289	0.440	0.98324	0.55180	0.44	2.37261	0.40130	0.458
12	0.30234	0.51405	0.480	0.98341	0.55405	0.48	2.37261	0.40130	0.500
13	0.30234	0.51405	0.520	0.98702	0.60013	0.52	2.37993	0.42147	0.542
14	0.30272	0.52422	0.560	0.98702	0.60013	0.56	2.42834	0.55749	0.583
15	0.30350	0.54494	0.600	0.99082	0.64715	0.60	2.42834	0.55749	0.625
16	0.30585	0.60606	0.640	0.99104	0.64979	0.64	2.43774	0.58361	0.667
17	0.30813	0.66336	0.680	0.99371	0.68149	0.68	2.44684	0.60855	0.708
18	0.30813	0.66336	0.720	0.99487	0.69494	0.72	2.46364	0.65340	0.750
19	0.30813	0.66336	0.760	0.99754	0.72474	0.76	2.46576	0.65891	0.792
20	0.31049	0.71866	0.800	0.99754	0.72474	0.80	2.55271	0.84735	0.833
21	0.31927	0.87794	0.840	1.00139	0.76502	0.84	2.60856	0.92227	0.875
22	0.32297	0.92097	0.880	1.01303	0.86481	0.88	2.62868	0.94097	0.917
23	0.32297	0.92097	0.920	1.01507	0.87876	0.92	2.65915	0.96235	0.958
24	0.32297	0.92097	0.960	1.01894	0.90237	0.96	2.68997	0.97708	1.000
25	0.32671	0.95168	1.000	1.01894	0.90237	1.00			

D.2.5. Step 5: Plot Sample Data versus CDF and F values

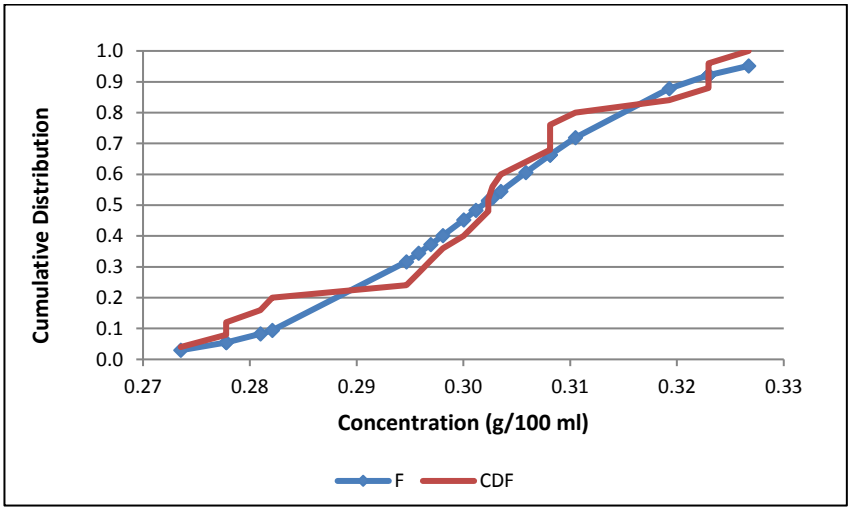

Figure 4: Plot of fluoride concentration (g/100 ml) versus F and CDF for the LC data

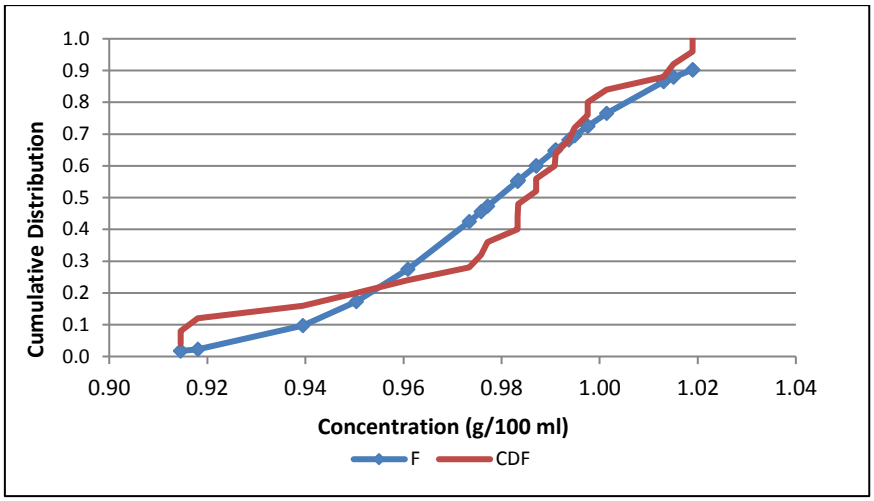

Figure 5: Plot of fluoride concentration (g/100 ml) versus F and CDF for the MC data

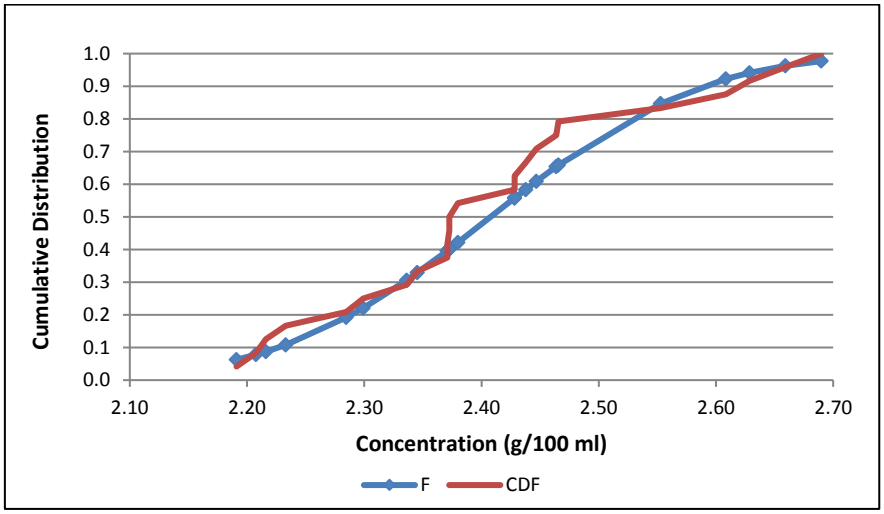

Figure 6: Plot of fluoride concentration (g/100 ml) versus F and CDF for the HC data

D.2.6. Step 6: Calculate Differences Between CDF and F values, D

Table 12: Control data for the Low Control (LC), Medium Control (MC) and High Control (HC) fluoride internal quality controls ordered in ascending order, and corresponding differences between CDF and F values, D

	LC	D_{LC}	MC	D_{MC}	HC	D_{HC}
Theoretical Concentration (g/100 ml)	**0.300**		**1.00**		**2.75**	
1	0.27353	0.01045	0.91456	0.02259	2.19099	0.02063
2	0.27777	0.02566	0.91456	0.06259	2.20775	0.00512
3	0.27777	0.06566	0.91808	0.09702	2.21617	0.03770
4	0.28100	0.07755	0.93948	0.06275	2.23312	0.05873
5	0.28208	0.10599	0.95037	0.02695	2.28474	0.01675
6	0.29465	0.07627	0.96088	0.03451	2.29907	0.02954
7	0.29579	0.06382	0.97342	0.14461	2.33633	0.01446
8	0.29693	0.05234	0.97584	0.13579	2.34537	0.00440
9	0.29808	0.04169	0.97713	0.11253	2.37081	0.02138
10	0.30003	0.05257	0.98324	0.15180	2.37081	0.02028

11	0.30117	0.04289	0.98324	0.11180	2.37261	0.05704
12	0.30234	0.03405	0.98341	0.07405	2.37261	0.09870
13	0.30234	0.00595	0.98702	0.08013	2.37993	0.12019
14	0.30272	0.03578	0.98702	0.04013	2.42834	0.02585
15	0.30350	0.05506	0.99082	0.04715	2.42834	0.06751
16	0.30585	0.03394	0.99104	0.00979	2.43774	0.08305
17	0.30813	0.01664	0.99371	0.00149	2.44684	0.09978
18	0.30813	0.05664	0.99487	0.02506	2.46364	0.09660
19	0.30813	0.09664	0.99754	0.03526	2.46576	0.13276
20	0.31049	0.08134	0.99754	0.07526	2.55271	0.01402
21	0.31927	0.03794	1.00139	0.07498	2.60856	0.04727
22	0.32297	0.04097	1.01303	0.01519	2.62868	0.02430
23	0.32297	0.00097	1.01507	0.04124	2.65915	0.00402
24	0.32297	0.03903	1.01894	0.05763	2.68997	0.02292
25	0.32671	0.04832	1.01894	0.09763		

D.2.7. Step 7: Find Largest Difference

Table 13: Largest Differences for the LC, MC and HC fluoride internal quality controls

	LC	**MC**	**HC**
Theoretical Concentration (g/100 ml)	0.300	1.00	2.75
D_T	0.10599	0.15180	0.13276

D.2.8. Step 8: Compare Largest Difference With Critical Difference Value

For data sets of under 50, the critical difference value, D_C, at 95% confidence is read from tables in literature. When D_T is less than D_C the sample set is considered to be normally distributed.

Table 14: Largest Differences and Critical Differences for the LC, MC, and HC fluoride internal quality control values

	LC	**MC**	**HC**
Theoretical Concentration (g/100 ml)	0.300	1.00	2.75
D_T	0.10599	0.15180	0.13276
D_C	0.26404	0.26404	0.26404
Distribution	**Normal**	**Normal**	**Normal**

Appendix E

Certificates of Analysis

1. **Aqueous Ethanol**
2. **Aqueous Sodium Fluoride**
3. **Anhydrous Sodium Fluoride**

nmisa
National Metrology Institute of South Africa

Private Bag X34, Lynnwood Ridge, Pretoria, 0040
Calibration office: +27 12 841 4623
Reception: +27 12 841 4152
Fax: +27 12 841 4458
E-mail enquiries: info@nmisa.org
www.nmisa.org

Certificate of Analysis

Analysis of:	Aqueous ethanol (Batch 0321/16)
Certified Reference Material:	NML-ORG-001
Analytical procedure:	ORG\MTD-0001
Date/s analysed:	11 - 12 August 2016

Certified value 20,909 ± 0,251[1] g/ 100 g

Notes:

1. The reported expanded uncertainty of measurement is stated as the standard uncertainty of measurement multiplied by a coverage factor of k=2, which for a normal distribution approximates a level of confidence of 95,45%.

   The expanded uncertainty is based on the combination of uncertainties associated with each individual operation involved in preparation and characterization of the material.

The reported uncertainties of measurement were calculated and expressed in accordance with the BIPM, IEC, IFCC, ILAC, ISO, IUPAC, IUPAP and OIML document entitled "A Guide to the Expression of Uncertainty in Measurement" (International Organisation for Standardisation, Geneva, Switzerland, 2008).

Analysed by R Visser **Metrologist (Technical Signatory)**	Checked by D Marajh **Metrologist**	For Chief Executive Officer
Date of issue **22 August 2016**	**Page 1 of 3**	Certificate number **Batch 0321/16** **Samples 01 - 14** **Copy no.** 3

1. PROCEDURE

1.1 Material Preparation

The solution was prepared by mixing known masses of pure ethanol and ultra pure water. The solution was thoroughly mixed and sub-sampled into amber glass bottles. The bottles were closed with tamper evident screw caps.

The ethanol in eight aliquots of the solution from selected containers was oxidised with known quantities of potassium dichromate in the presence of sulphuric acid. The excess dichromate was measured by titrimetry and the concentration of the ethanol determined according to procedure ORG\ MTD-0001.

1.2 Homogeneity assessment

The material was tested for homogeneity by analysis of selected samples. Eight aliquots of 2,6 grams each were analysed. The relative standard deviation of the eight results was 0,36%.

1.3 Stability assessment

Similar solutions were tested for stability over at least the certification period on samples kept unopened between 4°C and 10°C and on samples kept unopened at 20°C ± 2°C. No instability was observed.

2. INSTRUCTIONS FOR USE

The solutions are intended for the calibration of instruments and techniques used for the determination of ethanol in breath or blood. A minimum sample aliquot of 0,5 millilitre is recommended.

The solution should be allowed to equilibrate to ambient temperature (20 ± 3 °C) and mixed by inversion prior to use. Care should be exercised when handling the solution after the bottle has been opened to prevent evaporation and consequent changes in the concentration of the solution. The solution should be used within eight weeks of opening the bottle.

3. CERTIFICATION

The solution was certified at the National Metrology Institute of South Africa (NMISA) and involved gravimetric preparation of the solution, followed by the titrimetric determination of the concentration of eight sample aliquots. The certified value is the average of the prepared concentration and the eight titrimetric results.

4. ANALYTICAL METHODS

The ethanol solutions were prepared gravimetrically. Certification of ethanol and amount of substance content were established by titrimetry. The certified concentration is the mean of the eight titrimetric and gravimetric (20,825 g/ 100 g) values. The measurement values obtained are traceable to the kilogram.

5. STORAGE

The material should be stored in the dark at temperatures between 4°C and 10°C.

Analysed by R Visser **Metrologist (Technical Signatory)**	Checked by D Marajh **Metrologist**	For Chief Executive Officer
Date of issue **22 August 2016**	**Page 2 of 3**	Certificate number **Batch 0321/16** **Samples 01 - 14** **Copy no. 3**

6. SHELF LIFE

Provided that the sample is stored under the appropriate conditions, the certification will remain valid until 11 August 2018 (24 months from date of preparation). The certified reference material will be monitored over the period of its certification. If technical changes occur that affect the certification before the expiry date, the purchaser will be notified.

