DEVELOPMENT AND VALIDATION OF A METHOD FOR THE DETERMINATION OF NINE BENZODIAZEPINES AND METABOLITES IN DRIED BLOOD SPOTS (DBS) USING UPLC-QTOF-MS

By

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ABSTRACT:

In forensic toxicology benzodiazepines are common compounds implicated in drug impaired driving cases. Detection of these compounds is ideally determined from whole blood collected from the individual after impairment has been established and qualified personnel are available to draw blood. This study focused on the development and validation of a screening method that could also be applied to quantification of a set of nine benzodiazepines and metabolites extracted from dried blood spots and analyzed using UPLC-QTOF-MS: diazepam (DZP), temazepam (TMZ), oxazepam (OXZ), nordiazepam (NOZ), lorazepam (LRZ), clonazepam(CLZ), 7-aminoclonazepam (7CLZ), alprazolam (APZ), and α-hydroxyalprazolam (aHAM). Advantages of DBS over whole blood sampling are less invasive sampling, potential for increased stability of analytes in the card matrix, small sample volumes, simplified extraction and ease of storage and transport. All dried blood spots were 20 µL of sheep blood spiked with a 2.86µg/mL mixture of the compounds included in this study and left to try overnight at ambient temperature. The optimized method immersed a ½" diameter punch of card containing the entire blood spot in an extraction solvent of 1:1 (v/v) methanol:acetonitrile and sonicated for 30 minutes. The extraction solvent was separated from the spot, 1 mL of acetonitrile was added followed by protein filtration. Filtrate was evaporated down to dryness and analytes were then reconstituted in 1:1 (v/v) acetonitrile:water before analysis. Each step of extraction and sample preparation was optimized for this study. All analytes were stable within the card matrix for 14 days under refrigeration at 4°C. All analytes were stable up to 12 hours in the autosampler of the instrument. Hematocrit over a range of 20 - 70% did not affect interpretation of results. Validation produced calibration curves over a range of 7.8 – 500 ng/mL that had R^2 values ranging from 0.998-1.00 with a quadratic line of best fit. Bias was <20% for high blind samples and ≤25.6% for low blind samples.

A screening method using UPLC-QTOF-MS to analyze DBS extracts able to identify all benzodiazepines of interest was developed and validated. Calibration curves representing impairment ranges of the analytes of interest predicted concentrations within a reliable range that allowed for quantification of the analytes.

KEYWORDS: forensic science, toxicology, benzodiazepines, dried blood spots, UPLC-QTOF-MS

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ACRONYMS AND ABBREVIATIONS

ACN – Acetonitrile

aHAM – α-hydroxyalprazolam

aHAM-d_5 – α-hydroxyalprazolam-d_5

APZ – Alprazolam

APZ-d_5 – Alprazolam-d_5

CLZ – Clonazepam

CV%- Coefficient of variation

DBS- Dried Blood Spots

DZP – Diazepam

DZP-d_5 – Diazepam-d_5

FA – Formic acid

LRZ – Lorazepam

MeOH – Methanol

NDZ – Nordiazepam

NDZ-d_5 – Nordiazepam-d_5

OXZ – Oxazepam

OXZ-d_5 – Oxazepam-d_5

SD – Standard deviation

SWGTOX – Scientific Working Group of Forensic Toxicologists

TMZ – Temazepam

UPLC-QTOF-MS- Ultra-performance liquid chromatography-quadrupole time of flight-mass spectrometry

7CLZ – 7-aminoclonazepam

7CLZ-d_4 – 7-aminoclonazepam-d_4

CHAPTER 1: INTRODUCTION

1.1 Introduction

Forensic toxicology is the study of interactions between exogenous compounds, such as drugs and poisons, with the biological systems of the body applied within a legal context. This is a crucial discipline within forensic science that can produce results with high impact in court. There are a few principle case types that are encountered in forensic toxicology: death investigations where various post-mortem biological fluids are analyzed, sexual assaults, and impaired driving [1]. An increasingly important application of forensic toxicology is to impaired driving cases. There has always been a large incidence of cases involving impairment by alcohol but over time a steady increase in the number of impairment cases involving drugs has been observed [2]. The ideal biological sample collected for these cases is blood. Blood is usually the preferred matrix for quantitative analysis, as the drug concentration measured in blood is typically best correlated to the concentration of drug at neural receptors. Therefore, the impairing effects and the level of impairment are often best correlated with blood drug concentration [3-5]. Thus, blood is preferred to urine for cases alleging impairment [4].

Often the collection of samples is complicated due to an individual's legal rights, where reasonable grounds must be first established before drawing blood. Drawing blood is an intravenous technique; considered highly invasive and requiring trained personnel [5,6]. Increases in analytical sensitivity through tandem (MS/MS) and high resolution (e.g. qTOF) mass spectrometry now mean that lower sample volumes are required for toxicological analysis [1,6,7]. This allows minimally invasive microsampling (i.e. < 100 μL) methods such as the use

of dried blood spots (DBS). This study presents the detection and quantification of benzodiazepines from dried blood spots (DBS) by UPLC-qTOF-MS.

1.2 Benzodiazepines

Benzodiazepines are sedative-hypnotic drugs commonly prescribed for anxiety, insomnia, and seizure management for conditions such as epilepsy. They are also used for an anesthesia and sedation for certain medical procedures [8]. There are close to 35 benzodiazepines approved for clinical use. Although the different drugs vary in potency, absorption, half life, and metabolism, they have similar general structures and mechanisms of action [9-11]. The initial interest in the use of this class of drugs was the relatively quick effect and low toxicity [10]. All benzodiazepines have the same basic structure. The range in potency has helped in a wide range of cases as the clinical effective dose is variable across prescribed benzodiazepines and use can range from anxiety relief to sedation [9,11]. Benzodiazepine effects are subject to tolerance in the effects causing sedation, anti-convulsant action and anxiolytic properties. These drugs are subject to dependence, and may have substantial abuse potential with many patients suffering difficult withdrawal [9-11]. Co-administration with alcohol or other drugs has been observed [8]. Recently studies, such as one by Hoiseth *et al.* have examined the prevalence of new designer benzodiazepines in drug impaired driving and other criminal cases [12]. Of 77 cases where designer benzodiazepines were detected over a 3-year period, only 6 cases were absent of any other drug. There is a distinct lack of research about the blood concentrations of these new drugs and how they correlate to impairment. The ability to detect and interpret concentrations both older,

commonly prescribed, and newer, designer, benzodiazepines is crucial in driving impairment cases [12].

There are many methods available for the detection of benzodiazepines from whole blood and urine, as well as from hair. The most common methods appear to be liquid chromatography mass spectrometry (LC-MS), liquid chromatography tandem mass spectroscopy (LC-MS/MS), with some gas chromatography (GC) methods reported [1,12-16]. Varied success had been achieved in quantitative analysis of benzodiazepines using GC/MS methods [1]. These compounds have been shown to be highly susceptible to thermal degradation and rearrangement, with varied reaction products produced in unpredictable patterns, limiting accurate application of GC/MS [1,13]. The variable nature of the potency of these drugs has complicated the choice of method, as sensitivity is required for those with high potency but resolution needs to be maintained with high concentrations of those with low potency [13]. Benzodiazepines also show a range of polar characteristics varying the behaviour of different species during extraction and chromatographic separation. This polarity also requires derivatization to induce nonpolar qualities before chromatographic separation [13,15]. LC/MS is less restrictive of chemical properties accommodating a wider range of polarities, molecular weights, and temperature dependence. High-pressure systems such as HPLC and UPLC have been introduced that allow higher resolution, and quicker analysis time [7,15,18-22]. The use of a quadrupole time of flight-mass spectrometer (qTOF-MS) as a detector has increased in popularity. There is the potential to apply it to both screening and quantitative analyses. This method allows the determination of accurate mass and retention time as identifying features of a given unknown compound [17,19,20,22]. This can be combined with modes, such as MS^E that alternates between low and high energy

ionizations to induce varying degrees of fragmentation to allow high-resolution measurement of the mass of both the parent ion and ion fragments [7]. Distinguishes between compounds with similar retention times and/or same molecular weight for confirmation of identity [7]. The measurement of exact mass has also been cited to decrease both the matrix effects and interferences as it allows reduction of noise from compounds of the same nominal mass [7,17,20]. Both solid phase extraction and liquid-liquid extraction have been reported for the sample preparation of whole blood suspected to contain benzodiazepines. This is required especially with small concentrations of analyte when efficient removal of interfering compounds is necessary [15,18,21]. The basic properties of benzodiazepines influence the extraction solvents and mobile phases used. The tendency is to use polar organic extraction solvents to remove the drug from the sample matrix along with the plasma proteins that bind with the benzodiazepines [23]. This allows high protein binding affinity to be overcome for benzodiazepines that tend to stay associated with plasma proteins [23]. There is also the tendency to use electrospray ionization in positive mode with acidic mobile phases when analyzing basic compounds as it has been shown to ionize most basic drugs; it appears to be the most widely used [15,19,20, 23]. The pairing of UPLC/QTOF/MS using ESI has the potential to be a good method for identifying and quantifying benzodiazepines.

1.2.1 Pharamacokinetics and Pharamacodynamics of Benzodiazepines

The main site of action for benzodiazepines is one of the main inhibitory receptors in the body: γ-aminobutyric acid, $GABA_A$, receptors. In binding to these receptors they increase the inhibitory effect of GABA therefore increasing the permeability of the plasma membrane to chloride ions, allowing an influx into the cell and decrease in the action potential of the

neuron deactivating activity [8,10,24,25]. Benzodiazepines have a specific binding spot at the junction of the α and γ subunits on the receptor [24]. Although the exact mechanism is still not clearly understood, binding causes ion gates to open more frequently. They do not act directly on the GABA receptor to induce the effect but rather alter the shape by binding to the benzodiazepine binding spot to cause the increase in frequency of opening [25]. This action is what depresses the central nervous system. Two different forms of the GABA receptor have been found: the α-1-GABA receptor is connected to the sedative, memory, and anticonvulsant activity, whereas the α-2-GABA receptor is more linked to anxiety relief [10].

The activity of benzodiazepines in maintaining the activation of GABA receptors for long periods of time prompts the body to compensate for this increase in inhibitory action to maintain homeostasis. This results in the development of tolerance, whereby the mechanism is not completely understood [26, 27]. The rate at which tolerance occurs is most likely linked to factors such as the type of benzodiazepine, dose, and route of administration [26]. It has been proposed that tolerance results from the conformational change of the GABA receptor induced by the binding of benzodiazepines being reduced over time so that that the drug does not cause the same effect at the same concentrations. It has also been reported that with repeated use there is a reduction in the number of receptors being produced in the body to regulate the increased inhibitory GABA activity produced by benzodiazepines [26]. Similar changes can also be induced by the presence of other drugs that enhance GABA activity such as ethanol and barbiturates. This allows for the development of cross tolerance, when having developed tolerance for compounds from one class may offer a degree of tolerance to another similarly acting drug [27].

The most common route of administration for benzodiazepines is oral, but they can also be taken however the user chooses such as intravenously or intramuscularly [24]. There is a large variety in the properties of benzodiazepines based on the variable structure from addition of different functional groups to the common backbone. Lipid solubility is a variable factor that affects the rate of absorption and diffusion into the body [25,26]. Most tend to have a high bioavailability even when taken orally. Benzodiazepines with a higher lipophilicity have a quicker rate of absorption and distribute more within tissue. The duration of effect of these compounds and their bioavailability is also varied, and are grouped into short, intermediate, and long acting [25].

A wide variety of cytochrome P450 enzymes are used for metabolism of different benzodiazepines, the main enzyme being CYP 3A [28]. These enzymes either hydroxylate, demethylate, or nitro-reduce to alter the structure to facilitate elimination from the body [10,28]. These metabolic enzymes are primarily found in the liver, but also occur in other tissues [28]. Many of these drugs have active metabolites that continue to affect the body, such as diazepam with its three active metabolites: nordiazepam, temazepam, and oxazepam [10]. There is a second pathway in benzodiazepine metabolism where diphosphase glucoronosyltrasnferase adds a glucoronide to the metabolite or drug to aid in metabolism [28]. This is seen in 3-hydroxybenzodiazepines such as lorazepam and oxazepam, which are not acted on by CYP enzymes [29].

Benzodiazepines are commonly used in combination with many different drugs, which produces a high potential for drug interaction. This action can either inhibit or enhance the rate of metabolism of the drug or the effect of the drug. As the metabolism of these drugs is mediated largely by CYP enzymes, there is a potential for drug interactions with other

classes of drugs [10,30]. Some include certain antidepressants such as tricyclic anti-depressants and selective serotonin re-uptake inhibitors (SSRIs), opiates, antipsychotics, and alcohol. The interaction of benzodiazepines with other drug classes is entirely dependent on their route of metabolism [30]. For example, fluvoxamine, a SSRI, inhibits CYP3A and CYP2C19, affecting the metabolism of benzodiazepines such as alprazolam and diazepam that are oxidized and metabolized by CYP3A4. SSRIs also interact with the enzyme and slow the action, decreasing the rate of metabolism and therefore the extending the time the drug is in circulation in the body [31]. Co-administration of fluvoxamine and alprazolam resulted in a significant increase in plasma alprazolam concentrations and an extended half-life [31]. Pharmacodynamic interactions have also been proposed, such as an enhancing effect of benzodiazepines on opioids, and *vice versa* [32]. The effect of benzodiazepines on the delta opioid receptor, an important site of action of opiates, was found to mediate the anxiolytic effect of benzodiazepines as well, indicating a potential explanation for the combined use of these drugs [33]. An additive effect from the action of both benzodiazepines and opiates on this receptor is experienced when taken together. An additive effect of not only the anxiolytic properties of benzodiazepines but also the general analgesic effects of opioids has also been suggested [32,33].

1.2.2 Benzodiazepines and Impairment of Faculties Required for Safe Driving

Benzodiazepines are commonly prescribed to the general population for their effects as sedatives and hypnotics. They have also been regularly abused [8]. An obvious concern is the ability of those individuals taking them both therapeutically or recreationally to drive. There has been a noticeable increase in drug impaired driving in the last two decades, with a

high frequency of benzodiazepines being observed [34]. In a recent study of fatal motor vehicle accidents in Ontario, benzodiazepines were one of the most commonly encountered compounds after cannabis and alcohol [35]. This was also presented in an Australian study from 2016, where benzodiazepines were detected in 7.0% of fatally injured drivers from 2000-2013 [36]. The odds ratio was given to be 5.2 although stated as not statistically significant due to low number of incidences [36]. There has also been a high incidence of benzodiazepines in combination with other substances observed, for example in Norway in 1995; of cases where benzodiazepines were found in suspected impaired drivers, 92% included other substances of which 73% involved alcohol [34]. The impairing effects of benzodiazepines have been well studied. A well-correlated relationship after an acute dose between the effect of benzodiazepines and the decline of psychomotor and cognitive ability has been shown [36-41,43]. Impairment has been shown to have a dependence on blood drug concentration that was also determined to be present in chronic administration [41]. Multiple studies under controlled conditions have found impairment to visual perception, information processing, coordination reaction time, and memory [36-41,43]. A recent retrospective study found that an observable decrease in performance in common field sobriety tests focused on balance control and motor function correlated to blood concentration whereas pupil size, nystagmus, and orientation of time and place could not be correlated [39]. A meta-analysis study compiled the data from clinical and toxicological data and established the range of concentrations across which people have been found to be impaired. These values are seen in Table 1.1 for the drugs of interest in this project [42]. There is a distinct overlap observed between the therapeutic and the impairment ranges. Overlap of therapeutic and impairment ranges is a common observation, according to a study in which blood concentrations of

lorazepam and determination of impairment after detention in Washington were analyzed [43]. The mean concentration of individuals found impaired by lorazepam alone was 0.051 mg/mL within the therapeutic range of 0.018 mg/L to 0.240 mg/mL [43]. Therefore individuals taking benzodiazepines at therapeutic doses may still exhibit signs of impairment, but there is also the consideration of tolerance. A study from Norway found that there is a direct relationship between the increase in impairment and an increase in blood benzodiazepine concentration [38]. There has been disagreement about the ability to establish a set blood concentration above which individuals are impaired when taking benzodiazepines especially when considering that users are susceptible to development of tolerance to this drug class [44]. However, the authors of the latter study believed they presented data supporting potential for legal limits as the odds ratio of being determined as impaired by benzodiazepines increased across a range of 4 levels from therapeutic to highly elevated blood concentration ranges [38]. There is still research to be done regarding a limit above which most individuals are impaired, but it has been extensively shown that benzodiazepines do cause impairment of skills required for driving [36-41,43]. Currently individuals undergo field sobriety tests to establish reasonable and probable grounds to believe an individual is impaired before samples of are taken of blood, urine, or oral fluid to measure the concentration of the impairing substance. A recent study published in 2016 looked at the use of point-of-contact oral fluid drug screening devices as a presumptive test to identify and determine recent drug use at detention in suspected drug impaired driving cases [45]. This study showed acceptable performance with certain drugs of abuse, however the average sensitivity of the benzodiazepine tests was 0.592, indicating that true-drug positive individuals were only identified 59.2% of the time [45]. This result suggests difficulties in

establishing recent exposure to benzodiazepines using oral fluid, further supporting the use of blood as the sample of choice for drug impaired driving cases. Collection of blood must be done by a medical practitioner or qualified technician [5]. A method that could be used to mitigate this need while reducing the delay between incident and sampling would be beneficial to establishing concentration at the time of detention. It would be advantageous to confirm impairment established by standard roadside tests closer to time of detention.

Table1.1: List of nine benzodiazepines and metabolites of interest with their half-life, common dose, therapeutic drug concentration range and impairing drug concentration range [42].

Drug of Interest	**Half life (hours)**	**Common Dose (mg)**	**Therapeutic Blood Concentration (ng/mL)**	**Impairing Blood Concentration Range (ng/mL)**
Diazepam	50-120	4-40	100-800	8-2500
Oxazepam	6-20	30-120	200-1500	6-10000
Temazepam	6-25	7.5-30	20-900	**
Nordiazepam†	**	N/A	20-800	6-10000
Lorazepam	`10-40	2-10	3-250	<10-630
Alprazolam	6-20	0.5-10	5-55	20-650
α-hydroxyalprazolam†	**	N/A	<10% of alprazolam concentration	**
Clonazepam	20-60	1.5-20	20-80	15-125
7-aminoclonazepam†	**	N/A	**	11-68

**unable to find the information
† metabolite not prescribed on its own as medication
N/A not applicable as a metabolite not prescribed independently; no dose information

1.3 Dried Blood Spot Analysis

Techniques utilizing dried blood spots (DBS) have been used since the early 1960s, initially used in neonatal blood screening for metabolic disorders. It is an alternative to drawing venous blood [46-48]. Analysis of DBS extracts has been used to detect a wide variety of biological molecules such as DNA, various amino acids, other organic acids, proteins, and hormones [47,48]. Studies have also been published showing results from the analysis of smaller molecules such as drugs and other toxins. Although initially the interest was focused within neonatal testing there has been an increase in interest in DBS within fields such as therapeutic drug monitoring and toxicology [46-54]. The principle behind the technique is fairly simple; the heel or finger of the individual being tested is pricked and a small quantity of blood is removed and placed onto a card with distinct areas to spot the blood that will be left to dry and then that spot can be analyzed. The card is usually made up of a cellulose material but the composition can vary between companies such as the presence and nature of added preservatives [47,48].

In order for this to be advantageous for use in forensic toxicology, sensitive analytical methods able to detect the small amounts of drug present in the smaller volumes of blood collected using DBS, and with a high level of accuracy need to be available. With the continual development of separation and detection methods such as MS and MS/MS the use of DBS within this discipline has become more plausible [49]. These detection methods are commonly attached to instruments performing HPLC or UPLC to separate the various compounds in a given sample before they reach the detector. Many methods have been developed and validated for quantifying individual drug and multidrug mixes from DBS extraction analyzed using MS/MS [14,46-50,52,54-56]. More recent research has identified

extraction and screening methods for larger groups of drugs with varying properties to optimize the amount of drugs they can detect in one run. A recent study proposed a method of identification and quantification of 64 psychoactive drugs from multiple classes extracted from DBS using UPLC-MS/MS [55]. The authors were successful in meeting the standards required for reliable quantitative methods with precision and accuracy below a coefficient of variation below 20%. They were able to apply this method to real case samples detecting most of the drugs reported to be taken by the individual and identified by immunoassay [55]. A comparison of concentration determined with DBS compared to venous blood concentration was not reported; an important consideration in correlating DBS to possible effects of the drug [55]. Another method recently developed used LC-MS/MS to optimize detection and quantification of a large mix of benzodiazepines, their metabolites, and a z-drug from DBS [14]. They spotted 30 μL of blood onto the card before extracting with a 1:1 volume to volume methanol-acetonitrile mix before vortexing for 1 minute. All standard curves reported coefficient of determinations, R^2, of 0.99 with precision and accuracy measurements all below the 20% requirement from the Scientific Working Group of Toxicology (SWGTOX) for quantitative analytical methods [14]. These are all positive results for a reliable method to determine the amount of drug in the sample. A concern in this study was the high amount of matrix effects that were reported, with most values above the 20% cutoff recognized by SWGTOX [14,46,54]. However, a good starting point for successful methods that allow the development and simplification of extraction and quantification of benzodiazepines from DBS was found [14]. A newer method of detection, UPLC quadrupole time of flight mass spectrometer (qTOF-MS), has more recently been

proposed for the analysis of small molecules but presents minimal research available in applying this method to DBS analysis [17,20,21,46,54,57].

1.3.1 Advantages and Disadvantages

The emergence of interest within forensic toxicology communities in using DBS is based on the many advantages that have been associated with this technique. One of these advantages is a less invasive collection technique [48]. The collection of a blood sample from living individuals is associated with many legal considerations to respect the basic rights of the individual, making collection of venous samples a more laborious process as qualified individuals trained in drawing blood must be found. This prolongs the time the individual is waiting to give the sample. Over this time, metabolism of the compound of interest will continue to occur within the individual. This potentially reduces the interpretative value of the sample, as the blood drug concentration level is lowered and less representative of the initial concentration at the time of detention [47-49]. DBS may allow law enforcement to overcome this issue. Another important advantage is the stability of the compounds once spotted on the card matrix. There have been studies that test the stability of drugs including drugs such as cocaine and benzodiazepines that are structurally prone to degradation in aqueous environments [50,51,55,58,59]. The integrity of the drugs appears to be well maintained over prolonged periods of time. In one particular study concerning cocaine and benzodiazepines, three different storage temperatures were compared. At all temperatures there was less than a 20% reduction in the concentration after a month, with less than 10% reduction in the freezer for all drugs and in the refrigerator for all benzodiazepines when compared to initial concentrations of samples spotted on DBS [58]. A limitation to this study

was they did not compare degradation between whole blood vials and DBS to show a difference in degradation percent. Testing also did not continue beyond a month. The precision of the method was within a range of 1.6%-18.3% for intra- and interday experiments. However, because of this, DBS have been suggested to offer a faster and simpler way to obtain a blood sample, as it only requires a finger prick and the cards to can be shipped without refrigeration. There is also a simplification in storage and transportation of DBS compared to blood vials [47].

Another advantage is the small volumes that can be collected. This was originally a limitation but now with improvement of the sensitivity of the instrumentation this is viewed as beneficial when little sample is available and also allows the use of the less invasive technique discussed above. The volumes spotted on the card for toxicological studies range from 10-40 μL. Multiple study results have been published using DBS methods that satisfy all the requirements for quantitative analytical methods using small volumes within this range [14,46-50,52,54-56].

