QUANTITATIVE ANALYSIS OF ACETONE IN BLOOD VIA HEADSPACE GAS CHROMATOGRAPHY/ FLAME IONIZATION DETECTION

By

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My grandfather, Jimmie Jones, always said that I could do anything I set my mind to. I would like to dedicate my thesis to him.

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NOMENCLATURE

ANOVA	analysis of variance
ASCLD-LAB	American Society of Crime Laboratory Directors - Laboratory Accreditation Board
ATP	Adenosine triphosphate
Conc	concentration
dL	deciliter; 0.1 liter
DNA	deoxyribonucleic acid
FBI	Federal Bureau of Investigation
FID	flame ionization detector
g	gram
GC	gas chromatography
HS	headspace
IS	internal standard
ISO/IEC	International Organization of Standardization/International Electrotechnical Commission
LDR	linear dynamic range
LOD	limit of detection
LOQ	limit of quantitation
mg	milligram; 0.001 gram
ND	not detected

NIDA	National Institute on Drug Abuse
R	resolution
RSD	relative standard deviation (%)
SD	standard deviation
t_m	retention time for unretained compound in selectivity study
t'_r	apparent retention time in selectivity study, calculated as (t_r-t_m)
t_r	retention time in selectivity study
W_b	width at base in selectivity study

CHAPTER I

INTRODUCTION

Acetone is normally present in the body at low levels (United States Public Health Service, 1994). High levels of acetone can be toxic, leading to coma and even death (Levine, 2006). At the same time, an increased blood-acetone concentration could indicate a toxic condition, such as diabetic ketoacidosis. Acetone levels may also be elevated as a result of ketogenic diets (Kalapos, 2003).

Acetone has a high vapor pressure, or volatility, which means that it evaporates readily. Because acetone is volatile, quantitation in biological samples can be challenging. Headspace Gas Chromatography with Flame Ionization Detection (HS-GC/FID) has been successfully utilized for the analysis of volatile chemicals (Barua, Chi, Fitzpatrick, Gillard, & Kostyniak, 2008). The HS autosampler allows for the analysis of volatiles in blood, the GC separates and identifies the sample components, and the FID measures each component (Levine, 2006).

The purpose of this research project was to develop and validate a method for the quantitation of acetone in blood using HS-GC/FID. Method validation serves as a quality assurance measure and is required for accredited laboratories (Federal Bureau of Investigation, 2009). The Portland Metropolitan Forensic Laboratory (Portland Lab) is

accredited through the American Society of Crime Laboratory Directors - Laboratory Accreditation Board (ASCLD-LAB), one of the main national accrediting bodies for forensic laboratories. Without a validated method for acetone quantitation on-site, the Portland Lab had to subcontract acetone quantitations to a laboratory with an operational validated method, as per ASCLD-LAB standards.

Precision, accuracy, selectivity, sensitivity, repeatability, and stability are the key aspects of a method validation (Federal Bureau of Investigation, 2009). Therefore, the following items were included in this method development: preparation of standards and positive controls, testing the necessity of using salts, establishment of a suitable internal standard concentration, determination of an appropriate sample size, limit of detection calculation, establishment of the linear dynamic range, checking for carryover, checking the stability of samples containing acetone, and method validation including postmortem case samples.

Standards are used to create a calibration curve from which the concentrations of samples and controls may be determined. As such, the standard concentrations should be based on known normal, toxic, and disease-state acetone levels. Testing acetone standards from two different sources will confirm that the provided standards are pure and that the instrument is functioning properly while determining the mean, standard deviation, and percent error for each of the acetone standards will test for accuracy and precision. The correlation coefficient from the calibration curve measures how well the data fits the line and should be as close to one as possible (Levine, 2006). The Portland Lab requires that the correlation coefficient be at or above 0.99 in their validated quantitation methods (Oregon State Police Forensic Services Division, 2008).

When preparing standard calibration curves, generally a ratio of analyte to internal standard peak area is plotted on the y-axis with analyte concentration on the x-axis. The peak area ratio is an accurate reflection of the total amount of a compound in the original sample. Peak height ratios are sometimes used instead of peak area, but only when the peaks are broad or overlapping (Levine, 2006).

Salts can affect the degree to which a volatile partitions into the headspace (Kolb & Ettre, 2006). A greater amount of volatile present in the headspace means there is more analyte available for analysis. Therefore, salts have the potential of increasing the sensitivity for detecting certain analytes.

The Portland Lab uses 1.5 mL of 0.05% (v/v) n-propanol in water internal-standard solution for blood-alcohol analysis. The same concentration was used as the starting point for the internal standard concentration experiment. An appropriate internal standard concentration will be low enough to allow peak detection at low analyte concentrations. The amount of internal standard added should also result in detectable and reproducible peak regardless of the sample characteristics like clots. A high internal standard concentration will result in a large internal standard peak, making the analyte peak appear smaller. Thus, internal standard concentration can affect the visibility of the analyte peak.

Sample size may affect peak visibility and/or accuracy of calculated analyte concentrations. The smallest sample size that still produces accurate results should be used. Using a method with a small sample volume will ensure that enough of the sample is present for duplicate analyses. This also preserves samples for subsequent testing by outside laboratories if needed.

The limit of detection corresponds to the lowest detectable signal. Thus, a method with a lower limit of detection can "see" a smaller concentration of drug and therefore has a higher sensitivity. The limit of detection can be estimated from blank samples as mean plus three times the standard deviation of the noise (Levine, 2006). A blank sample is not expected to contain the analyte of interest. The mean concentration of the blank samples is determined by integrating the chromatogram along the analyte's expected retention time range. Generally, the mean is close to zero.

A method should at least be linear over the expected normal and toxic ranges of an analyte. The linear dynamic range identifies the concentrations between which an analyte may be quantitated. Any quantitations performed outside the linear dynamic range will not produce accurate or precise results (Skoog, Holler, & Crouch, 2007). In any case, quantitative assays require that the unknown value be determined in the linear range of the sample curve for accurate quantitation and the sample should be compared with standards at higher and lower values within that particular analysis.

Carryover occurs when a sample containing a large amount of analyte is not completely eluted from the column and is "carried over" to the next sample. The result of carryover from one sample to another is artificially high concentrations calculated for samples that contained the analyte already and false-positives in samples that actually had no analyte whatsoever (Levine, 2006).

Validation using controls and actual postmortem case samples ensures that the method leads to accurate and precise results with high degrees of sensitivity and selectivity. Accuracy is measured by calculating the percent error between expected and calculated control concentrations. Standard deviations can be used to measure the

method's precision. The limits of detection and quantitation illustrate the sensitivity of the method while baseline separation of several components can demonstrate selectivity (Federal Bureau of Investigation, 2009).

Sample degradation may occur over time as a result of storage temperature (Levine, 2006). Samples are generally stored in the refrigerator but other options include storage in the freezer or at room temperature. These storage alternatives could vastly increase sample storage space.

With the acetone quantitation parameters optimized and the method validated, forensic toxicologists at the Portland Metropolitan Forensic Lab may conduct acetone quantitations themselves, rather than having to send the samples to another laboratory for analysis. Having a validated acetone-quantitation method on-site may save time and money. In terms of time, the Portland Lab will be able to control when acetone-containing samples are analyzed. The costs associated with an in-house acetone-quantitation method include analysts' salaries, instrument maintenance, training, and purchasing calibrator, internal standard, and control chemicals.

CHAPTER II

REVIEW OF LITERATURE

II. A. Acetone

Acetone is a volatile chemical with a pungent, “characteristic odor and sweetish taste” (O’Neil, Smith, Heckelman, & Obenchain, Jr., 2001). The chemical structure of acetone is shown in Figure 1.

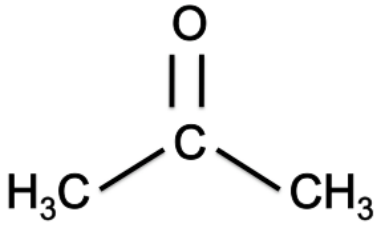

Figure 1. Chemical Structure of Acetone

Volatiles have the ability of existing in "a form that may be inhaled" (Levine, 2006). Therefore, acetone and other volatile chemicals may also be referred to as inhalants. Inhalant abuse is common among teenagers and is “one of the most prevalent drug abuse problems in the world” (Bowen, Daniel, & Balster, 1999). According to the 2007 National Survey of Drug Use and Health, 775,000 people over the age of 12 had abused inhalants for the first time in that year while 66.7% of those first-time abusers were under the age of 18 (National Institute on Drug Abuse, 2009). Acetone abusers desire euphoria and loss of inhibition, but other effects include confusion and vomiting as well as

irritation of the nose, throat, trachea, and lungs (United States Public Health Service, 1994). A full description of the toxic effects of acetone may be found in section II. A. ii.