7. SAFETY

Please refer to the attached material safety data sheet (MSDS).

8. REMARKS

8.1 The analyses were carried out at an ambient temperature of 20°C ± 2°C and a relative humidity of 45% RH ± 20% RH.

8.2 The ethanol concentrations at different temperatures are as follows:

Temperature (°C)	Concentration (g/ 100 g)	Concentration (g/ 100 ml)
15	20,909	20,270
20	20,909	20,225
25	20,909	20,176
35	20,909	20,067
40	20,909	20,007

8.3 The results in this certificate relate only to the samples mentioned herein. The final certificate will be the property of the client and may be published by him, provided that it is published in full, or where only extracts therefrom or a summary or an abridgement thereof is published, the NMISA's prior written approval of the extracts, summary or abridged certificate be obtained.

8.4 Certain of the NMISA certificates are consistent with the capabilities that are included in appendix C of the MRA (Mutual Recognition Arrangement) drawn up by the CIPM. Under the MRA, all participating institutes recognise the validity of each other's calibration and measurement certificates for the quantities and ranges and measurement uncertainties specified in Appendix C. For details see http://www.bipm.org.

8.5 The results of the measurement are traceable to the relevant national measuring standards.

end of certificate

Analysed by R Visser **Metrologist (Technical Signatory)**	Checked by D Marajh **Metrologist**	For Chief Executive Officer
Date of issue **22 August 2016**	**Page 3 of 3**	Certificate number **Batch 0321/16** **Samples 01 - 14** **Copy no.** 3

MATERIAL SAFETY DATA SHEET REFERENCE MATERIAL NML-ORG-0001	May 2007

MANUFACTURER INFORMATION/ INFORMATION ON INGREDIENTS

Manufacturer: Chemical Metrology National Metrology Institute of South Africa (NMISA) P/ Bag X34, Lynnwood Ridge, 0040 Telephone number: +27 12 841 4078 Fax number: +27 12 841 2131	These solutions are intended for the calibration of instruments and techniques used for the determination of ethanol in breath or blood. They consist **of at least 99.5% water** with less than 0.5% of ethanol.

HAZARDS IDENTIFICATION

The product consists of at least **99.5% water** with a small percentage of ethanol (less than 0.5%) packed in amber glass bottles. At this concentration, the solutions are **non-flammable and non-explosive. The properties of the solution are mainly those of water**.

Main hazards	None
Other hazards	Not intended for human consumption

FIRST AID MEASURES (SYMPTOMS)

Skin contact	There may be mild irritation at the site of contact
Eye contact	There may be irritation and redness
Ingestion	There may be irritation of the throat
Inhalation	There may be irritation of the nose and throat

FIRST AID MEASURES (ACTION)

Skin contact	Wash with soap and plenty of water
Eye contact	Bathe the eye with running water for 15 minutes
Ingestion	Wash out mouth with water

FIRE FIGHTING MEASURES

Extinguishing media	Suitable extinguishing media for the surrounding fire should be used
Exposure hazards	Does not emit toxic fumes
Protection of fire fighters	Suitable protection for the surrounding fire should be used

ACCIDENTAL RELEASE MEASURES	
Personal precautions	See exposure controls/ personal protection. Turn leaking containers leak-side up to prevent escape of liquid
Environmental precautions	Contain the spillage with sand or earth. Not expected to cause problems to the environment
Clean-up procedures	Absorb into dry earth or sand. Transfer to a closable, labelled salvage container for disposal by an appropriate method

HANDLING AND STORAGE	
Handling	Do not smoke. Avoid the formations of mists in air
Storage	Store in a cool area. Keep container tightly closed. When used as a CRM, the produced should be stored between 4°C and 10°C to avoid change of composition
Packaging	Keep in original packaging

EXPOSURE CONTROLS/ PERSONAL PROTECTION	
Respiratory protection	Not required
Hand protection	Protective gloves
Eye protection	Safety glasses. Ensure eye bath is at hand
Skin protection	Protective clothing

PHYSICAL AND CHEMICAL PROPERTIES	
State	Liquid
Colour	Colourless
Oxidising	Non-oxidising
Soluble in water	Miscible with water in all proportions
Boiling point, °C	100
Melting point	n/a
Danger of explosion	Not explosive
Self-ignitability	Not self-igniting

STABILITY AND REACTIVITY	
Stability	Stable under normal conditions
Hazardous decomposition products	None
Hazardous polymerization	Will not occur
Conditions to avoid	Heat (to prevent evaporation and change in composition)

TOXICOLOGY	
Routes of exposure	See above

ECOLOGICAL INFORMATION	
Mobility	Readily absorbed into soil
Persistence and degradability	Biodegradable
Bioaccumulative potential	No data available
Other adverse effects	Negligible ecotoxicity

DISPOSAL CONSIDERATIONS	
Disposal operations	See accidental release measures. Consult regional or national regulations regarding disposal

TRANSPORT INFORMATION	
ADR/ RID	
UN no	None
Shipping name	Not subject to ADR
IMDG/ IMO	
UN no	None
IATA/ ICAO	
UN no	None

REGULATORY INFORMATION	
Hazardous symbols	None
Risk phases	None
Safety phases	Not classified as dangerous for supply

nmisa
National Metrology Institute of South Africa

Certificate of Analysis

Private Bag X34, Lynnwood Ridge, Pretona, 0040
Calibration office: +27 12 841 4623
Reception: +27 12 841 4152
Fax: +27 12 841 4458
E-mail enquiries: info@nmisa.org
www.nmisa.org

Analysis of:	Aqueous sodium fluoride solution (Batch 0154/15)
Certified Reference Material:	NML-ORG-002
Analytical procedure:	NML-ORG\MTD-0023
Date/s analysed:	25 August 2015

Certified value 2,997 ± 0,075[1] g/ 100 ml (at 20°C ± 2°C)

Notes:

[1] The reported expanded uncertainty of measurement is stated as the standard uncertainty of measurement multiplied by a coverage factor of k = 2 which for a normal distribution approximates a level of confidence of 95%.

The expanded uncertainty is based on the combination of uncertainties associated with each individual operation involved in preparation and characterization of the material.

The reported uncertainties of measurement were calculated and expressed in accordance with the BIPM, IEC, ISO, IUPAP, OIML document entitled "A Guide to the Expression of Uncertainty in Measurement" (International Organisation for Standardisation, Geneva, Switzerland, 1995).

Analysed by **DN Marajh** **Metrologist**	Checked by **M Archer** **Metrologist (Technical Signatory)**	For Chief Executive Officer
Date of issue **1 September 2015**	**Page 1 of 3**	Certificate number **Batch 0154/15** **Sample nos. 01 – 09** **Copy no.** 04

Conditions under which the NMISA will perform work

In this document, reference to a service or services will include: calibration, measurement, analysis or conformance work performed by NMISA on behalf of the Applicant.

1. The NMISA is empowered by the Measurement Unit and Measurement Standards Act, Act No. 18 of 2006.
2. Services are carried out at the discretion of the NMISA, which reserves the right to decline any application for performance of services when deemed to be outside the scope of services of the NMISA.
3. Through acceptance of the original quotation, the Applicant agrees to the quoted fee and the conditions stated herein. In cases where the NMISA has not published the amount of the fee, the NMISA will in good faith give estimates of the time and cost of the service based upon its previous experience.
4. Payment is strictly 30 days from the date of invoice; or as mutually agreed in writing between the Applicant and the NMISA before the service commenced. The NMISA retains the right to ask for a deposit for international services.
5. Regarding certificates and reports:
    a. A certificate or report, as appropriate, will be furnished to the Applicant on completion of the service;
    b. Reports or certificates may be freely published by the Applicant provided that such publication is verbatim and in full;
    c. The NMISA reserves the right after the termination of a period of one year or any period agreed upon, to publish or report in whole or in part together with any comments or additional matter which is considered desirable but will not in general expect to exercise that right except as regards service results deemed to be of general interest;
    d. Additional certified copies of certificates or reports, or re-issued certificates or reports will be subject to an additional fee, as determined on a case by case basis.
6. All gauges, instruments, items of equipment, etc. sent by the Applicant for performance of services at the NMISA shall be delivered and collected at the Applicant's own cost and risk.
7. The NMISA cannot guarantee to complete the work within the estimated time and cost but will consult the Applicant if it becomes apparent that either estimate will be exceeded.
8. If a service is not completed because of defects or deficiencies in the item submitted by the applicant, an appropriate reduction in the fee may be allowed depending on the amount of work already performed. The normal practice will be to charge the fee in full.
9. The Applicant hereby consents that the legal liability of the NMISA with regard to any damage whatsoever or a mistake made by the NMISA in services performed for the Applicant will be limited to the original quoted fee.

VALIDITY OF CALIBRATION

The values in this certificate are correct at the time of the calibration. Subsequently the accuracy will depend on such factors as the care exercised in handling and use of the instrument and the frequency of its use. Recalibration should be performed after a period which has been chosen to ensure that the instrument's accuracy remains within the desired limits.

ANALYSIS OF SODIUM FLUORIDE SOLUTION BATCH 0154/15
2,997 ± 0,075 g/ 100 ml (at 20°C ± 2°C)

1. PROCEDURE

1.1 Material Preparation

The sodium fluoride used for the preparation of the solution was certified by titrimetry, a primary method, at NMISA using method ANA\MTD-0023. This involved the precipitation as lead chlorofluoride with a known excess of lead nitrate solution. The lead nitrate remaining in the solution after filtration was back-titrated with standard EDTA solution and the purity of the sodium fluoride was calculated.

The solution was prepared by mixing known masses of pure sodium fluoride and ultra-pure water. The solution was thoroughly mixed and sub-sampled into polyethylene bottles. The bottles were closed with tamper evident screw caps.

The sub-divided batch was assayed by method NML-ANA\MTD-0023. The fluoride was precipitated as lead chlorofluoride by treating four aliquots from four separate sample bottles with a known excess of standard lead nitrate solution. The lead nitrate remaining in the solution after filtration was back-titrated with standard EDTA solution and the concentration of the sodium fluoride was calculated.

The results of the measurement are traceable to the relevant national measurement standards.

1.2 Homogeneity assessment

The material was tested for homogeneity by analysis of four separate aliquots of 1,0 millilitres each. The relative standard deviation for the four results was 0,3%.

1.3 Stability assessment

Similar solutions were tested for stability over at least the certification period on samples kept at 20°C ± 2°C. No instability was observed.

2. INSTRUCTIONS FOR USE

The solution is intended for the calibration of fluoride ion selective electrodes. A minimum sample aliquot of 1,5 millilitres is recommended.

The solution should be allowed to equilibrate to ambient temperature (20 ± 3 °C) and mixed by inversion prior to use. Care should be exercised when handling the solution after the bottle has been opened to prevent contamination of the solution.

3. CERTIFICATION

The solution was certified at the National Metrology Institute of South Africa (NMISA) and involved gravimetric preparation of the solution, followed by the titrimetric determination of the concentration of four sample aliquots to confirm the prepared concentration. The certified value is the prepared (gravimetric) concentration.

Analysed by	Checked by	For Chief Executive Officer
DN Marajh **Metrologist**	**M Archer** **Metrologist (Technical Signatory)**	
Date of issue **1 September 2015**	**Page 2 of 3**	Certificate number **Batch 0154/15** **Sample nos. 01 – 09** **Copy no.** 04

4. ANALYTICAL METHODS

The sodium fluoride solutions were prepared gravimetrically. Certification of fluoride content and amount of substance content were established by titrimetry. The measurement values obtained are traceable to recognised national standards and to units realised at international measurement institutes.

5. STORAGE

The material should be stored tightly closed at room temperature (20 ± 4ºC).

6. SHELF LIFE

Provided that the sample is stored under the appropriate conditions, the certification of the unopened container will remain valid until 20 August 2017 (24 months from date of preparation). The certified reference material will be monitored over the period of its certification. If technical changes occur that affect the certification before the expiry date, the purchaser will be notified.

7. SAFETY

Please refer to the attached material safety data sheet (MSDS).

8. REMARKS

8.1 The analyses were carried out at an ambient temperature of 20°C ± 2°C and a relative humidity of 45% RH ± 20% RH.

8.2 The results in this certificate relate only to the samples mentioned herein. The final certificate will be the property of the client and may be published by him, provided that it is published in full, or where only extracts taken from or a summary or an abridgement thereof is published, the NMISA's prior written approval of the extracts, summary or abridged certificate be obtained.

8.3 Certain of the NMISA certificates are consistent with the capabilities that are included in appendix C of the MRA (Mutual Recognition Arrangement) drawn up by the CIPM. Under the MRA, all participating institutes recognise the validity of each other's calibration and measurement certificates for the quantities and ranges and measurement uncertainties specified in Appendix C. For details see http://www.bipm.org.