Although there are clear advantages, there are also some limitations to consider in terms of this method of collection that may impair the ability of a researcher to determine accurate and reliable analyte concentrations. The small volume spotted onto the card introduces sources of error due to variation in properties such as blood viscosity, hematocrit, drug distribution, spotting technique, and the card matrix itself [47,48]. This introduces the uncertainty of the distribution of the drug throughout the card. Even if the extract volume added to the card is controlled, how much does the determined concentration deviate from the venous concentration. A uniform distribution of the drug would be ideal but that is not always the case [60-62]. This has raised a concern mostly in cases where it is not the entire

blood spot being punched out but rather a small portion. Solutions have been proposed for this problem. One is punching an area that encompasses the entire spot and controlling the volume that is being added to the card, and the other is precutting the card before spotting it with a particular volume [63,64]. The precut technique showed results that were comparable to the common method of punching out a known area of the spot but without the mathematical equation that is necessary when using the more common method to determine the total blood volume of the spot itself [64]. Published studies removing the entire spot have reported results that were comparable to results taken when a sample of the spot is taken [63]. This was a proposed method used in a paper by Youhnovski *et al.* that was comparing a new pre-cut dried blood spot technique to the conventional smaller diameter sampling. The precision and accuracy of the results were all within the required parameters that ensure a method that is adequate for reliable quantification [64]. Therefore there are two simplified approaches that require no mathematical equation step to determine spot volume when back-calculating concentration.

Another limitation that relates to a disruption in the equal distribution of drug throughout the spot that would interfere with DBS quantification methods is the effect of hematocrit [47,49]. Hematocrit is the percentage of erythrocytes in a given volume of blood. This crucial property of blood is directly related to the viscosity of blood causing it to have a direct effect on the diffusion through the card. Within humans there is a normal range of 0.37-0.51 for adults but can reach between 0.2 and 0.8 and above in cases of disease [47]. A higher percentage of red blood cells may restrict movement of the blood into the card and therefore distribution. This may also have a significant effect when the drugs are highly protein bound, such as with benzodiazepines, causing the drugs to be concentrated in areas

where the erythrocytes remain, and not where the serum may have spread beyond; this would most likely also have a profound effect with a lower hematocrit [16, 65]. This also offers a challenge in interpretation as drugs that are more highly bound to erythrocytes may show an artificially high concentration compared to those with lower hematocrits, as more drug will be concentrated on the spot [65]. Therefore this is an important parameter to test experimentally. As previously mentioned, the study by Youhnovski *et al.* showed an increase in the bias of the measured quantity of drug at the higher and lower ends of the range of variation in humans [64]. A study that was conducted to quantify dexamethasone in DBS showed that there was a difference across different hematocrits in quantification but within the normal range of adults the accuracy did not change over 20%; it appeared that the extremes in variation are where the problems would lie [66]. Similarly, effect of hematocrit on 25-hydroxy vitamin D2 and D3, found that there was no significant difference in their ability to measure the concentration of the compound from DBS across a range of hematocrit from 0.4-0.6 [67]. Although comparable results were found with the alternate spot sampling methods, in the study by Leuthold *et al.* a loss of accuracy was determined to occur at hematocrit levels that deviated from the normal range of humans [61].

A major limitation in terms of using DBS in forensic toxicology to assess impairment at the time that the sample was taken is the correlation of venous blood, the preferred matrix allowing the best estimation of drug concentration, and capillary blood [48]. In the simplified sample method that is highlighted, an advantage of DBS is the ability to collect the sample from a finger prick which will involve the collection of capillary blood rather than blood directly from the vein [47]. Possible problems may arise from the tendency of capillary blood to have a higher hematocrit than venous blood [47]. Before a quantification method can be

interpreted reliably it will have to show that the concentrations that are being determined in capillary blood correlate to venous blood, so that interpretation could be made regarding the impairment of the individual. If not, the method may only serve as an identification method, only being able to confirm if the compound was present or not in the blood [48]. An experiment with this goal was conducted in a study seeking the correlation of capillary blood cocaine concentrations and plasma blood concentrations [51]. It was found that the concentrations correlated well after hematocrit was taken into effect, as it was comparing plasma to whole blood, allowing interpretation of cocaine concentrations in venous blood from DBS [51]. Recent research has looked to optimize the advantages and minimize the disadvantages of DBS. One paper from Saussereau *et al.* looked to minimize these effects as well as simplify the procedure by using on-line sample cleaning methods where the extraction solvent is immediately injected into an on-line system after extraction [68]. This method measured a selection of common illicit drugs. Using this system the hope is to allow a quick and simple process that further eliminates transfer steps and reduces the potential for contamination. Using this on-line method, precision and accuracy for all drugs had a relative standard deviation and bias measurements below 20%. The recoveries obtained for the group were all above 75% at a low concentration of 20 ng/mL [68]. Within real samples a coefficient of determination was 0.98 and above [68]. Results such as those above show potential for the development of accurate and precise methods that allow the quantification of drugs from DBS, with considerations of hematocrit and capillary-venous blood correlations needed for proper interpretation of results.

1.3.2 Forensic Relevance of DBS

It is evident from the current state of research within drug detection and quantification using DBS methods that this has a high potential for use in forensic toxicology. Considering the advantages of a less invasive sampling procedure and small sample volumes as well as stability of the compounds within the matrix, DBS lends itself well to be considered as a road-side collection technique. There has already been a wide range of success with identifying drugs from a wide range of classes from antemortem and postmortem blood [14,46-50,52,54-56]. Currently, road side tests to measure the level of impairment due to drugs in Canada is limited to psychomotor tests, and the collection of biological samples required for confirmation of the presence and concentration of the drug happens later, after qualified personnel has been found to draw blood [5]. This long period between detention and the sampling of the blood; does not offer a complete idea of the individual's level of impairment when pulled over, as the body has had time to metabolize more of the drug, producing a lower concentration of the drug at the time of sampling. The continued development of extraction methods for different drug classes, with different detection techniques such as UPLC-MS/MS and UPLC-qTOF-MS to include a wider range of drugs that can be identified as well as inclusion of on-line methods to speed up the process also offer the potential for more efficient and quick analysis of impaired driving cases.

1.4 Main Objective of Study

The main objective to be reached in this study was the development and validation of a screening method for the detection of nine benzodiazepine and benzodiazepine metabolites from dried blood spots that could also function to quantify the identified analyte. The aim

was to develop a simple extraction method followed by detection with UPLC-QTOF-MS. The nine compounds were drugs commonly prescribed from this class worldwide. The list consists of diazepam and its three metabolites, temazepam, oxazepam, and nordiazepam, as well as lorazepam, alprazolam, its metabolite α-hydroxyalprazolam, clonazepam, and its metabolite 7-aminoclonazepam. Being able to detect and quantify the drugs at blood concentrations relevant to impairing blood concentrations determined through both experimental and retrospective studies was important. A number of parameters were modified in regards to the sample preparation and extraction method applied to the dried blood spots. This was necessary to optimize matrix effects present due to the chromatography method, within the ±20% range required by the SWGTOX acceptable for quantitative methods, while maximizing recovery of the analytes of interest. The precision, determined through the calculation of the coefficient of variation, was also required by SWGTOX to be within 20% for methods acceptable for quantitative analysis. If these parameters were not met, an alternative semi-quantitative analysis would be proposed.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Materials

All benzodiazepine standards (diazepam, lorazepam, oxazepam, alprazolam, clonazepam, nordiazepam, temazepam) and benzodiazepine metabolites (7-aminoclonazepam, α-hydroxyalprazolam) at a concentration of 1.0 mg/mL as well as deuterated internal standards (diazepam-*d*5, oxazepam-*d*5, alprazolam-*d*5, nordiazepam-*d*5, 7-aminoclonazepam-*d*4, α-hydroxyalprazolam-*d*5) at a concentration of 100µg/mL were obtained from Cerilliant (Round Rock TX, USA). LCMS-grade methanol, acetonitrile, and water used in sample preparation, extraction, and separation steps were purchased from OmniSolv Inc (Charlotte, NC, USA). LCMS- grade formic acid and ammonium formate were purchased from Fisher Chemical(Fair Lawn, NJ, USA). The dried blood spots cards were Whatman 903^{TM} Protein Saver DBS cards (GE Healthcare Ltd, Cardiff, UK). A 1/2" hole punch (EK Success Ltd, US) was used. All dried blood spots were made using sterile, drug free sheep blood supplied by the Niagara Police Services (Canada).

2.2 Dried Blood Spot (DBS) Preparation

Benzodiazepine stock solutions were prepared at concentrations of 40µg/mL, with diazepam, clonazepam, nordiazepam, oxazepam, temazepam, alprazolam, and α-hydroxyalprazolam prepared in methanol, and lorazepam and 7-aminoclonazepam prepared in acetonitrile. A stock mix of all benzodiazepine standards was prepared to a concentration of 2.86 µg/mL per drug. This mix was used to make all calibrators. Stock solutions of internal standards were prepared to a concentration of 4 µg/mL with diazepam-*d*5, oxazepam-*d*5,

nordiazepam-*d*5, alprazolam-*d*5, and α-hydroxyalprazolam-*d*5 prepared in methanol and 7-aminoclonazepam-*d*4 prepared in acetonitrile. The stock solutions were combined to produce a final concentration of 0.5 µg/mL per drug. Consideration of therapeutic and impairment ranges of the benzodiazepines under investigation led to the following blood concentrations to be assessed for all method development experiments: 15 ng/mL, 150 ng/mL and 450 ng/mL. Appropriate volumes of 2.86 µg/mL benzodiazepine mix were combined in clean test tubes with 1 mL of blood to produce spiked blood samples of each desired concentration. The solution was inverted and vortexed to ensure uniform distribution.

For method validation, concentrations ranging from 0 ng/mL to 500 ng/mL were produced. Spiked blood standards for curves were prepared by serial dilution. Sufficient aliquots of benzodiazepine mix was measured out and added to 2 mL of blood to make a 500 ng/mL sample, which was inverted and vortexed. A serial dilution followed to make 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.3 ng/mL, 15.6 ng/mL and 7.8 ng/mL calibrator solutions.

Preparation of DBS was performed the day before extraction was performed. Aliquots of blood (20 µL), both spiked, benzodiazepine standards and drug-free controls, were spotted onto Whatman 903™ cards using a 20 µL micropipette (Drummond Scientific Co. Broomall, PA, USA). Blood samples were spotted in triplicate for controls and calibrators. Samples were dried overnight under ambient conditions for a minimum of 12 hours between spotting and extraction.

2.3 Method Development

Before proceeding with method validation steps and extracting target compounds from authentic samples, there are parameters that had to be analyzed and optimized. These included assessment of solvent and spot agitation methods to optimize extraction time, optimization of extraction solvent, method of internal standard application, sample clean up steps (protein filtration and different solvent systems) to optimize matrix effects and recovery, and reconstitution solvent. Three solvent-spot agitation methods were assessed: vortexing, sonication, and rapid agitation utilizing a Tissuelyzer® (Qiagen). Four extraction solvents were tested: methanol; 1:1 v/v methanol:acetonitrile; 1% formic acid in methanol; and 1% formic acid in 1:1 v/v methanol:acetonitrile. Acetonitrile was assessed as an extraction solvent in preliminary experiments, but recovery was sufficiently poor, that it was not tested further (data not shown). Internal standard addition methods tested included adding the internal standard mix to the spot on the card before removing the spot; removing the spot and placing in a clean test tube before adding the internal standard mixture. Both methods were tested either immediately or 2 hours post-addition. Adding the internal standard directly to the extraction solvent was also tested. Inclusion of protein filtration, using Clean Screen FASt® 96 well plates (100 mg, United Chemical Technologies, Bristol, PA) for sample clean up, was tested to improve matrix effects. To optimize recovery and matrix effects for this step, four different solvent systems were tested: extraction in 1:1 methanol:acetonitrile; extraction in 1:1 methanol acetonitrile with 1% formic acid; extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile before filtration; and extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile with 1% formic acid before filtration. To improve recovery, reconstitution in mobile phase A was compared to

reconstitution in 1:1 v/v water:acetonitrile. Analyte stability within the card was assessed at 1, 2, 7, 14, and 28 days post-spotting at both ambient temperature and 4°C. Hematocrit effects were measured using a hematocrit range of 20-70%. Accuracy, precision, and concentration dependence were assessed over a concentration range of 0-500 ng/mL benzodiazepine mix in blood. Matrix effects, recovery, accuracy, and precision were all measured and optimized to meet parameters outlined in SWGTOX guidelines.

2.4 Sample Preparation

Each spot was removed using a ½" hole punch after waiting the minimum dry time of 12 hours and placed in a clean test tube. Internal standard mix (20 µL) was then added directly to each spot, using a 20 µL microdispensor. Methanol:acetonitrile (1 mL of 1:1 v/v) was added directly to the test tube, completely immersing the spot. This was sonicated for 30 minutes. The extraction solvent was removed from the spot, and placed in a clean test tube, where 1 mL of acetonitrile was added to the test tube. Vortexing was used to mix the solution. Using a Pasteur pipette, the extraction solvent was introduced into one of the 96 wells of the Clean Screen FASt plate to filter. The filtrate was transferred to a clean test tube and evaporated to dryness at 70°C by vacuum centrifugation and reconstituted in 200 µL of H_2O:ACN (1:1 v/v). The reconstituted filtrate was transferred to micro-centrifuge tubes (VWR Radnor, PA, USA) and centrifuged at 13,000x*g* for 10 minutes at 4°C. The reconstituted extract was then placed in a clean insert and vial to prepare for injection for analysis by ultra-performance liquid chromatography-quadrupole time of flight-mass spectroscopy (UPLC-qTOF-MS).

2.5 UPLC- qTOF-MS System

Analysis of DBS extracts, injected at a volume of 2 μL, was performed using an Acquity UPLC equipped with a Xevo-G2-XS qTOF-MS (Waters, Milford, MA). The UPLC employed a Waters Acquity HSS C18 column (150 mm×2.1 mm, 1.8 μm particle diameter). The elution gradient, Table 2.1, was used fro UPLC separation. The UPLC and qTOF MS conditions are summarized in Table 2.1. Mobile phase A was 5mM ammonium formate in 1% formic acid in LCMS grade water. Mobile phase B was 1% formic acid in LCMS grade acetonitrile (OmniSolv Billerica, MA, USA).

Table 2.1 Settings under which DBS extracts were sampled, separated and analyzed under using Waters AQUITY UPLC equipped with a Xevo-G2-XS qTOF-MS.

Chromatography	
Liquid Chromatography system	Waters ACQUITY UPLC
Column	Waters ACQUITY UPLC® HSS C18 (2.1 mm x 150 mm, 1.8 µm)
Column Temperature	50 °C
Injection Volume	2 µL
Solvent A	Water with 5mM ammonium formate, 0.1% formic acid
Solvent B	Acetonitrile with 0.1% formic acid
Gradient	0-1 min 20% solvent B 1-2 min 20-30% solvent B 2-4.5 min 30-65% solvent B 4.5-7 min 65%-70% solvent B 7-8.5 min 70-77 % solvent B 8.5-9 min 77-95% solvent B 9-9.5 min 95-20% solvent B 9.5 -11 min 20% solvent B
Total Run Time	11 min
Flow Rate	0.400 mL/min
Mass Spectrometry	
Mass Spectrometer	Waters XEVO-G2-XS
Ionization Mode	Electrospray +ve
Capillary Voltage	0.80 kV
Cone Voltage	40 kV
Desolvation temperature	500 °C
Desolvation Gas	1000 L/h
Source temperature	150 °C
Data acquisition	TOF MS
Mass range	50- 601 Da
Lock spray	Leucine Encephalin m/z= 278.1171
Sensitivity Mode	

2.6 Extraction Time Study

Three different spot-solvent agitation methods (vortexing, sonication, and rapid agitation (Tissuelyzer®)) were compared to select a method that would optimize extraction time, recovery, and throughput. The choice of agitation method was the first parameter determined for this study. DBS were prepared as outlined in section 2.2, at 0, 15, 150, and 450 ng/mL. Internal standards were added to each spot just prior to extraction. Methanol (1 mL) was added to each clean test tube and used as the extraction solvent for this preliminary experiment. A set of each concentration in triplicate was extracted over a 60-minute period using each of the three spot-solvent agitation methods. The entire volume of extraction solvent was removed and transferred to a clean test tube after 5 minutes and replaced with fresh methanol (1 mL). This was repeated at 10, 15, 30, 45 and 60 minutes. The extraction solvent from each time point was then evaporated to dryness at 70°C by vacuum centrifugation, reconstituted in 200 µL of MeOH and then centrifuged at 13,000x*g* for 10 minutes at 4°C. Analysis of each set of time points for all concentrations using each spot-solvent agitation method followed the procedure described in section 2.5 to observe the extraction of the analytes of interest over time.

2.7 Matrix Effect and Recovery Measurements

To develop the final method that was used in producing, extracting and analyzing the DBS for benzodiazepines, a number of conditions for each step of the method were tested. The performance of each of these conditions was assessed based primarily on the calculated matrix effects and recovery. Matrix effects and recovery were measured during method development for extraction solvent comparison, addition of protein filtration, comparison of

solvent systems for protein filtration, and reconstitution solvent. To measure matrix effects and recovery samples spiked pre-extraction and post-extraction were required as well as neat, matrix free, standards. Post-extraction spiked samples were produced by adding drug to drug-free DBS extract at concentrations corresponding to those expected in extracted samples, presuming 100% extraction efficiency of both compounds of interest and internal standard. Extraction is achieved by following the same procedure as that of the pre-spiked samples. 20 µL drug-free aliquots were produced in triplicate on Whatman 903™ cards. No internal standard was added to the post-extraction spots prior to extraction. Spiked post-extraction samples were produced by reconstituting the matrix evaporated to dryness in 200 µL of reconstitution solvent prepared to concentrations expected with 100% extraction efficiency. After the post-extraction addition of the benzodiazepine mixture and internal standards, the same procedure was followed as with the pre-spiked, with centrifugation at 13,000x*g* for 10 minutes at 4°C. A neat standard, of the expected concentration, if 100% of the drug was extracted, without matrix, is required. This is prepared using the reconstitution solvent and adding the necessary aliquot for the desired concentrations corresponding to 100% of each analyte being obtained at the end of the procedure. This is added to a clean vial and injected directly. Neat standards require no extraction step or sample preparation steps as no matrix is introducing into these samples. Comparison of neat standard samples to pre-spiked samples allows quantification of matrix effects. Comparison of pre-spiked and post-spiked samples allows calculation of recovery.

2.8 Analyte Stability in DBS

Stability of the benzodiazepines of interest in this study was assessed in dried blood on the Whatman 903™ card over a 1-month period at both ambient temperature and 4°C. DBS were prepared using the procedure outlined in Section 2.2 at concentrations of 0, 15, 150, and 450 ng/mL. Spots were prepared in triplicate at each concentration and analyzed after 1, 2, 7, 14, and 28 days, at each temperature. All spots, both for the ambient temperature group and the 4°C group, were left overnight at ambient temperature to allow the blood to dry. The cards dried for 24 hours were analyzed after this step as the base measurement, represented as the day 1 measurement. All other spots were separated into clean plastic bags grouped by concentration, sampling time and storage temperature. All bags were sealed and remained sealed until analysis. The samples at ambient temperature were all placed in the same fume hood where they remained undisturbed until sampling. All samples at 4°C were placed within the same refrigerator all in the same general area, where they remained until sampling. At each time interval all spots were subjected to the same extraction and analysis procedure. Extraction followed the method described in Section 2.4 and analysis as described in Section 2.5.

2.9 Hematocrit Effect

The effect of different hematocrit levels on measured results was assessed. A range of 20-70% was chosen to cover both a healthy range for humans and hematocrit changes seen along with disease. This was achieved by separating the components of drug-free whole sheep blood using a clinical centrifuge (VWR, Radnor, PA, USA) at 5000x*g* for 15 minutes in clean glass test tubes. The supernatant, plasma component, was removed and the pellet

consisting of red blood cells was set aside for testing, this was repeated until the volume of red blood cells satisfied the aliquots needed to make 1 mL of each hematocrit to be tested. Using an adjustable 1 mL microdispensor (Drummond Scientific Co. Broomall, PA, USA), sufficient red blood cells were added to clean test tubes to make 1 mL of each hematocrit being tested (20%, 35%, 45%, 55%, 70%) at 4 analtye concentrations (0, 15, 150, and 450 ng/mL). The remainder of each 1 mL volume was completed with physiological saline, 0.9% NaCl in LCMS grade water. The benzodiazepine mix was then added to make one of each concentration at each hematocrit level. Each mixture was vortexed and inverted thoroughly to ensure uniform distribution of the red blood cells and the benzodiazepine mix. Each hematocrit and concentration pair were spotted (20 μL) in triplicate onto the Whatman 903™ card and left to dry overnight at ambient temperature. They were then extracted and analyzed as described in Sections 2.4 and 2.5.

2.10 Autosampler Stability

The stability of the benzodiazepines of interest in this study was also assessed over a 36-hour period within the autosampler of the instrument. The autosampler is a part of the Waters AQUITY UPLC, and the chamber was set to maintain a constant temperature of 10°C. A set of concentrations, 15.6, 125, and 500 ng/mL were produced using the procedure outlined in Section 2.2 run with the final procedure determined from method development. Samples were run at 0, 12, 24, and 36-hour time points. The injections were made from the same sample vials, and were used to assess the stability of the drugs within the reconstitution solvent in the autosampler if samples were to remain in the chamber for an extended period of time before analysis.

2.11 Analysis Procedure

Identification of analytes was determined using the QuantLynx® software (Waters, Milford, MA). Using the retention time and accurate mass of each benzodiazepine of interest and corresponding internal standard, the software identified and calculated the peak integration. For each step of method development the peak integration was determined at a mass resolution of 0.05 Dalton (Da) from the specified accurate mass of each analyte. Mass resolution was reduced to 0.005 Da for identification of the compound and 0.01 Da for quantification from the specified accurate mass for all method validation steps. To correct for variation in conditions of each run, a response ratio was calculated. The analyte response ratio was calculated as follows:

$$\textit{Response Ratio (RR)} = \frac{Peak\ area\ of analyte}{Peak\ area\ of\ internal\ standard}$$

Response ratio was used for comparison of each of the different parameters that were assessed during method development and was the value used to calculate accuracy, and precision for method validation. The different parameters of each step of method development were assessed based on precision (assessed by the calculated coefficient of variation), matrix effects, and recovery. As outlined in the SWGTOX guidelines, coefficients of variation that exceed 20% do not fit the precision standard for good methods, therefore sample measurements must not vary more than 20% from the calculated mean of the sample. Coefficient of variation was calculated using the following equation:

$$\textit{Coefficient of variation (CV)} = \frac{Standard\ deviation\ of\ RR}{Mean\ RR} \times 100\%$$

Matrix effect is a required measurement when using LCMS. By SWGTOX guidelines, all matrix effects must be ±25% for a quantitative method. This was measured as described in Section 2.7. The value was calculated using the equation:

$$Matrix\ Effects = \frac{Post\ extraction\ spiked\ RR}{Neat\ standard\ RR - 1} \times 100\%$$

In order the optimize each step and assess the amount of each drug for each condition tested, recovery was also calculated using response ratios calculated using the integrated peaks of the drugs of interest and their corresponding internal standard. The value of recovery was determined using the equation:

$$Recovery = \frac{Pre\ extraction\ spiked\ RR}{Post\ extraction\ spiked\ RR} \times 100\%$$

CHAPTER 3: RESULTS

3.1 Method Development

3.1.1 Extraction Optimization: Time Point Comparison

Detector response measured as analyte peak area was collected for each benzodiazepine analyte included in this study after extraction with methanol using three agitation methods (rapid agitation (Tissuelyzer®), sonication, vortexing). Detector response was measured over a total time period of 60 minutes with the response of each analyte measured after 5, 10, 15, 30, 45 and 60 minutes. Response was interpreted as the quantity of drug extracted at a particular time as the extraction solvent was collected for each extraction time and fresh solvent was added between each collection time. The mean response was compared between the three agitation methods at each concentration tested to evaluate the amount of drug extracted by each method. Figure 3.1 shows the difference in response at each concentration for each of the methods tested for lorazepam. The general trends observed in this figure were comparable to the other analytes measured. Rapid agitation tended to have the highest measured response for the 150 and 450 ng/mL trials, followed by sonication and then vortexing. The values at 15 ng/mL showed similar trends on a smaller scale. Rapid agitation demonstrated the highest response and sonication and vortexing tended to be very close in response across the agitation methods. Figures for other analytes and values used are found in Appendix A. The intra-assay precision, calculated by the coefficient of variation (CV%) of the measured triplicate values, was also compared between the three agitation methods. Figure 3.2 shows the difference in coefficients of variation for each of the benzodiazepines detected. The figure shows that data from extracted DBS spotted with blood at 15 ng/mL, is consistent with trends observed with DBS at 150 ng/mL. Sonication had the

lowest coefficient of variation over the majority of analytes, at values below the 20% threshold recommended by SWGTOX for reliability of a method. Vortexing and rapid agitation showed a greater variability of results with some values exceeding the 20% threshold. LRZ and NDZ exceeded 20% CV for rapid agitation, and DZP, 7CLZ, NDZ, and aHAM exceeded 20% for vortexing. At 150 ng/mL, sonication displayed the lowest coefficient of variation for all benzodiazepines except aHAM where vortexing instead had the lowest calculated value. Vortexing resulted in the highest calculated values for DZP, 7CLZ, LRZ, APZ, CLZ, NDZ, and TMZ while rapid agitation had the highest value for OXZ and aHAM. At the highest concentration, vortexing resulted in the highest values above 20% for all drugs. Rapid agitation showed the smallest values, all below 20%, at 450 ng/mL, except for aHAM. Sonication resulted in values all below 20% except for TMZ. Results for all analytes are presented in Appendix A. Sonication appeared to display the lowest coefficient of variation over the majority of analytes. The relative recovery of each analyte was calculated for each extraction time as the percent of response at each time to total response over the 60 minutes tested. Figure 3.3 illustrates relative recovery of lorazepam at each time point analyzed comparing the three agitation methods of interest. The trend of the data in Figure 3.3 is consistent with the trends observed with the other benzodiazepines measured as well as the internal standards used; these results can be found in Appendix A. The majority of extracted benzodiazepines were removed after the first 5 minutes with calculated relative responses between 81.1-92.6% for rapid agitation; 83.1-100% for sonication; and 80.8-100% for vortexing, across all concentrations. After 10 minutes the relative responses across all concentrations are 6.8-18.9% for rapid agitation, 5.4-16.9% for sonication and 5.0-19.1% for vortexing. The majority of each benzodiazepine was extracted completely by 15 minutes;

only DZP was extracted at the lowest concentration with a 1.4% relative recovery. Between 150 and 450 ng/mL the relative recovery was 0-1.7% for rapid agitation, 0-1.2% for sonication and 0.4-1.0% for vortexing. Negligible amount of drug was extracted after that time point.

