Title

Determination of Variation in Forensic Ethanol Analysis Results Using a Deuterated Internal Standard for Samples Collected at the Lower Limit of Sufficient Volume for Analysis: A Cost Benefit Perspective

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Determination of Variation in Forensic Ethanol Analysis Results Using a Deuterated Internal Standard for Samples Collected at the Lower Limit of Sufficient Volume for Analysis: A Cost Benefit Perspective

By

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THESIS

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2024

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Abstract

Forensic alcohol analysis of ante-mortem blood samples is a common service performed in many laboratories across the country. 1-Propanol is a commonly used internal standard for the detection and quantification of ethanol in these samples. Ethanol-d_6 could potentially be a more reliable internal standard due to sharing a similar structure and vapor pressure to ethanol and because it would also be similarly affected by outside factors as ethanol. However, ethanol-d_6 is more expensive and its use may require adjusting the parameters of a gas chromatograph to achieve adequate separation from ethanol for quantitation by flame ionization detection (FID). In this study, aqueous ethanol samples were individually spiked with either 1-propanol or ethanol-d_6 and run through a headspace GC-MS to quantify the concentration of ethanol present and to compare the results between the two internal standards. Samples drawn from 0.080 g/dL ethanol solutions of various sodium fluoride and potassium oxalate salt concentrations were also spiked with the internal standards, run, and compared to test the salting-out effect on the internal standards. This study found that while 1-propanol was more accurate than ethanol-d_6 in the salt-free samples, ethanol-d_6 was more accurate when salts were present. It also found that 1-propanol was more precise at lower ethanol concentrations while ethanol-d_6 was more precise at higher concentrations. However, ethanol-d_6 is over 300 times more expensive than 1-propanol on a cost per run basis, so this may outweigh any potential improvements in assay performance.

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1. Introduction

Determining the concentration of ethanol in different biological matrices, such as whole blood, is important for both scientific studies of alcohol metabolism and forensic laboratories (Dorubeţ et al., 2009). Forensic scientists use this analysis to determine if an individual's blood alcohol concentration (BAC) was below, at, or above the legal limit when they were driving. In California, this limit is 0.080 g/dL ethanol by weight for general drivers and 0.040 g/dL by weight for commercial and passenger-for-hire drivers (Vehicle Code, section 23152 b, d, & e). There are multiple analytical approaches to conduct these analyses, but forensic scientists generally use headspace gas chromatography (HS-GC) with either a flame ionization detector (FID) or a dual flame ionization detector/mass spectrometer (FID/MS) instrumentation.

HS analysis takes defined volume of the vapors above the liquid sample after equilibration to a controlled and maintained temperature, such as 50°C (Dubowski, 1980; Snow & Slack, 2002). At this heated equilibrium, the concentration of volatile substances in the headspace vapor is proportional to the substances' concentration in the liquid sample (Kolb & Ettre, 2006). HS sampling is often preferred over direct injection of the sample because it is not likely to contaminate the GC inlet and column (Dubowski, 1980; Tagliaro et al., 1992) and because it reduces matrix effects (Tagliaro et al., 1992). A GC is used to separate the analytes in this analysis because of its high throughput, sensitivity, selectivity, minimal sample preparation requirements, and relatively low cost of analysis (Dubowski, 1980). It can also be used to simultaneously detect other volatile analytes (Tagliaro et al., 1992), if so desired. An FID is universally used to determine the quantitative concentration of ethanol because it is simple and robust (Tiscione et al., 2011) as well as cost effective (Tagliaro et al., 1992). However, as the

FID cannot differentiate between co-eluting compounds, it lacks clear specificity to ethanol in case another volatile is eluting at the same time (Haißer et al., 2009). Due to this lack of specificity, the FID can be paired with a MS in forensic laboratories to qualitatively identify ethanol at the same time (Tiscione et al., 2011). According to industry experts using this approach, this methodology provides robust and reliable forensic alcohol results (C. Triebold, personal communication, May 19, 2022). It may be possible to use a GC-MS instrument methodology for this analysis, though it is not generally used in the field. This is because MS is less precise than FID when using full scan and selected ion monitoring (SIM) might not be able to differentiate between interfering, low boiling point compounds like those used in this analysis (B. Miller, personal communication, August 26, 2022).

Most BAC analyses also utilize an internal standard (IS) to measure the relative response of the peak areas of ethanol to the IS and to compensate for operating condition fluctuations and matrix effects (Dubowski, 1977). Forensic scientists generally use 1-propanol as the IS because it is inexpensive and reliable to use. However, there may be some issues with its use. 1-propanol has an additional carbon in its structural chain compared to ethanol, as depicted in Figure 1. While small, this structural difference can lead to 1-propanol and ethanol responding to outside factors and matrix effects in different ways, thus affecting the analysis and

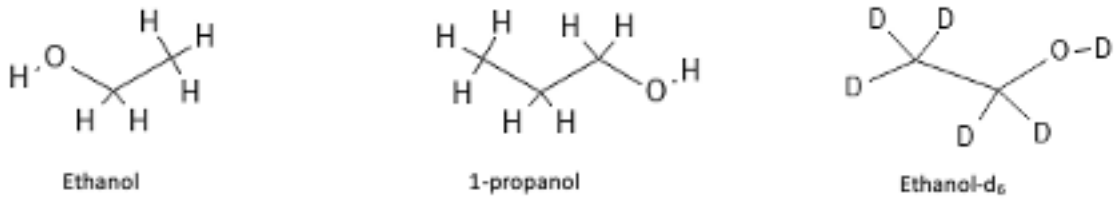

Fig. 1: Chemical structure of ethanol, 1-propanol, and ethanol-d_6

concentration determination. The salting-out effect is one such concern, particularly since blood samples are required to have a preservative and anticoagulant mixed in (17 CCR **§ 1219.1**). Sodium fluoride salt serves as the preservative and potassium oxalate as the anticoagulant in the commonly-used Vacutainer tubes (Jones & Fransson, 2003). The salting-out effect, according to Watts and McDonald (1990) and Watts and McDonald (1987), occurs when inorganic salts, such as sodium fluoride, are added to miscible organic/aqueous liquids. The water in the solution hydrates the salt ions disrupting the weak hydrogen bonds between the water and the organic in favor of stronger bonds between the water and salt ions. This results in the partition coefficient between the vapor and liquid phases changing, leading to an increase in the concentration of the organic in the vapor headspace above the liquid sample. Excess salt concentrations in the liquids lead to elevated vapor pressures of non-electrolytes like ethanol and 1-propanol, thus increasing their concentrations in the headspace above the sample (Watts & McDonald, 1990; Watts & McDonald, 1987). Using an IS helps to mitigate the salting-out effect because both the target analyte and the IS are salted out (Miller et al., 2004). However, as Jones and Fransson (2003) state, uncooperative subjects can lead to varying amounts of blood being collected from sample to sample, from 0.5 mL to 10 mL collected at the extreme ends. Samples collected at the low extreme end may be insufficient due to the difficulty of drawing out the 100 µL required for analysis, thus affecting reproducibility, precision, and accuracy. Salt concentrations in the blood samples will also vary widely at the extremes (Jones & Fransson, 2003).