----------end of certificate----------

Analysed by	Checked by	For Chief Executive Officer
DN Marajh **Metrologist**	**M Archer** **Metrologist (Technical Signatory)**	
Date of issue **1 September 2015**	**Page 3 of 3**	Certificate number **Batch 0154/15** **Sample nos. 01 – 09** **Copy no.** 04

Certificate of Analysis

1.06449.0250 Sodium fluoride for analysis EMSURE® ACS,ISO,Reag. Ph Eur
Batch B1114149

	Spec. Values		Batch Values	
Assay (precipitative titration)	≥ 99.5	%	100.1	%
In water insoluble matter	≤ 0.01	%	≤ 0.01	%
Free alkali (as NaOH)	≤ 0.04	%	0.02	%
Free acid (as hydrofloric acid)	≤ 0.05	%	< 0.01	%
Chloride (Cl)	≤ 0.003	%	≤ 0.003	%
Hexafluorosilicate (SiF_6)	≤ 0.1	%	≤ 0.1	%
Sulphate (SO_4)	≤ 0.01	%	≤ 0.01	%
Sulfite (SO_3)	< 0.005	%	< 0.005	%
Heavy metals (as Pb)	≤ 0.001	%	< 0.001	%
Ca (Calcium)	≤ 0.004	%	< 0.001	%
Cu (Copper)	≤ 0.0002	%	< 0.0001	%
Fe (Iron)	≤ 0.002	%	< 0.001	%
K (Potassium)	≤ 0.02	%	≤ 0.02	%
Loss on drying (150 °C, 4 h)	≤ 0.2	%	< 0.1	%

Date of release (DD.MM.YYYY) 19.09.2014
Minimum shelf life (DD.MM.YYYY) 30.09.2019

Dr. Andreas Lang
Responsible laboratory manager quality control

This document has been produced electronically and is valid without a signature

SAFETY DATA SHEET
according to Regulation (EC) No. 1907/2006

Revision Date 04.08.2011 Version 19.0

SECTION 1. Identification of the substance/mixture and of the company/undertaking

1.1 Product identifier

Catalogue No.	106449
Product name	Sodium fluoride for analysis EMSURE® ACS,ISO,Reag. Ph Eur
REACH Registration Number	A registration number is not available for this substance as the substance or its use are exempted from registration according to Article 2 REACH Regulation (EC) No 1907/2006, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.

1.2 Relevant identified uses of the substance or mixture and uses advised against

Identified uses	Reagent for analysis For additional information on uses please refer to the Merck Chemicals portal (www.merck-chemicals.com).

1.3 Details of the supplier of the safety data sheet

Company	Merck KGaA * 64271 Darmstadt * Germany * Phone:+49 6151 72-0
Responsible Department	EQ-RS * e-mail: prodsafe@merckgroup.com

1.4 Emergency telephone number

Please contact the regional company representation in your country.

SECTION 2. Hazards identification

2.1 Classification of the substance or mixture

Classification (REGULATION (EC) No 1272/2008)

Acute toxicity, Category 3, Oral, H301
Skin irritation, Category 2, H315
Eye irritation, Category 2, H319

For the full text of the H-Statements mentioned in this Section, see Section 16.

Classification (67/548/EEC or 1999/45/EC)

T	Toxic	R25
Xi	Irritant	R36/38

For the full text of the R-phrases mentioned in this Section, see Section 16.

2.2 Label elements

Labelling (REGULATION (EC) No 1272/2008)

Hazard pictograms

Signal word
Danger

Hazard statements
H301 Toxic if swallowed.
H315 Causes skin irritation.
H319 Causes serious eye irritation.
EUH032 Contact with acids liberates very toxic gas.

Precautionary statements
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P302 + P352 IF ON SKIN: Wash with plenty of soap and water.
P309 + P310 IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

Reduced labelling (≤125 ml)

Hazard pictograms

Signal word
Danger

Hazard statements
H301 Toxic if swallowed.
EUH032 Contact with acids liberates very toxic gas.

Precautionary statements
P309 + P310 IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

Index-No. 009-004-00-7

Labelling (67/548/EEC or 1999/45/EC)

Symbol(s)	T	Toxic
R-phrase(s)	25-32-36/38	Toxic if swallowed. Contact with acids liberates very toxic gas. Irritating to eyes and skin.
S-phrase(s)	22-36-45	Do not breathe dust. Wear suitable protective clothing. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
EC-No.	231-667-8	EC Label

Reduced labelling (≤125 ml)

Symbol(s)	T	Toxic
R-phrase(s)	25-32	Toxic if swallowed. Contact with acids liberates very toxic gas.
S-phrase(s)	45	In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

2.3 Other hazards
None known.

SECTION 3. Composition/information on ingredients

Formula	NaF	FNa (Hill)
CAS-No.	7681-49-4	
Index-No.	009-004-00-7	

SECTION 4. First aid measures

4.1 Description of first aid measures

After inhalation: fresh air. Get medical attention.

After contact with skin: Rinse with plenty of water for at least 10 minutes. Immediately remove contaminated clothes. Apply calcium gluconate gel (preparation: boil 5 g of calcium gluconate in 85 ml of hot distilled water, add 10 g glycerol. Allow 5 g of Carmellose-sodium to swell in the hot solution. Stable for 6 months, store in a cool place) and massage into the skin until the pain subsides, in between rinse with water and apply fresh gel. Continue gel therapy for another 15 minutes after the pain has subsided. If no calcium gluconate gel is available, apply several dressings thoroughly moistened with 20 % calcium gluconate solution. Medical advice absolutely required!

After eye contact: rinse out with plenty of water. Immediately call in ophthalmologist.

After swallowing: Immediately give to drink plenty of water, add calcium (in the form of calcium gluconate or calcium lactate). Caution: In the case of vomiting risk of perforation! Administer more calcium gluconate solution. Laxative: Sodium sulfate (1 tablespoon/1/4 l water). Seek medical advice immediately. Ensure that injured persons remain calm and protect them against heat loss.

4.2 Most important symptoms and effects, both acute and delayed

Irritation and corrosion, irritant effects, Cough, respiratory arrest, Convulsions, death
Risk of corneal clouding.
The following applies to soluble inorganic fluorides in general: may cause irritations to burns in contact with eyes, skin, mucous membranes. Systemic effect: drop in blood calcium level, agitation, spasms, cardiovascular disorders, CNS disorders.

4.3 Indication of any immediate medical attention and special treatment needed

Note for the doctor: It is recommended to consult a doctor with experience in the treatment of lesions caused by hydrofluoric acid. If a systemic effect is suspected, monitoring and treatment in an intensive care unit is urgently required. Caution, ventricular fibrillation due to electrolyte imbalance.

SECTION 5. Firefighting measures

5.1 Extinguishing media

Suitable extinguishing media
Use extinguishing measures that are appropriate to local circumstances and the surrounding environment.

Unsuitable extinguishing media
For this substance/mixture no limitations of extinguishing agents are given.

5.2 Special hazards arising from the substance or mixture

Not combustible.
Development of hazardous combustion gases or vapours possible in the event of fire.
Fire may cause evolution of:
Hydrogen fluoride

5.3 Advice for firefighters

Special protective equipment for firefighters
Stay in danger area only with self-contained breathing apparatus. Prevent skin contact by keeping a safe distance or by wearing suitable protective clothing.

Further information
Suppress (knock down) gases/vapours/mists with a water spray jet. Prevent fire extinguishing water from contaminating surface water or the ground water system.

SECTION 6. Accidental release measures

6.1 Personal precautions, protective equipment and emergency procedures
Advice for non-emergency personnel: Avoid substance contact. Avoid inhalation of dusts. Ensure adequate ventilation. Evacuate the danger area, observe emergency procedures, consult an expert.

Advice for emergency responders: Protective equipment see section 8.

6.2 Environmental precautions
Do not empty into drains.

6.3 Methods and materials for containment and cleaning up
Cover drains. Collect, bind, and pump off spills.
Observe possible material restrictions (see sections 7.2 and 10.5).
Take up dry. Dispose of properly. Clean up affected area. Avoid generation of dusts.

6.4 Reference to other sections
Indications about waste treatment see section 13.

SECTION 7. Handling and storage

7.1 Precautions for safe handling
Observe label precautions.

7.2 Conditions for safe storage, including any incompatibilities
Tightly closed. Dry. Keep in a well-ventilated place. Keep locked up or in an area accessible only to qualified or authorised persons.

Store at +5°C to +30°C.

7.3 Specific end uses
Apart from the uses mentioned in section 1.2 no other specific uses are stipulated.

SECTION 8. Exposure controls/personal protection

8.1 Control parameters

8.2 Exposure controls

Engineering measures
Technical measures and appropriate working operations should be given priority over the use of personal protective equipment.

See section 7.1.

Individual protection measures
Protective clothing needs to be selected specifically for the workplace, depending on concentrations and quantities of the hazardous substances handled. The chemical resistance of the protective equipment should be enquired at the respective supplier.

Hygiene measures
Immediately change contaminated clothing. Apply preventive skin protection. Wash hands and face after working with substance. Work under hood. Do not inhale substance.

Eye/face protection
Safety glasses

Hand protection
full contact:

Glove material:	Nitrile rubber
Glove thickness:	0,11 mm
Break through time:	> 480 min

splash contact:

Glove material:	Nitrile rubber
Glove thickness:	0,11 mm
Break through time:	> 480 min

The protective gloves to be used must comply with the specifications of EC Directive 89/686/EEC and the related standard EN374, for example KCL 741 Dermatril® L (full contact), KCL 741 Dermatril® L (splash contact).
The breakthrough times stated above were determined by KCL in laboratory tests acc. to EN374 with samples of the recommended glove types.
This recommendation applies only to the product stated in the safety data sheet<(>,<)> supplied by us and for the designated use. When dissolving in or mixing with other substances and under conditions deviating from those stated in EN374 please contact the supplier of CE-approved gloves (e.g. KCL GmbH, D-36124 Eichenzell, Internet: www.kcl.de).

Other protective equipment
protective clothing

Respiratory protection
required when dusts are generated.
Recommended Filter type: Filter B-(P3)
The entrepeneur has to ensure that maintenance, cleaning and testing of respiratory protective devices are carried out according to the instructions of the producer. These measures have to be properly documented.

Environmental exposure controls
Do not empty into drains.

SECTION 9. Physical and chemical properties

9.1 Information on basic physical and chemical properties

Form	crystals
Colour	colourless
Odour	odourless
Odour Threshold	No information available.

pH	ca. 10,2 at 40 g/l 20 °C
Melting point	993 °C
Boiling point/boiling range	1.704 °C
Flash point	not applicable
Evaporation rate	No information available.
Flammability (solid, gas)	No information available.
Lower explosion limit	not applicable
Upper explosion limit	not applicable
Vapour pressure	1 hPa at 1.077 °C
Relative vapour density	No information available.
Relative density	2,8 g/cm³ at 20 °C
Water solubility	42 g/l at 20 °C
Partition coefficient: n-octanol/water	No information available.
Autoignition temperature	No information available.
Decomposition temperature	No information available.
Viscosity, dynamic	No information available.
Explosive properties	No information available.
Oxidizing properties	No information available.

9.2 Other data

Ignition temperature	not applicable

SECTION 10. Stability and reactivity

10.1 Reactivity

See section 10.3.

10.2 Chemical stability

The product is chemically stable under standard ambient conditions (room temperature) .

10.3 Possibility of hazardous reactions

Generates dangerous gases or fumes in contact with:

acids, Formed could be:
Hydrogen fluoride

10.4 Conditions to avoid
Heating (decomposition).

10.5 Incompatible materials
glass

10.6 Hazardous decomposition products
in the event of fire: See chapter 5.

SECTION 11. Toxicological information

11.1 Information on toxicological effects

Acute oral toxicity
LDLO human: 71 mg/kg (RTECS)

LD50 rat: 31 mg/kg (RTECS)

Symptoms: Convulsions
absorption

Acute inhalation toxicity

Symptoms: mucosal irritations, Cough

Acute dermal toxicity
absorption

Skin irritation
Causes poorly healing wounds.
Causes skin irritation.

Eye irritation
Risk of corneal clouding.
Causes serious eye irritation.

Genotoxicity in vitro
Mutagenicity (mammal cell test): micronucleus.
Result: negative
(National Toxicology Program)
Ames test
Salmonella typhimurium
Result: negative
(National Toxicology Program)

Carcinogenicity
Did not show carcinogenic effects in animal experiments. (External MSDS)

Specific target organ toxicity - single exposure
The substance or mixture is not classified as specific target organ toxicant, single exposure.

Specific target organ toxicity - repeated exposure
The substance or mixture is not classified as specific target organ toxicant, repeated exposure.

Aspiration hazard
Based on available data the classification criteria are not met.

11.2 Further information

Decomposition of the substance with tissue moisture.
Systemic effects:
Cardiac irregularities, respiratory arrest, death
Other information
The following applies to soluble inorganic fluorides in general: may cause irritations to burns in contact with eyes, skin, mucous membranes. Systemic effect: drop in blood calcium level, agitation, spasms, cardiovascular disorders, CNS disorders.
Further data:
Handle in accordance with good industrial hygiene and safety practice.

SECTION 12. Ecological information

12.1 Toxicity

Toxicity to fish
LC50 Gambusia affinis (Mosquito fish): 925 mg/l; 96 h (IUCLID)

Toxicity to daphnia and other aquatic invertebrates.
EC50 Daphnia magna (Water flea): 338 mg/l; 48 h (IUCLID)

EC5 E.sulcatum: 101 mg/l(maximum permissible toxic concentration) (Hommel)

Toxicity to algae
IC50 Desmodesmus subspicatus (green algae): 850 mg/l; 72 h (IUCLID)

Toxicity to bacteria
EC0 Pseudomonas putida: 231 mg/l; 16 h (referred to the anion) (maximum permissible toxic concentration) (IUCLID)

EC50 activated sludge: 2.930 mg/l; 3 h
ISO 8192 (IUCLID)

12.2 Persistence and degradability

No information available.

12.3 Bioaccumulative potential

No information available.

12.4 Mobility in soil

No information available.

12.5 Results of PBT and vPvB assessment

PBT/vPvB assessment not available as chemical safety assessment not required/not conducted.

12.6 Other adverse effects

Additional ecological information
Forms corrosive mixtures with water even if diluted.
Hazard for drinking water supplies.
Further information on ecology
Discharge into the environment must be avoided.