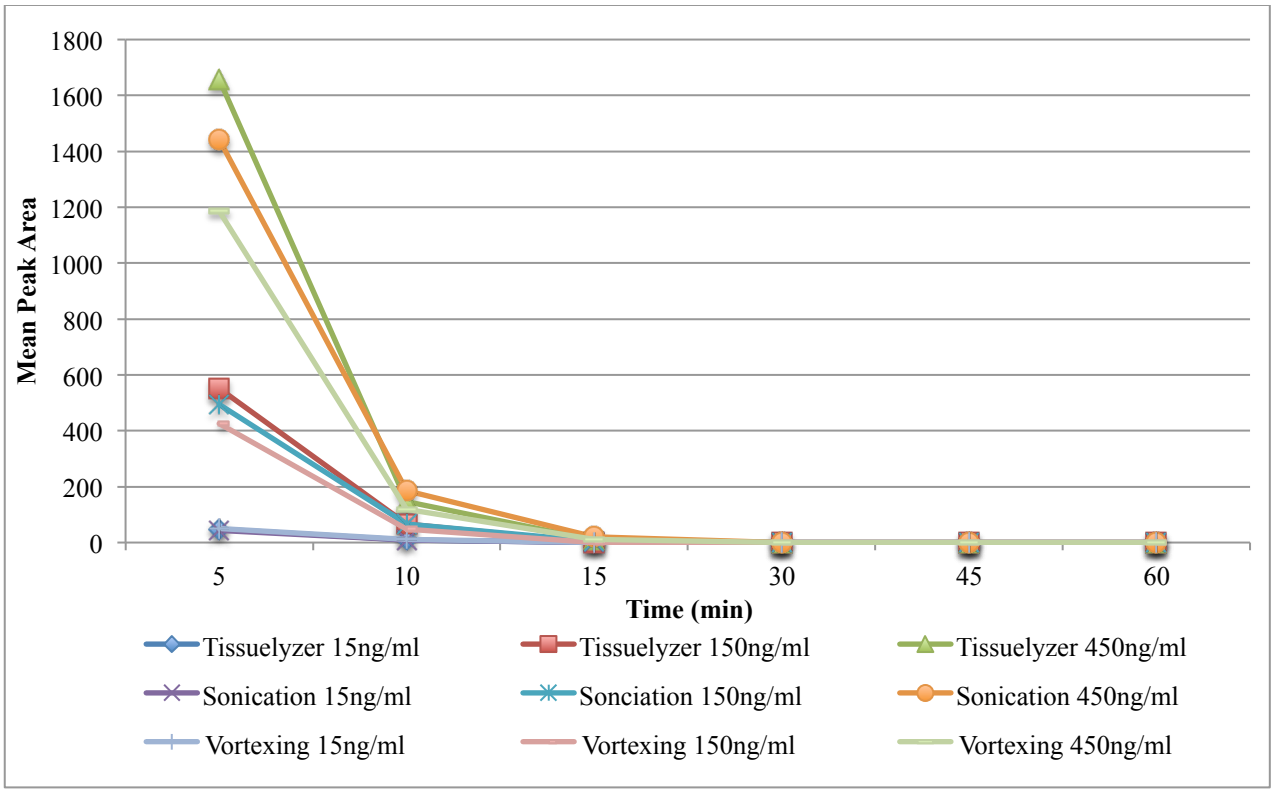

Figure 3.1: Measured response of lorazepam at each of the 3 concentrations tested (15, 150, 450 ng/mL) and at each of the three different agitation methods (Tissuelyzer®, sonication, vortexing). Each measurement was performed in triplicate (n=3).

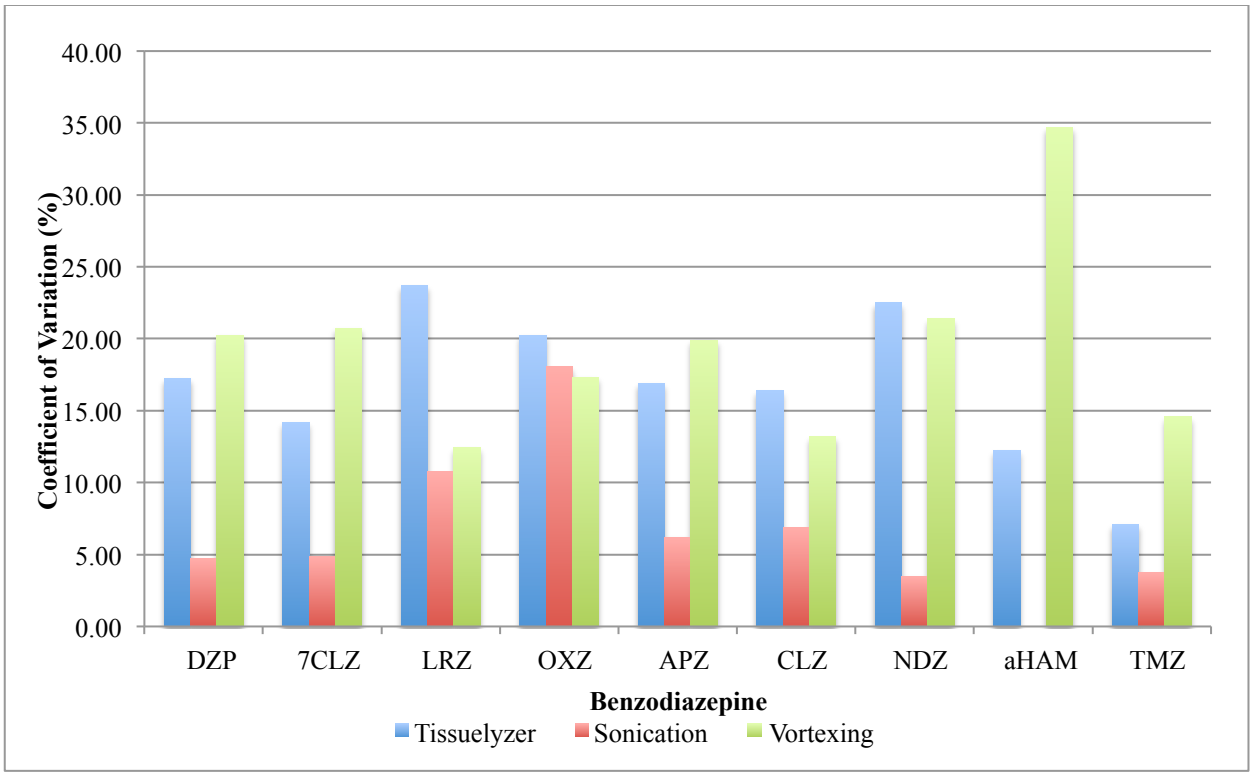

Figure 3.2: Coefficient of variation in percent of each analyte (DZP, 7CLZ, LRZ, OXZ, APZ, CLZ, NDZ, aHAM, TMZ) measured from DBS at 15 ng/mL after 5 minutes of extraction comparing each of the three agitation methods (Tissuelyzer®, sonication, vortexing). Each value was made from measurements made in triplicate (n=3).

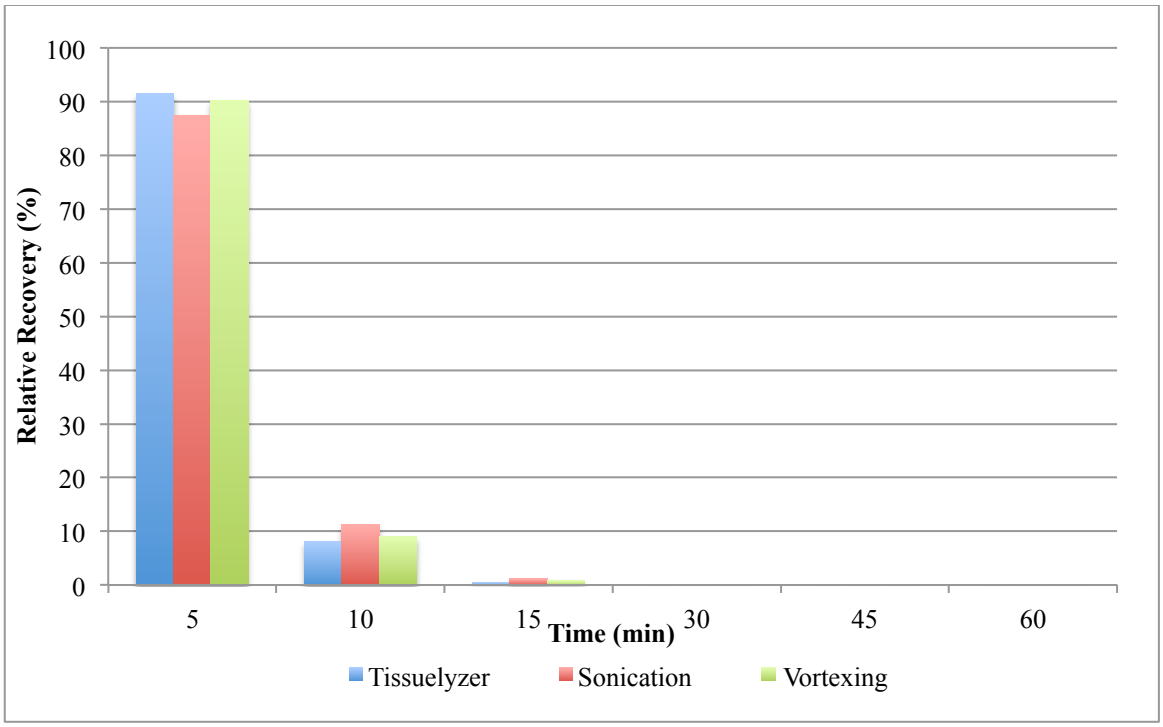

Figure 3.3: The relative recovery to the total amount of analyte extracted after 60 minutes in percent of lorazepam measured at each time point after extraction from a DBS spotted with 450 ng/mL using each of the three different agitation methods (Tissuelyzer®, sonication, vortexing) extracting from a DBS of 450 ng/mL. Each measurement was made in triplicate (n=3).

3.1.2 Extraction Optimization: Solvent Comparison

Four different solvents were assessed for optimization of extraction from DBS. Optimization of an extraction method includes selecting conditions producing high yield of the analytes, measured by calculating recovery. Acceptable precision was indicated by a CV of 20% or less. Maximization of precision is achieved by minimizing the coefficient of variation value. Optimization of matrix effects is also required for LC methods. SWGTOX standards require that matrix effects be within ±25%. The four solvents were; methanol, methanol:acetonitrile (1:1 v/v), methanol with 1 % formic acid, and methanol:acetonitrile (1:1 v/v) with 1 % formic acid. The response ratio of each benzodiazepine was measured and used to calculate the parameters compared. These values were calculated using response from DBS spiked pre-extraction, spiked post-extraction and neat standards. The recovery, precision, and matrix effects were compared for each of the nine drugs of interest for each solvent. Table 3.1 presents the matrix effects calculated for each drug at each concentration measured, 15, 150 and 450 ng/mL. Extraction solvents including formic acid presented ion suppression for more analytes and those without presented ion enhancement for all drugs except LRZ extracted with MeOH at 15 and 450 ng/mL. Most values for all extraction solvents considering the standard deviation (SD) exceeded the limit of ±20% presented by SWGTOX. MeOH as an extraction solvent resulted in calculated coefficients of variation < 20% for all drugs at 15 and 150 ng/mL; the coefficient of variation was 30.6-40.7% for all drugs at 450 ng/mL. Using MeOH with 1% formic acid, the range of CV% was < 20% for all drugs at all concentrations except aHAM at 15 ng/mL with a CV% of 32.3%. All CV% values for both MeOH:ACN and MeOH:ACN with 1 % formic acid were < 20% for all concentrations. All average recovery values for all drugs, with the exception of 7CLZ, for all

extraction solvents assessed at all concentrations exceeded the lowest recovery calculated of aHAM of 118.5% after extraction using MeOH with 1 % formic acid. The metabolite 7CLZ had a range of average recovery of 45.3 - 64.8% with MeOH, 45.6 - 58.3% for MeOH and 1 % formic acid, 75.5 - 83.6% with MeOH:ACN, and 59.6 - 70.9% with MeOH-ACN with 1 % formic acid. Specific values can be found in Appendix A.

Table 3.1: Calculated matrix effects values (±SD) of each of the nine analytes at each concentration (ng/mL) for each of the four different extraction solvents assessed; methanol (MeOH), methanol with 1% formic acid, 1:1 (v/v) methanol: acetonitrile (MeOH:ACN), and 1:1 (v/v) methanol: acetonitrile with 1% formic acid. Each condition was repeated in triplicate (n=3)

Extraction Solvent	**MeOH**			**MeOH with 1% Formic Acid**			**MeOH:ACN (1:1, v/v)**			**MeOH:ACN (1:1, v/v) with 1% Formic Acid**		
Conc. (ng/mL)	**15**	**150**	**450**	**15**	**150**	**450**	**15**	**150**	**450**	**15**	**150**	**450**
DZP	31±12	39±8	19±1	-28±7	-28±5	-42±27	26±24	13±5	11±5	-16±5	-22±10	-29±8
7CLZ	15±11	19±7	2±3	-27±6	-27±4	-41±8	34±12	16±5	16±3	-31±2	-31±9	-38±5
LRZ	-10±20	8±10	-7±2	-45±10	-53±5	-61±26	43±22	14±7	11±6	-22±12	-17±8	-28±5
OXZ	59±18	85±15	59±5	-42±10	-28±5	-38±20	12±31	24±8	21±3	-21±2	-8±9	-16±5
APZ	86±24	97±18	35±3	-1±11	-8±4	-39±15	42±20	29±6	30±10	-9±4	-18±14	-22±16
CLZ	74±14	62±10	33±3	-37±3	-47±4	-58±30	28±28	15±5	14±5	-36±7	-39±7	-49±6
NDZ	31±4	42±11	20±2	-47±4	-43±24	-56±33	27±24	16±6	12±4	-37±10	-37±9	-45±8
aHAM	134±29	119±15	77±1	101±38	61±10	29±15	73±43	44±7	39±4	159±17	66±19	39±7
TMZ	45±28	54±14	23±3	-31±16	-32±18	-47±26	62±13	12±4	12±5	-37±25	-29±8	-36±6

3.1.3 Internal Standard Application Comparison

The addition of internal standard is an important requirement that allows for correction in extraction efficiency and intra-sample injection variability. Three different application conditions were measured: the addition of the internal standard to the spot with a dry time of 2 hours, no dry time after addition to the spot after it had been punched out, and adding the internal standard directly to the extraction solvent. Variability in precision was the parameter being measured. Figure 3.4 shows the calculated coefficient of variation for each of the 3 methods that were measured for each of the six internal standards that were used for this method: oxazepam-d_5(OXZ-d_5), 7-aminoclonazepam-d_4(7CLZ-d_4), nordiazepam-d_5 (NDZ-d_5), alprazolam-d_5(APZ-d_5), α-hydroxyalprazolam-d_5 (aHAM-d_5), and diazepam-d_5 (DZP-d_5). The coefficient of variation observed in adding internal standard to the extraction solvent was much lower than the other two methods; the CV% was < 20% for all standards. No dry time produced CV% values all below 20% with the exception of 7CLZ-d_4. The CV% values with a 2-hour dry time were below 20% for 7CLZ-d_4, NDZ-d_5, and DZP-d_5. Table 3.2 shows results of student t-tests performed to measure the difference between each pair of conditions. A p-value of < 0.05 was considered to demonstrate a significant difference. No significant difference was found between the coefficients of variations comparing no dry time and 2 hour dry time methods. Significant differences were found between adding the internal standard to the extraction solvent and the two other application methods.

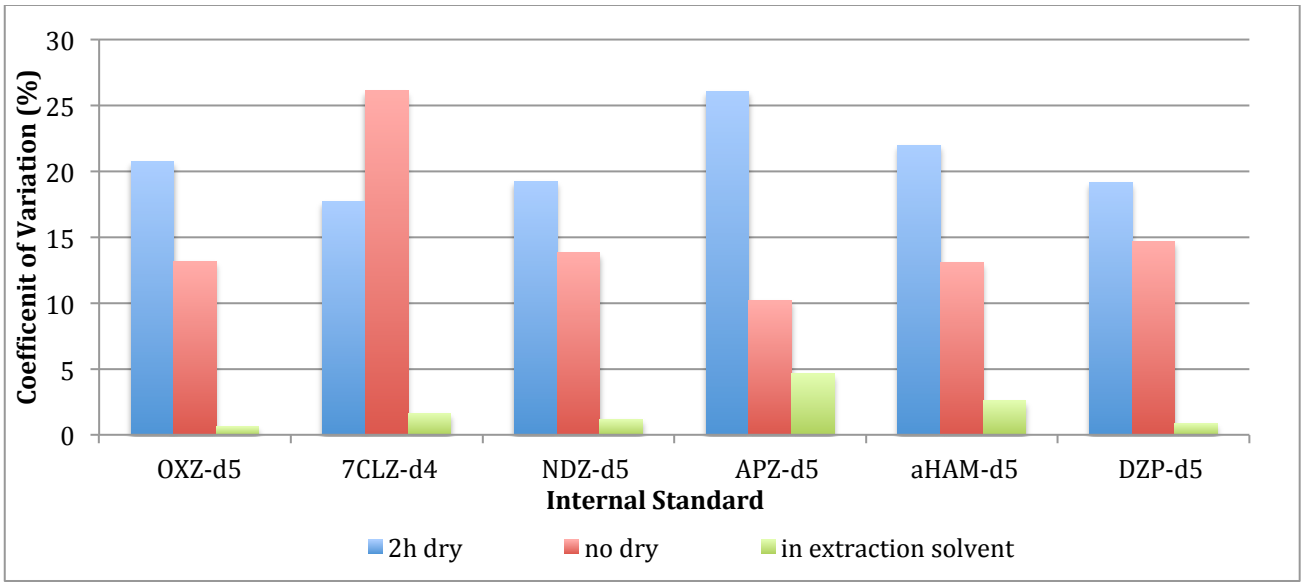

Figure 3.4: Calculated coefficient of variation in percent of each of the six deuterated internal standards used; oxazepam-d_5 (OXZ-d_5), 7-aminoclonazepam-d_4 (7CLZ-d_4), nordiazepam-d_5 (NDZ-d_5), alprazolam-d_5 (APZ-d_5), alpha-hydroxyalprazolam-d_5 (aHAM-d_5), and diazepam-d_5 (DZP-d_5). Each resulting from 20 µL of a 0.5µg/mL mixture added to each DBS extracted. Each measurement was made in triplicate (n=3)

Table 3.2: Calculated p-values for each internal standard analyzed by Student t-tests comparing each pair of parameters of internal standard application: i) addition of internal standard 2 hours dry time before extraction, ii) no dry time for internal standard before extraction, iii) adding the internal standard directly to the extraction solvent. Coefficient of variation was the point of comparison, calculated from response collected in triplicate (n=3) for each parameter. Significant differences are indicated.

Application Conditions Compared	**p-value (ISTD)**					
	DZP-d_5	**OXZ-d_5**	**NDZ-d_5**	**7CLZ-d_4**	**APZ-d_5**	**aHAM-d_5**
2h dry vs no dry	0.371	0.427	0.385	0.853	0.345	0.369
No dry vs extraction solvent	0.004*	0.004*	0.004*	0.006*	0.00005*	0.000096*
2h dry vs extraction solvent	0.004*	0.006*	0.005*	0.000043*	0.00047*	0.00025*

***** significant statistical difference calculated (p-value<0.05)

3.1.4 Solvent Clean-up: Addition of Protein Filtration Step

Addition of a solvent clean-up step during sample preparation was assessed to try to reduce matrix effects within the ±25% threshold outlined in SWGTOX for method reliability for a quantitative method. The addition of a protein filtration step using a 96-well Clean Screen® FASt plate to filter out macromolecules >1μm in size was compared to a sample preparation without. Figure 3.5 shows the comparison of matrix effects between sample preparation with and without protein filtration. The figure shows the matrix effects at 15 ng/mL for all drugs measured; the same trend is observed across all concentrations that were assessed. Across all drugs there was a decrease in the measured matrix effects when including protein filtration by Clean Screen® FASt plate. The recovery of each analyte was also measured and compared between sample preparation with and without the protein filtration step (Figure 3.6). Again, the same trend is observed across all concentrations; Figure 3.6 presents the results from 15 ng/mL DBS. The recovery calculated was found to be lower for all analytes when protein filtration was included.