In fact, there was a case in the United Kingdom where the defense experts claimed that an insufficient sample of blood was collected. They argued that this resulted in an

oversaturation of salt, and thus the salting-out effect caused the determined ethanol concentration to be higher than the true value (*Gregory v. Director of Public Prosecution,* 2002). However, a few different authors dispute this claim. Miller et al. (2004) found that the salting-out effect did not increase the measured ethanol concentration. In fact, they found that the ethanol concentration decreased slightly when sodium fluoride concentrations increased (Miller et al., 2004). Jones and Fransson (2003) also found this with small amounts of sample collected that thus had an increased sodium fluoride concentration. They went on to explain that this is because the 1-propanol IS dilutes the sample some, thus decreasing the sodium fluoride concentration in the sample. They also stated that 1-propanol is salted out more effectively than ethanol, leading to a larger 1-propanol peak area and thus a lower analyte/IS response ratio between the two alcohols. This then leads to a lower apparent ethanol concentration (Jones & Fransson, 2003). Fung (2018) explored this further and found that sodium fluoride and potassium oxalate separately had a larger influence on 1-propanol than they did on ethanol. He also found that combining the two in solution enhanced this effect (Fung, 2018). A different IS may need to be explored for this analysis to determine the true ethanol concentration instead of an underestimate.

Ethanol-d_6 could potentially be used instead of 1-propanol in this analysis. Its physical and chemical properties are more comparable to ethanol due to having the same number of carbons in the chain, as seen in Figure 1. In fact, Haißer et al. (2009) developed a mass spectrometric procedure for this analysis using ethanol-d_6 as an IS. They found that deuterated ethanol's nearly identical vapor pressure and similar retention time to ethanol was advantageous as it led to a shorter run time. They found ethanol-d_6 to be both fairly accurate to

the target value, and their method was comparable to the GC-FID method (Haißer et al., 2009). However, ethanol-d_6 is more expensive than 1-propanol. It could also prove more difficult to achieve adequate separation between ethanol and ethanol-d_6 due to their similar structures and physical and chemical properties. Neither Haißer et al. (2009) nor Dean, Thomasson, Dumaual et al. (1996) were able to achieve full resolution between ethanol and a deuterated version. This could be an issue when quantifying with either an FID or an MS. As stated previously, FIDs cannot differentiate between co-eluting compounds, so there would be overlap between the ethanol and ethanol-d_6 signals if they are not fully separated. For MS quantification, there would be ionic interference since both ethanol and ethanol-d_6 produce some m/z 45 ions, requiring further mathematical steps to determine the concentration of ethanol in solution using abundance ratios (Dean et al., 1996). Full separation may require a different column or chromatographic conditions than what is commonly used in forensic alcohol analysis. This is less of a worry with 1-propanol, which is different enough in structure and volatility from ethanol to ensure adequate separation. With the dual detection of an FID and MS, the mass spectral data would be used primarily for identification (Haißer et al., 2009), and one can resolve ethanol and ethanol-d_6 by extracting chromatographic data based on dissimilar ions (Dean, et al., 1996).

This study explores the use of ethanol-d_6 as an IS for blood alcohol analysis, specifically comparing its accuracy and precision to 1-propanol, particularly in relation to the salting-out effect. This study found that 1-propanol was more accurate than ethanol-d_6 in the salt-free samples while ethanol-d_6 was more accurate when sodium fluoride and potassium oxalate salts were present. It also found that 1-propanol had better precision at lower ethanol

concentrations while ethanol-d_6 had better precision at higher ethanol concentrations. However, ethanol-d_6 is much more expensive than 1-propanol on a per run basis, about 300 times more.

2. Materials and Methods

2.1 Solutions

A test was conducted to determine if there would be adequate separation between ethanol and its deuterated version using Sigma-Aldrich 200 proof ethyl alcohol and Sigma-Aldrich ethanol-1,1,2,2,2-d_5, 99.5 atom% D. The ethyl alcohol was used to prepare a 0.100 L 0.100% ethanol base solution while the ethanol-1,1,2,2,2-d_5 was used to prepare a 0.50 L IS solution with a concentration of 0.200 mL of IS compound in 1.00 L of solution. These prepared solutions and deionized water were then used to prepare 0.600 mL samples of the following: a blank sample with neither ethanol present, a sample with 0.100 mL of the ethanol solution, a sample with 0.500 mL of the ethanol-1,1,2,2,2-d_5 solution, and a sample with 0.100 mL of the ethanol solution and 0.500 mL of the ethanol-1,1,2,2,2-d_5 solution. Each of these samples were run using GC-MS parameters similar to those used in the field, and the separation of the two was found to be adequate enough to not warrant purchasing a different column than what was available at the Mondavi lab on campus.

Two separate IS solutions were prepared, one for 1-propanol and one for ethanol-d_6, with concentrations of 0.200 mL of the IS compound in 1.0000 L of water/IS solution. These solutions were made using Sigma-Aldrich ACS reagent 1-propanol ≥99.9% and Sigma-Aldrich anhydrous ethanol-d_6 with ≥99.5 atom% D. Based on their differing densities, the 1-propanol IS solution had a concentration of approximately 0.0161 g/dL, while the ethanol-d_6 IS solution had

a concentration of approximately 0.0178 g/dL. A 10. mL low salt concentration solution with a concentration of 0.080 g/dL alcohol, meant to mimic the real-life situation where 10 mL of blood ("more than enough for forensic alcohol") is collected from a subject, was prepared using 4.0 mL of the 200 mg/dL Cerilliant ethanol standard, 0.101 g of Sigma-Aldrich ≥99% sodium fluoride, 0.020 g of Sigma-Aldrich ≥98.5% potassium oxalate monohydrate, and deionized water. A 10. mL high salt concentration solution with a concentration of 0.080 g/dL alcohol, meant to mimic the real-life situation where 500 μL of blood ("insufficient") is collected from a subject, was also prepared using 4.0 mL of the 200 mg/dL Cerilliant ethanol standard, 2.002 g of Sigma-Aldrich sodium fluoride, 0.403 g of Sigma-Aldrich potassium oxalate monohydrate, and deionized water.

2.2 Sample Preparation

For each IS evaluated, a Millipore Sigma Cerilliant E-034 ethanol calibration kit and blank stock solution consisting of deionized water were used to create a calibrant and 5 samples for each of the following ethanol concentrations: 0.000 g/dL, 0.050 g/dL, 0.080 g/dL, 0.100 g/dL, 0.200 g/dL, and 0.300 g/dL. These samples were prepared, one concentration at a time, by drawing 0.100 mL of the Cerilliant standard, or 0.100 mL of the blank stock solution for 0.000 g/dL, and depositing it into each individual sample vial. Then, 0.500 mL of IS solution was drawn and deposited into each individual sample vial, as illustrated in Table 1, and the vials were capped and sealed.

Salt samples were also prepared using each IS. Using the prepared low and high salt concentration solutions, 0.100 mL aliquots of each solution were drawn and deposited into five individual sample vials for low salt concentration samples and five for high salt concentration

samples. Then, as illustrated in Table 2, 0.500 mL of IS solution was drawn and deposited into each individual sample vial, and the vials were capped and sealed.

All 1-propanol samples, including the calibrants, salt-free samples, low salt concentration samples, and high salt concentration samples, were prepared in one day. Then, all ethanol-d_6 samples, including the calibrants, salt-free samples, low salt concentration samples, and high salt concentration samples, were prepared the following day. The samples were stored in their sealed headspace vials at room temperature for approximately a week prior to analysis.

	0.000 g/dL	0.050 g/dL	0.080 g/dL	0.100 g/dL	0.200 g/dL	0.300g/dL
Spiked with 1-propanol	5	5	5	5	5	5
Spiked with ethanol-d_6	5	5	5	5	5	5
Created for 1-propanol calibration curve	1	1	1	1	1	1
Created for ethanol-d_6 calibration curve	1	1	1	1	1	1
Total collected	12	12	12	12	12	12
Grand total: 72						

Table 1: Number of samples spiked with each internal standard.

	"More than enough for forensic alcohol" (Low salt concentration)	"Insufficient" (High salt concentration)
Spiked with 1-propanol	5	5
Spiked with ethanol-d_6	5	5
Total collected	10	10
Grand Total: 20		

Table 2: Number of salt solution samples spiked with each internal standard.