Low concentrations of acetone, an endogenous ketone, build up in the body as a result of the breakdown of fat. The mechanism of fat breakdown, or lipolysis, is generally beta-oxidation to acetyl-CoA. Acetyl-CoA can then either enter the Citric Acid Cycle or it can be converted to acetone and two other ketones via ketogenesis (Berg, Tymoczko, & Stryer, 2002). Ketogenesis generally occurs in the liver. After excretion into the blood, ketones are carried throughout the body to be used as a source of energy (United States Public Health Service, 1994). In the blood, acetone concentrations around 1 mg/dL are considered normal (Ashley, 1994).

When acetone is inhaled, the majority is absorbed after 15 minutes (United States Public Health Service, 1994). Acetone is distributed to tissues and organs with high water content. The volume of distribution for acetone, or the amount of body water in which the chemical is distributed if the whole dose remains in the blood (Levine, 2006), is 0.8 L/kg (Baselt, 2000), which is consistent with other hydrophilic substances. Acetone is excreted from the body into urine and air, either as parent compound or as metabolite(s). Inhaled acetone is mainly eliminated via the lungs. The half-life of acetone is between 3 and 6 hours (Baselt, 2000).

II. A. i. Metabolism

Acetone metabolism does not depend on the route of exposure (i.e. naturally-occurring acetone versus inhaled acetone), and generally takes place in the liver. Acetone is intermediately catabolized to glucose via gluconeogenesis (Figure 2).

Glucose is then broken down via glycolysis to produce acetone's final metabolic products of carbon dioxide and adenosine triphosphate (ATP). ATP is a major energy source in cellular biology and is often referred to as the "currency of energy" (Berg, et al., 2002).

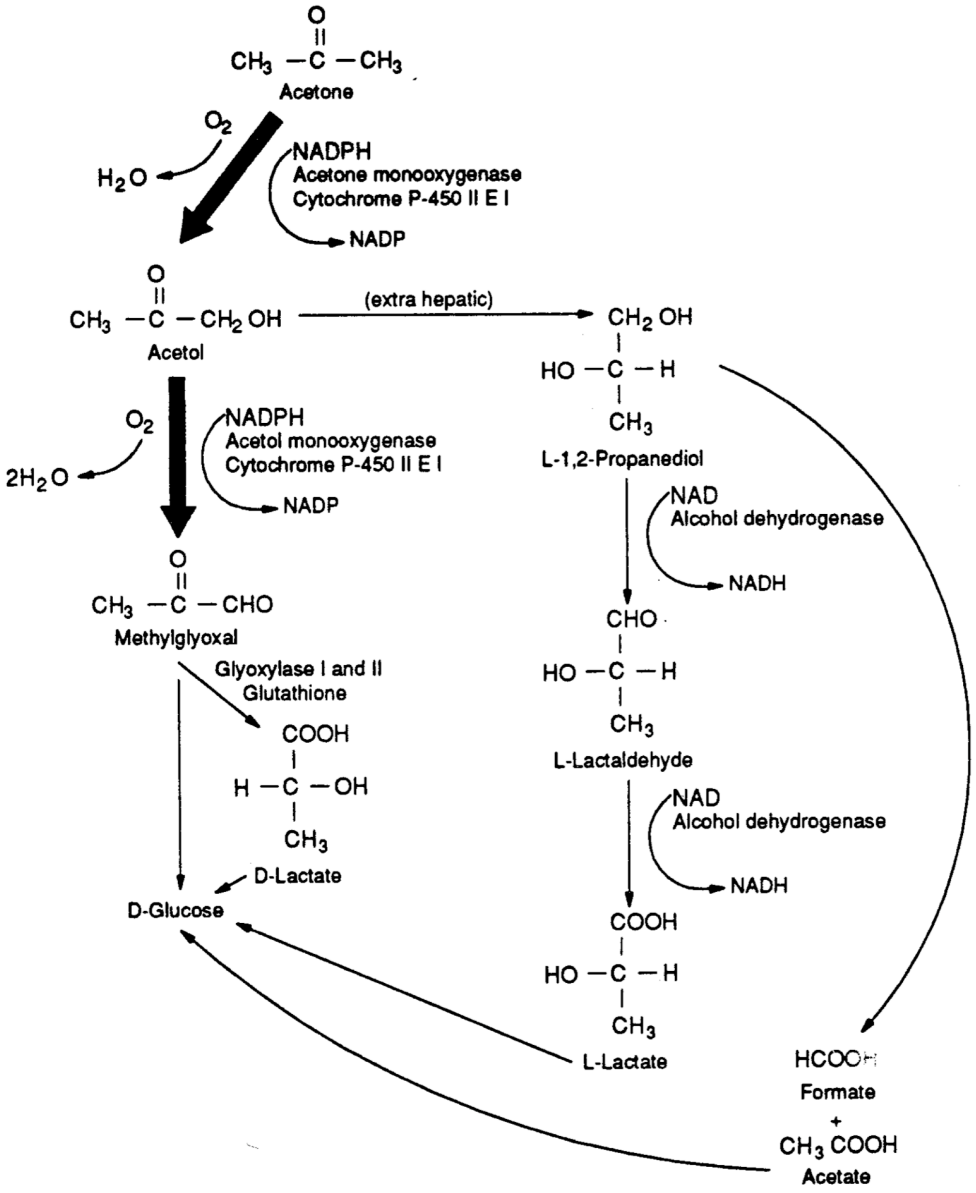

Figure 2. Intermediary Metabolic Pathways of Acetone (Kalapos, 2003)

As seen in Figure 2, the primary step in acetone catabolism to glucose is oxidation to acetol. This is the rate-limiting step (United States Public Health Service, 1994). In

the liver, acetol is further broken down to methylglyoxal and ultimately produces glucose. When metabolism takes place outside the liver, acetol is converted to 1,2-propanediol then lactaldehyde then lactate and finally glucose. Glucose can also be synthesized from acetate and formate, the third gluconeogenic pathway of acetone metabolism (Kalapos, 2003). Metabolism to acetate and formate occurs at a rate of 1 to 3 mg/kg/hr (Baselt, 2000). However, diabetes and fasting can "alter the pattern of metabolism" (United States Public Health Service, 1994). Other factors that affect the rate of metabolism include "respiratory rate, blood flow, proportion of body fat, and metabolic clearance rate" (Levine, 2006).

Metabolites are eliminated in both exhaled air and urine while acetone itself may also be eliminated in exhaled air. The average blood to breath acetone concentration ratio is 330, with a range from 322 to 339 (Haggard, Greenberg, & Turner, 1944). Metabolism is the chief route of elimination and accounts for 70 to 80 percent of acetone elimination from the body (United States Public Health Service, 1994).

II. A. ii. Toxicology

Establishing a toxic level of acetone and other volatile chemicals can be difficult due to individual variation in metabolism and other factors. Chronic acetone abusers may develop a high threshold. For example, Levine reported a toxic concentration for the volatile chemical toluene of 70 mg/L while the majority of deaths attributed to toluene inhalation occurred at concentrations less than 5 mg/L (2006). Death after acetone (or another volatile chemical) intoxication is often a result of cardiac arrest or pulmonary complications, including asphyxiation and suffocation.

Acetone is toxic in the blood at levels greater than 33 mg/dL (Burns et al., 1998). Symptoms include confusion, vomiting, drowsiness, and hyperventilation (Sulway & Malins, 1970). Larger doses can lead to convulsions, coma, or death. Prolonged abuse of acetone as an inhalant negatively affects the central nervous system. Loss of coordination, loss of cognitive function, and gait disturbance are only some of the effects. Worse still, volatile chemicals such as acetone can lead to a decrease in brain mass because they "literally dissolve brain cells" (Levine, 2006).

II. A. iii. Ketoacidosis/ketogenic diets

According to Deng, “acetone is an important volatile disease marker” (2004). However, acetone is not the causative agent in disease. Acetone levels are elevated in people with type-I diabetes, especially during ketoacidosis. Ketoacidosis occurs when diabetes is uncontrolled leading to an increase in acidic ketone concentrations, including acetone, with the final result of a lowered serum pH (Miekisch, Schubert, Vagts, & Geiger, 2001). The increased acetone concentrations can be “traced to the increased mobilization and utilization of free fatty acids in the liver” (United States Public Health Service, 1994). Ketoacidosis can cause diabetic coma and/or death.