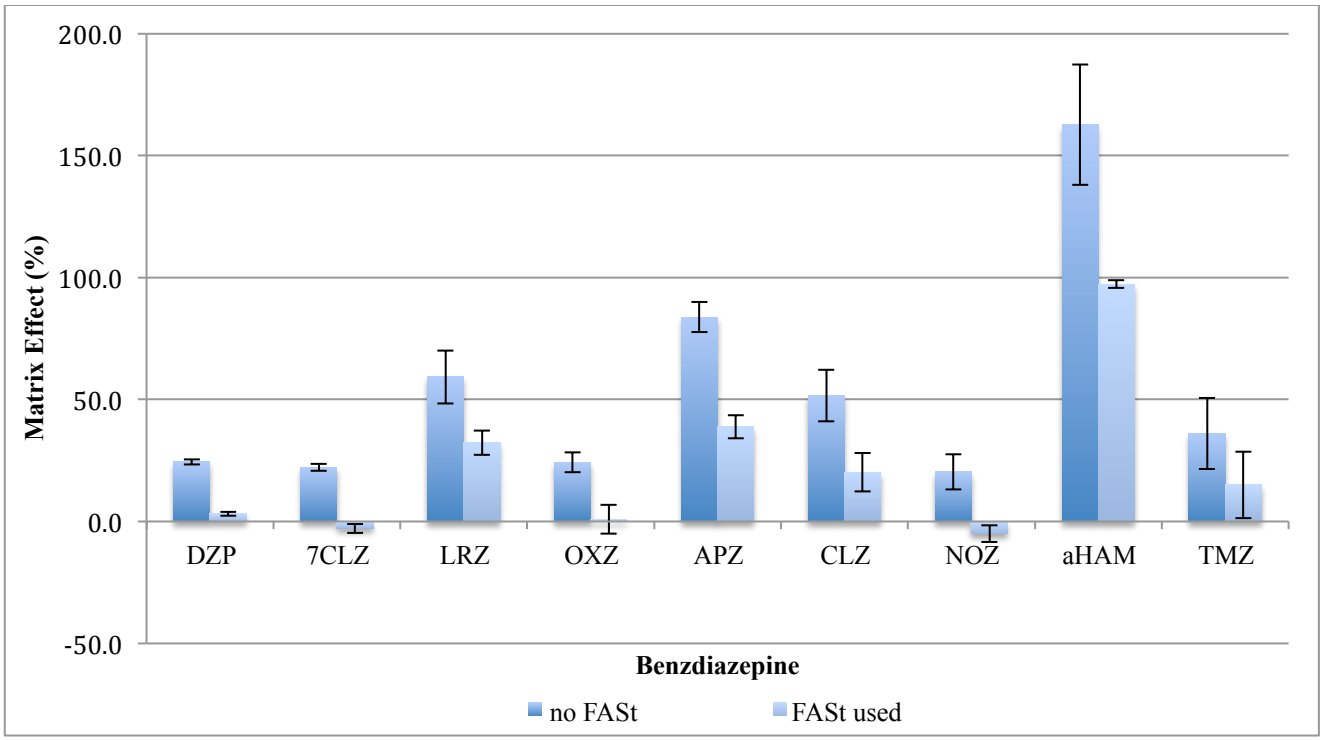

Figure 3.5: Calculated matrix effects of each of the benzodiazepines analyzed (±SD) comparing the addition of protein filtration of the extraction solvent using a 96-well Clean Screen® FASt plate to a procedure without. These data are calculated from DBS at 15 ng/mL, each measurement was made in triplicate (n=3).

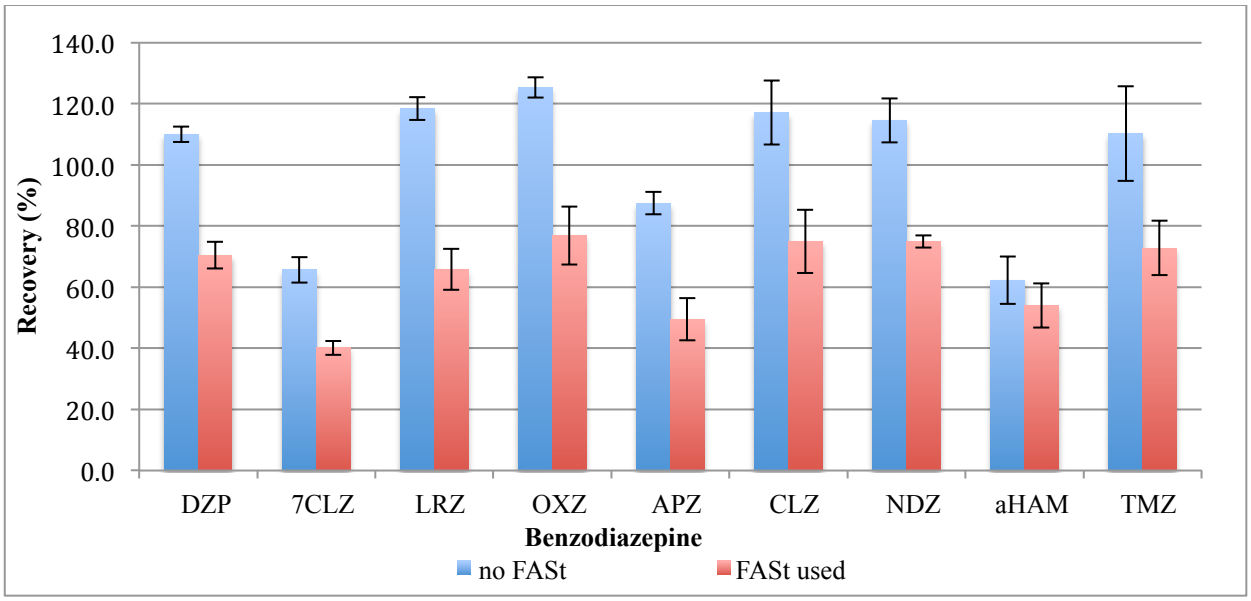

Figure 3.6: Calculated recovery (±SD) of each of the analytes comparing the use of the 96 well Clean Screen® FASt plate for protein filtration of the extraction solvent to a procedure excluding this step. These data are calculated from DBS at 15 ng/mL, results were collected in triplicate (n=3).

3.1.5 Optimization of Protein Filtration: Comparison of Solvent Systems

Matrix effects for the inclusion of protein filtration still demonstrated high matrix effects outside the ±25% threshold that was established by SWGTOX guidelines for reliable quantitative methods and the recovery also had an observable decrease with the inclusion of this step. Four different solvent systems were then tested to decrease matrix effects and increase recovery: extraction in 1:1 methanol:acetonitrile; extraction in 1:1 methanol acetonitrile with 1% formic acid; extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile to the extraction solvent before filtration; and extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile with 1% formic acid to the extraction solvent before filtration. Precision, recovery, and matrix effects were compared for all analytes in order to optimize the protein filtration step. Precision, measured using coefficient of variation (CV%), met the < 20% SWGTOX criterion for all drugs with all solvent systems assessed, with the exception of aHAM using MeOH:ACN with the additional 1mL of ACN, with a CV% of 22.0%. Figure 3.7 shows the calculated matrix effects extracted from a 15 ng/mL DBS for all benzodiazepines in this study. The graph shows both suppression and enhancement of the analytes. Similar trends were observed at 150 and 450 ng/mL (presented in Appendix A). Matrix effects when extracted in MeOH:ACN were consistently outside the ±25% range given by SWGTOX; all values demonstrated ion suppression above 25%. Using MeOH:ACN with 1% formic acid (FA) as the extraction solvent, 7CLZ and APZ exceeded the SWGTOX threshold at both 15 ng/mL and 150 ng/mL, 7CLZ displayed ion enhancement and APZ displayed ion suppression. At 450 ng/mL APZ and CLZ both demonstrated ion suppression beyond 25%. This solvent system predominantly resulted in ion suppression, more evident at the higher concentrations than at 15 ng/mL.

Using the resulting matrix effects, the solvent systems were narrowed to a choice between two. Average recovery was compared between extraction in 1:1 MeOH:ACN with addition of 1 mL of ACN before filtration; and extraction in 1:1 MeOH:ACN with addition of 1 mL of ACN with 1% FA. Figure 3.8 compares the recovery between the two solvent systems from 15 ng/mL DBS; a similar trend was observed at the other three concentrations (presented in Appendix A). At 15 and 150 ng/mL the average recovery from MeOH:ACN with 1 mL ACN added post-extraction before filtration appeared to be higher for most analytes however the standard deviation for most drugs overlaped, suggesting the two were not significantly different in recovery. However at 450 ng/mL there was no overlap of average recovery and respective standard deviations between the two conditions tested for 7CLZ, LRZ, OXZ, APZ, CLZ, NDZ, and TMZ when MeOH:ACN with 1 mL ACN was added post-extraction before filtration for all compounds with a higher recovery value (presented in Appendix A).

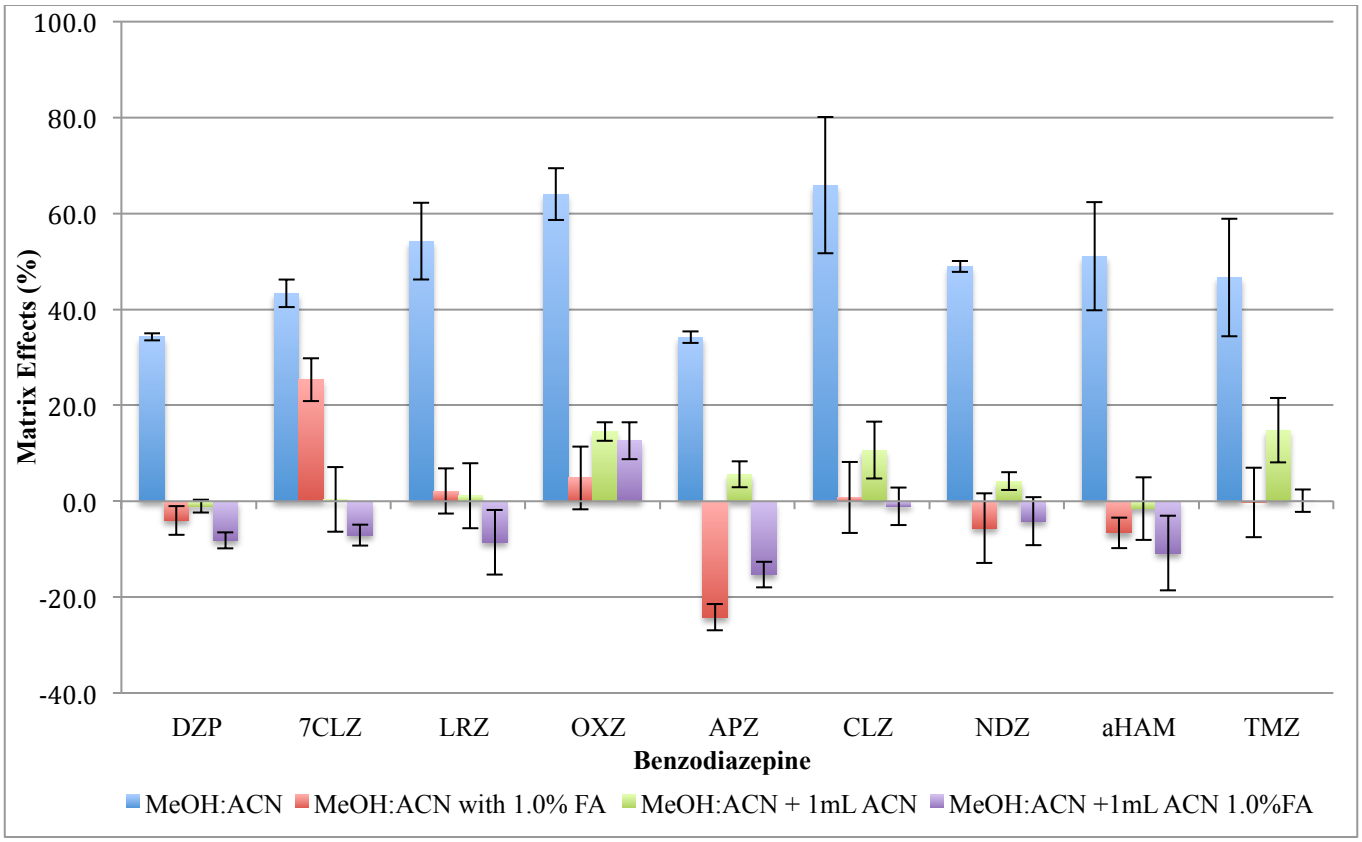

Figure 3.7: Calculated matrix effects (±SD) of each benzodiazpine from each solvent system measured to optimize protein filtration with a Clean Screen® FASt plate: 1:1 (v/v) MeOH:ACN (methanol: acetonitrile); MeOH:ACN with 1% formic acid (FA); 1:1 (v/v) MeOH:ACN with 1 mL ACN added post-extraction before filtration; 1:1 (v/v) MeOH:ACN with 1 mL ACN with 1% FA added post-extraction before filtration. These data are calculated from 15 ng/mL DBS measured in triplicate (n=3).

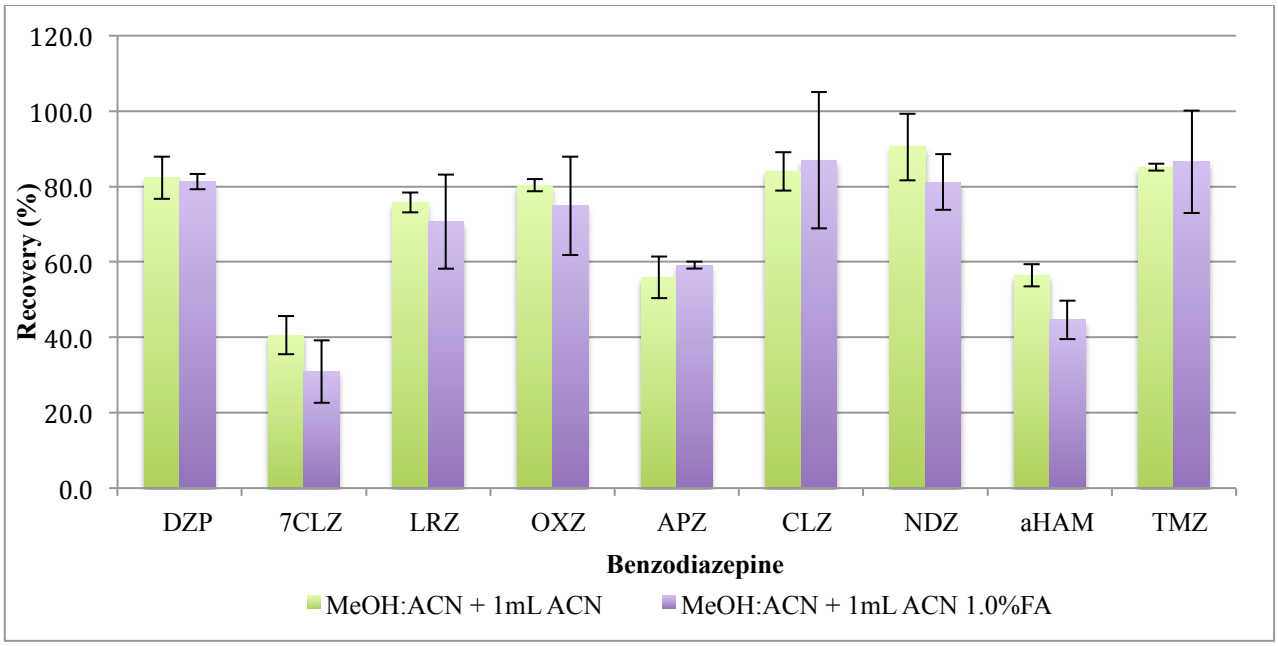

Figure 3.8: Calculated recovery (±SD) of each benzodiazepine extracted with MeOH:ACN with the addition of 1 mL of ACN to the extraction solvent before filtration compared to extraction with MeOH:ACN with the addition of 1 mL of ACN with 1% of formic acid (FA) to optimize the solvent system. These data are calculated from DBS at 15 ng/mL collected in triplicate (n=3).

3.1.6 Optimization of Reconstitution Solvent

Further optimization to increase recovery was continued to compare the reconstitution in mobile phase A (MPA) of 5 mM ammonium formate in water with 1% formic acid to reconstitution in 1:1 (v/v) acetonitrile:water (ACN:H_2O). Figure 3.9 shows the comparison of the calculated average recovery of each benzodiazepine between the two reconstitution conditions. The same trend was observed at both 150 and 450 ng/mL. After evaporating the extraction solvent to dryness, reconstituting the analytes in the ACN:H_2O demonstrated a higher percentage of recovery than with MPA. The only analyte that showed a decrease in recovery was 7CLZ, and this was observed at each of the three concentrations of extracted DBS analyzed.

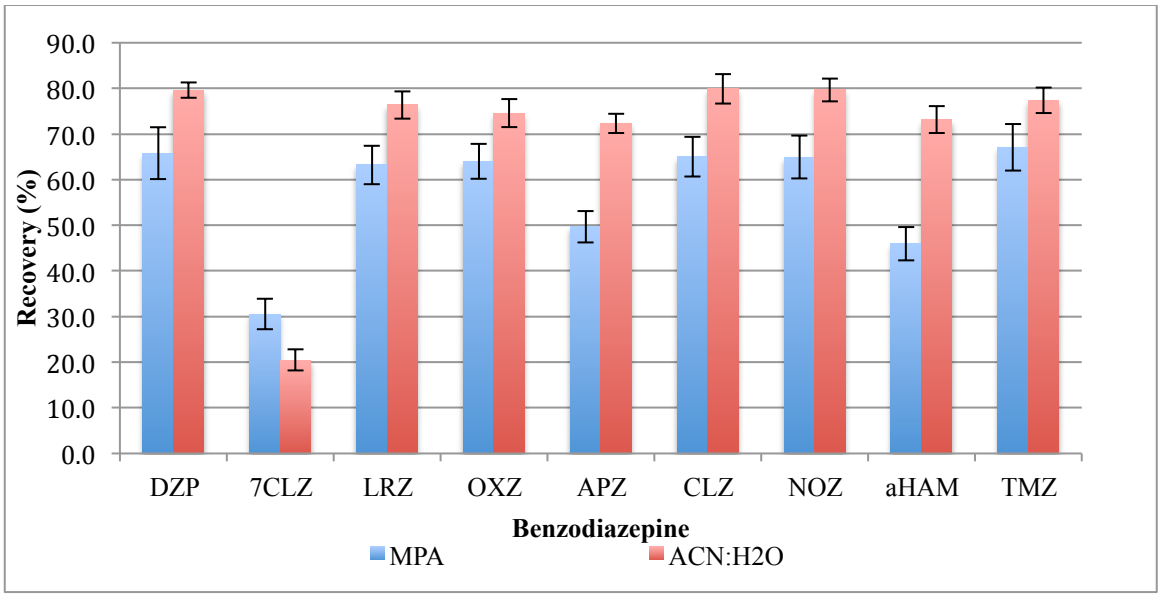

Figure 3.9: Recovery of each benzodiazepine comparing reconstitution after evaporation dryness in mobile phase A (MPA) and reconstitution in 1:1 (v/v) acetonitrile: water (ACN:H_2O) to optimize the recovery of the final step. These data show results from DBS at 150 ng/mL collected in triplicate (n=3).

3.1.7 Analyte Stability in DBS

The stability of each analyte within the Whatman 903 Protein Saver cards was evaluated over 28 days with measurements of response being made at 1, 2, 7, 14, and 28 days. Measurement was made to quantify degradation that may occur over time and compare two storage temperatures, ambient temperature and refrigeration at 4°C. The response ratio was measured and compared over time at three concentrations (15, 150, and 450 ng/mL). Any change of response ±20% from the initial response on day 1, was considered a significant change, indicating a notable change in the concentration of the analyte. Figure 3.10 illustrates the relative response over time to response on day 1 of temazepam at each time point measured, calculated compared to the initial day 1 response and represented in comparison to day 1 as 100% of the expected response at ambient temperature. Figure 3.11 illustrates the relative response to the initial response measured on day 1 of temazepam while refrigerated. Under both conditions a decrease in response at 28 days at all 3 concentrations was observed that dropped below a 20% change from the initial measured response ratio. This trend was only observed for temazepam. Figure 3.12 shows the change over the observed time period of the relative response to response measured on day 1 ± standard deviation (SD) of alprazolam under ambient conditions and Figure 3.13 shows the same parameters measured from DBS stored at 4°C. Day 1 represented 100% response and all measured responses being measured relative to that value. There was an observed increase in the relative response over time of 15 ng/mL DBS both at ambient temperature and refrigeration. Under both conditions the relative response change extended above a 20% change in response indicating a significant change. This was a common trend observed. Analytes that demonstrated this trend after 14 days are indicated in Table 3.3 while those

showing this trend after 28 days are indicated in Table 3.4. The tables indicate the percent change in response above or below the measured response relative to that on day 1. The metabolite 7CLZ showed a significant increase over 20% at 14 days at room temperature not seen when stored by refrigeration. DZP, APZ, and aHAM demonstrated significant increases in response change at 15 ng/mL at 28 days under both conditions. LRZ showed a significant increase at 28 days at room temperature and no significant change under refrigeration, while CLZ demonstrated a significant increase at 15 ng/mL and 150 ng/mL under both conditions. Both tables give the value of relative response change ± SD for each compound. Values from 2 and 7 days were not presented as all changes in response were < 20% for all compounds, therefore no significant changes in analyte response were seen at all concentrations up to 7 days.

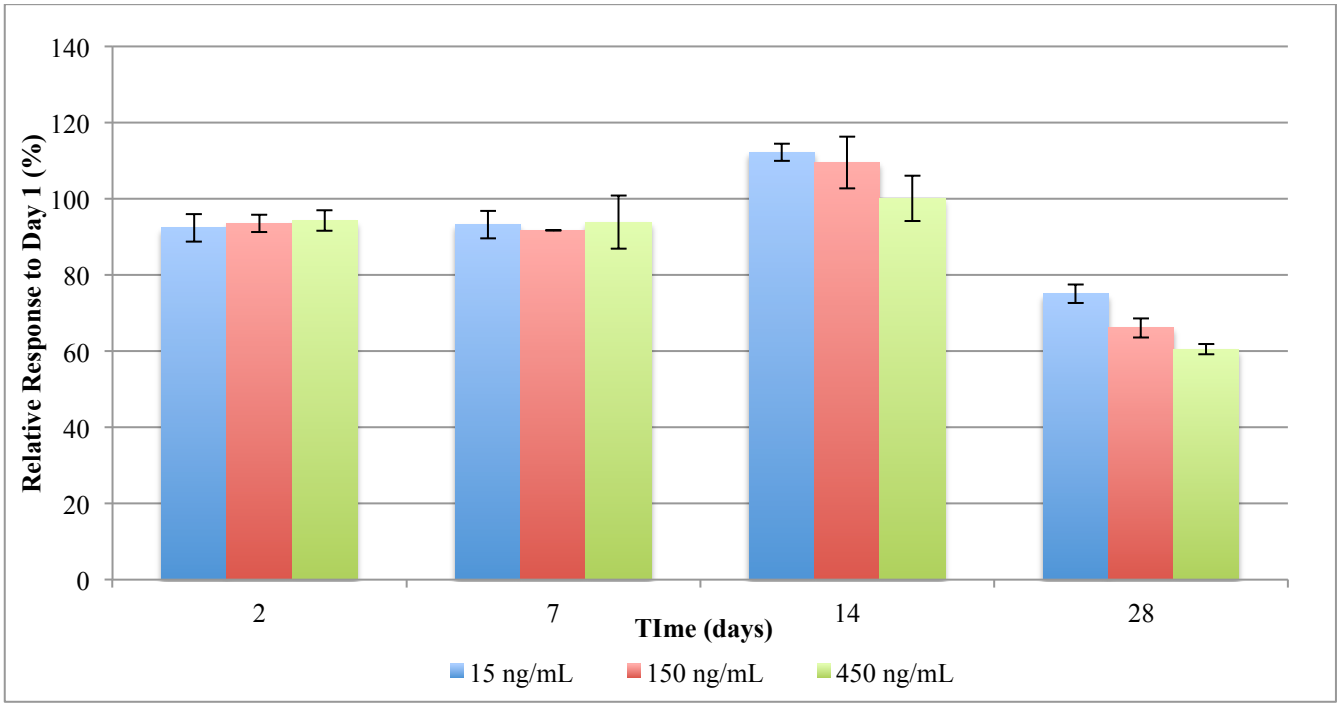

Figure 3.10: Relative response change in percent at 3 concentrations (15, 150, 450 ng/mL) of temazepam over time after storage at ambient temperature after 2, 7, 14, and 28 days measured relative to the response on day 1. Measurements were collected in triplicate (n=3).