2.3 Instrumentation

All samples were run on a HS-GC-MS in the Mitchell Laboratory in the UC Davis Food, Science, & Technology Laboratory, located in the Robert Mondavi Institute South building on the UC Davis campus. An Agilent (7890A) GC equipped with an Agilent (80) GC Sampler and an Agilent (5975C) MS with Triple-Axis Detector in the lab was used for this experiment.

Each sample was incubated for 15 min at 60°C with an agitator speed of 500 rpm. The headspace autosampler, using a 2.5 mL syringe heated at a temperature of 70°C, sampled a 250 μL aliquot from each sample vial with a fill speed of 500 μL/s and injector penetration depth of 20mm. Each sample was then injected on the GC column at 500 μL/s at an injector penetration depth of 54mm. The syringe was then flushed after each analysis with helium gas for 1 min. The GC run cycle time was 8.7 min.

Each sample was injected on the column in split mode with a split ratio of 20:1. The GC analysis was conducted isothermally at 40°C with a constant flow of helium carrier gas at 1 mL/min on an Agilent DB-WaxETR 30 m capillary column with an inner diameter of 250 μm and a film thickness of 0.25 μm. Ethanol-d_6 eluted at about 3.5 min, ethanol at about 3.6 min, and 1-propanol at about 4.9 min. The inlet, transfer line, quadrupole, and source were kept at 250°C, 250°C, 150°C, and 230°C, respectively. The mass spectrometer was simultaneously run in SIM and scan modes with SIM dwell times and scan frequency optimized to give approximately 20 points across the peaks. The scan was conducted over a mass range of *m/z* 29-300 with a threshold of 0. The SIM mode was programmed to focus on ions with masses *m/z* of 31, 33, 42, 45, 46, 49, 51, and 59 and had a sample acquisition/dwell time of 30 ms for each ion.

2.4 Analysis

Agilent MassHunter Workstation Quantitative Analysis version 10.0 was used to analyze the raw data acquired from the instrument and to calculate the concentrations of each sample vial. The software allowed me to assign ions to the different compounds of interest and specify which ions were quantifiers and qualifiers for each compound. For ethanol, m/z 31 was used as the quantifier with m/z 45 and 46 as qualifier ions; for ethanol-d_6, m/z 33 was used as the quantifier with m/z 49 and 51 as qualifier ions; and for 1-propanol, m/z 31was used as the quantifier with m/z 42 and 59 as qualifier ions. We are able to use *m/z* 31 to quantify both ethanol and 1-propanol because they elute at separate times, ethanol at approximately 3.6 min and 1-propanol at approximately 4.9 min, so the compounds are fully resolved and there is no risk of interference.

Unweighted and weighted calibration curves were created for both IS compounds. These four curves were then used to calculate the ethanol concentration of the samples spiked with the corresponding IS compound. The raw data, including the concentrations calculated with and without the weighting, was downloaded from the quantitative software and underwent further statistical analysis using Microsoft Excel for Mac version 16.77.1. The means and standard deviations for each IS, both unweighted and weighted, were calculated at each individual concentration for the salt-free samples (at 0.050 g/dL, 0.080 g/dL, 0.100 g/dL, etc.), as well as for the low and high salt concentration samples. The means of each IS standard at each salt-free concentration were graphed with their individual standard deviations as error bars for visual comparison, and the same treatment was given to the low and high salt concentration samples as well. The means were then statistically compared to their respective

expected values using hypothesis testing t-tests on a TI-84 Plus Silver Edition calculator. The null hypothesis (H_0) for these statistical comparisons was that there is no statistical difference between the expected value at the specified concentration and the sample mean at that concentration. Accepting H_0 means there is no statistical difference between the expected value and the sample mean. Rejecting H_0 means there is a statistical difference between the expected value and the sample mean.

Excel's t-test for two samples assuming unequal variances was used to compare the IS salt-free sample sets at each concentration (0.050 g/dL, 0.080 g/dL, 0.100 g/dL, etc.) to determine if the samples and their means could have come from the same hypothetical population. The following comparisons were conducted with this method: unweighted ethanol-d_6 to unweighted 1-propanol, unweighted ethanol-d_6 to weighted 1-propanol, weighted ethanol-d_6 to unweighted 1-propanol, and weighted ethanol-d_6 to weighted 1-propanol. H_0 for these statistical comparisons was that the two data sets' sample means came from the same hypothetical population. Accepting H_0 means that the two compared sample means could have come from the same population. Rejecting H_0 means that the two compared sample means could not have come from the same population. This t-test was also conducted between the low and high salt concentration samples of each IS compound to determine if their samples and means could have come from the same hypothetical population. H_0 for these statistical comparisons was that the low and high salt concentration data sets' sample means came from the same hypothetical population. Accepting H_0 means that the two compared sample means could have come from the same population. Rejecting H_0 means that the two compared sample means could not have come from the same population.

Lastly, a cost per run analysis was conducted using the prices of ethanol-d_6 and 1-propanol from 3 different companies to determine how much more expensive it is to use ethanol-d_6 as an IS for this analysis compared to 1-propanol. The three companies included were Millipore Sigma/Sigma-Aldrich (Merck KGaA, 2024a; Merck KGaA, 2024b), Fisher Scientific (Thermo Fisher Scientific Inc., 2024a; Thermo Fisher Scientific Inc., 2024b), and Capitol Scientific (Capitol Scientific, n.d.a; Capitol Scientific, n.d.b).

3. Results and Discussion

The following section discusses the results of the statistics tests described in the Materials and Methods section. It will also provide an overview of the cost-benefit analysis conducted, as well as further points of discussion and the limitations of this analysis.

3.1 Statistical Results

The quantitative software allowed for the selection of which compounds were analytes and which were internal standards, and it also allowed for the selection of which samples would define the calibration curve. Using these capabilities and the ion abundances described in the methods section, two calibration curves were created, one for ethanol-d_6 (Figure 2) and one for 1-propanol (Figure 3). The fit of the 1-propanol curve, based on the curves' R^2 values, is slightly better than the fit of the ethanol-d_6 curve, with a value of about 0.99988 for 1-propanol and about 0.99959 for ethanol-d_6. These curves were used to calculate the concentration of the salt-free, low salt concentration, and high salt concentration samples.

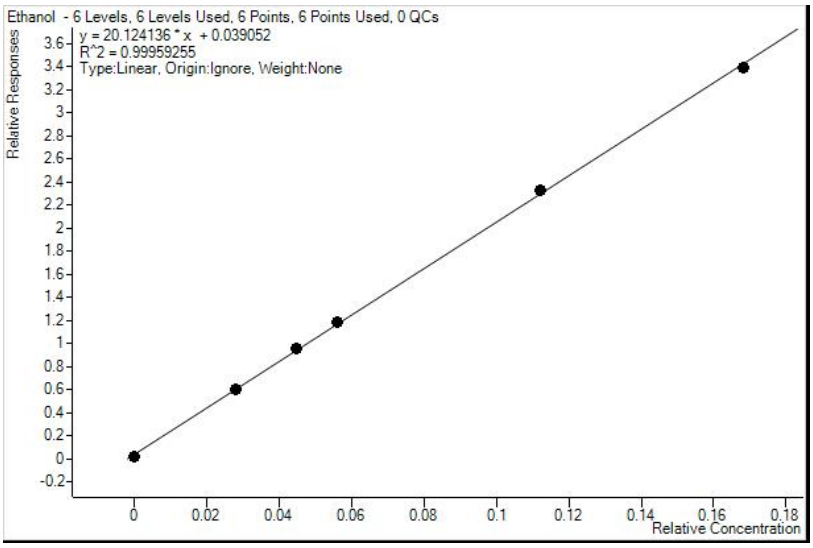

Fig. 2: Ethanol-d_6 calibration curve, no weighting applied.