In controlled diabetes, the blood acetone level is normally less than 3 mg/dL (Jones, Sagarduy, Ericsson, & Arnqvist, 1993). Ketoacidotic individuals will exhibit a higher blood acetone level. During ketoacidosis, the concentration of acetone may range from 10 to 70 mg/dL (Ramu, Rosenbaum, & Blaschke, 1978).

Acetone levels can also be higher than average for people on ketogenic diets. Ketogenic diets are low in carbohydrates so fat is the primary source of energy. They

have been implemented for the prevention of epilepsy (Kalapos, 2006). Such diets elevate the level of acetone in the brain. These elevated acetone levels are what make ketogenic diets effective in controlling epilepsy, although the mechanism is not well understood (Likhodii, 2003).

Diabetes and ketogenic diets "place high energy demands upon the body which result in increased fatty acid utilization and higher than normal blood levels of acetone" (United States Public Health Service, 1994). Children and pregnant women also have higher-than-average acetone blood levels due to their higher energy expenditures.

II. B. Validation

Validation is used to evaluate a quantitation procedure for "efficacy and reliability for forensic casework analysis" (Federal Bureau of Investigation, 2009). For developmental validation, the appropriate conditions for analysis as well the limitations of the method must be determined. Precision, accuracy, specificity, and sensitivity are the key components to be investigated during development. Stability and reproducibility should also be considered.

Precision is a gauge of the variability between samples while accuracy is a "measure of the degree to which the experimental mean agrees with the true or theoretical concentration" (Levine, 2006). Both precision and accuracy are considered when determining the error associated with a method.

Accurate gas chromatographic quantitative methods generally require the inclusion of an internal standard with each sample analyzed. An internal standard will improve precision and accuracy by normalizing any variations in recovery due to analyte

behavior during processing and analysis. Prior to analysis, an equal amount of internal standard must be added to each prepared sample, standard, and control vial. An appropriate internal standard should be similar in structure to the analyte so that it behaves similarly during chemical analysis. The internal standard should not be expected to be present in the sample and should be resolved chromatographically (i.e. have a different retention time than the analyte). Taking these qualifications into account, n-propanol should serve as a good internal standard for the quantitation of acetone in blood (Figure 3).

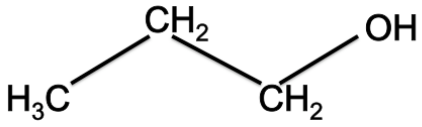

Figure 3. Chemical Structure of n-Propanol

Methods capable of measuring an analyte while differentiating that analyte from other compounds present in the sample are considered specific. For a gas chromatographic method, specificity refers to the ability to completely separate, or resolve, several compounds. Selectivity can be illustrated through baseline resolution of components. Resolution may be calculated using Equation 2.1.

$$R = \frac{\left(t'_{rb} - t'_{ra}\right)}{\frac{1}{2}\left(W_{ba} + W_{bb}\right)} \qquad (2.1)$$

From Equation 2.1, R stands for resolution, t'_r represents adjusted retention time, and W_b represents width at base of peak. To determine the adjusted retention time (t'_r), the retention time for the unretained compound (t_m) is subtracted from the actual retention time (t_r) from both compounds "a" and "b" (Levine, 2006). A chromatogram further delineating these variables is shown in Figure 4.

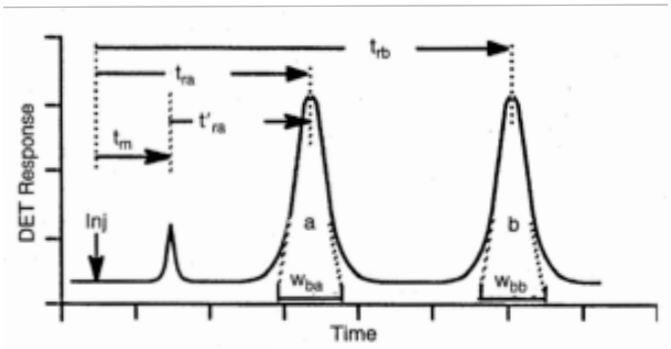

Figure 4. Example Chromatogram (Levine, 2006, Chapter 7, Figure 1)

Sensitivity may be defined as the ability to detect an analyte. In terms of sensitivity, the limit of detection (LOD) is the smallest analyte concentration that may be discerned from the noise while the limit of quantitation (LOQ) is the lowest analyte concentration that may be accurately measured (Skoog et al., 2007).

The addition of salt to samples has been demonstrated to improve the sensitivity when detecting certain analytes by increasing the partitioning of the analyte into the headspace. This process is known as the "salting out effect" (Chang, 2000). In order to increase the analyte concentration in the headspace, the partition coefficient must be reduced. The partition coefficient relates the "solubility of the analyte in the matrix" (Kolb & Ettre, 2006). The partition coefficient is inversely proportional to the vapor pressure and the activity coefficient, which relates the intermolecular interaction of the analyte and the solvent, or matrix. Therefore, to reduce the partition coefficient, the activity coefficient and/or the vapor pressure must be increased (Chang, 2000). Vapor pressure increases with increasing temperature. As for the activity coefficient, the addition of salt changes the sample matrix by forming a bond between the salt and the solvent, thereby decreasing the bond strength that previously existed between the analyte

and the solvent and thus allowing a volatile analyte to enter the headspace (Kolb & Ettre, 2006).

The degree to which a salt increases the partitioning of an analyte into the headspace depends not only on the analyte and the sample matrix, but also the salt itself. Salts are made up of positively-charged cations and negatively-charged anions that interact with the solvent. Salts containing either monovalent or divalent anions may be used (Kalra, Tugcu, Cramer, & Garde, 2001).

The stability of samples in a specific matrix (e.g. blood) must be tested as part of a method validation. Over time, chemical degradation may occur, and temperature may play a role in how quickly degradation occurs (Levine, 2006). Reproducibility takes into account the precision and accuracy of the method after use of the method over time (interday) and by different analysts (interindividual).

Method validation is required for forensic laboratories accredited through the American Society of Crime Laboratory Directors-Laboratory Accreditation Board (ASCLD-LAB). ASCLD-LAB is one of the main national accrediting bodies for forensic testing laboratories. According to their website, ASCLD-LAB is "dedicated to providing excellence in forensic science through leadership and innovation" (American Society of Crime Laboratory Directors, 2009). ASCLD-LAB adheres to the standards for testing and calibration laboratories prepared by the International Organization for Standardization/International Electrotechnical Commission (ISO/IEC). In accordance with the ISO/IEC standards, until an on-site method has been validated, laboratories must sub-contract to another laboratory with a validated method in place (Federal Bureau of Investigation, 2009).

II. C. Headspace Gas Chromatography/Flame Ionization Detection

Volatility is a measure of how quickly a substance changes from a liquid to a gas and highly volatile substances tend to evaporate quickly. Because acetone is volatile, determining the acetone concentration in biological samples can be difficult. Gas chromatography is an analytical method used for separating volatile and semivolatile substances in a mixture in order to identify and quantitate the substances (Skoog et al., 2007).

Gas chromatography works well with these substances since the separation of the volatile mixture is mediated by a carrier gas flowing past a stationary phase that will interact with the mixture components based on solubility. Chromatographic separation of mixture components relies on the partitioning of the components between the stationary and mobile phases. For gas chromatography, the stationary phase is the column coating while the mobile phase is a gas, often called a carrier gas. After sample injection into the injection port of the GC, the sample is vaporized and the carrier gas then pushes the sample components through the column to the detector. Sample components with a higher affinity for the stationary phase (i.e. components of a similar polarity to the stationary phase) will remain on the column longer. The time required for a component to elute off the column, also known as retention time, is used to identify the component. A simplified scheme illustrating how gas chromatography works is given in Figure 5.

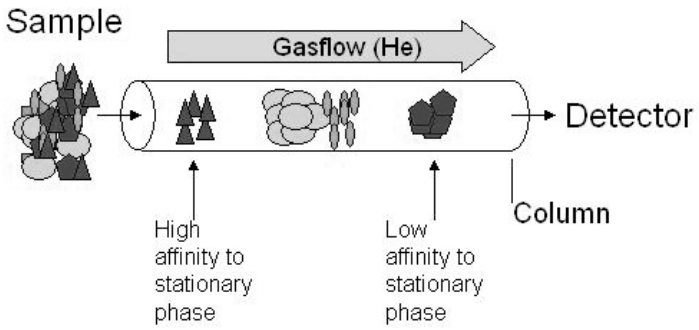

Figure 5. Schematic Diagram of Gas Chromatograph (Buchinger, 2006)

As a volatile chemical, acetone obeys Henry's law, which states that at a given temperature there is a direct relationship between the amount of a volatile in a liquid and the amount of the volatile in the vapor above the solution (see Figure 6).