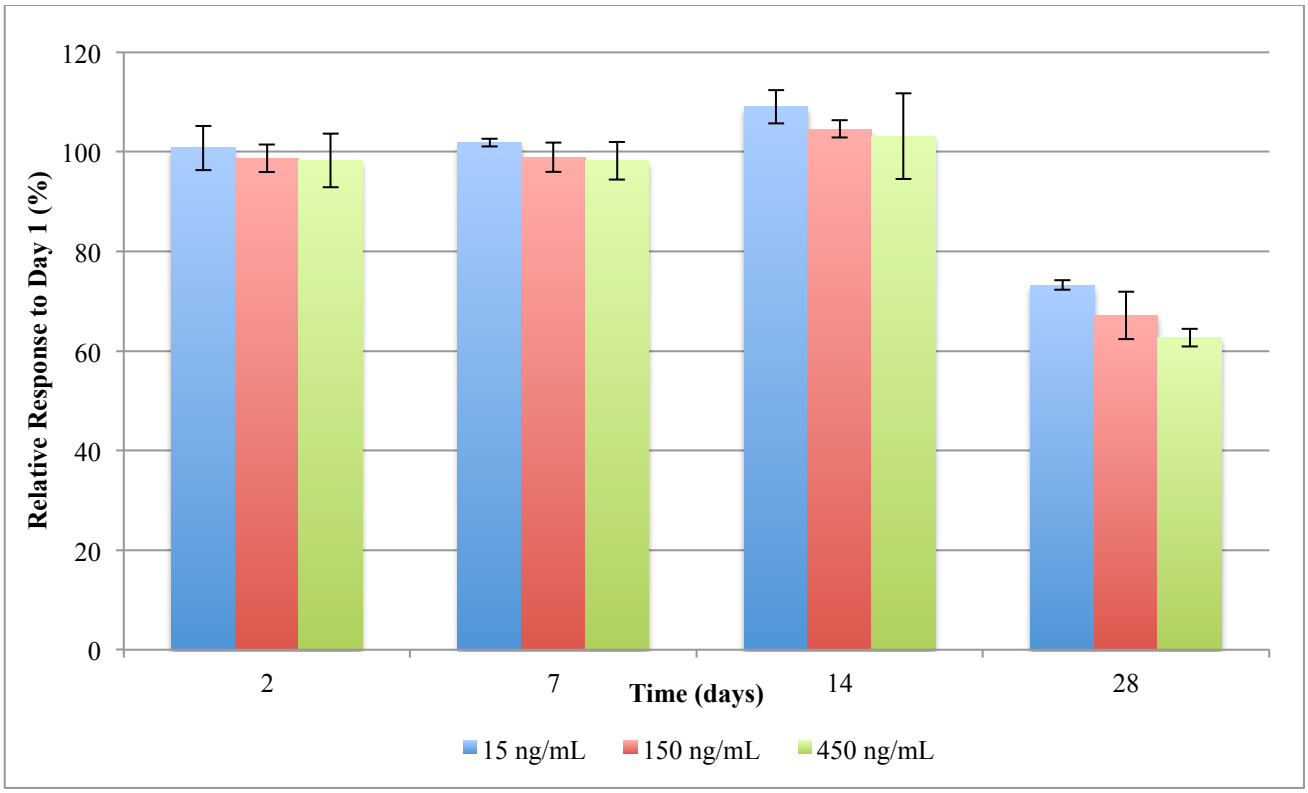

Figure 3.11: Relative response change in percent at 3 concentrations (15, 150, 450 ng/mL) of temazepam over time after storage in a refrigerator at 4°C after 2, 7, 14, and 28 days measured relative to the response on day 1. Measurements were collected in triplicate (n=3).

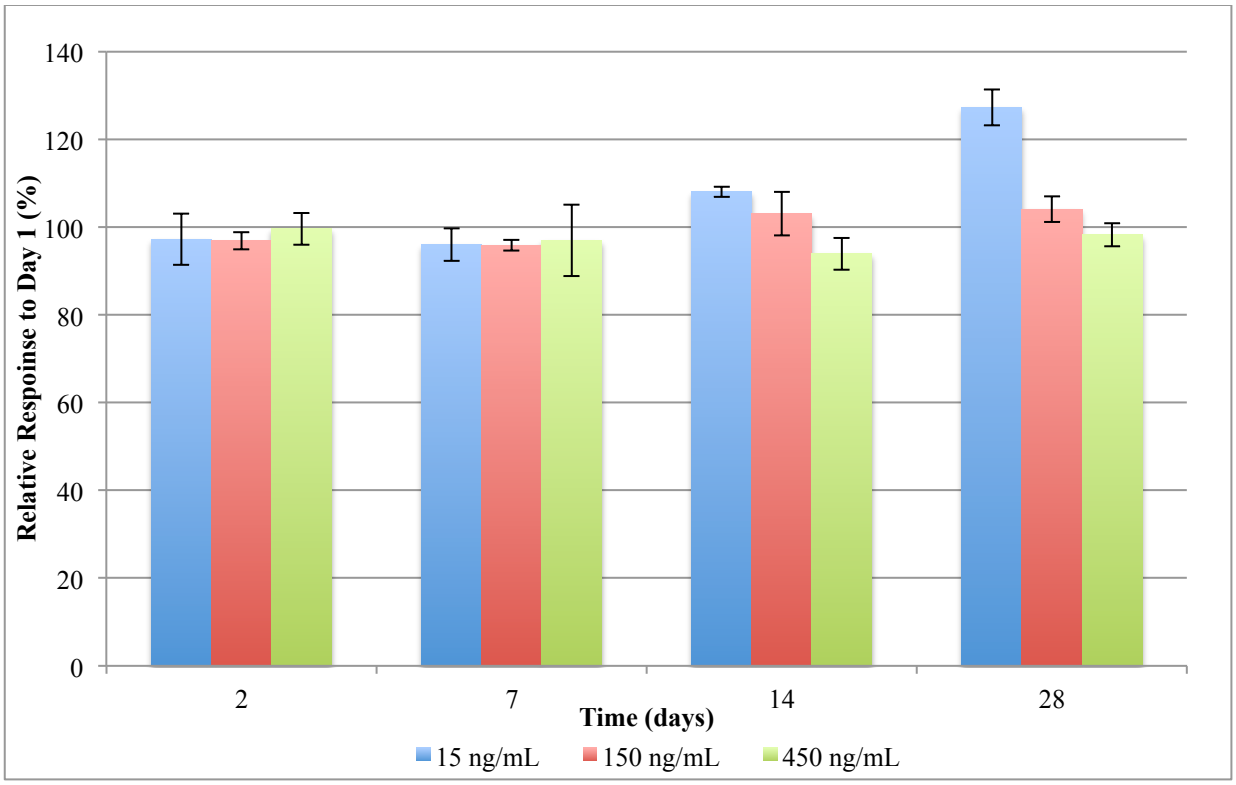

Figure 3.12: Relative response change in percent at 3 concentrations (15, 150, 450 ng/mL) of alprazolam over time after storage at ambient temperature after 2, 7, 14, and 28 days measured relative to the response on day 1. Measurements were collected in triplicate (n=3).

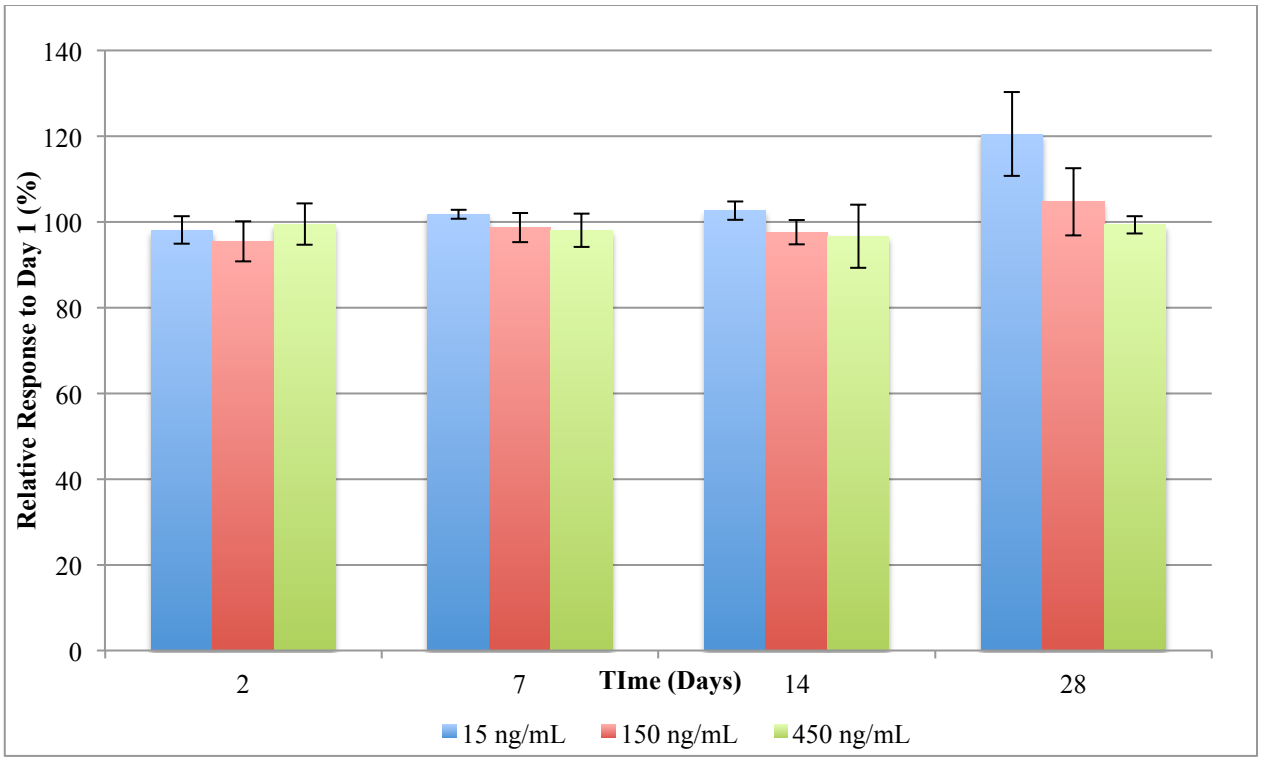

Figure 3.13: Relative response change in percent at 3 concentrations (15, 150, 450 ng/mL) of alprazolam over time after storage in a refrigerator at 4°C after 2, 7, 14, and 28 days measured relative to the response on day 1. Measurements were collected in triplicate (n=3).

Table 3.3: Relative response change (±SD) of each benzodiazepine measured relative to the measurement of response on day 1 after 14 days in both conditions assessed; ambient temperature and refrigeration at 4 ºC. Incidences of significant change in relative response are indicated. Measurements were collected in triplicate (n=3).

	Relative Response Change (%) after 14 days					
	Ambient Temperature			**Refrigeration (4ºC)**		
Conc. (ng/mL)	**15**	**150**	**450**	**15**	**150**	**450**
DZP	11.9±2.4	6.4±5.1	-3.5±3.1	7.5±3.0	2.4±2.7	0.9±7.4
7CLZ	31.6±5.4**	13.1±10.8**	9.0±3.8	-2.7±6.8	-8.5±1.4	-3.6±4.4
LRZ	3.4±5.1	-1.7±4.8	-9.9±3.3	-5.1±3.3	-4.6±2.8	-5.6±5.6
OXZ	3.0±2.1	-2.8±5.3	-8.5±4.4	-8.2±5.1	-6.7±1.6	-6.3±7.0
APZ	8.0±1.1	3.1±5.0	-6.1±3.6	2.6±2.1	-2.4±2.8	-3.4±7.4
CLZ	12.6±0.1	13.7±4.4	-0.8±3.6	6.3±4.0	8.8±4.1	2.4±8.5
NDZ	6.8±9.7	-0.4±4.8	-5.8±4.3	-7.9±2.6	-7.5±4.4	-3.6±7.3
aHAM	13.2±3.1	4.8±5.1	-10.1±3.0	5.1±4.2	-1.7±1.7	-8.3±7.4
TMZ	12.2±2.3	9.5±6.8	0.05±5.9	9.0±3.3	4.5±1.7	3.1±8.6

** Increase in relative response (%) exceeds ±20% threshold indicative of significant change

Table 3.4: Relative response change (±SD) of each benzodiazepine measured relative to the measurement of response on day 1 after 28 days in both conditions assessed; ambient temperature and refrigeration at 4 ºC. Incidences of significant change in relative response are indicated. Measurements were collected in triplicate (n=3).

	Relative Response Change (%) after 28 days					
	Ambient Temperature			**Refrigeration (4ºC)**		
Conc. (ng/mL)	**15**	**150**	**450**	**15**	**150**	**450**
DZP	30.3±3.0**	-10.8±3.7	6.2±3.6	23.8±6.7**	13.2±6.3	7.6±2.4
7CLZ	-3.2±8.9	-2.3±5.4	-11.6±2.7	3.3±8.5	-6.5±9.2	-13.0±3.3
LRZ	22.9±9.0**	4.6±5.1	-3.4±2.3	7.8±10.1	3.2±5.6	-1.7±1.8
OXZ	5.2±4.2	-5.0±5.0	-10.8±2.3	8.8±3.2	-0.4±4.9	-7.4±2.3
APZ	27.2±4.1**	4.1±2.9	-1.8±2.6	20.4±9.8**	4.61±7.8	-0.7±2.0
CLZ	41.7±5.6**	20.4±5.5**	8.1±2.8	23.0±3.6**	23.2±8.9**	12.2±2.3
NDZ	14.4±4.1	2.6±5.6	-6.7±3.3	8.9±7.5	0.2±5.3	-6.2±2.7
aHAM	27.9±4.2**	8.1±4.3	-3.9±4.3	16.6±6.3**	8.5±7.1	-7.4±1.5
TMZ	-25.0±2.4*	-33.9±2.5*	-39.5±1.4*	-26.8±0.9*	-32.9±4.8*	-37.3±1.8*

***** Decrease in relative response (%) exceeds ±20% threshold indicative of significant change
****** Increase in relative response (%) exceeds ±20% threshold indicative of significant change

3.1.8 Autosampler Stability

Stability of each of the analytes at three concentrations (15, 150, and 450 ng/mL) was assessed not only within the card matrix but also within the autosampler of the instrument. Each analyte was assessed over a 36 hour period to measure stability within the reconstitution solvent of ACN:H_2O at the constant 10°C to assess the length of time samples can wait to be sampled. The response ratio was calculated at 0, 12, 24, and 36 hours after reconstitution, and the relative response was calculated in relation to the initial response at 0 hours. Any change ±20% from the initial response from 0 hours was considered a significant change in response ratio indicating a notable change in the stability of the compound. Table 3.5 shows the relative response change ±SD calculated at 12, 24, and 36 hours at all concentrations of DBS extracts assessed. The change in relative response that exceeds ± 20% is indicated in Table 3.5. TMZ demonstrated an increase in response at the high concentration at 24 and all concentrations at 36 hours. CLZ also showed an increase with a SD extending above a 20% change after 12 and 24 hours. LRZ demonstrated a decrease with a SD extending below a 20% change in response at 24 hours. OXZ showed a response above 20% at 24 hours. 7CLZ showed a decrease in response at 24 hours.

Table 3.5: Relative response change (±SD) of each benzodiazepine measured relative to the measurement of initial response at 0 hours after 12, 24, and 36 hours in the autosampler held at a constant 10°C. Incidences of significant change in relative response are indicated. Measurements were collected in triplicate (n=3).

	Relative Response Change (%)								
	12 hours			**24 hours**			**36 hours**		
Conc. ng/mL	**15**	**150**	**450**	**15**	**150**	**450**	**15**	**150**	**450**
DZP	4.2±3.7	2.3±3.8	-0.9±5.4	-6.3±4.2	-1.5±9.7	-1.0±6.3	-5.4±4.6	0.3±4.3	-2.7±2.8
7CLZ	3.6±7.6	-6.1±4.5	-8.0±6.1	-18.9±4.5*	-5.5±4.7	-2.5±6.6	3.4±7.1	-0.2±5.0	-4.3±5.2
LRZ	-5.8±3.3	-0.2±6.9	4.4±5.3	-13.4±6.9*	-3.8±6.0	3.7±4.4	-4.9±6.2	-2.2±2.7	1.0±2.0
OXZ	14.3±2.8	1.4±3.3	1.2±4.9	20.2±7.7*	1.2±3.8	2.8±4.8	9.2±10.6	-1.6±3.2	2.1±3.6
APZ	6.1±4.7	1.1±8.8	4.4±3.9	-3.6±1.4	2.9±4.0	1.9±3.1	-6.1±1.0	-1.4±6.0	4.6±8.2
CLZ	10.9±10.6*	-3.6±7.5	1.2±4.9	16.5±4.4*	-8.1±4.0	1.7±1.7	3.6±8.2	-10.7±5.2	-3.2±4.2
NDZ	-1.1±3.1	-9.0±5.9	0.5±5.3	3.6±6.2	-5.5±3.5	0.8±3.3	2.1±3.4	-2.5±3.8	3.2±4.4
aHAM	-1.0±6.6	-0.1±3.6	3.1±2.7	11.2±5.5	-1.5±6.5	2.3±5.0	3.0±6.2	-2.6±4.0	1.2±4.7
TMZ	0.4±3.5	3.4±10.6	0.4±4.1	4.7±5.8	16.0±3.6	18.0±6.5*	9.7±12.0*	16.0±4.6*	18.1±5.3*

***** Change in relative response (%) exceeds ±20% threshold indicative of significant change

3.1.9 Effect of Hematocrit Variation

The effect of hematocrit on the measured response ratio was assessed over a reported range of variability in humans. The response ratio was calculated for each analyte of interest at five different hematocrit levels: 20, 35, 45, 55, and 70%. Figure 3.14 shows the response ratio ±SD of each benzodiazepine compared at each hematocrit level tested. A Kruskal-Wallis non-parametric test was performed for each drug to test if there was a significant difference between the response ratios of each drug measured from each hematocrit sample. Table 3.6 gives the calculated p-value for each drug and each concentration. A p-value that is < 0.05 is considered to be statistically significant. All drugs except LRZ and NDZ were found to have no significant difference across all hematocrit assessed at all benzodiazepine concentrations. LRZ at a concentration of 15 ng/mL and NDZ at a concentration of 150 ng/mL each had statistically significant differences between hematocrit levels, with the other concentrations showing no significant differences. A post-hoc Nemenyi style test was performed to identify which hematocrit values had significant statistical differences. For LRZ a significant difference was found between 20% and all other hematocrit values and between 70% and all other hematocrit values. NDZ was calculated to have statistically significant differences between 20% and all other hematocrit values and 55% from all other hematocrit values.

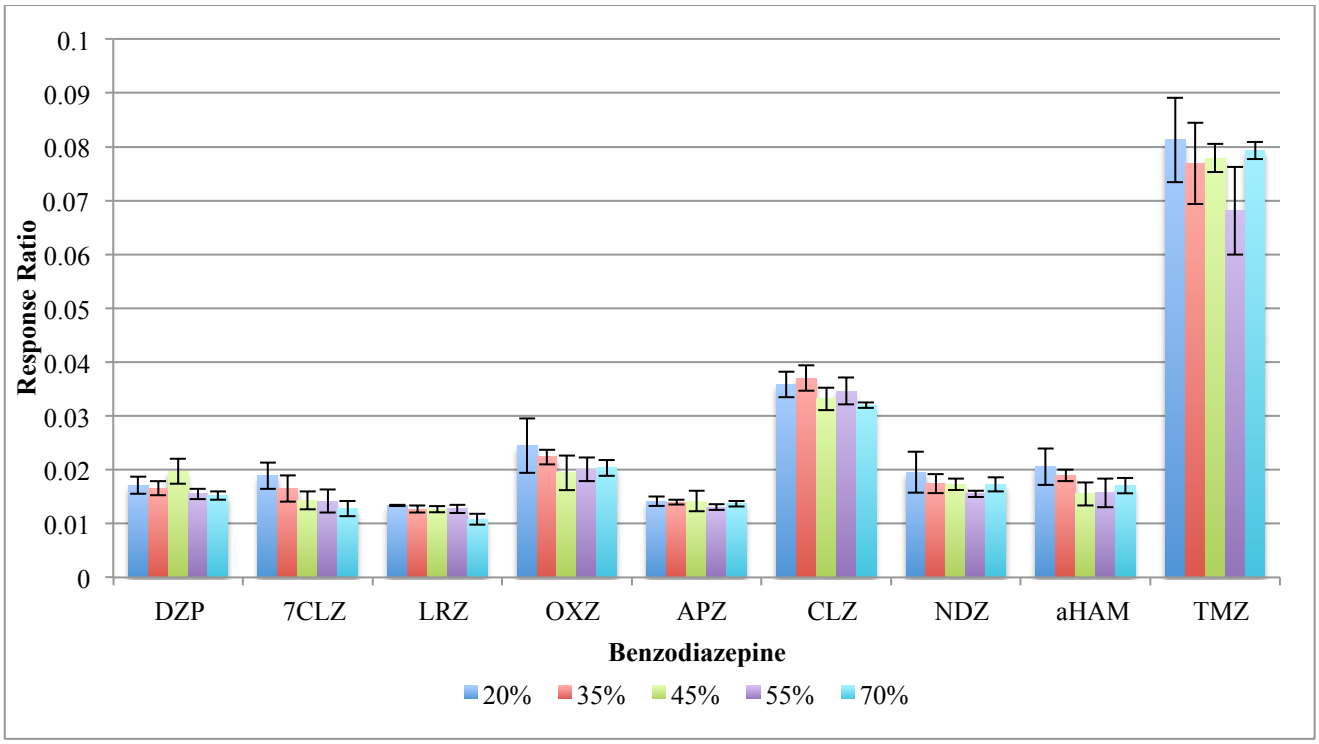

Figure 3.14: Measured response ratio (±SD) of each benzodiazepine comparing the response at each of the five hematocrit percentages; 20, 35, 45, 55, and 70%. These data present the results from DBS at 15 ng/mL. Measurements were collected in triplicate (n=3) at each concentration and hematocrit.

Table 3.6: Calculated p-values from Kruskal-Wallis non-parametric test performed to assess significant difference in response ratio between hematocrit levels measured for each drug. Results for drug concentrations of 15, 150, and 450 ng/mL presented. Each treatment was measured in triplicate (n=3).

Benzodiazepine Concentration (ng/mL)	**p-value of comparison between 5 hematocrit levels**								
	DZP	**7CLZ**	**LRZ**	**OXZ**	**APZ**	**CLZ**	**NDZ**	**aHAM**	**TMZ**
15	0.054	0.099	0.045*	0.384	0.569	0.153	0.223	0.075	0.274
150	0.545	0.106	0.197	0.129	0.160	0.479	0.048*	0.151	0.265
450	0.082	0.098	0.099	0.095	0.067	0.099	0.067	0.151	0.061

***** p-value<0.05 indicating a statistically significant difference between groups

3.2 Method Validation

To validate this method for quantitative use, calibration curves for each analyte were produced on five separate days. Response ratio for seven data points, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 ng/mL was measured. Figure 3.13 is a calibration curve completed for diazepam. Each curve was fit with a quadratic line of best fit, and the equation of the line and the R^2 were given for each graph. A calibration curve was produced for each of the drugs on each of the five days curves were analyzed. The R^2 value for all curves on all days was 1.00 with the exception of 7CLZ, LRZ, and OXZ for curve 1, producing R^2 of 0.998, 0.999, and 0.999 respectively. Precision was assessed as intra-assay with replicate analysis (n=3) measured using coefficient of variation. All drugs at all concentrations met the SWGTOX guidelines of a CV% of less than 20% except TMZ from the first curve at 7.8 ng/mL at 22.6%. Across all curves the range of CV% was 0.8 – 18.1% for DZP; 0.9 - 19.2% for 7CLZ; 0.8 - 19.9% for LRZ; 1.7 - 18.8% for OXZ; 1.4 - 10.7% for APZ; 0.2 - 13.6% for CLZ; 0.8 - 11.5% for NDZ; 0.3 - 15.6% for aHAM; and 0.9 - 22.6% for TMZ. CV% for all blind accuracy samples at low and high concentrations had CV% < 20%. The equation of the line was used to calculate two blind accuracy samples that were analyzed at the same time as the curve, one low and one high concentration. This was completed for curves 2-5. This calculated concentration was then compared to the actual value of concentration to measure bias. Bias is the measurement of accuracy. SWGTOX sets a bias threshold of ≤ 20% difference between the calculated and actual value. The bias values for all high concentrations met this threshold. Across all drugs a range of 2.2-18.5% was calculated for the blind high concentration samples from all four curves where accuracy samples were included. The calculated values for the low concentrations are given in Table 3.7. Values

where the bias exceeded the 20% SWGTOX threshold are indicated in Table 3.7. DZP, 7CLZ, OXZ, CLZ, and TMZ all had one curve where the bias exceeded 20% while NDZ and aHAM had two curves with accuracy samples that exceeded 20%. All bias values can be found in Appendix A.