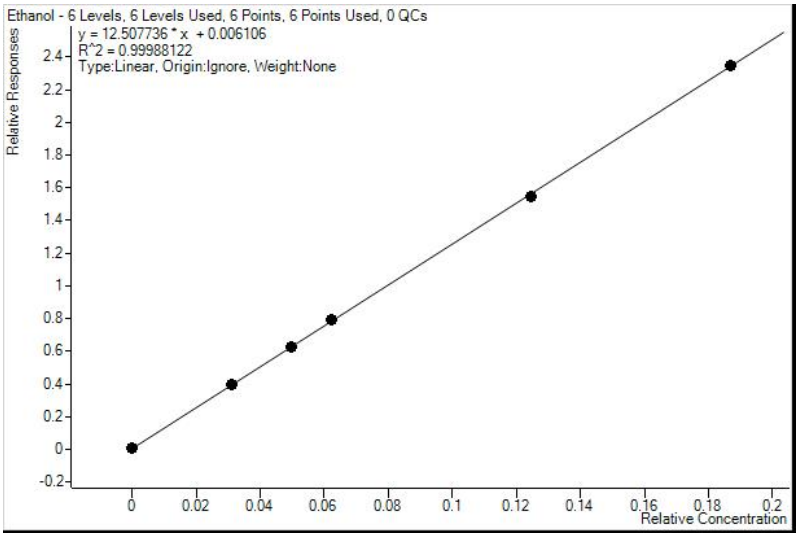

Fig. 3: 1-propanol calibration curve, no weighting applied.

A weighting factor of 1/y was also applied to each calibration curve, one for ethanol-d_6 (Figure 4) and one for 1-propanol (Figure 5), to create weighted calibration curves that gave greater priority to the lower end of the curve. The fit of the weighted 1-propanol curve, based on the curves' R^2 values, is slightly better than the fit of the weighted ethanol-d_6 curve, with a value of about 0.99993 for weighted 1-propanol and about 0.99969 for weighted ethanol-d_6. These curves were used to calculate the concentration of all the samples as well.

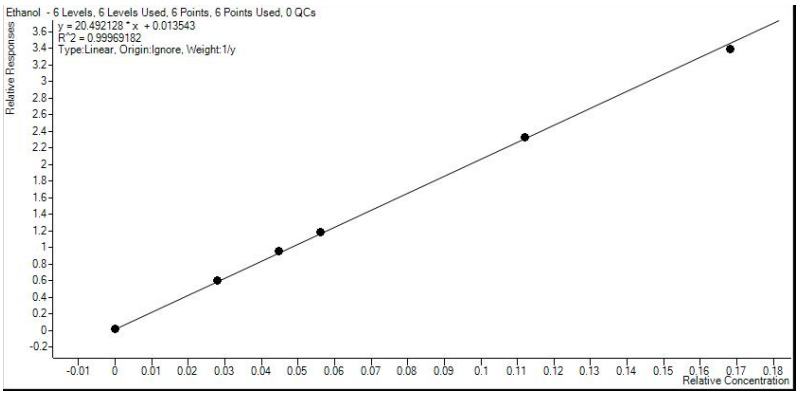

Fig. 4: Ethanol-d_6 calibration curve, weighting factor of 1/y.

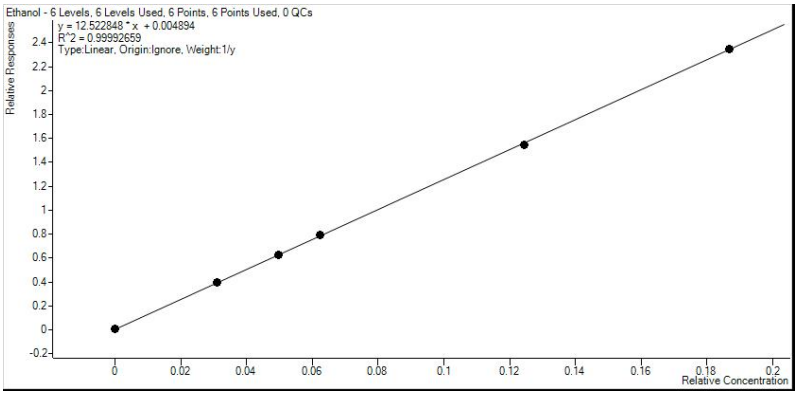

Fig. 5: 1-propanol calibration curve, weighting factor of 1/y

The mean and standard deviation of each sample type and concentration are included in Table 3. The table illustrates two trends in the data. The first is that the ethanol-d_6 means, both unweighted and weighted, tend to be overestimates of the expected concentration, with a few exceptions in the salt samples. The second is that the 1-propanol means, both unweighted and weighted, tend to be underestimates of the expected concentration. The salt sample means, which seemed the least accurate of the 1-propanol sample means, tended to be at least 0.005 g/ dL below the expected concentration of 0.080 g/dL.

	Ethanol-d6		Weighted Ethanol-d6		1-propanol		Weighted 1-propanol	
Concentration	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
0.000 g/dL	0	0	0	0	0	0	0	0
0.050 g/dL	0.0508	0.000563	0.0521	0.000563	0.0494	0.000409	0.0495	0.000409
0.080 g/dL	0.0840	0.00252	0.085	0.00249	0.0787	0.000537	0.0788	0.000505
0.100 g/dL	0.1023	0.000541	0.1027	0.000495	0.099	0.00173	0.099	0.00176
0.200 g/dL	0.207	0.00207	0.206	0.00204	0.197	0.00267	0.197	0.00269
0.300 g/dL	0.312	0.00208	0.309	0.00205	0.292	0.00593	0.292	0.00591
Low salt (0.080 g/dL)	0.0768	0.000918	0.0777	0.000870	0.0740	0.000488	0.0741	0.000488
High salt (0.080 g/dL)	0.0794	0.000537	0.0801	0.000522	0.0749	0.000776	0.0750	0.000757

Table 3: Means and standard deviations of all samples. The standard deviations are rounded to 3 significant figures for convenience. The means have as many significant figures as their individual standard deviations allow for.

The means of each IS at each salt-free concentration were graphed with their individual standard deviations as error bars for visual comparison in Figure 6. Analyzing the figure shows that 1-propanol was the most accurate IS compound at each concentration level, as shown from the distance between each point and the expected value at each level. The weighted 1-propanol curve was the most accurate at the 0.050 g/dL, 0.080 g/dL, and 0.100 g/dL concentrations while the unweighted 1-propanol was the most accurate at the 0.200 g/dL and 0.300 g/dL concentrations. 1-propanol was also the most precise IS at the 0.050 g/dL and 0.080 g/dL concentration levels, the lower range of the calibration curves, as shown from the smaller error bars at these concentrations compared to ethanol-d_6. On the other hand, ethanol-d_6 was the most precise IS at the 0.100 g/dL, 0.200 g/dL, and 0.300 g/dL concentration levels, the higher range of the calibration curves. This suggests that while 1-propanol has better precision at lower ethanol concentrations, ethanol-d_6 has better precision at higher ethanol concentrations. Figure 6 also further demonstrates the tendency for the ethanol-d_6 sample

means to be overestimates and the 1-propanol sample means to be underestimates of the expected concentrations.

The low salt concentration sample means (Figure 7) and the high salt concentration means (Figure 8) were also graphed in the same manner as the salt-free sample means. Analyzing Figure 7 shows that ethanol-d_6 was the most accurate IS compound at the low salt concentration level, as shown from the distance between each point and the expected value of 0.080 g/dL. The weighted ethanol-d_6 was the most accurate between the two ethanol-d_6 calibration curves. The figure also shows that 1-propanol overall was the most precise IS, shown by the smaller error bars. This is in line qualitatively with the precision results of the salt-free 0.080 g/dL samples, where 1-propanol was the most precise.