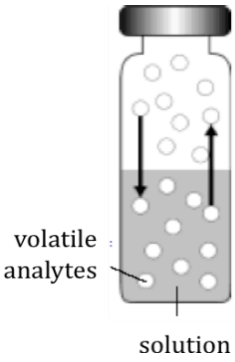

Figure 6. Depiction of Henry's Law (Koplow, 2006)

Equipping a gas chromatograph with a headspace analyzer will allow the analysis of acetone and other volatile materials in the blood with little to no sample processing. The headspace is the area between the liquid level and the top of the vial, and it will contain volatile substances that are in equilibrium with the blood. The headspace injector is designed to remove these volatiles from the headspace and place them onto a chromatographic column for analysis. First, the acetone is allowed to equilibrate between the headspace and the blood, sometimes with heating, which drives more acetone into the headspace. During this time, a carrier gas (usually helium or hydrogen) is used to flush

the injector loop. Next the vial septum is punctured and pressurization completed. Carrier gas flow is then halted so the headspace vapors may fill the sample loop. Finally, the sample enters the column via flushing by carrier gas (Levine, 2006).

Since acetone mainly consists of carbon, an appropriate detector would be the FID because it is known as a "carbon counter". This detector relies on the combustion or oxidation of carbon by a flame to signal its presence. Burning hydrogen and air produces the flame, and the analyte containing carbon becomes ionized as it is burned. There is a circuit set up near the flame such that without ions present in the flame, there is very little current flow. In this way, as carbon-containing analytes go by and are ionized, the current in the circuit will increase and this signal is recorded by a computer or chart recorder. The record of the signal versus time is called a chromatogram and the peaks will indicate when carbon compounds came out on the GC (Skoog, Holler, & Crouch, 2007). Flame ionization detectors are highly sensitive with a wide dynamic range. The negative aspect of using the FID is that it is a sample destructive detector (Levine, 2006). The schematic diagram of an FID is shown in Figure 7.

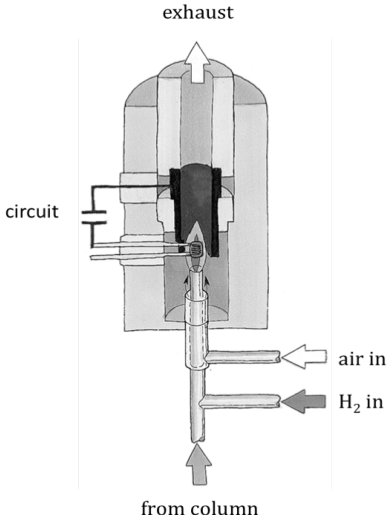

Figure 7. Diagram of Flame Ionization Detector (Linde Group, 2008)

Many articles have been published in the last twenty years detailing the use of HS-GC/FID for volatile chemical analysis (Barua et al., 2008; Zuba, Parczewski, & Reichenbacher, 2002; Streete, Ruprah, Ramsey, & Flanagan,1992). Moreover, HS-GC/FID has been used by other forensic laboratories in the United States for the quantitation of acetone in biological samples. The Utah Public Health Laboratories Bureau of Forensic Toxicology analyzes acetone as part of a volatile compound quantitation method mainly established for the analysis of ethanol (2008). The internal standard is 0.0001% (v/v) n-propanol in water, the sample size is 2 mL for either blood or urine, and samples are stored between 0 and 8 ºC prior to analysis. The limits of detection, quantitation, and linearity for this method are 2.0 mg/dL, 5.0 mg/dL, and 15.8 g/dL, respectively. At the Virginia Department of Forensic Sciences, HS-GC/FID is used to analyze ethanol and other volatiles, including acetone (2009). n-Propanol is used as the internal standard at a concentration of 0.03% (v/v) in water and at a volume of 0.45 mL. Sample volume for analysis is 0.05 mL blood.

Morris-Kukoski, Jagerdeo, Schaff, and LeBeau describe a volatile chemical quantitation method using a dual rail robotic autosampler (2007). For this method, one rail prepares samples while the other acts as the autosampler. The authors proposed the use of robotics as a way to streamline volatile analysis in forensic laboratories by reducing manual preparations. The limit of detection for this method is 5 mg/dL while the limit of quantitation is 17 mg/dL. However, this method considers acetone an interferent of ethanol analysis rather than an analyte of interest.

II. D. Analysis in blood

The body uses blood to transport drugs during their journey through the body. Testing for drugs in blood allows an analyst "to relate drug concentrations to pharmacological effects" (Levine, 2006). In other words, drug concentrations in the bloodstream may be used to interpret the degree of intoxication, either at the time the blood was drawn in nonfatal cases or at the time of death for fatal cases. Intoxication occurs when the level of drug exceeds either the normal level or the individual's tolerance, causing behavioral and/or physical impairment. Since acetone is highly water soluble, postmortem redistribution is possible from the liver to other areas of high water content, leading to high acetone concentrations in those areas (United States Public Health Service, 1994).

Over time, blood samples may become clotted or coagulated. Prior to sample preparation, clotted blood samples should be homogenized (Levine, 2006). Whenever testing blood samples there is a risk of exposure to disease such as HIV or Hepatitis B, and all samples should be treated as biohazards. In order to reduce the probability of exposure to blood-borne pathogens, the analyst should wear personal protective equipment including a lab coat, eye protection, and gloves.

CHAPTER III

METHODOLOGY

III. A. Headspace Gas Chromatography/Flame Ionization Detection

This work was carried out on a Perkin Elmer Clarus 500 Gas Chromatograph fitted with a flame ionization detector and a Perkin Elmer Turbomatrix 110 Automated Headspace Sampling Unit (Figure 8) (Perkin Elmer, Inc., Waltham, MA). High purity helium was the carrier gas while hydrogen was the fuel source for the detector. Helium was stored in a cylinder and introduced to the Gas Chromatograph via an inlet attached to the column head. Hydrogen was transferred to the FID from a Parker Balston H2-90 hydrogen generator (Parker Hannifin Corporation, Cleveland, OH).

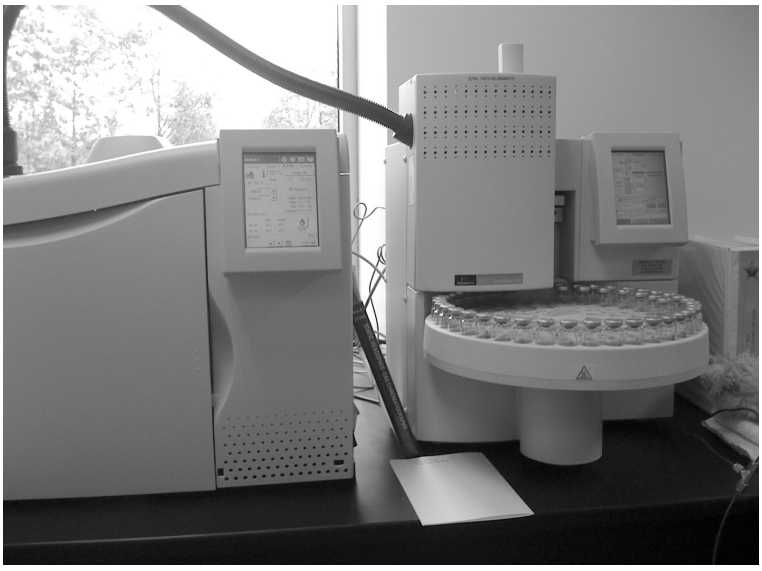

Figure 8. Perkin Elmer Clarus 500 GC with Turbomatrix Headspace Autosampler

III. A. i. Instrument conditions

Two columns, Restek Rtx-BAC1 (referred to as Channel A) and Rtx-BAC2 (Channel B), were used for the acetone-quantitation method (Restek Chromatography Products, Bellefonte, PA). According to Restek Corporation, "a dual-column configuration provides screening and confirmational data from the same injection" (1999). Both columns were fused silica wall-coated open tubular columns, 30 m in length, with an inner diameter of 0.32 mm, and a film thickness of 1.20 µm. The HS-GC/FID conditions were as follows (Oregon State Police Forensic Sciences Division, 2008):

- Headspace Conditions
  - Vial Oven Temperature: 70 °C
  - Needle Temperature: 100 °C
  - Transfer Line Temperature: 100 °C
  - Injection Time: 0.02 min
  - Pressurization Time: 1.0 min
  - Withdrawal Time: 0.2 min
  - Thermostating Time: 15.2 min
- GC Conditions
  - GC Cycle Time: 3.8 min
  - Oven Tempearture: 48 °C
  - Column Pressure: 20 psi
  - Carrier Gas Flow Rate: 20.0 mL/min

- FID Conditions
    - Detector Temperature: 220 °C
    - Gas Flow Rate: H_2 : 45mL/min
      Air : 450mL/min

III. A. ii. Techniques

III. A. ii. a. Sample preparation

Sample preparation was performed using a Hamilton ML 530 B AutoPipettor (Hamilton Company USA, Reno, NV) consisting of two syringes and two corresponding pipettes: one for internal standard and one for sample. The AutoPippettor was programmed to dispense 250 μL sample and 1500 μL internal-standard solution into 20 mL headspace vials.