SECTION 13. Disposal considerations

Waste treatment methods
See www.retrologistik.com for processes regarding the return of chemicals and containers, or contact us there if you have further questions.

SECTION 14. Transport information

ADR/RID
UN 1690 SODIUM FLUORIDE, SOLID, 6.1, III
Environmentally hazardous no

IATA
UN 1690 SODIUM FLUORIDE, SOLID, 6.1, III
Environmentally hazardous no

IMDG
UN 1690 SODIUM FLUORIDE, SOLID, 6.1, III
EmS F-A S-A
Marine pollutant no

The transport regulations are cited according to international regulations and in the form applicable in Germany. Possible national deviations in other countries are not considered.

SECTION 15. Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulations

Major Accident Hazard Legislation	96/82/EC Toxic 2 Quantity 1: 50 t Quantity 2: 200 t
Occupational restrictions	Take note of Dir 94/33/EC on the protection of young people at work. Take note of Dir 92/85/EEC on the safety and health at work of pregnant workers.

National legislation

Storage class 6.1B

15.2 Chemical Safety Assessment

For this product a chemical safety assessment was not carried out.

SECTION 16. Other information

Full text of H-Statements referred to under sections 2 and 3.

H301	Toxic if swallowed.
H315	Causes skin irritation.
H319	Causes serious eye irritation.

Full text of R-phrases referred to under sections 2 and 3

R25	Toxic if swallowed.
R36/38	Irritating to eyes and skin.

Training advice
Provide adequate information, instruction and training for operators.

Key or legend to abbreviations and acronyms used in the safety data sheet
Used abbreviations and acronyms can be looked up at www.wikipedia.org.

Regional representation
This information is given on the authorised Safety Data Sheet for your country.

The information contained herein is based on the present state of our knowledge. It characterises the product with regard to the appropriate safety precautions. It does not represent a guarantee of any properties of the product.

Appendix F

Ethical Clearance Documentation

Collection of blood specimens:

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national standards and in line with the 1964 Helsinki declaration and its amendments of comparable ethical standards. Ethical permission was obtained through the University of Pretoria with reference number: EC150618-013.

Voluntary written informed consent was obtained from all individuals included in the study, who supplied whole blood (approximately 200 mL each) for use as blank matrix samples.

The following permissions were obtained:

1. Faculty of Health Sciences Research Ethics Committee Endorsement Notice
2. Faculty of Natural and Agricultural Sciences Committee for Research Approval Letter
3. Deputy Dean of the Natural and Agricultural Sciences Permission Letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

9/12/2015

Endorsement Notice

Ethics Reference No.: EC150618-013

Title: Blood Alcohol: Forensic analysis by GC-MS and investigation of some pre-analytical factors that may influence the result

Dear Frances FJJ Sewell

The **New Application** as supported by documents specified in your cover letter for your research received on the 2/12/2015, was approved, by the Faculty of Health Sciences Research Ethics Committee on the 7/12/2015.

Please note the following about your ethics approval:
- Please remember to use your protocol number (**EC150618-013**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Conditional approval, pending blood banks consent form.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

☎ 012 354 1677 🖷 0866516047 deepeka.behari@up.ac.za http://www.healthethics-up.co.za
✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

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Reference Number: EC150618-013 11-Aug-2015

FJJ Sewell
Natural and Agricultural Sciences Dean's Office
UNIVERSITY OF PRETORIA

Dear Sewell,

FACULTY OF NATURAL AND AGRICULTURE SCIENCES COMMITTEE FOR RESEARCH

Your recent application to the > Faculty Of Natural And Agriculture Sciences Committee refers.

1. I hereby wish to inform you that the research project titled *"Blood Alcohol: Forensic analysis by GC-MS and investigation of some pre-analytical factors that may influence the result."* has been approved by the Committee.

   This approval does not imply that the researcher, student or lecturer is relieved of any accountability in terms of the Codes of Research Ethics of the University of Pretoria, if action is taken beyond the approved proposal.

2. According to the regulations, any relevant problem arising from the study or research methodology as well as any amendments or changes, must be brought to the attention of any member of the Faculty Committee who will deal with the matter.

3. The Committee must be notified on completion of the project.

The Committee wishes you every success with the research project.

Prof. Norman Casey
Chair: Faculty of Natural and Agriculture Sciences Committee for Research Ethics
FACULTY OF NATURAL AND AGRICULTURAL SCIENCES

29th Nov 2015

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Natural and Agricultural Sciences

To whom it may concern,

I hereby give permission to Ms Frances Sewell, an MSc student under Dr JB Laurens, to obtain blood from students of the Natural and Agricultural Sciences faculty per request of the Faculty of Health Sciences Research Ethics Committee.

This permission is granted provided that the blood is drawn by a licensed phlebotomist, and no more than 200ml of blood is taken at a time from each student.

Sincerely,

Prof B. Wingfield
Deputy Dean of the Natural and Agricultural Sciences

Appendix G

Candida albicans Study

What follows are the plots of ethanol concentration and colony count for the four concentrations of *C. albicans* not shown in *Chapter 4*. The trends seen in these plots mirror those observed in the plots given in *Chapter 4*. The raw data that was used to produce these plots can be found in *Appendix C*.