Analyzing Figure 8 shows that ethanol-d_6 was the most accurate IS compound at the high salt concentration level, as shown from the distance between each point and the expected value of 0.080 g/dL. The weighted ethanol-d_6 was the most accurate between the two ethanol-d_6 calibration curves at the high salt concentration like it was at the low salt concentration. The figure also shows that the weighted ethanol-d_6 was the most precise IS, shown by the smaller error bar, which is slightly smaller than the unweighted ethanol-d_6 error bar. Both Figure 7 and Figure 8 suggest that ethanol-d_6 may be better for accuracy for samples where salts are present, which in California would be all blood alcohol samples per Title 17 (17 CCR **§ 1219.1**).

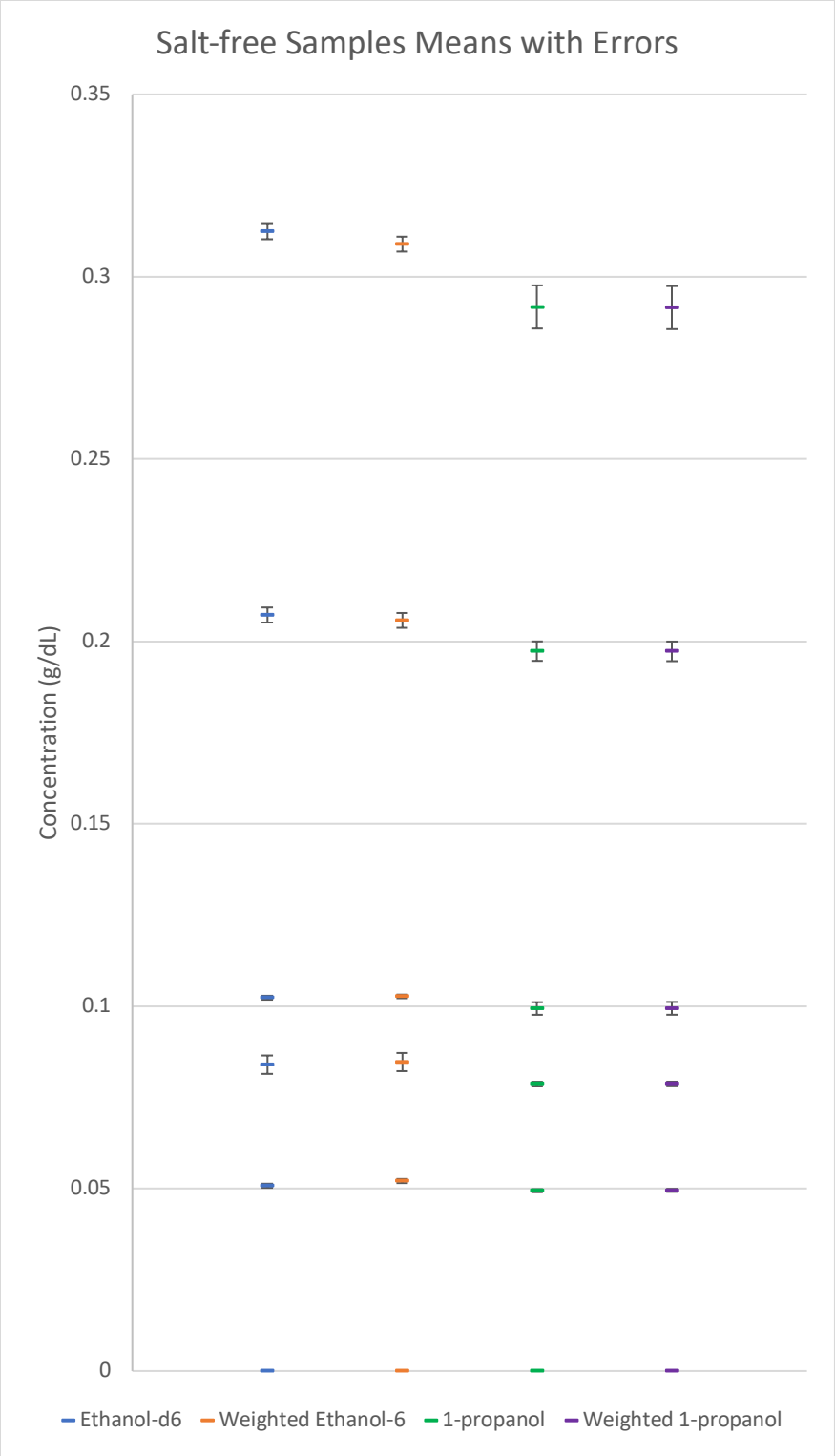

Fig. 6: Plot of the salt-free samples' means at each tested concentration, with individual error bars included. Each error bar was constructed using the standard deviations of the different sample sets, i.e., ethanol-d_6 error bar corresponding to the 0.100 g/dL ethanol-d_6 samples' mean has the value of the 0.100 g/dL ethanol-d_6 samples' standard deviation.

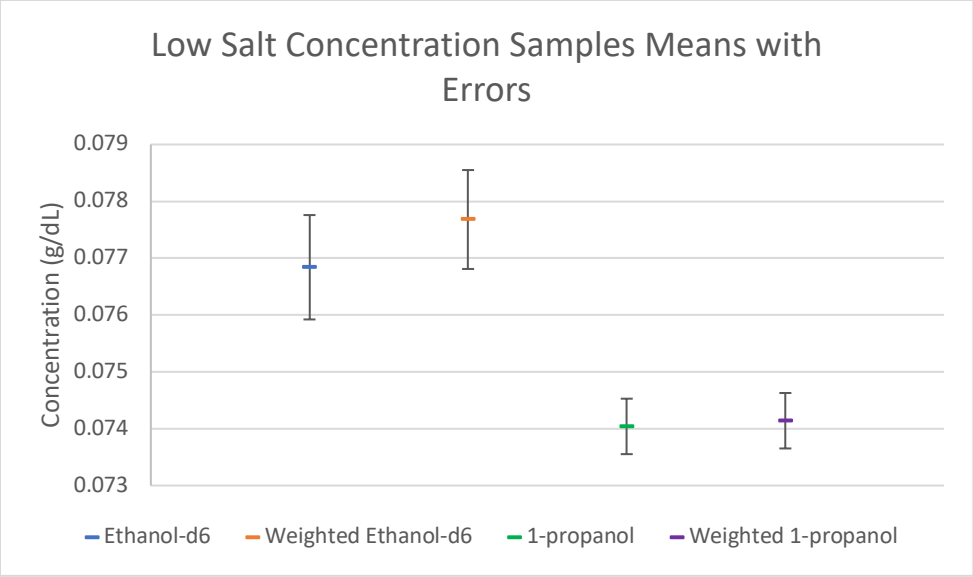

Fig. 7: Plot of the low salt concentration samples' means, with individual error bars included. Each error bar was constructed using the standard deviations of the different sample sets, i.e., ethanol-d_6 error bar has the value of the ethanol-d_6 low salt concentration samples' standard deviation.