When blood samples were clotted, it was necessary to manually pipette (using a positive-displacement pipettor) the samples into the headspace vials. After pipetting was complete, a rubber septum and a metal cap were then placed on the headspace-sample vial, the cap was crimped, and the sample was vortexed for roughly 10 seconds.

III. A. ii. b. Instrument operation

TurboMatrix Version 2.5.0.0125 was the software used to operate the headspace autosampler while TotalChrom (TC) Navigator Version 6.3.1 operated the GC (Perkin Elmer, Inc., Waltham, MA). The software was configured to automatically produce a five-point calibration curve based on the run sequence entered. The curve was generated

after manual verification of proper peak integration by the software for the samples and standards in each run.

III. B. Experiments

The blood-alcohol method in place at the Portland Metropolitan Laboratory served as a basis for determining where to start with the method development in terms of chromatography conditions, sample size and internal-standard concentration.

III. B. i. Development of standards and controls

Acetone standards from two different providers, Fisher Scientific (Thermo Fisher Scientific, Inc., Pittsburgh, PA) and Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO), were prepared at five concentrations: 1.0, 10.0, 50.0, 100.0, and 250.0 mg/dL. Three controls from each provider were also prepared at a low, medium, and high concentration: 3.0, 25.0, and 70.0 mg/dL. A certified reference from Cerilliant containing acetone at a concentration of 51.0 ± 0.21 mg/dL was included in several runs to demonstrate quantitative reliability in the method (Cerilliant Co., Round Rock, TX). A 0.25 mL aliquot of each standard and control from both providers was transferred in duplicate to a headspace vial. Data from the standards prepared from either provider were used to create calibration curves in order to determine control concentrations.

III. B. ii. Salting out effects

Several different salts were added to acetone samples at a constant concentration to see which salt, if any, increased sensitivity. Fifty milligrams of the following seven

salts was added to 0.25 mL of the 70.0 mg/dL acetone control from Fisher Scientific: sodium sulfate (Na_2SO_4), sodium acetate ($NaCH_3CO_2$), sodium fluoride (NaF), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), and calcium chloride ($CaCl_2$). The vials were capped immediately after adding the salt and were then vortexed for three seconds to dissolve the salt in the solution. The samples were run in triplicate and the standards were run in duplicate. Triplicate mixings of the 70.0 mg/dL acetone control not containing any salt were also included in the run.

To determine the optimal amount of salt, the following amounts of $MgCl_2$ were added to 0.25 mL of the 70.0 mg/dL acetone control from Fisher Scientic: 0.05, 0.10, 0.15, and 0.20 g. Samples and standards were run in duplicate. The batch also included duplicate mixings of a 70.0 mg/dL control without any salt.

III. B. iii. Internal standard concentration

Internal-standard solutions with 0.05, 0.04, and 0.03% (v/v) n-propanol in water were prepared. Three different sets of Fisher standards (one for each internal-standard concentration) were prepared and run in duplicate. The volume of internal standard was maintained at 1.5 mL.

III. B. iv. Sample size

For the blood-alcohol procedure, the Portland Lab uses a 0.25 mL sample size. The 0.25 mL sample size was compared to sample sizes of 0.50 mL and 0.75 mL. Fisher standards at each volume were prepared in duplicate. Fisher standards were also prepared in duplicate starting at a sample volume of 0.25 mL and decreasing by 0.05 mL

increments down to 0.05 mL. Duplicates of the 1.0 mg/dL control at each volume were also included in the run.

III. B. v. Limit of detection

For the limit of detection experiment, 12 water samples were spiked with internal standard solution and were subsequently run. The ratio of acetone to internal standard from each blank was determined and used to calculate the "acetone concentration" for each blank from the calibration curve. The mean and standard deviation were subsequently used to calculate the limit of detection.

III. B. vi. Linear dynamic range

In order to demonstrate linearity, acetone samples were prepared in duplicate and the data was used to create a calibration curve. Samples at the following Fisher-acetone concentrations were prepared: 0.01, 0.10, 1.0, 3.0, 10.0, 25.0, 70.0, 100.0, and 250.0 mg/dL.

III. B. vii. Carryover

In order to test the acetone method for carryover effects, five blank-blood samples were spiked with an extremely high acetone concentration (2500 mg/dL) and were run opposite five blank-blood samples. Blank blood was purchased from the Springfield Forensic Laboratory (Springfield, OR). Endogenous acetone was likely to be present in the blank blood, so a blank sample was run first, then a spiked sample, followed by a blank, then a spiked sample, and so on.

III. B. vii. a. Carryover statistical analysis

All statistical analyses were executed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA). At a threshold p-value, $p < 0.05$, the two measures being compared were found to be statistically different. One-way analysis of variance (ANOVA) was performed on the carryover data, followed by Tukey's multiple comparison posttest to establish whether or not the calculated acetone concentrations in the blank blood samples were statistically different.

III. B. viii. Stability study

To test the stability of samples containing acetone, three different case samples were tested several times over a two-month period. The following case samples were analyzed: 08M-1173, 08M-1179, and 08M-1182. The samples were first tested on January 23, 2009, hereafter referred to as time 0 days. Samples from each case were then stored at room temperature (23 ºC), in the refrigerator (4 ºC), and in the freezer (-11 ºC). The samples were tested again after 7, 28, and 56 days.

III. B. viii. a. Stability study statistical analysis

Two-way ANOVA was performed in order to compare the effect of both temperature and time on the stability of acetone-containing samples. After performing ANOVA, Bonferroni post tests were run to compare storage temperatures (i.e. freezer vs. refrigerator) as well as storage time (i.e. t=0 days vs. t=56 days) for each case.

III. B. ix. Method validation

To validate the method, three control concentrations were tested numerous times. The controls were made using Fisher acetone at the following concentrations: 3.0, 25.0, 75.0 mg/dL. Twelve runs were completed, each containing the following samples: the five standards, a negative sample, a certifiable control, and 33 of each the low, medium, and high controls. In order to test for interday and interindividual variations, two different analysts performed the runs (Susan L. Talbert and Michael D. Jackson) on separate days.

With the method developed, post-mortem case samples known to contain acetone were tested as part of the method validation. The following case samples were analyzed: 08M-256, 08M-271, 08M-587 08M-652, 08M-737, 08M-880, and 08M-903. The standards were run in singlet followed by the low, medium, and high controls, a certifiable Cerilliant control, a blank, a low control, the case samples in duplicate, and a medium control. The calculated acetone concentrations were compared to results from the external laboratory.

III. B. ix. a. Method validation statistical analysis

To calculate the interday variation, data for each control from all days (but excluding data from batches ran by Michael D. Jackson) was pooled. Column statistics were performed to determine the mean and standard deviation over all days. The percent variation for each control was then calculated using Equation 3.1.

$$\text{percent variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100 \qquad (3.1)$$

Percent variations from each control were averaged and reported as the final interday variation.

For the interindividual variation, the data for each control from both analysts was pooled and column statistics were performed to determine the mean and standard deviation at each control concentration. For each control, the percent variation was calculated then the average percent variation over all controls was determined. Since the analyses were performed on different days, the final interindividual variation was calculated by subtracting the interday variation from the averaged percent variation.

CHAPTER IV

RESULTS

IV. A. Selectivity

Selectivity was demonstrated using the compounds contained in the certifiable reference from Cerilliant. The elution profile from the Cerilliant volatile mixture is shown in Figure 9 while the calculated resolutions are given in Table 1.