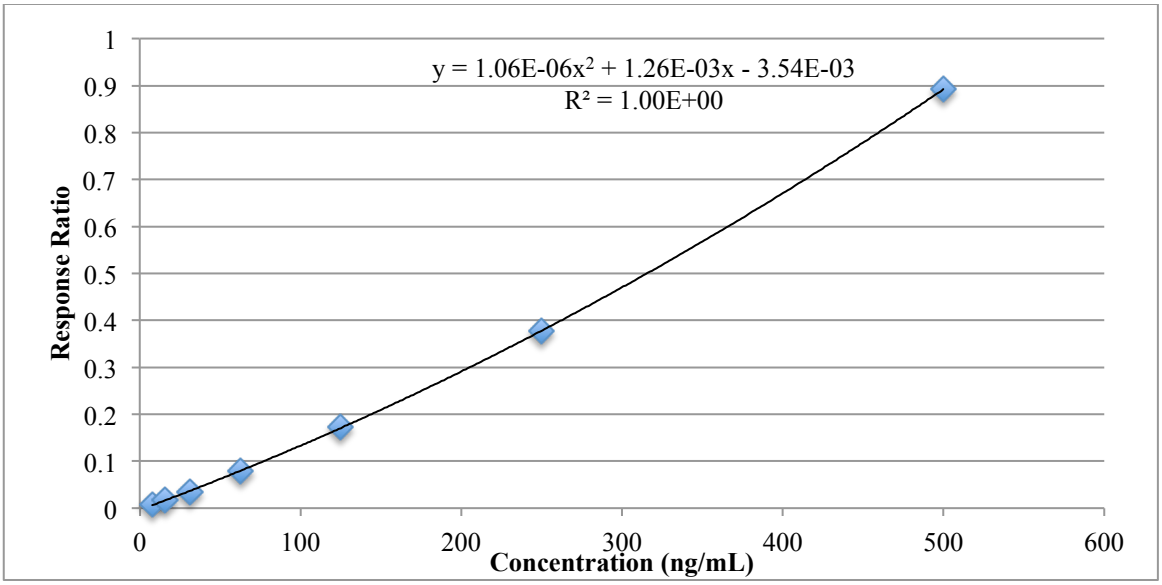

Figure 3.15: Calibration curve of diazepam measuring the relationship between response ratio and concentration across a range of 7.8 ng/mL – 500 ng/mL. (n=3) Line fit with a quadratic regression line. The equation of the line and the R^2 value are shown.

Table 3.7: Calculated bias of measured concentration to actual value of blind low concentration analyte sample for each benzodiazepine measuring the accuracy of the method for curves 2-5. Calculated from measurements made in triplicate (n=3). Values exceeding SWGTOX threshold indicated.

Curve	Calculated Bias (%)								
	DZP	**7CLZ**	**LRZ**	**OXZ**	**APZ**	**CLZ**	**NDZ**	**aHAM**	**TMZ**
2	14.80	16.58	12.27	16.91	11.75	16.30	16.56	21.66*	12.49
3	22.23*	11.02	10.01	21.80*	19.22	21.37*	23.14*	25.61*	14.80
4	15.42	25.43*	10.39	13.61	10.99	11.67	23.72*	18.32	20.16*
5	1.65	11.29	12.20	9.35	9.57	8.15	8.34	12.01	9.11

***** Bias exceeds 20% threshold established in SWGTOX guidelines

CHAPTER 4: DISCUSSION

This research was conducted in order to develop a screening method for the detection of nine benzodiazepine and benzodiazepine metabolites in blood after collection using dried blood spots (DBS) with analysis by ultra-performance liquid chromatography-quadrupole time of flight-mass spectroscopy (UPLC-QTOF-MS). Guidelines and thresholds supported by SWGTOX for reliable quantitative methods were followed to determine if this method could be classified as quantitative or semi-quantitative. This would allow quantification of each drug contained within the DBS either with a corresponding concentration or determining the concentration to be one of impairment or not. The method was developed completing a number of experiments in order to optimize the steps and minimize time and effort required for extraction. The stability of the analyte was assessed within the card matrix under different storage conditions as well as within the reconstitution solvent held in the autosampler. The potential effect of hematocrit on the interpretation of the concentration was also measured. After these steps the method was validated by producing calibration curves to measure the bias and therefore accuracy of the method within and between days as well as the precision.

Current legislation that controls the collection of biological samples from road side detentions in suspected drug impaired driving cases produces long delays between the time of detention and the time of sampling that can influence the interpretation of measured concentrations in blood. Current collection of blood, the sample of choice for drug impaired driving, is an invasive process that requires certified personnel to draw the sample, and more complicated storage considerations. DBS is proposed as a way to simplify collection and storage as well as extraction procedures.

4.1 Solvent-Spot Agitation Method

In order to determine the most effective method of solvent-spot agitation and the time that would optimize the recovery, precision, extraction time, and throughput of each benzodiazepine, three different agitation methods were compared (rapid agitation (Tissuelyzer®), sonication, and vortexing) over 60 minutes in triplicate. Extraction was achieved with methanol. The response of each of the analytes was compared between three different agitation methods at each time point where the extraction solvent was collected. This was compared at three different concentrations, a high (450 ng/mL), middle (150 ng/mL) and low (15 ng/mL) value that covered a comprehensive range of impairing concentrations of each benzodiazepine. Lorazepam was shown as an example of the comparison of the response, interpreted as the quantity of drug being extracted by a given method at a given time, between each of the extraction methods at three different concentrations (Figure 3.1). This response showed a similar trend in all drugs measured. Rapid agitation consistently demonstrated the highest response in peak area for each drug with the exception of OXZ, where sonication had the largest response. This was seen at the high and medium concentrations. At the low concentration the peak areas tended to be close in value, with rapid agitation and vortexing responses generally slightly above sonication. Based on response alone, rapid agitation showed the largest response and therefore most extracted drug. However the method itself was very variable in visible destruction of the spot, with some spots being pulverized by the motion of the Tissuelyzer® and others not showing any damage. The variability observed was suspected to be caused in part by non-uniform motion across plate over which the samples were distributed. Pulp present in the sample was

also a concern for interfering with later steps, and added the requirement of a second centrifugation step.

The precision of the measurements was the next parameter assessed to determine the best agitation method for DBS to extract the analytes of interest. The coefficient of variation was used to measure the precision of the calculated response ratios. Sonication appeared to have the best precision, with all CV% for the low and medium concentrations being below the 20% threshold specified by SWGTOX, and all CV% at 450 ng/mL but CLZ and TMZ were < 20%, as observed in Figure 3.2 with data after collection at 5 minutes. Beyond 5 minutes measurements tended to show more variation over all methods analyzed. The CV% for vortexing and sonication is variable at the low concentrations but values for vortexing are generally higher than those of sonication. All methods showed precision below 20% for all drugs at 150 ng/mL except rapid agitation that exceeded a CV% of 20% for aHAM. All CV% values for vortexing at the high concentration had precision that exceeded 20% deviation from the value whereas rapid agitation and sonication were all below a CV% of 20%, except for TMZ. Precision consistently was the best using sonication considering all concentrations. Sonication was the final choice made for the solvent-spot agitation method based on a high level of response measured at 150 and 450 ng/mL, and a comparable response to the other two methods at 15 ng/mL, with the most consistent precision below the SWGTOX cutoff, and no visible destruction of the spot. Relative recovery over time compared to the total amount of analyte recovered after 60 minutes of collection was analyzed. The same trend observed in Figure 3.3 was observed for all drugs. For all agitation methods the highest recovery occured after 5 minutes; 80.8 -100% of each drug in the mix was extracted after the first 5 minutes across all three methods and concentrations. After 10 minutes, 5.0 - 19.1% of

analyte was extracted depending on the drug and for most analytes (except aHAM) there was still a measured response at 15 minutes at the mid and high concentrations. This suggested that an extraction time of 15 minutes would extract the maximum amount of each analyte from DBS at concentrations relevant to impairment. Recovery of 99% of the total quantity extracted of each analyte occurred in the first 15 minutes at all three concentrations. Maximizing recovery is important in the case of dry blood spots when starting with such small volumes of sample. Preliminary testing of extraction solvent had already been performed (results not included). The highest response was seen using methanol, suggesting the highest recovery of analyte; however, further testing was required to optimize the solvent used considering matrix effects. These experiments included solvents demonstrating less efficient extraction time therefore to account for a potentially longer extraction with the optimized solvent, the extraction time was extended to 30 minutes.

Important limits to this experiment to be considered in choice of agitation methods are that the application method of internal standard had still not been optimized and higher variability was observed in internal standard values when this method was used for application than the final method determined. This variability in internal standard values could have misrepresented the coefficient of variation values calculated for each agitation method. It was also the response that was compared between methods for the first comparison, not the response ratio, therefore variation in response of the detector and extraction efficiency were not accounted for. However, the variability in the internal standard measurements, if response ratio was used, may have also misrepresented the solvent-spot agitation method with the highest yield, as variability in internal standard could have

distorted the average response ratio that would have been used for comparison. The final choice was extraction by sonication for 30 minutes.

4.2 Extraction Solvent

Matrix effects, recovery, and precision were all assessed in order to optimize the extraction solvent to use with the chosen solvent-spot agitation method. Four solvents - methanol; 1:1 v/v methanol:acetonitrile; 1% formic acid in methanol; and 1% formic acid in 1:1 v/v methanol:acetonitrile- at 3 concentrations- 15, 150, and 450 ng/mL- were compared in triplicate. Pre- and post-extraction spiked samples as well as neat standards were measured in order to calculate matrix effects and recovery. Matrix effects are an important consideration in methods that utilize liquid chromatography; ion suppression or enhancement must be restricted to a 25% change in response for quantitative methods by SWGTOX guidelines calculated from the neat standard with no matrix. Consideration must be given to matrix effects and recovery in the choice of extraction solvent due to the small quantity of analyte present, as only 20 μL of blood was used to produce each DBS. In this case any ion suppression of the response was to be avoided if possible. The majority of the matrix effects calculated for solvents including formic acid exceeded the 25% threshold given by SWGTOX for the appropriate amount of matrix effects for a quantitative method. The majority of the values were negative indicating ion suppression (Table 3.1). Therefore, MeOH with 1% formic acid and 1:1 (v/v) MeOH:ACN with 1% formic were no longer considered. Comparisons between the solvents MeOH and MeOH:ACN, at low drug concentrations showed no differences in matrix effects between solvents as there was an overlap in the error bars representing the standard deviation of the average matrix effect for each compound. This

suggested the variability in measurements overlapped for the two solvents although the average matrix effect value for MeOH:ACN was lower for more benzodiazepines. However as the drug concentration increased, differences in matrix effect between solvents was noted for LRZ, OXZ, CLZ, aHAM and TMZ. Matrix effects calculated for MeOH:ACN ± SD were found to be lower than those of MeOH. This supported MeOH:ACN as the extraction solvent.

Calculated precision values at all concentrations extracted using MeOH:ACN were < 20%, within the acceptable limits of variation outlined by SWGTOX. MeOH had acceptable precision CV% at 15 ng/ mL and 150 ng/mL but all precision values at the high concentration exceeded the 20% threshold. All average recovery values were significantly above 100%, most above 120%. Even in cases where the matrix effects suggested ion suppression, the recovery values were still well above 100%. This suggested a matrix-drug interaction beyond the matrix effects that enhanced the response well above that detected in a neat standard at the expected final concentration. It may have been the result of the method followed to produce the post-spiked sample, causing an unexpected interaction with glassware or perhaps a loss during evaporation, reducing the amount of analyte measured. A protein filtration step was considered to further reduce the observed matrix effects and elevated recovery. Based on this experiment, 1:1 (v/v) MeOH:ACN was chosen as the extraction solvent to minimize matrix effects measured, and increase precision of the measured values. A minimal volume of 1 mL was chosen to ensure the spot was completed submerged in solvent.

4.3 Internal Standard Application

Variability had been observed in the internal standard response that did not mirror the variability that was observed in response from the analytes extracted from the spot. Therefore different methods in applying internal standards were compared; addition of the internal standard to the spot with a dry time of 2 hours, no dry time after addition to the spot after it had been punched out, and adding the internal standard directly to the extraction solvent. The variability was observed in the coefficient of variation; a consistent volume (20 μL) was spotted to correct for differences in extraction efficiency and detector response that could occur due to subtle changes in conditions between samples. It was suspected that the initial way the internal standard was added, that is, addition to the spot before being punched out and with a 2 hour dry time, was causing variability as the blood spot already occupied much of the matrix of the filter paper. Therefore internal standard solvent diffused beyond the edge of the filter paper punched out for sampling, causing an uneven distribution of each internal standard, increasing the variation and causing misrepresentation of the variability of the response of each analyte. The CV% for each of the three methods was compared in Figure 3.4. Values derived from adding the internal standard directly to the extraction solvent had a much lower CV% indicating a more precise measurement. Overall, addition of internal standard to the spot before punching out and leaving to dry for 2 hours had the lowest amount of precision, suggested by the large CV%. Student t-tests were performed to assess if there was a significant difference between each of the three methods (Table 3.2). There was no significant difference between a 2-hour dry time adding to the card before spot removal and no dry time after spot removal. The CV% of adding directly to the extraction solvent was significantly different from the two other methods. Although adding the internal standard

directly to the extraction solvent before extraction did show the highest precision, it would no longer be able to be used to assess the extraction efficiency of the drugs from the spot as it is not being added directly to the spot on the card matrix, rather, directly to the extraction solvent, which negates this function of the internal standard. Although the internal standard is also not within the blood when added directly to the card, it is still being allowed to integrate within the matrix, and will be extracted to some extent along with the drugs of interest. This is also the approach that would need to be taken in casework, as the blood spotted from an individual would not include internal standard within the matrix. Therefore the final decision to increase the precision while still maintaining the functionality of the internal standard was to add internal standard to the spot after it had been removed.

4.4 Extract Clean-up by Protein Filtration

Optimization of the extraction solvent did not reduce the matrix effects within the SWGTOX standards of ±25% of the response of the neat standard absent of matrix of the expected concentration. Therefore the inclusion of a protein filtration step using a Clean Screen® FASt plate was included. The plate removes macromolecules that are >1μm in order to remove potential matrix components contributing to the observed matrix effects (e.g. phospholipids). Sample preparation with and without a protein filtration step were compared at three concentrations (15 ng/mL, 150 ng/mL, 450 ng/mL). There was a clear reduction in the calculated matrix effects (Figure 3.5) on all analytes for all compounds of interest, and this was observed at all concentrations. There was no overlap in standard deviation suggesting significant difference between matrix effects after protein filtration and matrix effects without filtration. However the matrix effect values all still appeared to be beyond the

±25% required by SWGTOX for quantitative methods. Recovery was also measured after the addition of the step. A clear drop in recovery can be seen for all analytes, in Figure 3.6 at all concentrations. This is expected as the addition of an extra filtration step produces opportunity where some analyte may be lost when associated with particles filtered out and interaction with additional materials may also cause a decrease in yield. In an effort to reduce the measured matrix effects and increase the recovery four different solvent systems were compared for protein filtration extraction - 1:1 methanol:acetonitrile; extraction in 1:1 methanol acetonitrile with 1% formic acid; extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile to the extraction solvent before filtration; and extraction in 1:1 methanol:acetonitrile with addition of 1 mL of acetonitrile with 1% formic acid to the extraction solvent before filtration. Similar to when the extraction solvent was chosen, avoidance of ion suppression was desired, in order to maximize response, as the quantity of analyte in each sample is reduced due to the small sampling volume. This eliminated MeOH:ACN with 1% formic acid; as extraction with this solvent showed a trend of increased ion suppression, indicated by negative matrix effect values, as the concentration increased. Suppression observed at 450 ng/mL extended beyond the -25% threshold of SWGTOX guidelines for multiple drugs, including LRZ, OXZ, and aHAM that had already demonstrated a lower signal on the instrument than other analytes. All matrix effect values calculated for extraction with MeOH:ACN were above the 25% threshold for ion enhancement at all concentrations, and therefore this combination was no longer considered. Figure 3.7 shows these trends at 15 ng/mL. This left extraction in 1:1 MeOH:ACN with addition of 1 mL of ACN or addition of 1 mL of ACN with 1% formic acid to the extraction solvent before filtration.

To further compare these two systems, the recovery of the two systems were considered. Between the recovery at 15 ng/mL and 150 ng/mL no significant difference was observed as the standard deviations overlapped between the two suggesting an overlap in the individual measurements of the replicates. However the average recovery tended to be higher for MeOH:ACN with 1 mL ACN. This same trend is seen at 450 ng/mL, where less overlap of the standard deviations for a number of drugs was observed showing a clear difference between the recoveries of the two systems. The higher average recovery and incidence of more ion enhancement than ion suppression across analytes and concentrations compared to extraction with 1:1 (v/v) MeOH:ACN with the addition of 1 mL of ACN with 1% formic acid lead to including a protein filtration step where extraction is completed using 1:1 (v/v) MeOH:ACN with the addition of 1 mL of ACN to the extraction solvent before filtration. The precision was acceptable for all analytes under all concentrations except TMZ with a CV% of 22.0 % from a 15 ng/mL DBS. As the precision over all other values was within < 20%, this was not a significant parameter to consider when choosing the system.

4.5 Reconstitution Solvent

Benzodiazepines show a high binding affinity to plasma proteins suggesting they are more soluble in organic solvent than in an aqueous solvent [23]. In order to increase the recovery of analyte extracted from each spot after evaporation to dryness, a reconstitution solvent of 1:1 (v/v) ACN:H_2O was compared to reconstitution in mobile phase A which was 5mM ammonium formate in water with 1% formic acid. The aim was to include an organic solvent to restore more analyte into solution. The retention time resulting from the use of either reconstitution solvents was compared and no significant change in the value was

determined, allowing the continued identification of each analyte by that parameter. For all analytes and at all concentrations there was an increase in the recovery with the use of 1:1 (v/v) ACN:H_2O with the exception of 7CLZ that showed a decrease in recovery, as seen in Figure 3.9. This could be the result of the chemical properties of the compound, as it is a metabolite of CLZ, a product of the body metabolizing the drug by producing a more polar molecule more soluble in aqueous media such as urine for ease of elimination. This could explain a higher solubility of 7CLZ in mobile phase A compared to ACN:H_2O. The observed increase in recovery for all drugs, especially those that already had a small observed signal such as LRZ and aHAM, resulted in the choice of ACN:H_2O as the reconstitution solvent.

4.6 Analyte Stability in DBS

Stability of each benzodiazepine within the matrix of the Whatman 903 Protein Saver Cards was important to assess the time each card can be stored before interpretation of the concentration is complicated by change in the amount of analyte. Potential variations between storage conditions are also important to measure. The change expected to occur over time was degradation of the compound into the known metabolites included in this study or others not included in this study. Therefore a reduction in the measured response over time that deviated from the initial response measured on day 1 by 20% was considered a significant change in response. Storage at ambient temperature and under refrigeration (4 °C) was compared at three levels (15, 150 and 450 ng/mL) in triplicate to see if there was a difference between the two storage conditions. It was expected that there would be less degradation observed under refrigeration as there would be less kinetic energy available in the system, slowing the spontaneous degradation of each analyte as well as lowering or

inhibiting enzyme activity within the blood. Figures 3.10 and 3.11 show the relative response change over time of temazepam compared to the initial measurement on day 1 at ambient temperature and under refrigeration respectively. The response fell below the 80% line at 28 days under both conditions indicating a significant reduction of over 20% in signal from temazepam. This result indicated interpretation of concentration of temazepam is not reliable after 28 days, and can only be reliably interpreted within 14 days of storage. The exact point between 14 days and 28 days where relative response falls below that of 14 days is unknown, therefore the last day of measurement is the last day it can be said with certainty that the results do not show significant degradation.

Temazepam is the only drug in the mix that demonstrated the expected degradation. The rest of the drugs exceeded a 20% change in the relative response demonstrated increases in signal. Demonstrated in Figure 3.12 and 3.13 showing the relative response change of alprazolam, at ambient temperature and refrigeration respectively. Over time the relative response change increases, until on day 28 the change in response exceeds 20%. This is observed at ambient temperature and refrigeration. Table 3.3 and Table 3.4 show the relative response change in percent for each analyte on day 14 and day 28 of storage respectively. Of the drugs that showed an increase in response, two , LRZ and 7CLZ, only changed at ambient temperature and with no significant change under refrigeration. This suggests that the analyte is stable for 28 days at 4°C so interpretation will not be affected up to 28 days, however, if left at ambient temperature, interpretation will only be reliable to 14 days. The other analytes showed an increase in response over time under both conditions after 28 days. Therefore interpretation of concentration would only remain unaffected up to 14 days for these compounds. All compounds that did not exhibit a change could be interpreted the same over

28 days if stored under both conditions. The increase of relative response over time to change beyond an additional 20% of the value on day 1 was an unexpected result that requires further testing to examine possible sources of the increase. An increase in signal would suggest an increase in the amount of analyte or increased recovery, however all spots of each concentration were made from the same aliquot of spiked blood, just stored within the cards over different time periods and extracted using the same method. There is the possibly that it is the result of a change in matrix effects, with compounds not measured in the blood degrading to new compounds that have a different effect on the analytes of interest. It could be certain compounds stabilizing in the card matrix and remaining held there rather than being removed with the extraction solvent, reducing background and therefore increasing the signal. Further experimentation is required. Most of the relative response changes observed were only observed at the low concentration, as the amount of sample spotted on the card is so small, and as an extension of that, the amount of analyte is limited. Modifications within the matrix may have significant effects whereas in larger quantities that effect is negligible. All compounds are stable up to 14 days under refrigeration. Refrigeration appears to stabilize more benzodiazepines included in this study than room temperature, and reliable interpretation is possible up to 14 days before compounds begin to show significant changes in relative response.

4.7 Autosampler Stability

The stability of each analyte must also be determined within the autosampler. This is assessing the stability of the analyte within the reconstitution solvent in the autosampler of the instrument while it waits to be sampled. It is the amount of time an analyte can remain

under those conditions before interpretation of the concentration is affected. The change expected, like with card stability, was degradation. A significant change was once again interpreted as a change in the relative response that is ±20% from the initial response measured at time zero. The relative change was measured at three concentrations (15.6, 125, and 500 ng/mL) in triplicate. Significant increases are indicated in the Table 3.6. The metabolite 7CLZ and LRZ showed a decrease in relative response at 15 ng/mL, standard deviation extended the variation beyond a 20% decrease in relative response, therefore interpretation of the concentration of these compounds can only reliably be made up to 24 hours in the autosampler. All other values once again show a relative response increase beyond a 20% increase of the original value; a repeat of this experiment as well as investigation of matrix effects are required to assess this observed increase in OXZ, CLZ, and TMZ. Until this can be investigated and adjustments can be made if necessary to mediate this change, measurement is reliable up to 12 hours for all compounds. Further testing is required for CLZ, as the response increases beyond 20% at the 12 hours mark and no measurements were made at time points before that to see where the stability waivers. Instability is once again seen primarily in the low concentration samples.