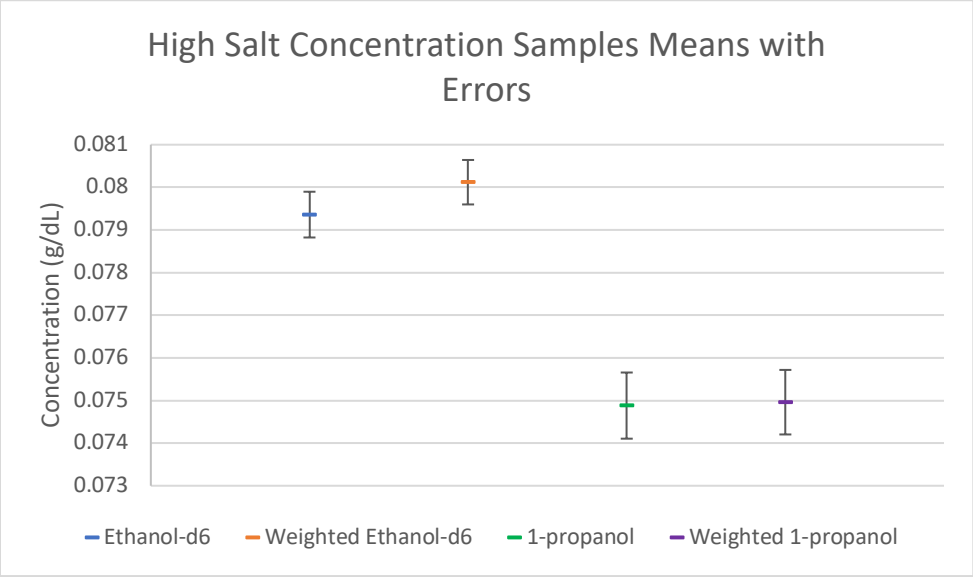

Fig. 8: Plot of the high salt concentration samples' means, with individual error bars included. Each error bar was constructed using the standard deviations of the different sample sets, i.e., ethanol-d_6 error bar has the value of the ethanol-d_6 high salt concentration samples' standard deviation.

A summary of the comparison between the IS compounds and which IS sample means were most accurate and which IS sample standard deviations were most precise is included in Table 4 for clarity.

	Ethanol-d6 vs. Weighted Ethanol-d6		1-propanol vs. Weighted 1-propanol	
Concentration	Most accurate	Most precise	Most accurate	Most precise
0.050 g/dL	Ethanol-d6	Neither weighted nor unweighted	Weighted 1-propanol	Neither weighted nor unweighted
0.080 g/dL	Ethanol-d6	Weighted Ethanol-d6	Weighted 1-propanol	Weighted 1-propanol
0.100 g/dL	Ethanol-d6	Weighted Ethanol-d6	Weighted 1-propanol	1-propanol
0.200 g/dL	Weighted Ethanol-d6	Weighted Ethanol-d6	1-propanol	1-propanol
0.300 g/dL	Weighted Ethanol-d6	Weighted Ethanol-d6	1-propanol	Weighted 1-propanol
Low salt	Weighted Ethanol-d6	Weighted Ethanol-d6	Weighted 1-propanol	Neither weighted nor unweighted
High salt	Weighted Ethanol-d6	Weighted Ethanol-d6	Weighted 1-propanol	Weighted 1-propanol

Table 4: Summary of the accuracy and precision comparisons between the IS compounds. The unweighted and weighted versions of the individual compounds were first compared to each other, then the most accurate and precise results of ethanol-d_6 and 1-propanol were compared at each concentration and sample type. Blue filled boxes indicate which was the most accurate overall at a given concentration, while green filled boxes indicate which was the most precise overall.

This study only explored how differing salt concentrations can affect the accuracy and precision of ethanol-d_6 and 1-propanol at a single ethanol concentration, 0.080 g/dL. Further research could explore and compare these IS compounds in low and high salt concentrations samples at various ethanol concentrations to see how the salt concentration can affect the IS accuracy and precision along an entire calibration curve.

The means of each IS, both unweighted and weighted, were then statistically compared to their expected values at each concentration level using hypothesis testing t-tests. Table 5 summarizes the hypothesis t-test results comparing the unweighted ethanol-d_6 means to the expected values at each concentration level. As demonstrated in Table 5, all the sample means were statistically different from their expected values, except for the high salt concentration solution sample mean.

Ethanol d6							
	0.050 g/dL	0.080 g/dL	0.100 g/dL	0.200 g/dL	0.300 g/dL	low salt	high salt
H_0	μ= 0.050 g/dL	μ= 0.080 g/dL	μ= 0.100 g/dL	μ= 0.200 g/dL	μ= 0.300 g/dL	μ= 0.080 g/dL	μ= 0.080 g/dL
t crit	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78
Mean	0.0508	0.084	0.1023	0.207	0.312	0.0768	0.0794
Standard deviation	0.000563	0.00252	0.000541	0.00207	0.00208	0.000918	0.000537
t calc	3.0978	3.509	9.6665	7.871	13.317	-7.6959	-2.6666
conclusion	reject H_0	reject H_0	reject H_0	reject H_0	reject H_0	reject H_0	accept H_0

Table 5: Results of the hypothesis testing used to compare the unweighted ethanol-d_6 calculated concentration means of each concentration group to their expected value. Each test was a two-tailed test where α=0.05 and the sample size n=5. All standard deviations t_{crit} values are rounded to 3 significant figures for convenience. All means and t_{stat} values have as many significant figures as their individual standard deviations allow.

Table 6 summarizes the hypothesis t-test results comparing the weighted ethanol-d_6 means to the expected values at each concentration level. All the sample means were statistically different from their expected values here as well, apart from the high salt concentration solution sample mean.

Weighted Ethanol d6							
	0.050 g/dL	0.080 g/dL	0.100 g/dL	0.200 g/dL	0.300 g/dL	low salt	high salt
H_0	μ= 0.050 g/dL	μ= 0.080 g/dL	μ= 0.100 g/dL	μ= 0.200 g/dL	μ= 0.300 g/dL	μ= 0.080 g/dL	μ= 0.080 g/dL
t crit	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78
Mean	0.0521	0.085	0.1027	0.206	0.309	0.0777	0.0801
Standard deviation	0.000563	0.00249	0.000495	0.00204	0.00205	0.000870	0.000522
t calc	8.2607	4.199	12.1975	6.359	9.813	-5.9625	0.5145
conclusion	reject H_0	reject H_0	reject H_0	reject H_0	reject H_0	reject H_0	accept H_0

Table 6: Results of the hypothesis testing used to compare the weighted ethanol-d_6 calculated concentration means of each concentration group to their expected value. Each test was a two-tailed test where α=0.05 and the sample size n=5. All standard deviations t_{crit} values are rounded to 3 significant figures for convenience. All means and t_{stat} values have as many significant figures as their individual standard deviations allow.

Table 7 summarizes the hypothesis t-test results comparing the unweighted 1-propanol means to the expected values at each concentration level. As demonstrated in the table, all the

sample means were statistically different from their expected values, except for the 0.100 g/dL and 0.200 g/dL sample means.

1-propanol							
	0.050 g/dL	0.080 g/dL	0.100 g/dL	0.200 g/dL	0.300 g/dL	low salt	high salt
H_0	μ= 0.050 g/dL	μ= 0.080 g/dL	μ= 0.100 g/dL	μ= 0.200 g/dL	μ= 0.300 g/dL	μ= 0.080 g/dL	μ= 0.080 g/dL
t crit	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78
Mean	0.0494	0.0787	0.099	0.197	0.292	0.0740	0.0749
Standard deviation	0.000409	0.000537	0.00173	0.00267	0.00593	0.000488	0.000776
t calc	-3.1736	-5.2500	-0.800	-2.213	-3.123	-27.3176	-14.7555
conclusion	reject H_0	reject H_0	accept H_0	accept H_0	reject H_0	reject H_0	reject H_0

Table 7: Results of the hypothesis testing used to compare the unweighted 1-propanol calculated concentration means of each concentration group to their expected value. Each test was a two-tailed test where α=0.05 and the sample size n=5. All standard deviations t_{crit} values are rounded to 3 significant figures for convenience. All means and t_{stat} values have as many significant figures as their individual standard deviations allow.

Table 8 summarizes the hypothesis t-test results comparing the weighted 1-propanol means to the expected values at each concentration level. All the sample means were statistically different from their expected values, apart from the 0.050 g/dL, 0.100 g/dL, and 0.200 g/dL sample means.