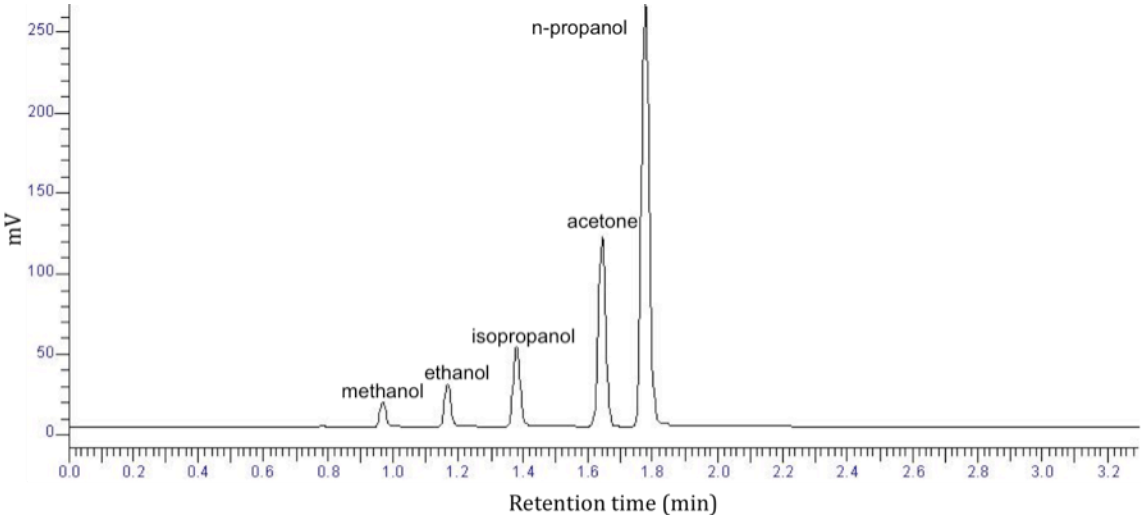

Figure 9. Elution profile from Cerilliant Volatile Mixture. The volatile mixture contained methanol, ethanol, isopropanol, and acetone at concentrations of 50.74 ± 0.39, 50.42 ± 0.31, 50.43 ± 0.16, and 51.04 ± 0.21 mg/dL, respectively. The internal standard, n-propanol, was included at 0.05% (v/v) in water.

Table 1. Component Resolution from Cerilliant Volatile Mixture

Compounds	Methanol - Ethanol	Ethanol - Isopropanol	Isopropanol - Acetone	Acetone - n-Propanol
Resolution	5.00	4.40	3.71	1.75

IV. B. Development of standards and controls

Acetone standards from both Fisher Scientific and Sigma-Aldrich were run on both the Rtx BAC-1 and BAC-2 columns (Channels A and B, respectively). After the data was processed, the acetone concentration was plotted versus the ratio of acetone peak area to n-propanol peak area to create calibration curves. Correlation coefficients for curves from both providers as well as both Channels were at or above 0.9998. Calibration curves from Channel A for Fisher Scientific and Sigma-Aldrich were combined and are shown in Figure 10.

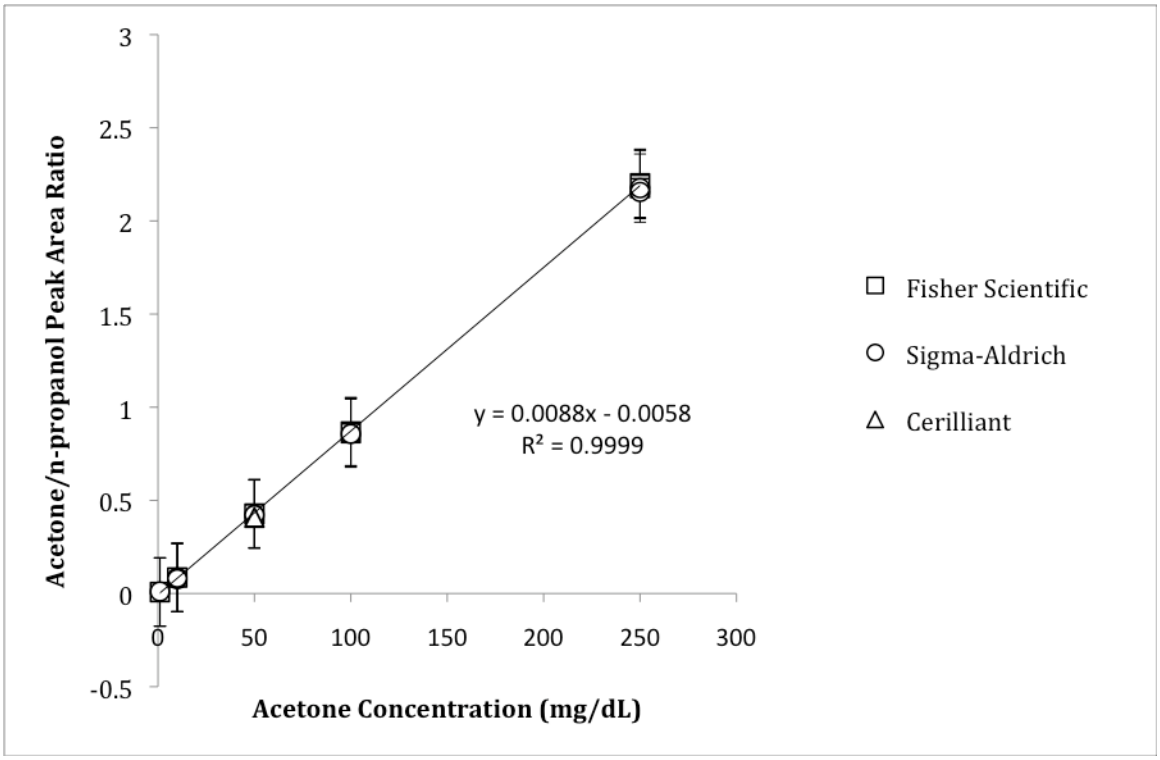

Figure 10. Standard Calibration Curve (n=2). The acetone/n-propanol peak area ratio was plotted versus the acetone concentration and the best-fit line yielded a correlation coefficient of 0.9999.

The best-fit line equation from Figure 7 was used to calculate acetone control concentrations from Fisher as well as a certifiable control from Cerilliant, as shown in Table 2.

Table 2. Acetone Control Concentrations (n=2)

Sample Name	Average Acetone/n-propanol Area Ratio	Average Calculated Acetone Concentration (mg/dL)	Percent Error
3.0 Control	0.025	3.51	15.8
25.0 Control	0.205	24.0	4.06
70.0 Control	0.573	65.9	6.10
51.0 Cerilliant Control	0.408	47.1	8.04

IV. C. Salting out effects

The effect of each salt on the analyte/internal standard peak area ratio, subsequently used to determine the acetone concentration, is shown in Figure 11.