G.1. 4°C and NaF

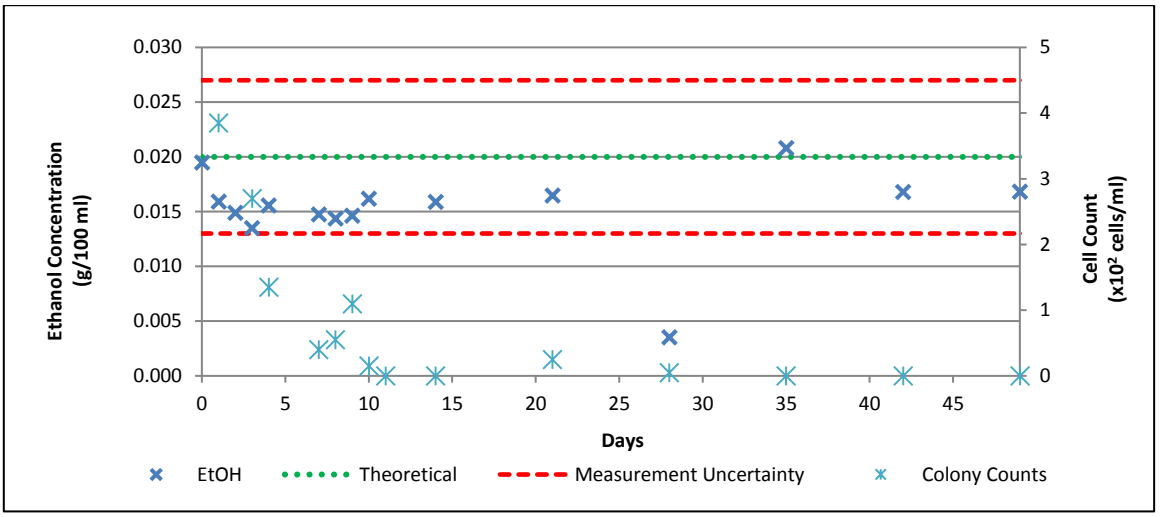

Figure 1: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

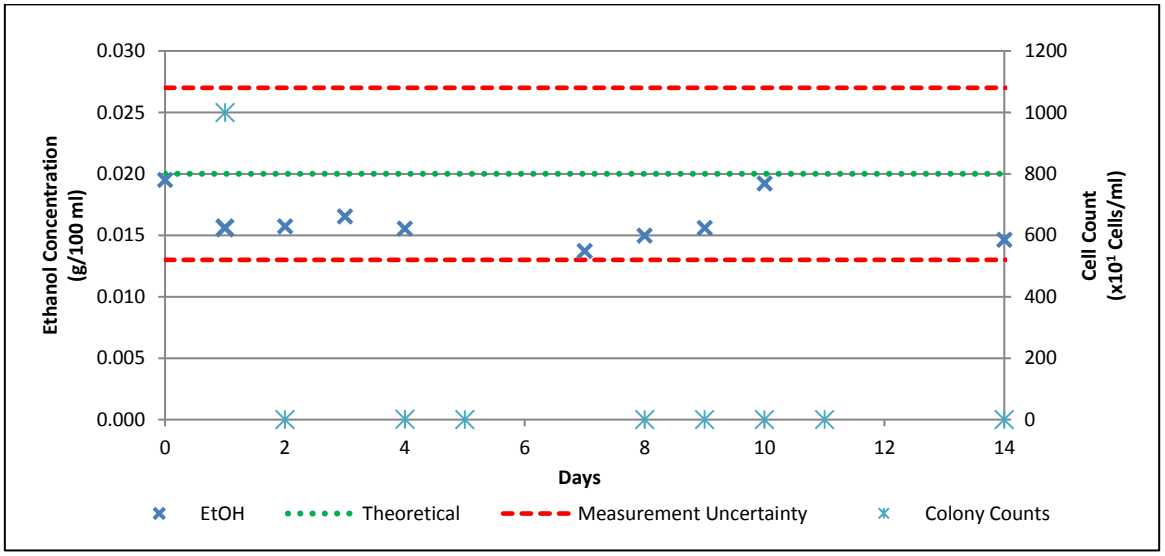

Figure 2: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

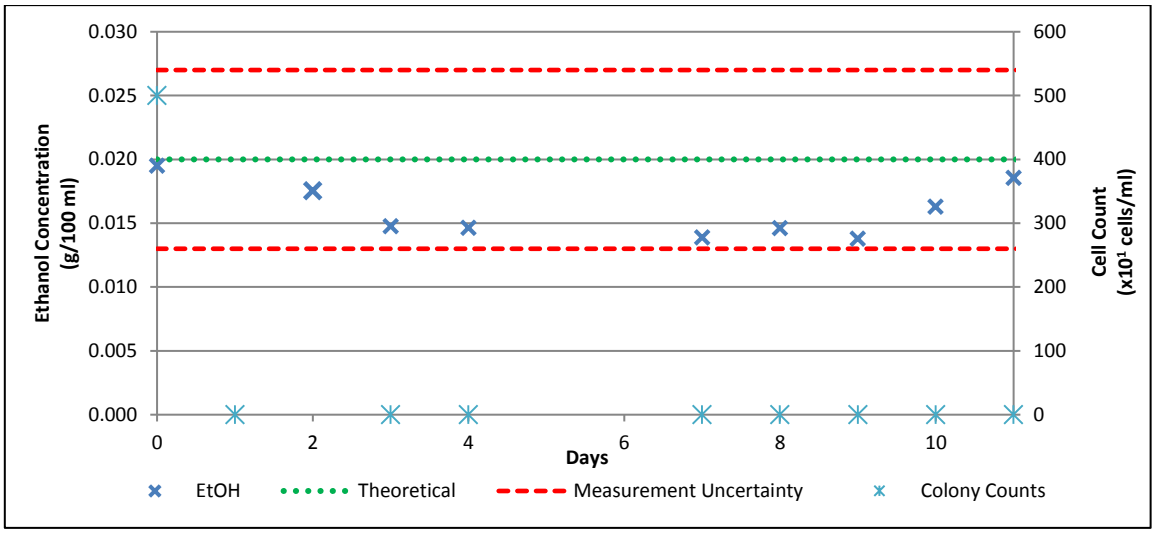

Figure 3: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

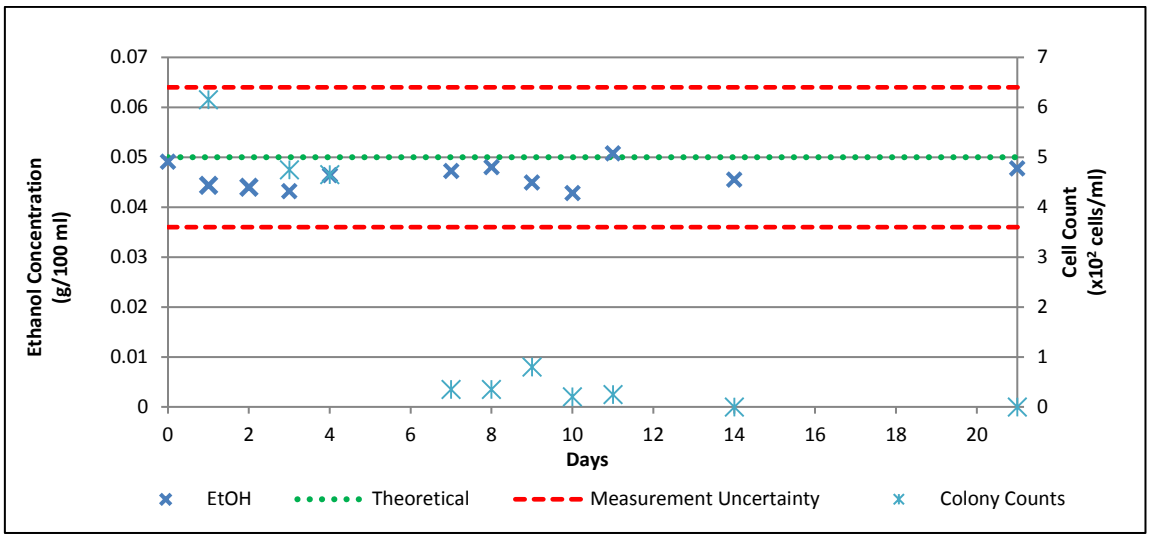

Figure 4: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

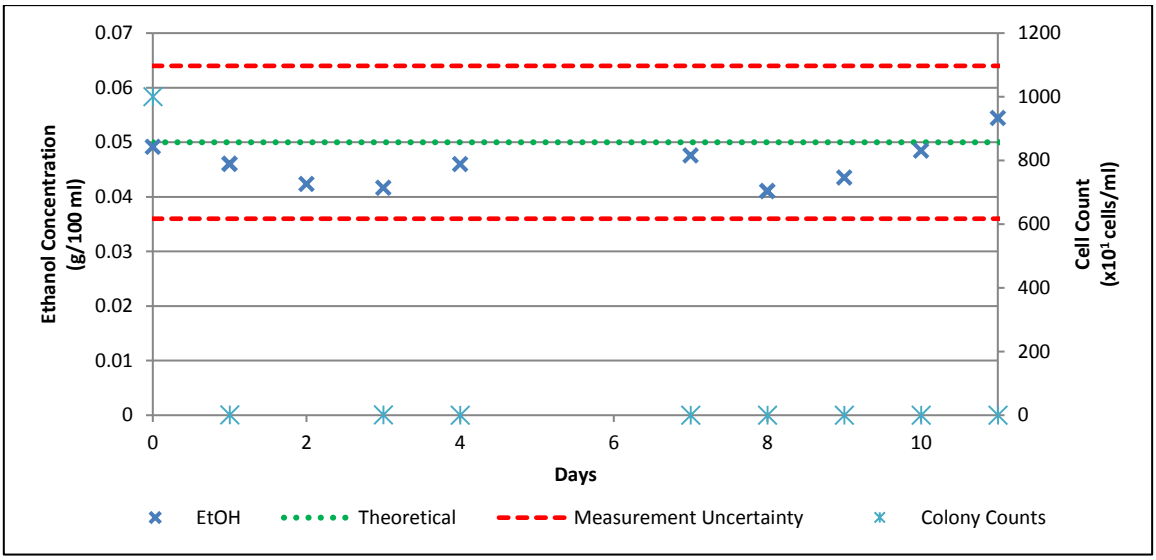

Figure 5: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

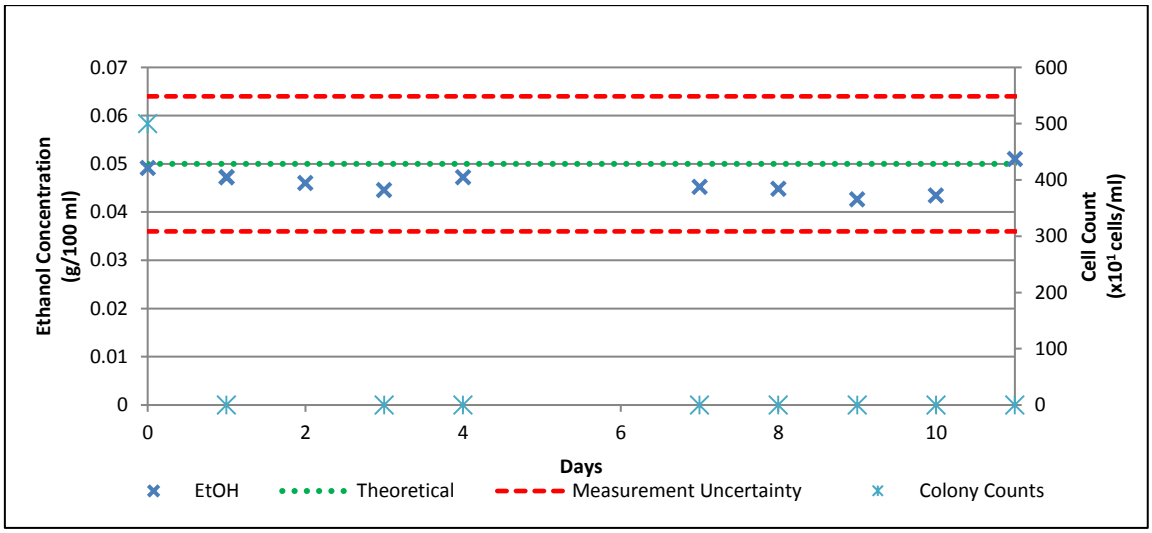

Figure 6: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 4°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