Weighted 1-propanol							
	0.050 g/dL	0.080 g/dL	0.100 g/dL	0.200 g/dL	0.300 g/dL	low salt	high salt
H_0	μ= 0.050 g/dL	μ= 0.080 g/dL	μ= 0.100 g/dL	μ= 0.200 g/dL	μ= 0.300 g/dL	μ= 0.080 g/dL	μ= 0.080 g/dL
t crit	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78	+/-2.78
Mean	0.0495	0.0788	0.099	0.197	0.292	0.0741	0.0750
Standard deviation	0.000409	0.000505	0.00176	0.00269	0.00591	0.000488	0.000757
t calc	-2.6264	-5.3137	-0.738	-2.242	-3.200	-26.8593	-14.8881
conclusion	accept H_0	reject H_0	accept H_0	accept H_0	reject H_0	reject H_0	reject H_0

Table 8: Results of the hypothesis testing used to compare the weighted 1-propanol calculated concentration means of each concentration group to their expected value. Each test was a two-tailed test where α=0.05 and the sample size n=5. All standard deviations t_{crit} values are rounded to 3 significant figures for convenience. All means and t_{stat} values have as many significant figures as their individual standard deviations allow.

It is interesting to note that, according to these statistical tests, most of the sample means at the different individual concentrations were statistically different from their expected values, regardless of the IS compound or weighting. This could be due to a few factors surrounding the experiment itself. It could be due to random and systematic errors introduced either during sample preparation or in the instrument method, particularly random human errors during the sample preparation. The small sample size at each concentration level could also be a factor, and future research could endeavor to have larger sample sizes, upwards of 20 or 30 samples, at each concentration, time and resources permitting.

The IS compounds were then compared statistically using a t-test for two samples assuming unequal variances at each concentration. The following comparisons were conducted in this way, and the results are recorded in Table 9: unweighted ethanol-d_6 to unweighted 1-propanol, unweighted ethanol-d_6 to weighted 1-propanol, weighted ethanol-d_6 to unweighted 1-propanol, and weighted ethanol-d_6 to weighted 1-propanol. Each test used the null hypothesis that the two compared sample means came from the same hypothetical population. As Table 9 shows, for each compound comparison at each concentration, the null hypothesis could be rejected. This means the ethanol-d_6 means, whether unweighted or weighted, at each concentration were statistically different from both the unweighted and weighted 1-propanol means at each concentration.

	Ethanol-d6 vs. 1-propanol	Ethanol-d6 vs. weighted 1-propanol	Weighted Ethanol-d6 vs. 1-propanol	Weighted Ethanol-d6 vs. weighted 1-propanol
0.050 g/dL				
t_{crit}	2.36			
t_{stat}	4.3712	4.0498	8.5496	8.2282
conclusion	reject H_0	reject H_0	reject H_0	reject H_0
0.080 g/dL				
t_{crit}	2.78			
t_{stat}	4.524	4.483	5.210	5.170
conclusion	reject H_0	reject H_0	reject H_0	reject H_0
0.100 g/dL				
t_{crit}	2.57			
t_{stat}	3.646	3.551	4.120	4.018
conclusion	reject H_0	reject H_0	reject H_0	reject H_0
0.200 g/dL				
t_{crit}	2.31	2.36	2.36	2.36
t_{stat}	6.571	6.571	5.620	5.626
conclusion	reject H_0	reject H_0	reject H_0	reject H_0
0.300 g/dL				
t_{crit}	2.57			
t_{stat}	7.358	7.443	6.153	6.234
conclusion	reject H_0	reject H_0	reject H_0	reject H_0

Table 9: Results of t-test comparisons between the IS compounds' salt-free samples. All t_{crit} values are rounded to 3 significant figures for convenience. All t_{stat} values have as many significant figures as the IS compound with the largest standard deviation at that concentration will allow.

This t-test was also conducted between the low salt concentration and high salt concentration samples of each IS compound to determine if their samples and means could have come from the same hypothetical population, indicating whether the IS maintained accuracy at varying salt concentrations. For instance, the unweighted ethanol-d_6 low salt concentration mean was compared to unweighted ethanol-d_6 high salt concentration samples using this statistical test. Each test used the null hypothesis that the low and high salt concentration sample means came from the same hypothetical population. The results are recorded in Table 10. As the table shows, both the unweighted and weighted ethanol-d_6

comparisons could reject the null hypothesis, indicating that ethanol-d_6 did not maintain its same accuracy at the different salt concentrations. On the other hand, both the unweighted and weighted 1-propanol could accept the null hypothesis, indicating that 1-propanol maintained what accuracy it had at the varying salt concentrations.

	Ethanol-d6	Weighted Ethanol-d6	1-propanol	Weighted 1-propanol
t_{crit}	2.45	2.36	2.36	2.36
t_{stat}	-5.30	-5.38	-2.05	-2.04
conclusion	reject H_0	reject H_0	accept H_0	accept H_0

Table 10: Results of the salt sample t-tests, comparing the individual IS compounds' low and high salt concentration sample means. All t_{crit} values are rounded to 3 significant figures for convenience. All t_{stat} values are rounded to 3 significant figures based on the precision of the experiment.

3.2 Cost-Benefit Analysis

As this is a bit of a cost-benefit analysis, the prices of ethanol-d_6 and 1-propanol from three different companies that sell both compounds were gathered and listed them in Table 11, along with the price paid for each compound when ordered through UC Davis. Purchasing the compounds through UC Davis provided for a significant discount, as the table demonstrates, one that other labs may not be privy to. Looking up the different prices at these different companies showed that ethanol-d_6 is more expensive to purchase than 1-propanol for a smaller amount of compound, as Table 11 demonstrates.

	Ethanol-d6		1-propanol	
	Cost	Amount	Cost	Amount
Through UC Davis	$43.17	1 gram ampoule	$11.82	100 mL
Millipore Sigma/Sigma Aldrich	$109.00	1 g vial	$61.50	100 mL
Fisher Scientific	$61.65	1 mL	$54.65	500 mL
Capitol Scientific	$114.57	1 g vial	$59.45	500 mL

Table 11: The prices and bottle or vial sizes of ethanol-d6 and 1-propanol from three different companies, as well as the price I paid for the two compounds through my university. Prices are before shipping and taxes are applied.

The cost per run for each IS compound was calculated and recorded in Table 12. The average cost/run for 1-propanol, including the price of the UC Davis purchase, was $\$2.33 \times 10^{-5}$; excluding the price of the UC Davis purchase, the average cost/run was $\$2.72 \times 10^{-5}$. The average cost/run for ethanol-d_6, including the price of the UC Davis purchase, was $\$7.49 \times 10^{-3}$; excluding the price of the UC Davis purchase, the average cost/run was $\$8.70 \times 10^{-3}$. By comparing the average costs/run of the IS compounds, we see that ethanol-d_6 is about 310 times more expensive to purchase than 1-propanol.

Cost of each IS compound per run						
	1-propanol			Ethanol-d6		
	Product Price	Product Amount (mL)	Cost/Run	Product Price	Product Amount (mL)	Cost/Run
Through UC Davis	$11.82	100	$0.0000118	$43.17	1.121076233	$0.00385
Millipore Sigma/Sigma Aldrich	$61.50	100	$0.0000615	$109.00	1.121076233	$0.00972
Fisher Scientific	$54.65	500	$0.0000109	$61.65	1	$0.00617
Capitol Scientific	$59.45	500	$0.0000119	$114.57	1.121076233	$0.0102
		Average cost	$0.0000240		Average cost	$0.00749
		Average cost (companies)	$0.0000281		Average cost (companies)	$0.00870

Table 12: Cost of each IS compound per run. Calculated using the constants of 0.2 mL of IS compound/1 L of IS solution, 0.5 mL IS solution/run, and the conversion factor of 1L/1000mL. For ethanol-d6, the product amount, except for the Fisher Scientific, is the projected volume based on the density of ethanol-d6 (0.892g/mL), since most of the companies only provided the amount of ethanol-d_6 they sold in grams as shown in Table 11.