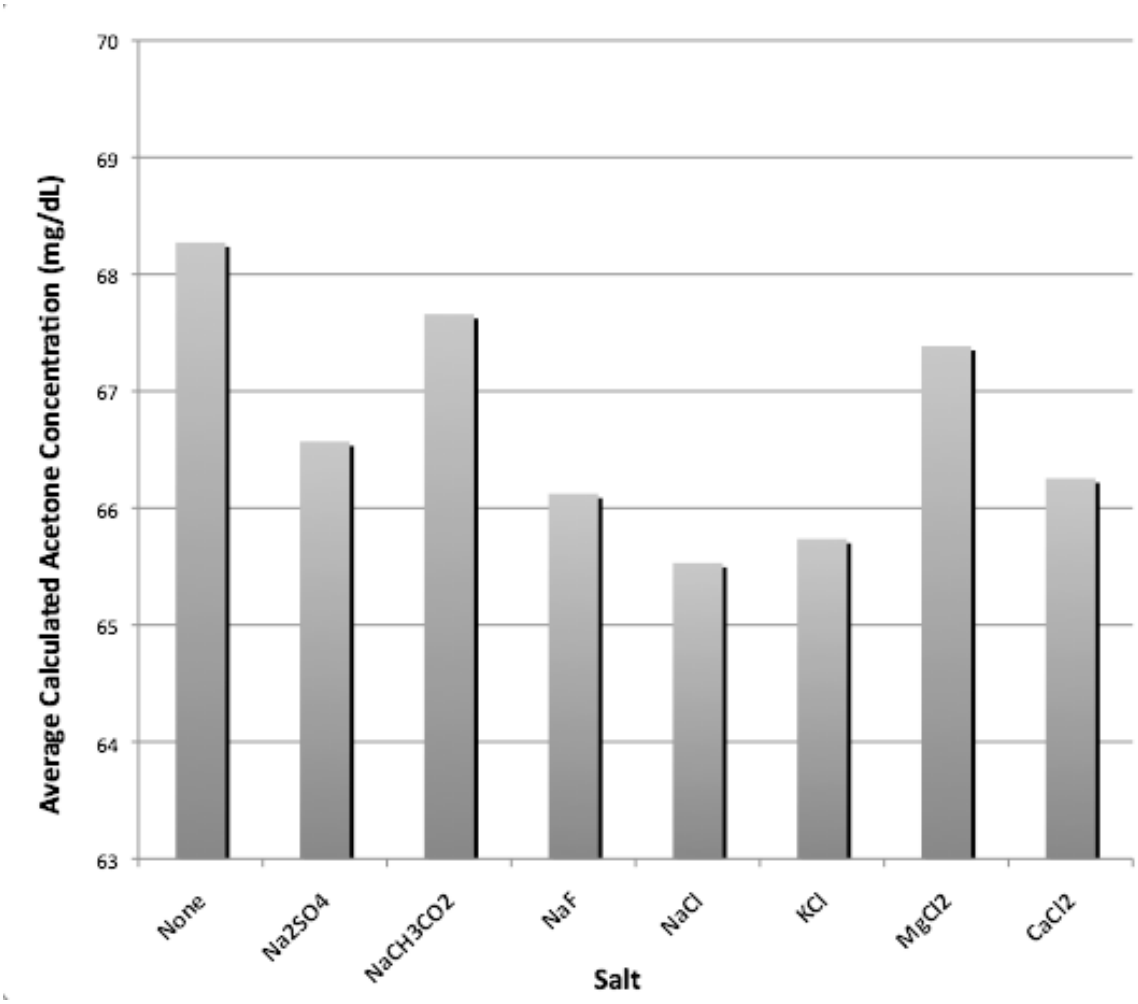

Figure 11. Effect of Salt on Calculated Acetone Control Concentration (n=3). For the salt study, 0.05 g of each salt was added to 0.25 mL of the 70 mg/dL acetone control from Fisher Scientific.

The addition of each of the salts slightly decreased the detector response (Figure 11). Lower ratios of acetone peak area to n-propanol peak area led to an apparent decrease in calculated acetone concentrations as shown in Table 3.

Table 3. Effect of Salt on Calculated Acetone Control Concentration (n=3)

Sample Name	Average Acetone/n-propanol Area Ratio	Average Calculated Acetone Concentration (mg/dL)	Percent Error
70 mg/dL Control	0.554	68.3	2.50
70 mg/dL Control + 0.05 g Na_2SO_4	0.540	66.6	5.02
70 mg/dL Control + 0.05 g $NaCH_3CO_2$	0.549	67.7	3.40
70 mg/dL Control + 0.05 g NaF	0.536	66.1	5.70
70 mg/dL Control + 0.05 g NaCl	0.532	65.8	6.13
70 mg/dL Control + 0.05 g KCl	0.533	65.7	6.28
70 mg/dL Control + 0.05 g $MgCl_2$	0.547	67.4	3.81
70 mg/dL Control + 0.05 g $CaCl_2$	0.537	66.3	5.50

Magnesium chloride was chosen at random to demonstrate the effect that varying the amount of salt has on calculated acetone concentrations, as shown in Figure 12.

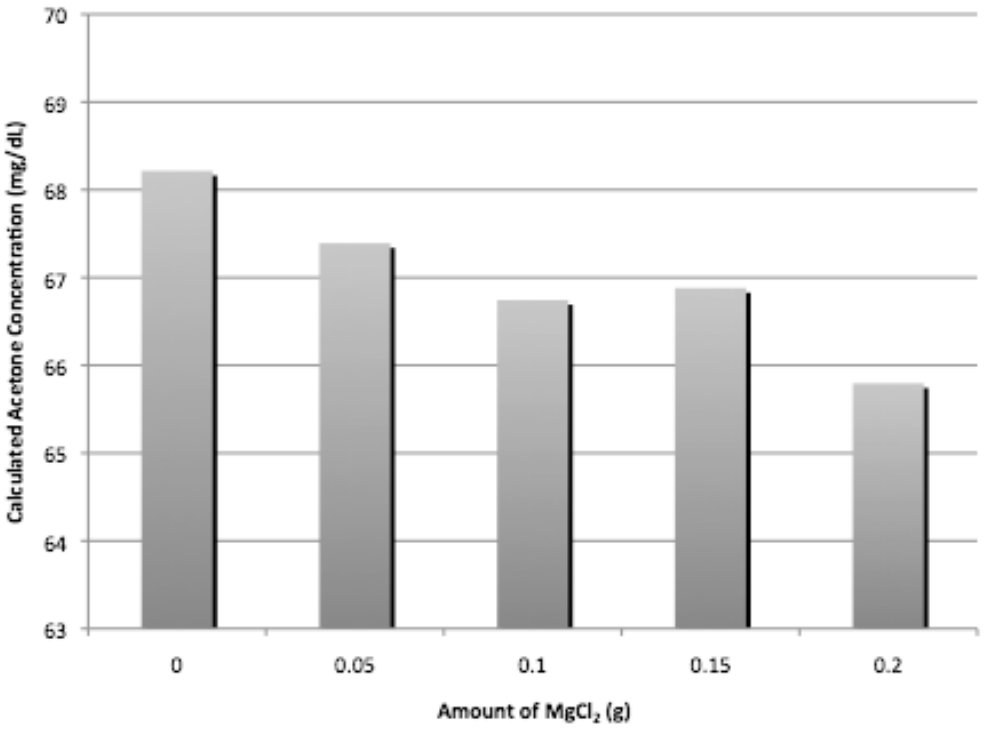

Figure 12. Effect of $MgCl_2$ on Calculated Acetone Control Concentration (n=2). A range of $MgCl_2$ from 0.05 to 0.20 g was added to 0.25 mL of the 70 mg/dL acetone control from Fisher Scientific.

Table 4. Effect of $MgCl_2$ on Calculated Acetone Control Concentration (n=2)

Sample	Acetone Area	n-Propanol Area	Average Calculated Acetone Concentration (mg/dL)
70.0 mg/dL Control	273083	493504	68.2
70.0 mg/dL Control + 0.05 g $MgCl_2$	275247	503551	67.4
70.0 mg/dL Control + 0.10 g $MgCl_2$	284125	524887	66.7
70.0 mg/dL Control + 0.15 g $MgCl_2$	277782	512104	66.9
70.0 mg/dL Control + 0.20 g $MgCl_2$	292469	548161	65.8

As demonstrated in Figure 12, an increased salt amount resulted in an apparent decreased calculated acetone concentration. Table 4 shows that as the salt amount increased, so did the n-propanol area, though the acetone peak area remained relatively constant.

IV. D. Internal standard concentration

Internal-standard solutions were prepared at 0.05, 0.04, and 0.03% (v/v) n-propanol in water. Figures 13a and 13b show chromatograms at the same concentration for the greatest and least concentrated internal standard solutions, respectively.

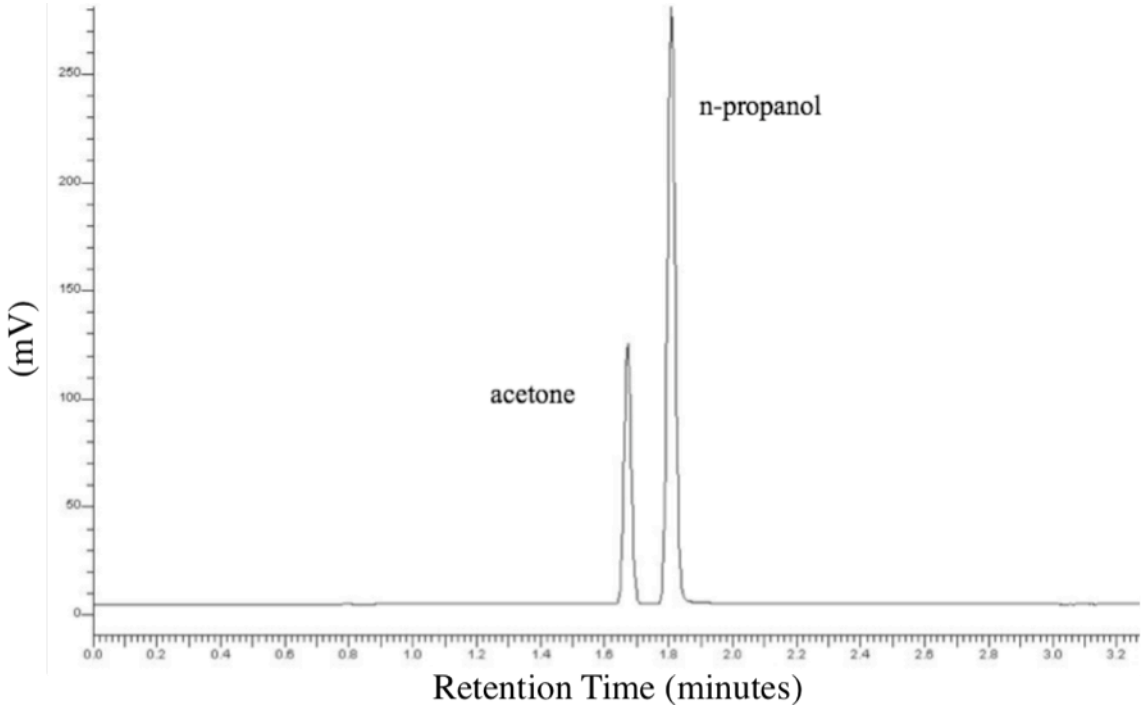

Figure 13a. Chromatogram 50 mg/dL Acetone Standard with 0.05% (v/v) Internal Standard. The chromatogram illustrates the relationship between the acetone and n-propanol peaks for the midrange calibrator at an internal standard concentration of 0.05% n-propanol in water.