G.2. 4°C and No NaF

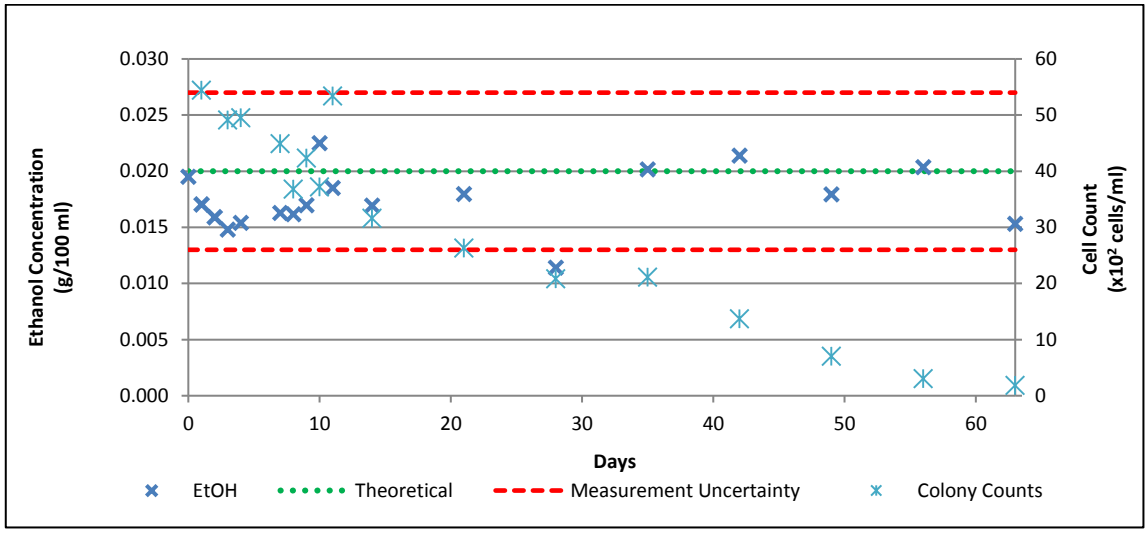

Figure 7: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

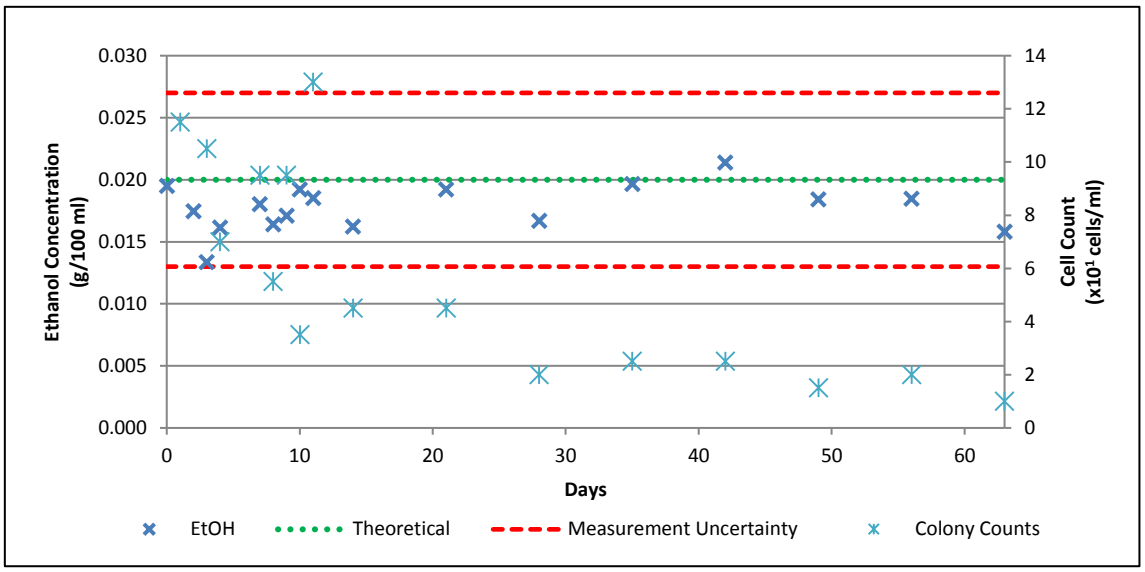

Figure 8: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

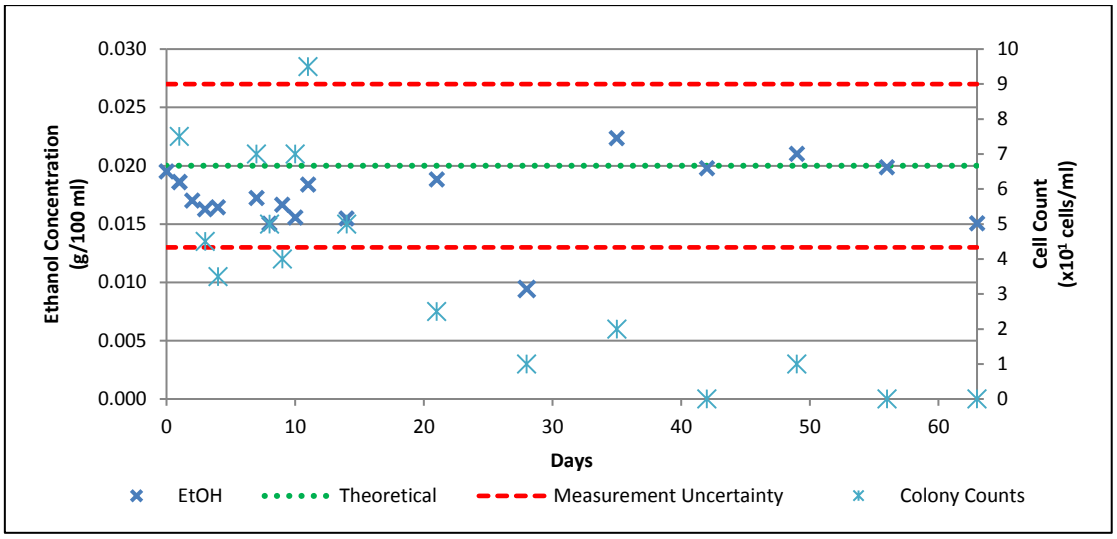

Figure 9: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

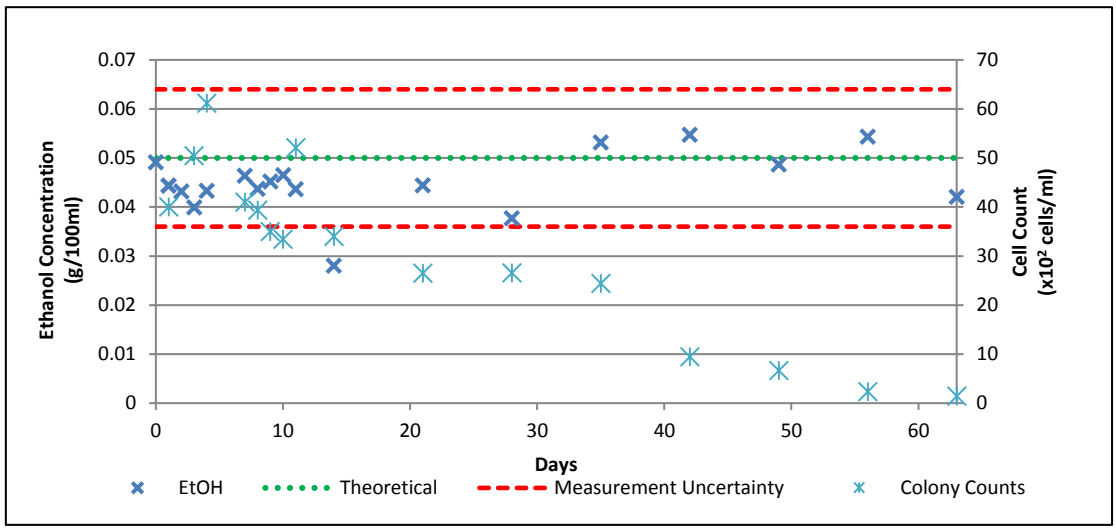

Figure 10: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

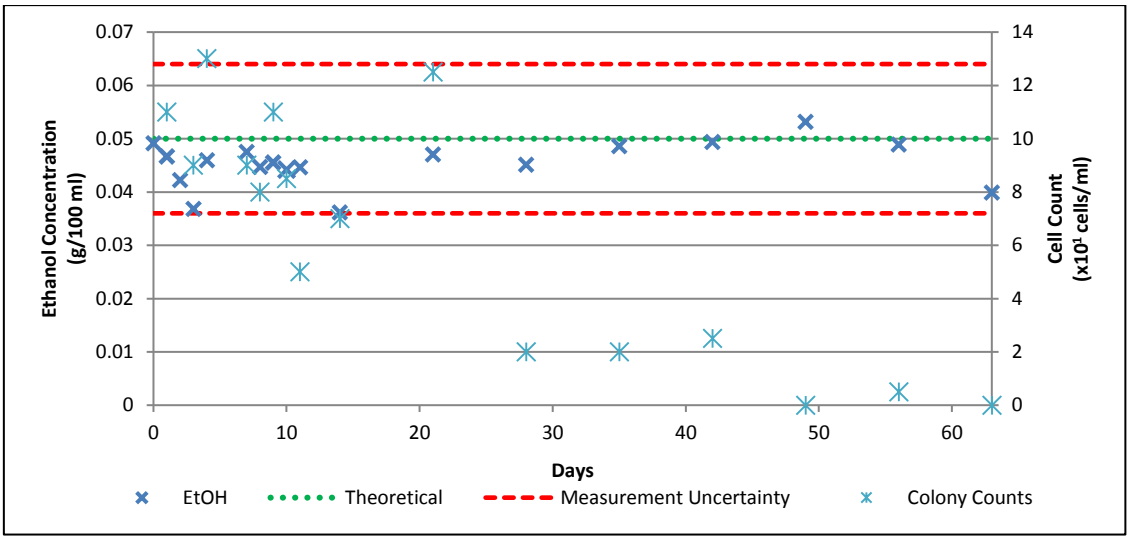

Figure 11: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

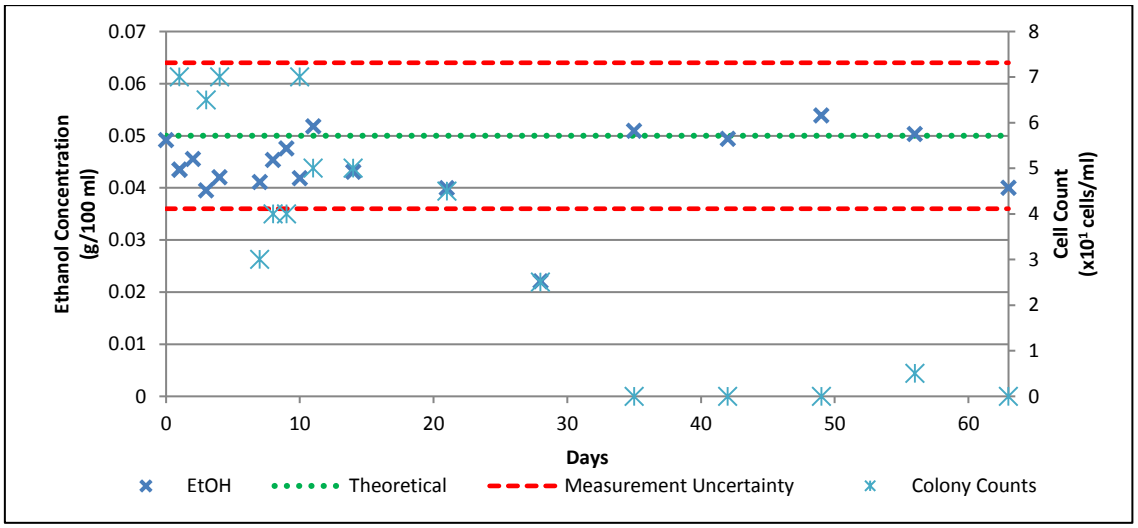

Figure 12: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 4°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

G.3. 22°C and NaF

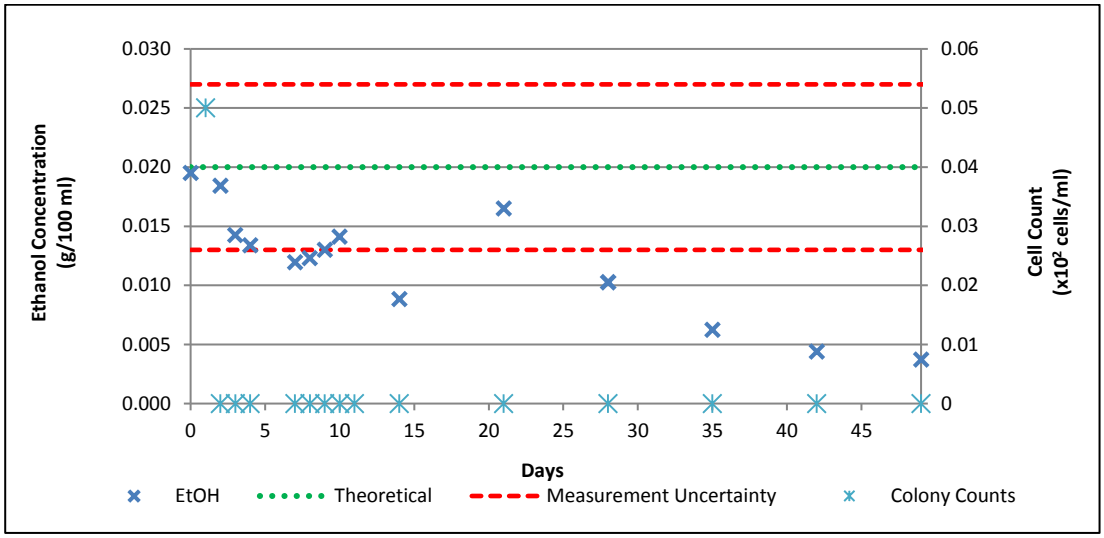

Figure 13: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

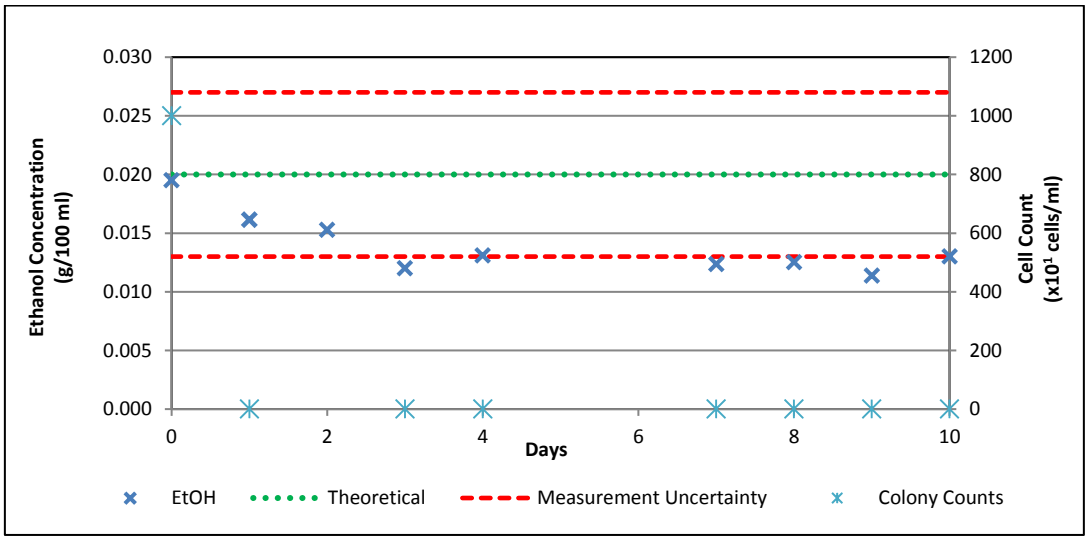

Figure 14: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

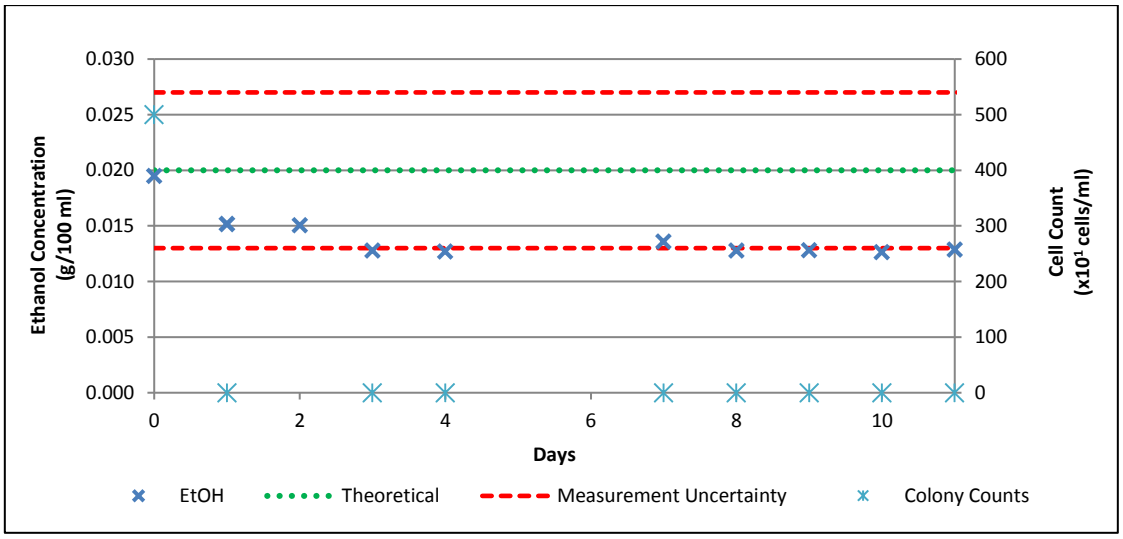

Figure 15: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

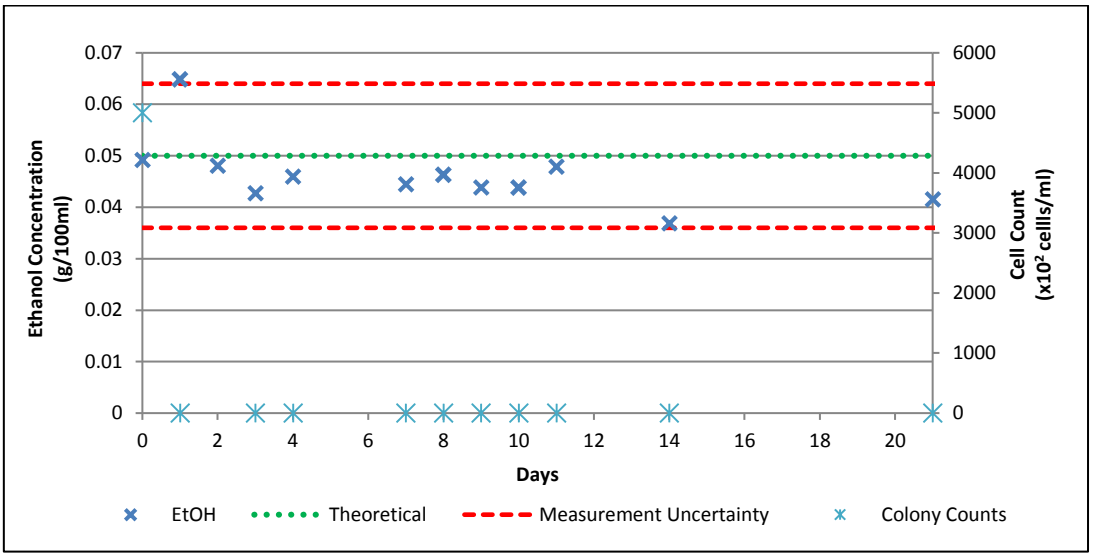

Figure 16: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

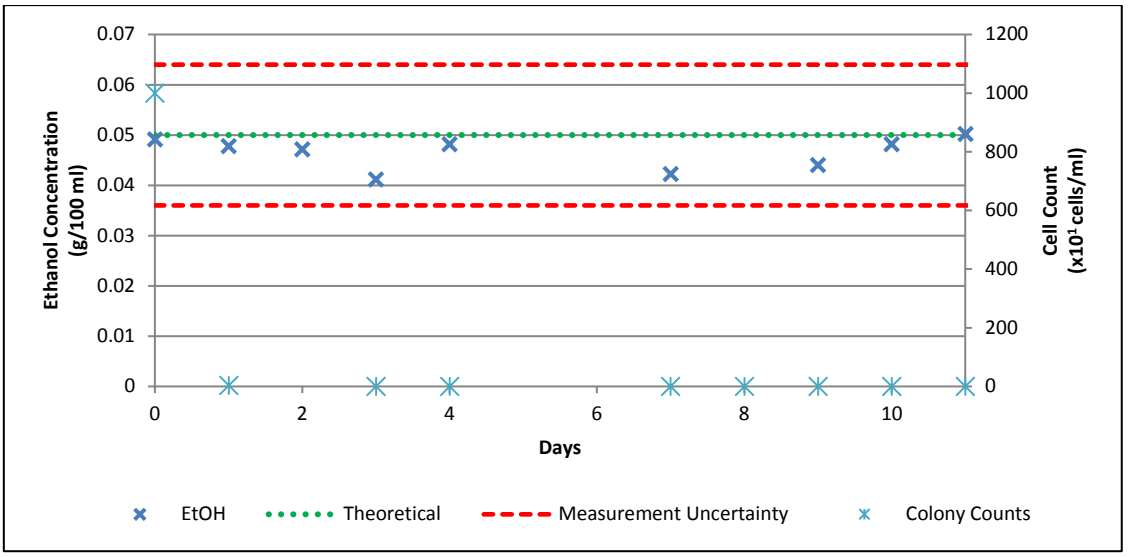

Figure 17: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

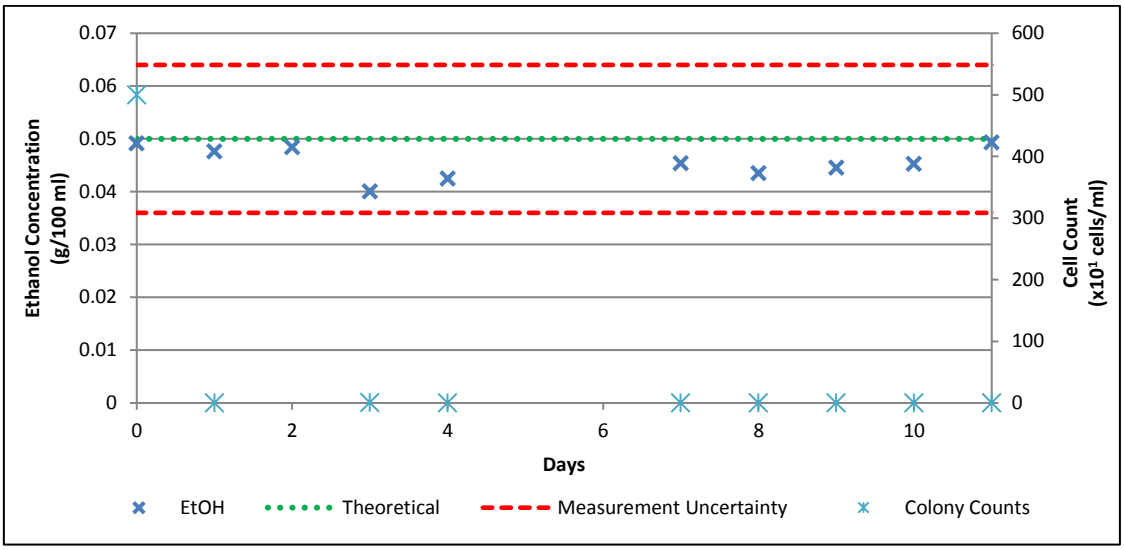

Figure 18: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 22°C in the presence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