4.8 Hematocrit Effect

The effect of hematocrit is an important consideration when it comes to dried blood spots (DBS). The volume fraction of blood occupied by red blood cells can affect the distribution of the blood components and analyte throughout the spot. Benzodiazepines are also a drug class demonstrating high binding affinity for plasma proteins, therefore the distribution of these components throughout the matrix and the quantity that are within a

DBS may have an effect on the measured response [23]. Within humans the normal range for hematocrit is 37-51% for adults but can reach between 20% and 80% of the total blood volume and above in cases of disease [46]. Therefore to cover the overall range of the human population response ratios at five different hematocrits were measured and compared: 20, 35, 45, 55, and 70%. Another factor compared within the hematocrit levels was different drug concentrations: 15, 150, and 450 ng/mL. A Kruskal-Wallis non-parametric test was performed to measure if there were significant differences between the hematocrit values at each concentration. Figure 3.14 shows response across hematocrit levels for each drug at 15 ng/mL. If a significant difference was calculated then a post-hoc Nemenyi style test was used to determine between which individual hematocrit values the significant differences were found. The Kruskal-Wallis non-parametric analysis found no significant differences for all drugs except LRZ and NDZ. LRZ had a statistically significant difference at 15 ng/mL between the two extremes of 20% and 70% and the rest of the hematocrits tested. NDZ had a calculated statistically significant difference that was determined at 150 ng/mL between 20% and 55% and all other hematocrit values. The values of all the differences calculated are found in Table 3.6. A p-value that was < 0.05 was considered to be significant; the calculated values were 0.045 and 0.048 respectively. The significant difference finding for NDZ at 150 ng/mL was not expected, and the lack of difference observed at the lower concentration and the higher concentration was not easily interpreted. Also the significant difference between 55% and all other hematocrit values occurring in the middle of the hematocrit range was difficult to rationalize. The mean values of the response ratios of both drugs that had a statistically significant value were compared. The mean response ratios upon comparison were not found to have although statistically significant were not significant differences. P-

values calculated were very close to the 0.05 value that determines significance. A nonparametric test was performed because of the small number of repetitions (n=3) collected for each drug and hematocrit level, and a normality test may not have properly represented the data. However had the data actually demonstrated normality upon larger sampling, this may have caused differences in response but not significantly difference values. There is also the possibility that the variability in precision on this particular day of sampling was low and all data points were clustered closely around the same response ratio for each hematocrit. Repeating the experiment on multiple days in order to better determine the inter-day precision, and better establish the variability in the measured values would likely support the conclusion that there is no significant difference across the measured response ratio for the entire hematocrit range. There is also an increased probability of calculating a statistically significant difference the more comparisons completed, therefore a correction factor to account for this increased probability may be more representative of the true variation in the data. The final conclusion of this experiment was that there was no significant difference across the whole range of hematocrit found in the human population, and therefore there is no effect of hematocrit on the interpretation of response. However further testing repeating this experiment is required to establish inter-day variability in measurement.

4.9 Method Validation: Calibration Curves and Accuracy

The final step after development of the method and the determination of the effect of stability and hematocrit on interpretation was method validation. This was achieved by producing calibration curves for each analyte on five separate days and running two blind accuracy samples with each curve, one high and one low unknown concentration. Curves

were produced with data points of 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 ng/mL. The limit of quantitation (LOQ) was determined to be the lowest point on the curve that demonstrated a precision of 20% and has a signal to noise ratio ≥10. The limit of detection (LOD) was determined in this study to be equal to the LOQ as the lowest concentration that was measured on the curve. The R^2 values that were produced for each curve showed a good fit with most having an R^2 of 1.00. Only three of forty-five curves deviated from an R^2 of 1.00 and they were 0.998, 0.999, and 0.999 respectively. Each curve was fit with a quadratic regression line. Bias was calculated using the equation of the line of best fit to determine calculated concentration of each unknown blind sample, and then comparing the calculated value to the theoretical value. SWGTOX allows up to 20% deviation of the calculated concentration from the theoretical value (i.e. bias) to meet the guidelines for a quantitative method. All calculated bias values for all benzodiazepines met this criterion for the unknown high concentration, indicating a reliable quantitative method at the high end of the curve. However, most drugs demonstrated at least one bias value that exceeded this threshold over four curves at the unknown low concentration. Bias values never exceeded 25.6 % for the low concentrations and although this exceeded the threshold indicated by SWGTOX guidelines, this still indicated a close estimate of concentration. These guidelines tend to refer to extraction from ante-mortem samples rather than other mediums of collection, especially since DBS as a collection medium is just recently experiencing an increase in interest and research in forensic toxicology this threshold may be too conservative. Variability and reliability of quantitation from a curve extracted from DBS has not yet been established. Considering the difference in value that is obtained by a 25% deviation from the actual value, the interpretation of impairment will remain unchanged. Therefore the method, though not

validated to SWGTOX standards, does present a method that can be used to reliably estimate concentration values from DBS from a standard curve. However if through further testing it is found that a 20% change is reliable for DBS methods and bias extends beyond that 20% change, this method can still be used as a semi-quantitative method, used to determine if an individual was within an impairing range or not. It is also important to note that the standard curves were produced using Microsoft Excel where weighting of the regression line is not possible, therefore if using a software where weighting (1/X) was possible this may improve the calculated concentrations of the samples of unknown concentration. It is also important to note the lack of legal limits established for benzodiazepines in Canada mean that any identification of a benzodiazepine in a sample taken from a suspected drug impaired driver that corroborate the observations of the officer of impairment could result in conviction.

4.10 Future Research

The method that was developed and validated for this project is a good starting point for analysis of dried blood spots (DBS) when benzodiazepines are the analyte of interest. Further experiments are needed to assess the bias of blind accuracy samples using a weighted (1/X) quadratic regression line to better assess the performance of the method as a quantitative method. This method was developed and validated using sheep blood that had been spiked with a drug mix to produce each concentration evaluated. Subsequent sets of experiments should assess the matrix effects, recovery and precision of the method when taken from human blood, which would have differing components within the matrix that could change the results observed. Also the sheep blood that was used for the majority of the experiments was closer in properties to post-mortem blood rather than the ante-mortem blood

that would be expected in drug impaired driving cases. The red blood cells appeared to have begun to undergo hemolysis, and degradation products may have been present that would not be seen in ante-mortem blood. Applying the method to real samples, collected from individuals that have ingested a therapeutic or impairing quantity of one of the benzodiazepines of interest is the next step. Ideally this would also include a venous sample taken at the same time as the DBS in order to look at the correlation of venous concentration and capillary concentration. A difference has been observed between the two sources, as well as possibility of the inclusion of interstitial fluid from the puncture for DBS collection. Experiments to establish if a consistent correction factor can be established would be important for interpretation of concentrations determined from DBS. Further experiments measuring the effect of other storage conditions, such as freezing and the effect of humidity should also be performed along with matrix effect experiments to explore the observation of increased response of the analyte over time. As the goal was a screening method that could also be used to quantify the concentration of drug in the DBS and by extension at the time of detention, increasing the number of benzodiazepines as well as other prescription drugs and drugs of abuse that can successfully be extracted and analyzed using this method would be a good extension of this method.

CHAPTER 5: CONCLUSION

Benzodiazepines are a class of drug that is commonly implicated in impaired driving cases. Blood is the preferred biological sample to be collected in order to establish blood concentration of the drug to support the finding of impairment by an officer. This study showed that nine benzodiazepine and benzodiazepine metabolites could be identified after extraction from DBS produced from only 20 μL of blood using a UPLC-QTOF-MS. The method functions as a screen for benzodiazepines, and also showed that it could be applied to quantification of concentration of a particular analyte.

The development of this method showed optimization of each extraction and sample preparation step to minimize time and steps required. Validation showed potential for the use of the method to both identify and quantify each drug to reliably say if the concentration is consistent with impairment ranges known for each drug. Stability of each drug of interest was determined under two storage conditions within the matrix of the cards, establishing over what time period and under what storage a response can be considered consistent with the concentration at the initial time of collection as well as the stability of the extracts within the autosampler of the instrument waiting for analysis.

A method with the potential for the simultaneous identification screen and quantification of identified analyte was developed, and with further research into important areas such as the method's efficacy applied to ante-mortem human blood and the correlation between capillary and venous blood, the method could transition to use of real samples collected in incidences of drug impaired driving. This research could be expanded to include other drugs of interest for impaired driving. This is provided that legislation is changed for the collection of biological tests at the roadside.

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APPENDIX: ADDITIONAL DATA FROM DBS ANALYSIS

Table I: Average peak area measured at each time point during extraction by rapid agitation by the Tissuelyzer® of the time point comparison study at all 3 concentrations analyzed: 15, 150 and 450 ng/mL.

Conc		5min	10min	15min	30min	45min	60min
15 ng/mL	DZP	1177.70	148.02	19.26	0.00	0.00	0.00
	7-CLZ	373.08	69.28	0.00	0.00	0.00	0.00
	LRZ	49.047	8.41	0.00	0.00	0.00	0.00
	OXZ	74.14	9.68	0.00	0.00	0.00	0.00
	APZ	428.97	65.31	0.00	0.00	0.00	0.00
	CLZ	120.78	18.71	0.00	0.00	0.00	0.00
	NDZ	224.89	26.59	0.00	0.00	0.00	0.00
	aHAM	59.75	13.92	0.00	0.00	0.00	0.00
	TMZ	277.90	0.00	0.00	0.00	0.00	0.00
	OXZ- D5	1697.51	187.26	23.14	0.00	0.00	0.00
	7CLZ- D4	8693.51	1445.74	146.61	0.00	0.00	0.00
	NDZ-D5	4955.17	599.83	66.25	0.00	0.00	0.00
	APZ-D5	10097.15	932.93	90.45	12.66	0.00	0.00
	aHAM- D5	1174.18	137.94	8.66	0.00	0.00	0.00
	DZP-D5	29482.37	3139.38	360.40	38.01	57.17	17.05
150 ng/mL	DZP	21440.79	2956.61	380.34	89.72	91.46	47.18
	7-CLZ	4413.81	710.16	90.96	0.00	0.00	0.00
	LRZ	551.54	65.42	0.00	0.00	0.00	0.00
	OXZ	25945.59	2075.24	173.85	0.00	20.76	14.64
	APZ	838.25	116.64	11.01	0.00	0.00	0.00
	CLZ	8504.39	986.34	77.62	6.21	16.05	0.00
	NDZ	964.85	134.82	6.80	0.00	0.00	0.00
	aHAM	5531.66	672.21	57.28	0.00	8.87	1.04
	TMZ	2420.48	308.20	0.00	0.00	0.00	0.00
	OXZ- D5	1710.54	202.98	0.00	0.00	0.00	0.00
	7CLZ- D4	10221.62	1414.24	110.96	0.00	0.00	0.00
	NDZ-D5	43960.15	5089.86	469.27	89.71	136.08	86.5
	APZ-D5	4766.03	580.37	35.89	0.00	3.84	0.00
	aHAM- D5	10243.70	899.92	88.65	9.40	0.00	0.00
	DZP-D5	963.44	125.03	0.00	0.00	0.00	0.00
450 ng/mL	DZP	41757.44	4141.03	438.84	66.58	45.54	30.22
	7-CLZ	51966.47	8227.72	930.05	208.05	169.58	187.28
	LRZ	13873.51	1798.56	187.41	0.00	0.00	0.00

	OXZ	1657.53	145.91	8.75	0.00	0.00	0.00
	APZ	57420.63	6884.14	318.55	29.65	46.95	36.11
	CLZ	1833.78	296.58	21.20	0.00	0.00	0.00
	NDZ	21455.51	2467.84	162.15	17.12	27.53	21.49
	aHAM	3135.95	291.91	11.31	0.00	0.00	0.00
	TMZ	8513.15	791.61	0.00	0.00	0.00	0.00
	OXZ- D5	1827.75	138.26	0.00	0.00	0.00	0.00
	7CLZ- D4	9835.40	1176.22	79.07	0.00	0.00	0.00
	NDZ-D5	44478.26	5155.91	365.93	82.64	155.68	97.98
	APZ-D5	4819.00	627.18	39.00	0.00	0.00	0.00
	aHAM- D5	9794.52	846.05	65.48	0.00	0.00	0.00
	DZP-D5	1001.63	106.42	0.00	0.00	0.00	0.00

Table II: Average peak area measured at each time point during extraction by sonication of the time point comparison study at all 3 concentrations analyzed: 15, 150 and 450 ng/mL.

Conc		5min	10min	15min	30min	45min	60min
15 ng/mL	DZP	971.79	81.78	0.00	0.00	0.00	0.00
	7-CLZ	1211.26	150.27	0.00	0.00	0.00	0.00
	LRZ	279.41	38.03	0.00	0.00	0.00	0.00
	OXZ	42.26	8.59	0.00	0.00	0.00	0.00
	APZ	1898.82	175.19	12.71	0.00	0.00	0.00
	CLZ	78.47	0.00	0.00	0.00	0.00	0.00
	NDZ	659.80	57.79	0.00	0.00	0.00	0.00
	aHAM	92.59	7.26	0.00	0.00	0.00	0.00
	TMZ	432.74	40.14	0.00	0.00	0.00	0.00
	OXZ- D5	1218.88	138.13	0.00	0.00	0.00	0.00
	7CLZ- D4	7859.39	995.02	58.08	0.00	0.00	0.00
	NDZ-D5	39172.40	3933.29	335.30	95.07	61.47	58.95
	APZ-D5	4162.53	344.62	14.43	0.00	0.00	0.00
	aHAM- D5	6054.64	496.68	34.75	0.00	0.00	0.00
	DZP-D5	13.01	0.00	0.00	0.00	0.00	0.00
150 ng/mL	DZP	11809.05	933.11	69.82	0.00	0.00	0.00
	7-CLZ	17578.20	1586.75	154.30	61.76	49.53	46.90
	LRZ	3899.08	496.62	61.40	19.66	14.30	12.03
	OXZ	493.93	67.18	4.70	0.00	0.00	0.00
	APZ	26218.56	2148.34	103.85	13.67	0.00	0.00
	CLZ	831.85	101.57	9.74	0.00	0.00	0.00
	NDZ	8432.84	547.26	21.66	0.00	0.00	0.00
	aHAM	1051.76	84.50	7.78	0.00	0.00	0.00
	TMZ	5243.96	431.28	31.80	0.00	0.00	0.00
	OXZ- D5	1176.70	133.10	0.00	0.00	0.00	0.00
	7CLZ- D4	9295.53	939.43	91.56	#DIV/0!	0.00	0.00
	NDZ-D5	42492.98	3737.39	331.51	99.78	67.94	67.32
	APZ-D5	4114.29	325.53	24.15	0.00	0.00	0.00
	aHAM- D5	5712.51	467.64	36.54	0.00	0.00	0.00
	DZP-D5	28.03	0.00	0.00	0.00	0.00	0.00
450 ng/mL	DZP	35778.20	2875.07	189.44	21.36	0.00	0.00
	7-CLZ	49556.10	5056.56	437.61	170.33	154.60	120.76
	LRZ	11799.70	1556.07	155.86	59.18	45.76	43.74
	OXZ	1443.84	185.57	20.13	0.00	0.00	0.00
	APZ	65787.40	6811.62	337.38	37.14	19.47	13.90
	CLZ	2499.69	270.80	13.10	0.00	0.00	0.00
	NDZ	26575.46	1668.23	73.85	7.35	0.00	0.00

	aHAM	3202.29	317.25	18.50	0.00	0.00	0.00
	TMZ	15954.87	1312.55	78.22	0.00	0.00	0.00
	OXZ- D5	1098.50	118.17	0.00	0.00	0.00	0.00
	7CLZ- D4	8457.44	926.78	72.66	0.00	0.00	0.00
	NDZ-D5	39238.90	3646.11	273.47	81.56	61.95	56.89
	APZ-D5	3810.11	311.01	18.64	0.00	0.00	0.00
	aHAM- D5	5462.45	438.98	30.59	0.00	0.00	0.00
	DZP-D5	6.26	0.00	0.00	0.00	0.00	0.00

Table III: Average peak area measured at each time point during extraction by vortexing of the time point comparison study at all 3 concentrations analyzed: 15, 150 and 450 ng/mL.

Conc		5min	10min	15min	30min	45min	60min
15 ng/mL	DZP	1013.53	52.83	0.00	0.00	0.00	0.00
	7-CLZ	1617.82	145.15	0.00	0.00	0.00	0.00
	LRZ	328.05	23.36	0.00	0.00	0.00	0.00
	OXZ	51.00	12.07	0.00	0.00	0.00	0.00
	APZ	2324.80	159.05	18.58	0.00	0.00	0.00
	CLZ	71.70	0.00	0.00	0.00	0.00	0.00
	NDZ	602.83	7.16	0.00	0.00	0.00	0.00
	aHAM	99.42	12.54	0.00	0.00	0.00	0.00
	TMZ	422.98	33.83	0.00	0.00	0.00	0.00
	OXZ- D5	1072.50	98.18	0.00	0.00	0.00	0.00
	7CLZ- D4	5654.71	505.04	46.99	0.00	0.00	0.00
	NDZ-D5	30673.30	2316.16	177.21	57.15	30.69	20.89
	APZ-D5	3254.32	166.62	20.11	0.00	0.00	0.00
	aHAM- D5	6936.49	445.21	39.86	3.31	0.00	0.00
	DZP-D5	850.32	63.44	0.00	0.00	0.00	0.00
150 ng/mL	DZP	9363.93	628.15	54.94	0.00	0.00	0.00
	7-CLZ	15517.19	1391.94	117.29	40.33	19.71	17.25
	LRZ	2824.94	311.37	31.73	9.97	0.00	0.00
	OXZ	426.55	47.36	#DIV/0!	0.00	0.00	0.00
	APZ	25380.78	1589.37	140.80	23.88	0.00	0.00
	CLZ	651.91	61.27	0.00	0.00	0.00	0.00
	NDZ	5500.00	99.93	9.55	0.00	0.00	0.00
	aHAM	950.52	90.43	8.28	0.00	0.00	0.00
	TMZ	3996.44	309.13	25.29	0.00	0.00	0.00
	OXZ- D5	1097.77	98.86	14.17	0.00	0.00	0.00
	7CLZ- D4	5594.25	551.96	42.64	0.00	0.00	0.00
	NDZ-D5	30544.76	2622.49	215.84	69.23	35.89	22.90
	APZ-D5	3303.43	237.44	23.70	0.00	0.00	0.00
	aHAM- D5	7341.45	507.76	44.11	0.00	0.00	0.00
	DZP-D5	881.88	65.97	10.94	0.00	0.00	0.00
450 ng/mL	DZP	27262.07	1624.33	119.68	18.19	18.19	0.00
	7-CLZ	45197.52	3618.56	266.15	109.81	59.38	37.05
	LRZ	8409.53	803.81	64.37	34.00	13.74	0.00
	OXZ	1186.64	118.03	10.99	0.00	0.00	0.00
	APZ	66400.31	4405.21	310.29	50.99	12.54	0.00
	CLZ	1797.06	165.17	0.00	0.00	0.00	0.00
	NDZ	16102.01	400.23	23.24	13.15	0.00	0.00

	aHAM	2784.99	220.96	17.52	0.00	0.00	0.00
	TMZ	11096.24	772.68	52.52	8.09	0.00	0.00
	OXZ- D5	1044.23	91.74	0.00	0.00	0.00	0.00
	7CLZ- D4	5593.47	498.35	43.80	0.00	0.00	0.00
	NDZ-D5	29776.07	2276.56	171.37	52.57	30.46	27.77
	APZ-D5	3171.61	213.03	18.38	0.00	0.00	0.00
	aHAM- D5	6945.40	425.42	34.29	0.00	0.00	0.00
	DZP-D5	864.53	59.60	0.00	0.00	0.00	0.00

Table IV: Relative recovery of each analyte at each time point sampled at each concentration after extraction by rapid agitation by Tissuelyzer®.

Conc.		Relative Recovery (%)					
		5 min	10 min	15 min	30 min	45 min	60 min
15 ng/mL	DZP	88.60	138.75	10.28	15.16	1.12	0
	NDZ	91.05	21.76	8.95	0.00	0.00	0.00
	TMZ	**100**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	OXZ	89.62	8.80	10.38	0.00	0.00	0.00
	LRZ	82.96	9.82	17.04	0.00	0.00	0.00
	APZ	88.72	54.92	11.28	0.00	0.00	0.00
	aHAM	82.55	14.93	17.45	0.00	0.00	0.00
	CLZ	86.17	19.23	13.83	0.00	0.00	0.00
	7CLZ	85.40	67.61	14.60	0.00	0.00	0.00
150	DZP	86.55	156.30	11.66	24.06	1.79	0.00
ng/mL	NDZ	87.96	30.23	12.04	0.00	0.00	0.00
	TMZ	88.71	11.29	0.00	0.00	0.00	0.00
	OXZ	86.50	10.82	13.50	0.00	0.00	
	LRZ	85.53	7.88	14.47	0.00	0.00	0.00
	APZ	84.57	75.20	15.43	0.00	0.00	0.00
	aHAM	84.29	10.51	15.71	0.00	0.00	0.00
	CLZ	87.80	17.20	12.20	0.00	0.00	0.00
	7CLZ	85.54	55.95	14.46	0.00	0.00	0.00
450	DZP	87.54	149.01	11.08	18.57	1.38	0.00
ng/mL	NDZ	89.31	27.79	10.69	0.00	0.00	0.00
	TMZ	91.49	8.51	0.00	0.00	0.00	0.00
	OXZ	89.12	9.42	10.88	0.00	0.00	0.00
	LRZ	87.50	7.54	12.50	0.00	0.00	0.00
	APZ	87.06	65.80	12.94	0.00	0.00	0.00
	aHAM	76.18	16.33	23.82	0.00	0.00	0.00
	CLZ	85.78	19.70	14.22	0.00	0.00	0.00
	7CLZ	82.33	84.29	17.67	0.00	0.00	0.00

Table V: Relative recovery of each analyte at each time point sampled at each concentration after extraction by vortexing.

Conc.		Relative Recovery (%)					
		5 min	10 min	15 min	30 min	45 min	60 min
15 ng/mL	DZP	95.05	4.95	0.00	0.00	0.00	0.00
	NDZ	91.55	8.45	0.00	0.00	0.00	0.00
	TMZ	92.59	7.41	0.00	0.00	0.00	0.00
	OXZ	100.00	0.00	0.00	0.00	0.00	0.00
	LRZ	80.86	19.14	0.00	0.00	0.00	0.00
	APZ	92.59	7.41	0.00	0.00	0.00	0.00
	aHAM	100.00	0.00	0.00	0.00	0.00	0.00
	CLZ	90.51	9.49	0.00	0.00	0.00	0.00
	7CLZ	93.35	6.65	0.00	0.00	0.00	0.00
150	DZP	93.20	6.25	0.55	0.00	0.00	0.00
ng/mL	NDZ	91.61	7.83	0.56	0.00	0.00	0.00
	TMZ	92.28	7.14	0.58	0.00	0.00	0.00
	OXZ	91.41	8.59	0.00	0.00	0.00	0.00
	LRZ	90.01	9.99	0.00	0.00	0.00	0.00
	APZ	92.28	7.14	0.58	0.00	0.00	0.00
	aHAM	90.20	9.80	0.00	0.00	0.00	0.00
	CLZ	90.37	8.63	1.00	0.00	0.00	0.00
	7CLZ	88.89	9.80	1.00	0.31	0.00	0.00
450	DZP	93.87	5.59	0.41	0.06	0.06	0.00
ng/mL	NDZ	92.53	6.92	0.55	0.00	0.00	0.00
	TMZ	93.01	6.48	0.44	0.07	0.00	0.00
	OXZ	91.58	8.42	0.00	0.00	0.00	0.00
	LRZ	90.19	8.97	0.84	0.00	0.00	0.00
	APZ	93.01	6.48	0.44	0.07	0.00	0.00
	aHAM	91.57	8.43	0.00	0.00	0.00	0.00
	CLZ	91.70	7.69	0.61	0.00	0.00	0.00
	7CLZ	90.18	8.62	0.69	0.36	0.15	0.00

Table VI: Relative recovery of each analyte at each time point sampled at each concentration after extraction by vortexing.