3.3 Further Discussion

Ethanol-1,1,2,2,2-d_5 was used to test the separation between ethanol and a deuterated version, as discussed in the methods section. This is because while both ethanol-d_6 and ethanol-

1,1,2,2,2-d_5 were ordered at the same time for this separation test, only the ethanol-1,1,2,2,2-d_5 arrived in time to run this particular test. This works just as well because in this IS solution, where 0.200 mL of IS compound is added to water to make a 1.0000 L solution, the exchangeable deuterium on the alcohol group in ethanol-d_6 has a mole fraction of approximately 3.08×10^{-5} in solution. This means there are about 30,000 more exchangeable hydrogens than deuterium ions in solution, so 1 in every 30,000 ethanol molecules will still be ethanol-d_6 instead of ethanol-1,1,2,2,2-d_5 in solution. Thus, while ethanol-d_6 may be used to prepare the solution, it is ultimately ethanol-1,1,2,2,2-d_5 that is detected on the instrument.

A study previously discussed in the introduction (Dean et al., 1996) described how further mathematical steps were necessary to determine the concentration of ethanol using the MS abundance ratio of *m/z* 45 because both ethanol and ethanol-d_6 produce some m/z 45 ions, leading to a bit of interference. However, in this experiment, the m/z 45 ion was used as a qualifier ion for ethanol rather than a quantifier, so there is less concern about a small amount of interference from ethanol-d_6. Instead, m/z 31 ion was used to quantify ethanol because it has a high abundance in ethanol but very low abundance in ethanol-d_6, while m/z 33 was used to quantify ethanol-d_6 because it has a high abundance in ethanol-d_6 but very low abundance in ethanol, as shown in Figure 9.

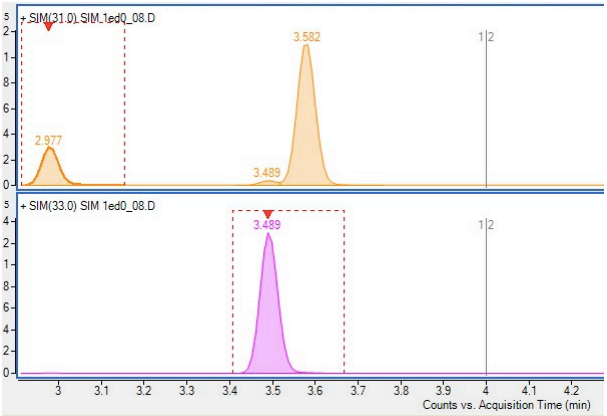

Fig. 9: Mass spectrometer SIM data for m/z 31 (top) and m/z 33 (bottom) from one of the salt-free samples. Ethanol-d_6 is the peak eluting at 3.489 min, while ethanol is the peak eluting at 3.582 min. There is some m/z 31 ion eluting at the ethanol-d_6 peak, but it is resolved enough from the ethanol peak to quantify the ethanol.

As stated previously, this study explored how differing salt concentrations can affect the accuracy and precision of ethanol-d_6 and 1-propanol at a single ethanol concentration, 0.080 g/dL. Further research could explore and compare these IS compounds in low and high salt concentrations samples at various ethanol concentrations along the calibration curve to see how the salt concentration can affect the IS compounds' accuracy and precision. Larger sample sizes, upwards of 20 or 30 samples, at each concentration should also be used, though this may depend on how prohibitive the cost of ethanol-d_6, both to purchase and on a cost per run basis, is for the researcher or laboratory interested in studying this. Further research could also investigate the possibility of a statistical method that could easily compare the two IS calibration curves to each other rather than just the curves' R^2 values or a pair-wise comparison of the means of each point on the curve. Both I and my principal investigator, Dr. Land, had trouble finding such a statistical test.

3.4 Limitations

The first limitation was that limited access to an FID for this experiment. The HS-GC on campus that was used had a MS attached instead. When forensic analysts use this detector in blood alcohol concentration analysis, they use it to qualitatively identify the analytes that the FID is quantifying instead of quantifying them (Tiscione et al., 2011) because the MS full scan is less precise than FID and the MS SIM might not be able to differentiate between interfering, low boiling point compounds like these alcohols (B. Miller, personal communication, August 26, 2022). Though the MS seemed to work adequately for this study, providing good data and performance, future research could utilize a HS-GC-FID setup instead. Forensic analysts and laboratories may also have access to an automated pipettor instrument to draw and distribute the samples and IS solutions into the headspace vials, whereas I only had access to adjustable pipettors. These automated instruments would not only save sample preparation time, but they would also decrease the uncertainty and potential error in that step of measurement.

The second possible limitation of this study is that aqueous samples were used rather than blood-based ones. Because of that, I was not able to observe any other potential matrix effects that may be present in an actual blood sample besides the salt concentration. This is notable because alcohol air-blood partition ratios can fluctuate, particularly when abnormal cell volumes or blood water contents are present (Harger et al., 1937; Harger et al., 1950). Matrix differences between aqueous calibrators and blood samples can also contribute a small uncertainty component in uncertainty calculations (Kristiansen & Petersen, 2004). However, there are aspects of the procedure that can reduce these matrix effects. For instance, the use of an internal standard dilutes the sample (Strassnig & Lankmayr, 1999). Headspace injectors also

pull from the vapor above the matrix rather than the matrix itself (Tagliaro & Lubli, 1992). Aqueous solutions are also used as calibrators for ethanol analysis in the field. For example, aqueous ethanol wet-bath simulator solutions are used to calibrate breath alcohol instruments (Hwang et. al, 2016), and Title 17 in California also specifically calls for aqueous ethanol solutions as calibration standards for blood alcohol tests (17 CCR § 1220.2). All in all, using aqueous solutions may have had minimal effects as a limitation. That said, future research should explore these IS compounds in blood-based samples in addition to the varying ethanol/salt concentrations mentioned previously.

Further limitations in this study occurred with the sample preparation and storage. As stated in the materials and methods section, they were prepared by drawing and dispensing first the 0.100 mL of Cerilliant ethanol standard and then the 0.500 mL of IS solution. This likely introduced error into the method due to the ethanol from the standard evaporating out of solution due to the small surface area of the liquid sample in the headspace vials. This may, along with the previously mentioned random and systematic errors in the sample preparation and the small concentration sample sizes, may have led to most of the sample concentration means being statistically different from their expected values.

4. Conclusion

This study found that while 1-propanol was more accurate than ethanol-d_6 in the salt-free samples, ethanol-d_6 was more accurate when sodium fluoride and potassium oxalate were present. This is notable because all blood alcohol samples in California should have a preservative and anticoagulant present under Title 17 (17 CCR **§ 1219.1**), and sodium fluoride and potassium oxalate usually fill these roles in the Vacutainers that are often used in this

analysis (Jones & Fransson, 2003). This study also found that 1-propanol had better precision at lower ethanol concentrations while ethanol-d_6 had better precision at higher ethanol concentrations. That said, ethanol-d_6 is over 300 times more expensive than 1-propanol on a cost per run basis, so this may outweigh any potential accuracy and precision benefits of ethanol-d_6 as an internal standard for this analysis. However, future research should explore and compare ethanol-d_6 and 1-propanol in low and high salt concentration samples at various ethanol concentrations instead of a single ethanol concentration to see how salt concentration can affect the IS compounds' accuracy and precision across the entire calibration curve.

5. References

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