G.4. 22°C and No NaF

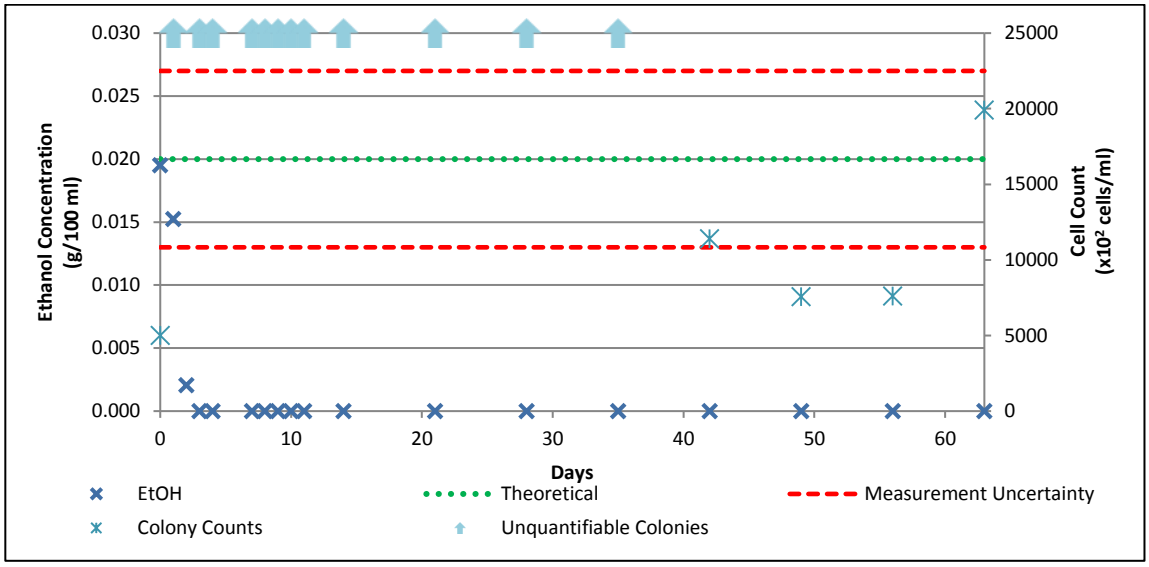

Figure 19: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

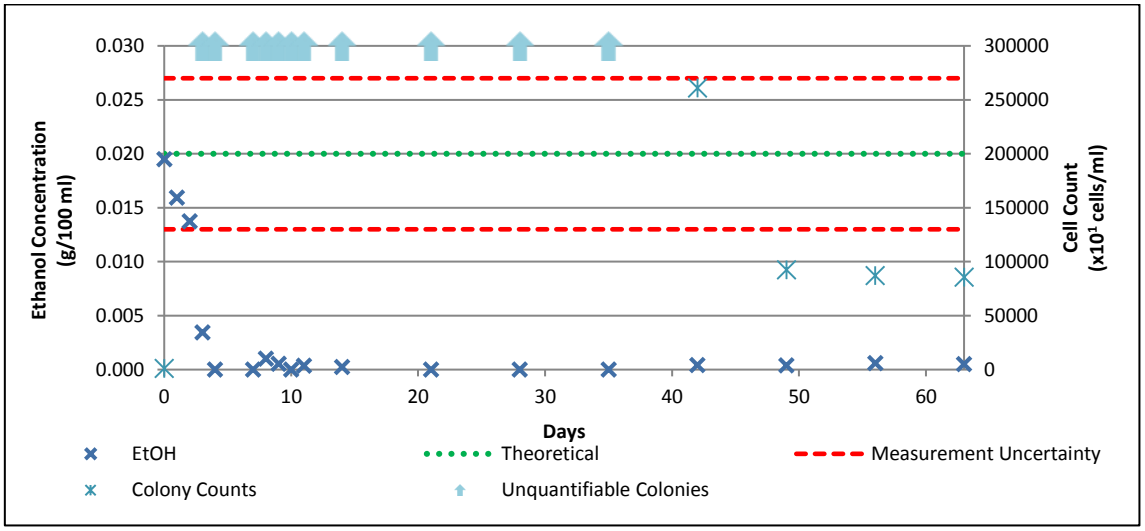

Figure 20: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

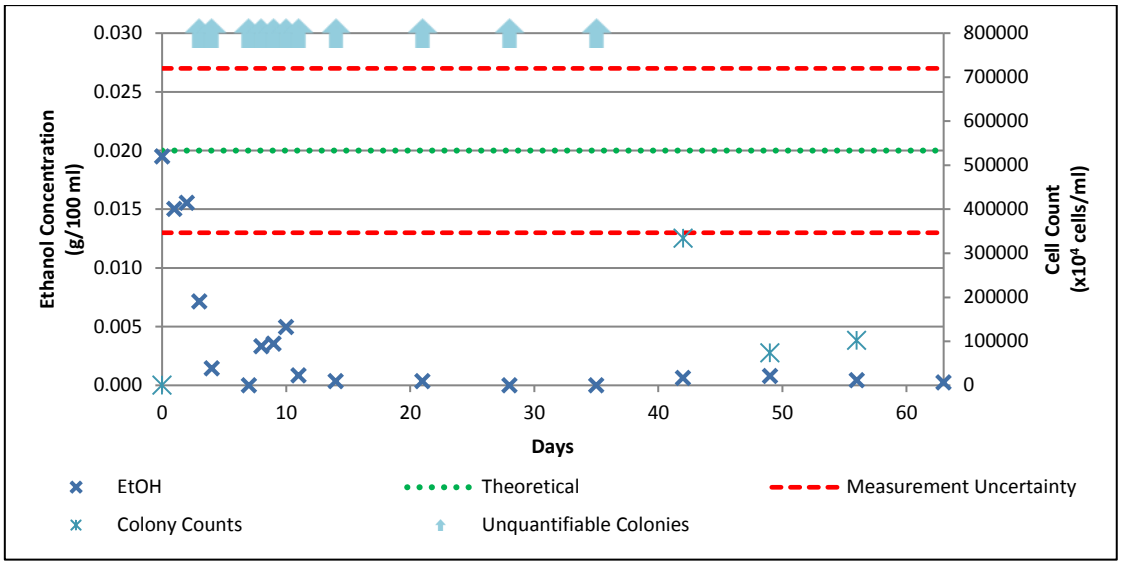

Figure 21: Ethanol concentrations and colony counts for specimens initially spiked at 0.02 g/100 ml ethanol and $5x10^4$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

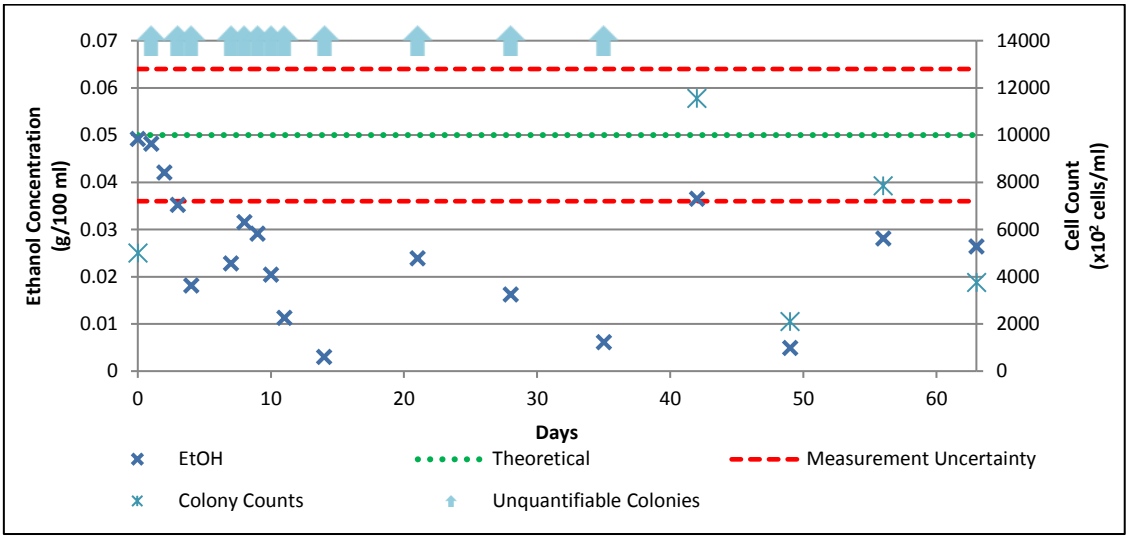

Figure 22: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^5$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

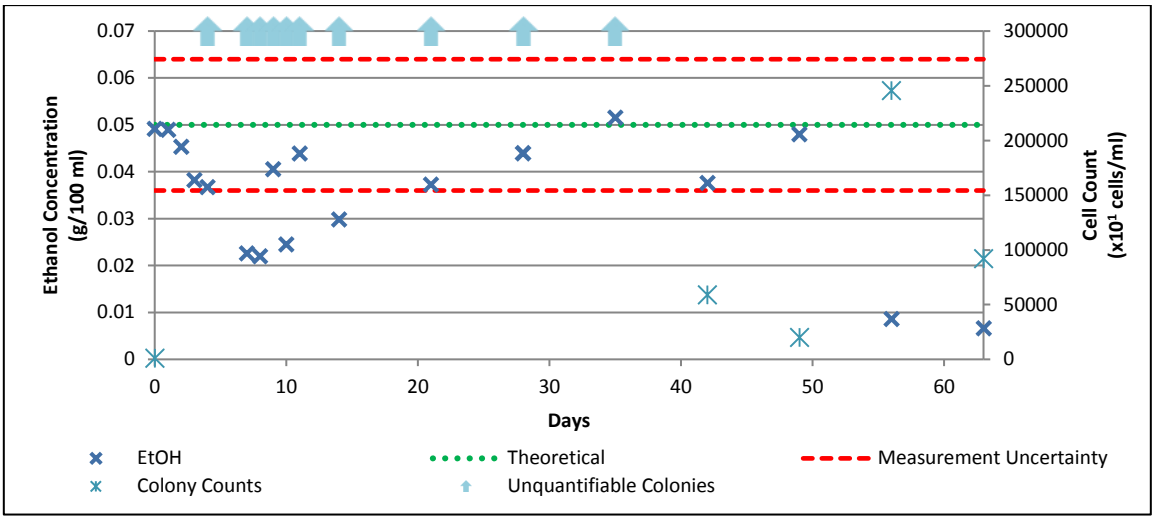

Figure 23: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $1x10^4$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

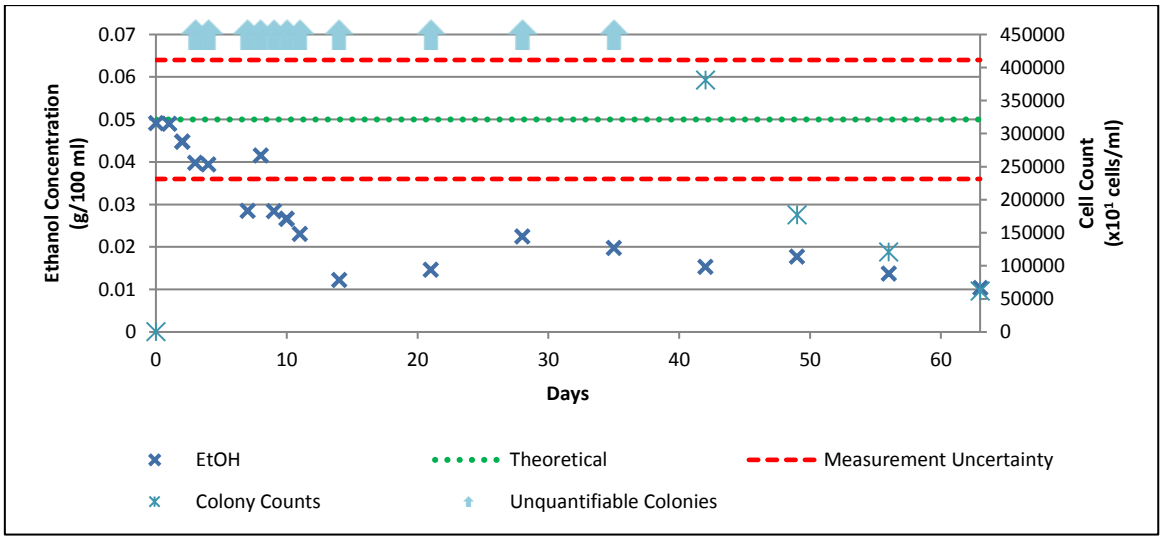

Figure 24: Ethanol concentrations and colony counts for specimens initially spiked at 0.05 g/100 ml ethanol and $5x10^3$ cells/ml *C. albicans*, and stored at 22°C in the absence of fluoride, with the 99% confidence interval around the theoretical ethanol concentration

Appendix H

Fluoride Concentrations

Time and Temperature Studies

Table 1: Experimental fluoride concentrations (g/100 ml) corrected for bias and the corresponding expanded measurement uncertainties (99% confidence) for specimens initially spiked at 0.02 g/100 ml (L1), 0.05 g/100 ml (L2), and 0.30 g/100 ml (L3) and stored at 22 ± 6°C for 29 weeks.