Conc.		Relative Recovery (%)					
		5 min	10 min	15 min	30 min	45 min	60 min
15 ng/mL	DZP	92.24	7.76	0.00	0.00	0.00	0.00
	NDZ	93.83	6.17	0.00	0.00	0.00	0.00
	TMZ	91.51	8.49	0.00	0.00	0.00	0.00
	OXZ	100.00	0.00	0.00	0.00	0.00	0.00
	LRZ	83.11	16.89	0.00	0.00	0.00	0.00
	APZ	91.51	8.49	0.00	0.00	0.00	0.00
	aHAM	100.00	0.00	0.00	0.00	0.00	0.00
	CLZ	94.57	5.43	0.00	0.00	0.00	0.00
	7CLZ	88.02	11.98	0.00	0.00	0.00	0.00
150	DZP	92.17	7.28	0.54	0.00	0.00	0.00
ng/mL	NDZ	91.18	8.33	0.49	0.00	0.00	0.00
	TMZ	91.89	7.56	0.56	0.00	0.00	0.00
	OXZ	88.20	10.77	1.03	0.00	0.00	0.00
	LRZ	87.30	11.87	0.83	0.00	0.00	0.00
	APZ	91.89	7.56	0.56	0.00	0.00	0.00
	aHAM	90.59	9.41	0.00	0.00	0.00	0.00
	CLZ	90.01	9.39	0.61	0.00	0.00	0.00
	7CLZ	86.59	11.03	1.36	0.44	0.32	0.27
450	DZP	92.06	7.40	0.49	0.05	0.00	0.00
ng/mL	NDZ	91.24	8.13	0.63	0.00	0.00	0.00
	TMZ	91.98	7.57	0.45	0.00	0.00	0.00
	OXZ	89.80	9.73	0.47	0.00	0.00	0.00
	LRZ	87.53	11.25	1.22	0.00	0.00	0.00
	APZ	91.98	7.57	0.45	0.00	0.00	0.00
	aHAM	90.32	9.56	0.12	0.00	0.00	0.00
	CLZ	92.48	6.83	0.69	0.00	0.00	0.00
	7CLZ	86.38	11.39	1.14	0.43	0.33	0.32

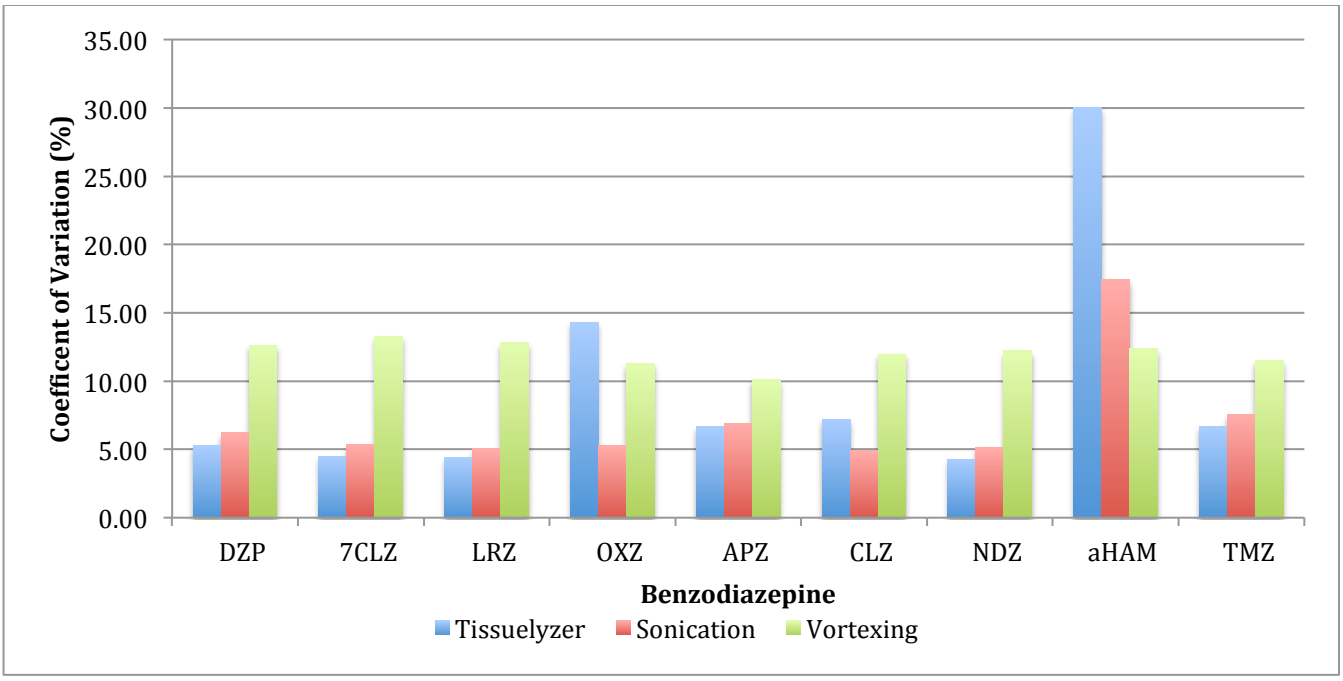

Figure I: Coefficient of variation in percent of each analyte (DZP, 7CLZ, LRZ, OXZ, APZ, CLZ, NDZ, aHAM, TMZ) measured from DBS at 150 ng/mL after 5 minutes of extraction comparing each of the 3 agitation methods (Tissuelyzer®, sonication, vortexing). Each value was made from measurements made in triplicate (n=3).

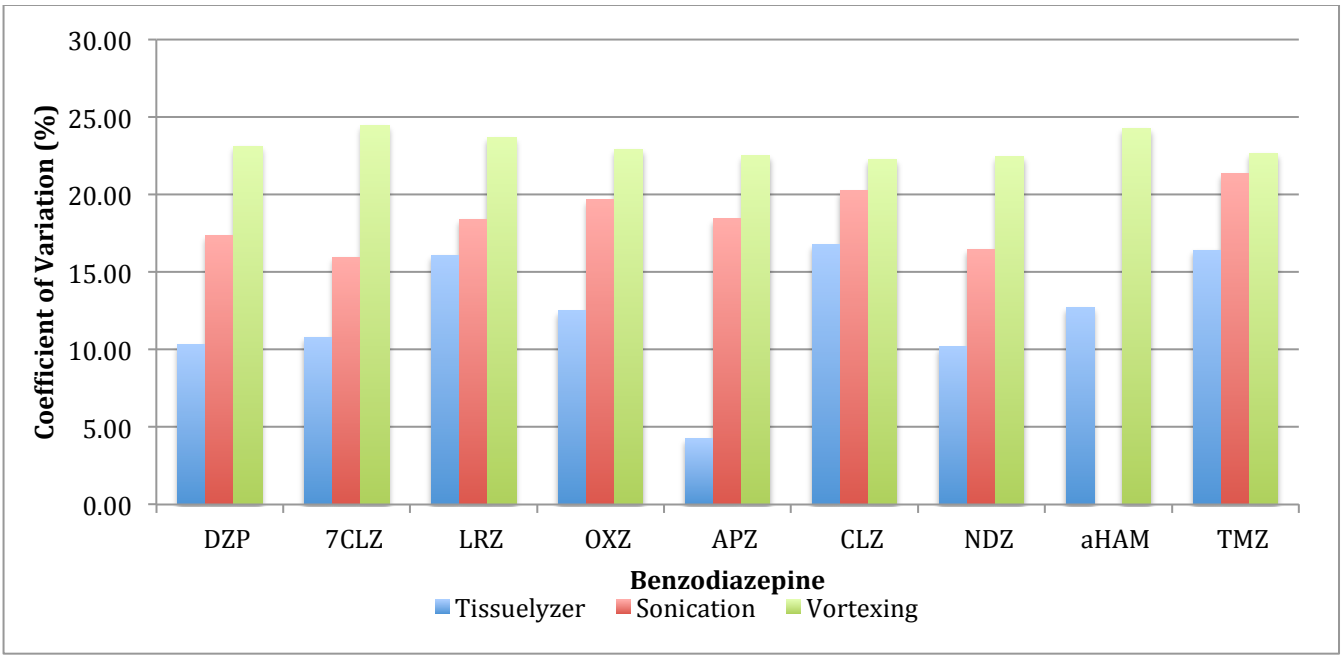

Figure II: Coefficient of variation in percent of each analyte (DZP, 7CLZ, LRZ, OXZ, APZ, CLZ, NDZ, aHAM, TMZ) measured from DBS at 450 ng/mL after 5 minutes of extraction comparing each of the 3 agitation methods (Tissuelyzer®, sonication, vortexing). Each value was made from measurements made in triplicate (n=3).

Table VII: Results of recovery for all analytes at all concentrations for all extraction solvents tested for optimization during method development.

Extraction Solvent	Analyte	Recovery (%)		
		15 ng/mL	150 ng/mL	450 ng/mL
MeOH	DZP	130.12	154.43	139.32
	7CLZ	63.76	79.08	74.14
	LRZ	61.73	75.26	64.80
	OXZ	121.44	149.42	137.99
	APZ	116.59	145.16	119.68
	CLZ	131.42	146.34	140.31
	NDZ	129.92	159.52	141.33
	aHAM	129.41	159.73	142.22
	TMZ	129.91	163.57	155.09
MeOH with 1% FA	DZP	181.6726797	155.2485994	182.8520653
	7CLZ	58.30743844	45.44657459	51.95697504
	LRZ	156.1293952	178.2396279	202.5519997
	OXZ	258.8449194	178.3271816	186.0537493
	APZ	177.0389713	148.1095431	173.4883736
	CLZ	221.8633634	203.1124231	225.1315936
	NDZ	210.7063427	175.4382271	202.7322019
	aHAM	141.306308	118.5313661	137.8555877
	TMZ	269.5012017	163.3414885	192.3839303
MeOH:ACN	DZP	168.0	152.5	157.5
	7CLZ	83.6	78.5	79.2
	LRZ	155.6	145.1	146.9
	OXZ	166.0	144.2	149.7
	APZ	161.3	152.2	154.3
	CLZ	152.2	147.1	148.2
	NDZ	173.8	152.7	160.5
	aHAM	169.6	154.2	153.9
	TMZ	163.3	146.1	153.8
MeOH:ACN with 1% FA	DZP	183.89	164.69	188.15
	7CLZ	70.88	59.63	64.17
	LRZ	189.22	183.80	207.01
	OXZ	205.46	180.60	206.56
	APZ	230.23	200.41	253.56
	CLZ	237.88	220.17	268.59
	NDZ	222.14	190.91	226.04
	aHAM	151.38	149.75	169.20
	TMZ	248.84	188.11	218.48

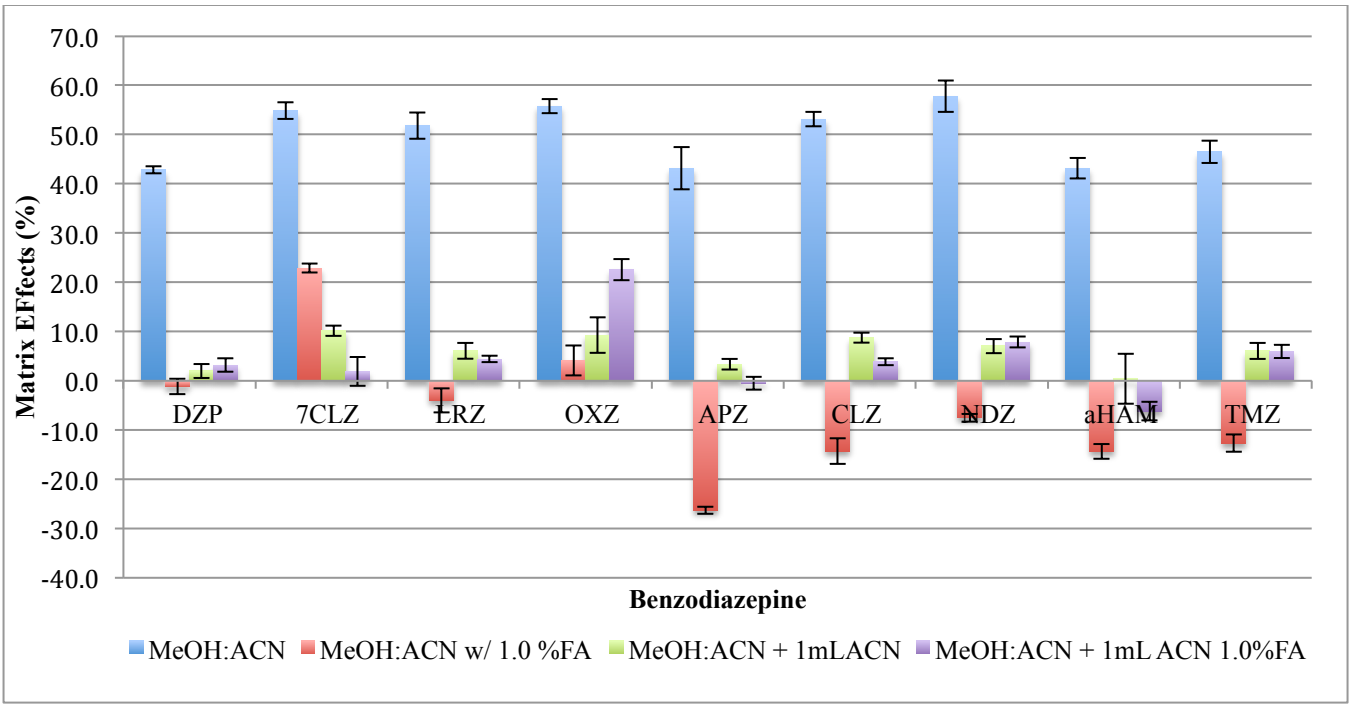

Figure III: Calcualted matrix effects (±SD) of each benzodiazpine from each solvent system measured to optimize protein filtration with a Clean Screen® FASt plate: 1:1 (v/v) MeOH:ACN (methanol: acetonitrile); MeOH:ACN with 1% formic acid (FA); 1:1 (v/v) MeOH:ACN with 1 mL ACN added post-extraction before filtration; 1:1 (v/v) MeOH:ACN with 1 mL ACN with 1% FA added post-extraction before filtration. These data are calculated from 150 ng/mL DBS measured in triplicate(n=3).

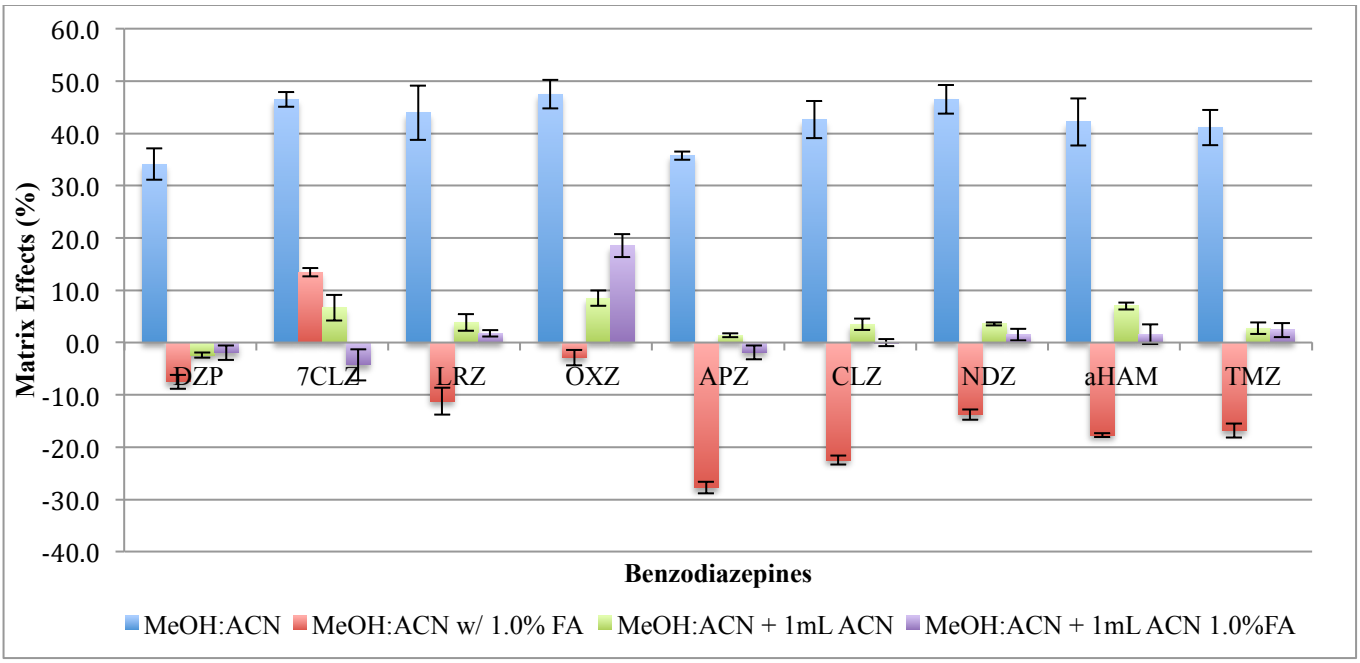

Figure IV: Calcualted matrix effects (±SD) of each benzodiazpine from each solvent system measured to optimize protein filtration with a Clean Screen® FASt plate: 1:1 (v/v) MeOH:ACN (methanol: acetonitrile); MeOH:ACN with 1% formic acid (FA); 1:1 (v/v) MeOH:ACN with 1 mL ACN added post-extraction before filtration; 1:1 (v/v) MeOH:ACN with 1 mL ACN with 1% FA added post-extraction before filtration. These data are calculated from 450 ng/mL DBS measured in triplicate(n=3).

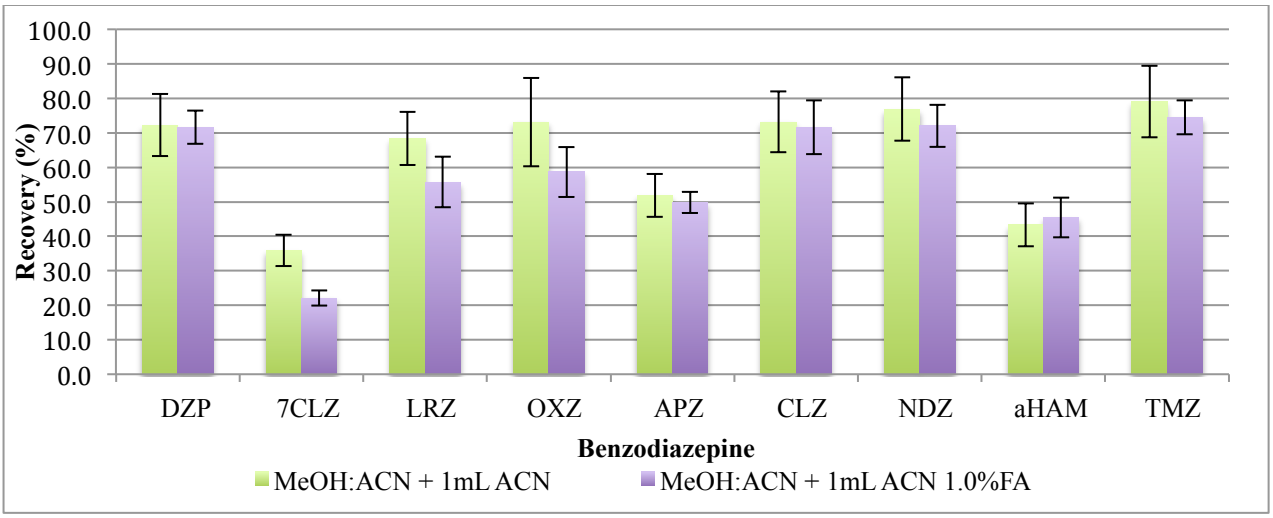

Figure V: Calculated recovery (±SD) of each benzodiazepine extracted with MeOH:ACN with the addition of 1 mL of ACN to the extraction solvent before filtration compared to extraction with MeOH:ACN with the addition of 1 mL of ACN with 1% of formic acid (FA) to optimize the solvent system. These data are calculated from DBS at 150 ng/mL collected in triplicate (n=3).

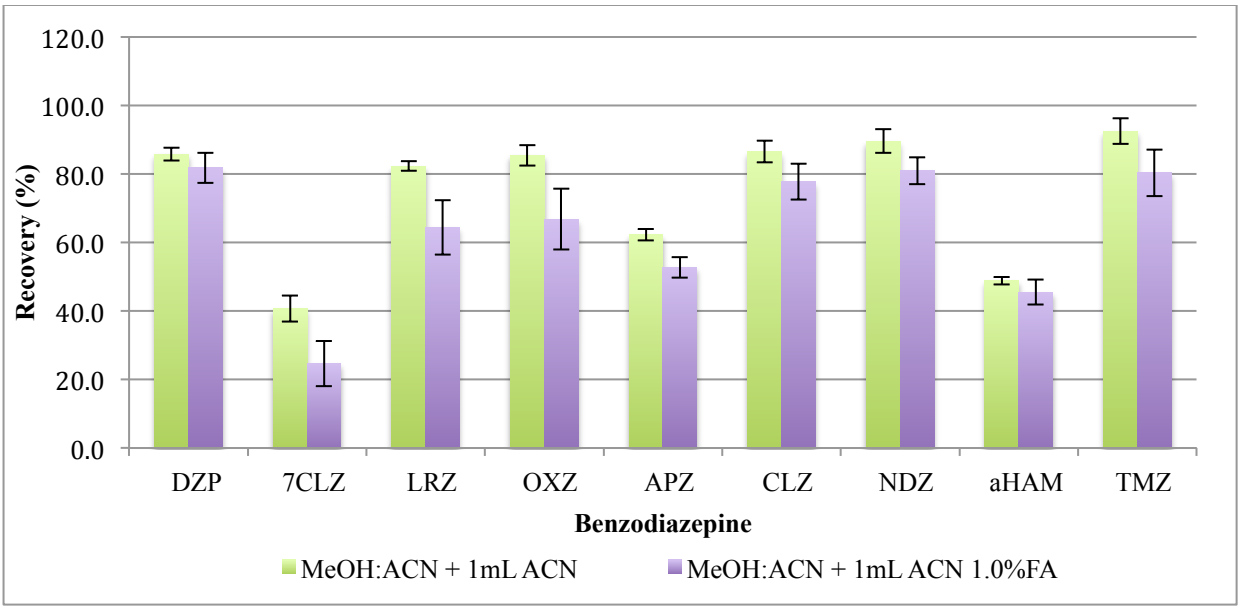

Figure VI: Calculated recovery (±SD) of each benzodiazepine extracted with MeOH:ACN with the addition of 1 mL of ACN to the extraction solvent before filtration compared to extraction with MeOH:ACN with the addition of 1 mL of ACN with 1% of formic acid (FA) to optimize the solvent system. These data are calculated from DBS at 450 ng/mL collected in triplicate (n=3).

Table VIII: Calculated bias for both the low and high blind accuracy samples determined from the equation of the line of best fit.

		Average Bias (%)			
Low Accuracy Sample	**Analyte**	**Curve 2**	**Curve 3**	**Curve 4**	**Curve 5**
	DZP	14.80	22.23	15.42	1.65
	7CLZ	16.58	11.02	25.43	11.29
	LRZ	12.27	10.01	10.39	12.20
	OXZ	16.91	21.80	13.61	9.35
	APZ	11.75	19.22	10.99	9.57
	CLZ	16.30	21.37	11.67	8.15
	NDZ	16.56	23.14	23.72	8.34
	aHAM	21.66	25.61	18.32	12.01
	TMZ	12.49	14.80	20.16	9.11
High Accuracy Sample	DZP	15.86	15.20	13.37	2.96
	7CLZ	16.21	15.76	16.25	4.16
	LRZ	16.20	16.32	3.30	4.30
	OXZ	12.76	13.38	10.09	3.09
	APZ	15.91	12.18	10.86	3.62
	CLZ	18.48	15.66	10.16	3.16
	NDZ	16.66	15.09	8.62	2.15
	aHAM	12.93	13.74	13.49	2.29
	TMZ	13.19	10.20	7.73	4.38