Week	L1 Concentration (g/100 ml)	L1 U (g/100 ml)	L2 Concentration (g/100 ml)	L2 U (g/100 ml)	L3 Concentration (g/100 ml)	L3 U (g/100 ml)
1	2.03	0.13	1.34	0.12	1.78	0.12
2	1.95	0.13	2.20	0.13	2.10	0.13
3	2.30	0.14	2.17	0.13	2.10	0.13
4	2.00	0.13	2.23	0.13	2.49	0.14
5	2.35	0.14	2.08	0.13	2.22	0.13
6	2.73	0.15	2.41	0.14	2.66	0.15
7	1.88	0.12	2.20	0.13	2.35	0.14
8	2.06	0.13	2.16	0.13	2.35	0.14
9	2.22	0.13	2.27	0.14	2.29	0.14
10	1.98	0.13	2.27	0.14	2.12	0.13
11	2.48	0.14	2.32	0.14	2.25	0.13
12	2.26	0.13	2.37	0.14	2.44	0.14
13	2.11	0.13	2.34	0.14	2.31	0.14
14	2.52	0.14	2.38	0.14	2.25	0.13
15	2.34	0.14	2.10	0.13	2.39	0.14
17	2.34	0.14	2.26	0.13	2.43	0.14
18	2.39	0.14	2.14	0.13	2.36	0.14
19	2.23	0.13	1.98	0.13	1.71	0.12
20	2.15	0.13	2.03	0.13	2.09	0.13
21	2.36	0.14	2.16	0.13	2.09	0.13
22	2.46	0.14	2.11	0.13	2.94	0.16
23	2.24	0.13	2.10	0.13	2.20	0.13
24	1.96	0.13	1.89	0.12	2.02	0.13
25	1.91	0.13	2.27	0.14	2.07	0.13
26	2.59	0.15	2.28	0.14	2.12	0.13
27	2.28	0.14	2.33	0.14	2.01	0.13
28	2.13	0.13	2.07	0.13	2.05	0.13
29	2.05	0.13	1.85	0.12	2.03	0.13

Table 2: Experimental fluoride concentrations (g/100 ml) corrected for bias and the corresponding expanded measurement uncertainties (99% confidence) for specimens initially spiked at 0.02 g/100 ml (L1), 0.05 g/100 ml (L2), and 0.30 g/100 ml (L3) and stored at 4 ± 3°C for 29 weeks.

Week	L1 Concentration (g/100 ml)	L1 U (g/100 ml)	L2 Concentration (g/100 ml)	L2 U (g/100 ml)	L3 Concentration (g/100 ml)	L3 U (g/100 ml)
1	2.12	0.13	1.91	0.13	2.11	0.13
2	2.05	0.13	2.07	0.13	2.11	0.13
3	2.25	0.13	2.06	0.13	2.11	0.13
4	2.35	0.14	2.04	0.13	2.35	0.14
5	2.05	0.13	2.15	0.13	2.28	0.14
6	2.29	0.14	2.26	0.13	2.22	0.13
7	2.39	0.14	2.05	0.13	2.21	0.13
8	2.03	0.13	2.21	0.13	2.06	0.13
9	2.12	0.13	2.24	0.13	2.30	0.14
10	2.47	0.14	2.20	0.13	2.46	0.14
11	2.37	0.14	2.35	0.14	2.42	0.14
12	2.55	0.14	2.48	0.14	2.49	0.14
13	2.62	0.15	2.12	0.13	2.29	0.14
14	2.50	0.14	2.54	0.14	2.39	0.14
15	2.35	0.14	2.59	0.15	2.44	0.14
17	2.29	0.14	2.55	0.14	2.34	0.14
18	2.60	0.15	2.54	0.14	2.36	0.14
19	2.32	0.14	2.28	0.14	2.11	0.13
20	2.32	0.14	2.23	0.13	2.40	0.14
21	2.23	0.13	2.03	0.13	2.21	0.13
22	2.39	0.14	2.25	0.13	2.30	0.14
23	2.06	0.13	2.04	0.13	2.14	0.13
24	2.13	0.13	2.24	0.13	2.20	0.13
25	2.25	0.13	2.16	0.13	2.44	0.14
26	2.24	0.13	2.07	0.13	2.26	0.13
27	2.10	0.13	2.09	0.13	2.10	0.13
28	2.39	0.14	2.17	0.13	2.20	0.13
29	1.90	0.13	2.02	0.13	2.04	0.13

Candida albicans Studies

Table 3: Experimental fluoride concentrations (g/100 ml) corrected for bias as well as the corresponding expanded measurement uncertainties (99% confidence) (g/100 ml) for specimens initially spiked at 0.02 g/100 ml, inoculated at five levels of *C. albicans*, and stored at 22 ± 6°C for up to 49 days.

Day	Level 1 Conc.	Level 1 U	Level 2 Conc.	Level 2 U	Level 3 Conc.	Level 3 U	Level 4 Conc.	Level 4 U	Level 5 Conc.	Level 5 U
***C. albicans* Conc. (cells/ml)**	**$5x10^1$**		**$5x10^3$**		**$1x10^4$**		**$5x10^5$**		**$1x10^6$**	
1	2.09	0.13	2.40	0.14	2.09	0.13	1.52	0.12	2.45	0.14
2	2.18	0.13	2.54	0.14	2.19	0.13	1.60	0.12	2.36	0.14
3	2.17	0.13	2.41	0.14	2.28	0.14	1.78	0.12	2.22	0.13
4	2.17	0.13	2.79	0.15	2.19	0.13	1.64	0.12	2.12	0.13
7	2.22	0.13	2.23	0.13	2.24	0.13	1.47	0.12	2.06	0.13
8	2.33	0.14	2.78	0.15	2.16	0.13	1.65	0.12	2.09	0.13
9	2.52	0.14	2.66	0.15	2.31	0.14	1.48	0.12	2.09	0.13
10	2.30	0.14	2.59	0.15	2.11	0.13	1.50	0.12	1.15	0.13
11	2.14	0.13	2.77	0.15	2.16	0.13	1.56	0.12	2.23	0.13
14	1.80	0.12	2.32	0.14	2.20	0.13	1.54	0.12	2.19	0.13
21	2.24	0.13	2.22	0.13	2.02	0.13	1.59	0.12	2.19	0.13
28	2.18	0.13	2.30	0.14	2.00	0.13	1.59	0.12	2.06	0.13
35	-	-	-	-	-	-	1.67	0.12	2.27	0.14
42	-	-	-	-	-	-	1.59	0.12	2.36	0.14
49	-	-	-	-	-	-	1.73	0.12	-	-

Table 4: Experimental fluoride concentrations (g/100 ml) corrected for bias as well as the corresponding expanded measurement uncertainties (99% confidence) (g/100 ml) for specimens initially spiked at 0.05 g/100 ml, inoculated at five levels of C. albicans, and stored at 22 ± 6°C for up to 56 days.

Day	Level 1 Conc.	Level 1 U	Level 2 Conc	Level 2 U	Level 3 Conc.	Level 3 U	Level 4 Conc.	Level 4 U	Level 5 Conc.	Level 5 U
***C. albicans* Conc. (cells/ml)**	**$5x10^1$**		**$5x10^3$**		**$1x10^4$**		**$5x10^5$**		**$1x10^6$**	
1	2.29	0.14	2.03	0.13	2.47	0.14	1.97	0.13	2.38	0.14
2	2.22	0.13	2.32	0.14	2.22	0.13	2.02	0.14	2.51	0.14
3	1.51	0.12	2.30	0.14	2.21	0.13	2.08	0.14	2.36	0.14
4	1.67	0.12	2.15	0.13	2.23	0.13	1.90	0.13	2.33	0.14
7	1.36	0.12	2.29	0.14	2.34	0.14	2.06	0.14	2.27	0.14
8	2.32	0.14	2.40	0.14	2.32	0.14	1.90	0.13	2.38	0.14
9	2.20	0.13	1.36	0.12	2.44	0.14	2.03	0.14	2.24	0.13
10	2.39	0.14	2.29	0.14	2.49	0.14	1.92	0.13	2.15	0.13
11	1.42	0.12	2.35	0.14	2.58	0.15	2.09	0.14	2.21	0.13
14	2.29	0.14	2.28	0.14	2.33	0.14	1.92	0.13	2.26	0.13
21	2.43	0.14	2.16	0.13	2.26	0.13	1.97	0.13	2.34	0.14
28	2.24	0.13	2.39	0.14	2.30	0.14	1.93	0.13	2.38	0.14

35	-	-	-	-	-	-	-	-	2.14	0.13
42	-	-	-	-	-	-	-	-	2.32	0.14
49									2.11	0.13
56									2.11	0.13

Table 5: Experimental fluoride concentrations (g/100 ml) corrected for bias as well as the corresponding expanded measurement uncertainties (99% confidence) (g/100 ml) for specimens initially spiked at 0.02 g/100 ml, inoculated at five levels of C. albicans, and stored at 4 ± 3°C for up to 49 days.

Day	Level 1 Conc.	Level 1 U	Level 2 Conc	Level 2 U	Level 3 Conc.	Level 3 U	Level 4 Conc.	Level 4 U	Level 5 Conc.	Level 5 U
***C. albicans* Conc. (cells/ml)**	**$5x10^1$**		**$5x10^3$**		**$1x10^4$**		**$5x10^5$**		**$1x10^6$**	
1	2.69	0.15	2.23	0.13	2.01	0.13	2.45	0.14	2.27	0.14
2	2.09	0.13	2.38	0.14	2.25	0.13	2.13	0.13	2.36	0.14
3	2.20	0.13	2.38	0.14	2.20	0.13	2.39	0.14	2.23	0.13
4	2.17	0.13	2.40	0.14	2.13	0.13	2.30	0.14	2.35	0.14
7	2.34	0.14	2.30	0.14	2.19	0.13	2.45	0.14	2.19	0.13
8	2.27	0.14	2.25	0.13	2.08	0.13	2.32	0.14	2.28	0.14
9	2.18	0.13	2.38	0.14	2.49	0.14	2.41	0.14	2.41	0.14
10	2.31	0.14	2.27	0.14	2.39	0.14	2.19	0.13	2.45	0.14
11	2.35	0.14	2.60	0.15	2.53	0.14	2.31	0.14	2.45	0.14
14	2.42	0.14	2.52	0.14	2.40	0.14	1.75	0.12	2.72	0.15
21	2.29	0.14	2.25	0.13	2.37	0.14	2.23	0.13	2.25	0.13
28	2.40	0.14	2.28	0.14	2.45	0.14	2.46	0.14	2.42	0.14
35	-	-	-	-	-	-	2.31	0.14	2.41	0.14
42	-	-	-	-	-	-	2.35	0.14	2.24	0.13
49	-	-	-	-	-	-	2.47	0.14	-	-

Table 6: Experimental fluoride concentrations (g/100 ml) corrected for bias as well as the corresponding expanded measurement uncertainties (99% confidence) (g/100 ml) for specimens initially spiked at 0.05 g/100 ml, inoculated at five levels of C. albicans, and stored at 4 ± 3°C for up to 56 days.

Day	Level 1 Conc.	Level 1 U	Level 2 Conc	Level 2 U	Level 3 Conc.	Level 3 U	Level 4 Conc.	Level 4 U	Level 5 Conc.	Level 5 U
***C. albicans* Conc. (cells/ml)**	**$5x10^1$**		**$5x10^3$**		**$1x10^4$**		**$5x10^5$**		**$1x10^6$**	
1	2.56	0.14	2.56	0.14	2.06	0.13	2.32	0.14	2.15	0.13
2	2.43	0.14	2.71	0.15	2.21	0.13	1.73	0.12	2.37	0.14
3	2.36	0.14	2.45	0.14	2.22	0.13	2.58	0.15	2.35	0.14
4	2.26	0.13	2.61	0.15	2.25	0.13	2.39	0.14	2.32	0.14
7	2.44	0.14	2.44	0.14	2.21	0.13	2.26	0.13	2.42	0.14
8	2.34	0.14	2.64	0.15	2.02	0.13	2.54	0.14	2.14	0.13
9	2.49	0.14	2.38	0.14	2.05	0.13	2.13	0.13	2.14	0.13
10	2.44	0.14	2.48	0.14	2.26	0.13	2.40	0.14	2.25	0.13
11	2.39	0.14	2.48	0.14	2.24	0.13	2.25	0.13	2.10	0.13

14	2.47	0.14	2.48	0.14	2.39	0.14	2.40	0.14	2.23	0.13
21	2.50	0.14	2.76	0.15	2.26	0.13	2.40	0.14	2.18	0.13
28	2.90	0.16	2.89	0.16	2.42	0.14	2.15	0.13	2.37	0.14
35	-	-	-	-	-	-	-	-	2.05	0.13
42	-	-	-	-	-	-	-	-	2.39	0.14
49	-	-	-	-	-	-	-	-	2.26	0.13
56	-	-	-	-	-	-	-	-	2.26	0.13