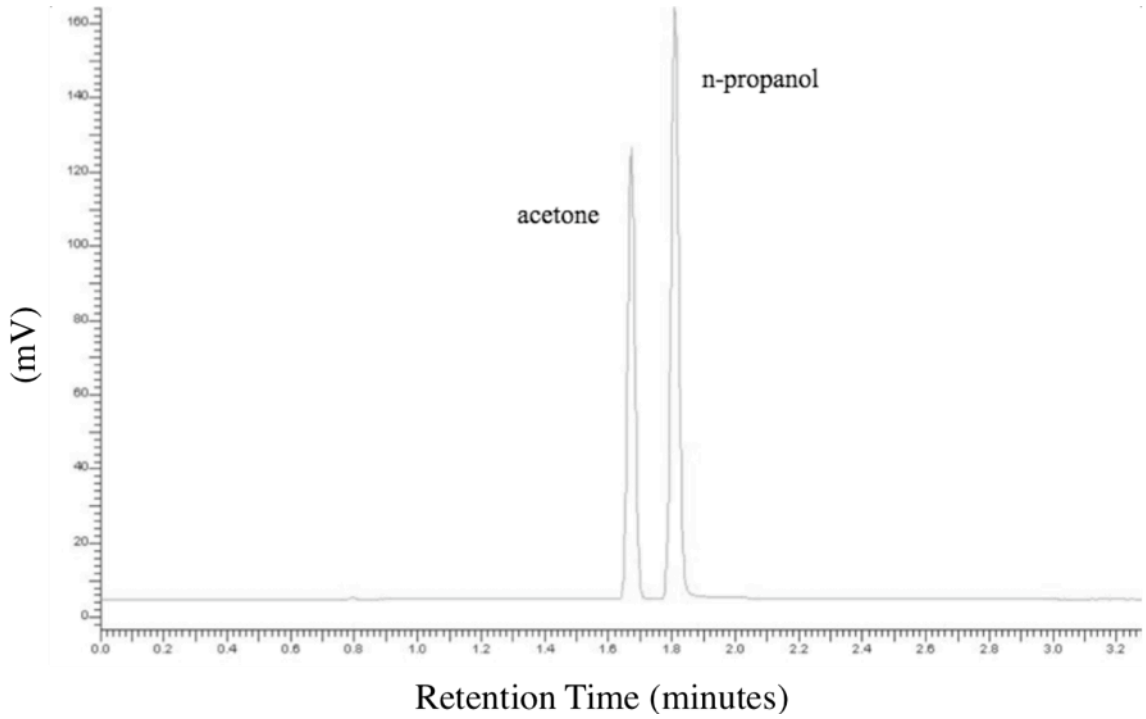

Figure 13b. Chromatogram 50 mg/dL Acetone Standard with 0.03% (v/v) Internal Standard. The chromatogram shows the relationship between the acetone and n-propanol peaks for the midrange calibrator at a decreased internal standard concentration, 0.03% n-propanol in water.

In Figures 13a and 13b, the peaks with retention times around 1.67 minutes represent acetone while the peaks with retention times around 1.81 minutes represent n-propanol. The peak for the internal standard in Figure 13a has a height roughly 100 units higher than the internal standard peak in Figure 13b. The acetone peak has a height around 130 units in either figure.

Three separate calibration curves were created, one for each internal standard concentration. The acetone concentrations were calculated from the corresponding calibration curve best-fit line and are shown in Table 5.

Table 5. Effect of IS Concentration on Calculated Acetone Concentration (n=2)

Sample	Acetone Area	n-Propanol Area	Acetone/n-Propanol Area Ratio	Calculated Acetone Concentration (mg/dL)
50.0 Calibrator with 0.05% IS	192264	474703	0.405	49.5
50.0 Calibrator with 0.04% IS	193664	375798	0.515	49.2
50.0 Calibrator with 0.03% IS	193987	274084	0.708	49.4

IV. E. Sample size

The effect of sample size on accuracy of the method was assessed. Volumes of 0.75, 0.50, 0.20, 0.25, 0.15, 0.10, and 0.05 mL containing the standard concentrations of acetone (i.e. 1.0, 10.0, 50.0, 100.0, and 250.0 mg/dL) were included in the assay. Figure 14 demonstrates the effect of a large sample size on the chromatogram, when the detector has been saturated.

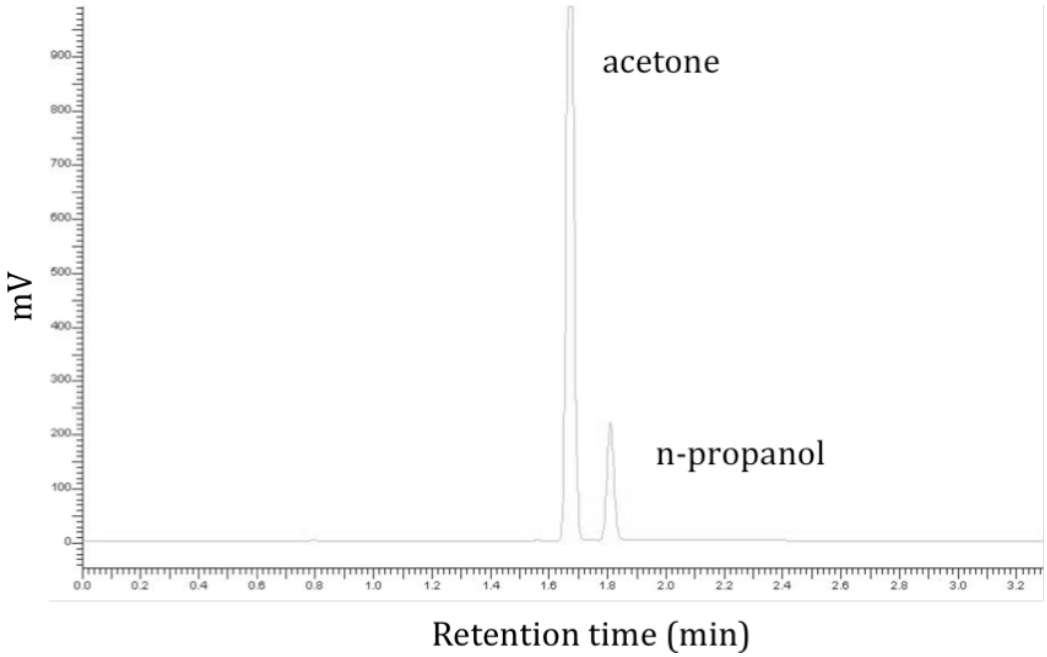

Figure 14. Chromatogram 250 mg/dL Acetone, 0.75 mL Sample. The chromatogram shows the effect of detector saturation as a result of an increased sample size.

Table 6 illustrates the effect of sample size on the acetone/n-propanol peak area ratio.

Table 6. Effect of Sample Size on Peak Area Ratio

Sample Volume of 250.0 mg/dL Calibrator (mL)	Average Acetone/n-Propanol Area Ratio
0.75	5.41
0.50	4.11
0.25	2.08
0.20	2.96
0.15	2.59
0.10	2.10
0.05	0.66

Table 6 demonstrates that, for the most part, as sample size increases so does the analyte/internal standard peak area ratio since the internal standard amounts stays constant. Linearity of data may also be considered when determining an appropriate sample size. Separate calibration curves were created for each sample volume using standards run in duplicate. The effect of sample size on the correlation coefficient from the corresponding calibration curve is shown in Table 7.

Table 7. Effect of Sample Size on Acetone Calibration Curve Data

Sample Volume (mL)	Correlation Coefficient
0.75	0.9956
0.50	0.9997
0.25	0.9999
0.20	0.9981
0.15	0.9995
0.10	0.9991
0.05	0.9986

From Table 7, there is no apparent trend in correlation coefficients as compared to sample volume.

IV. F. Limit of detection

Twelve blank water samples were injected with 1.5 mL of 0.05% (v/v) n-propanol in water internal standard solution. The data and results are presented in Table 8.

Table 8. Limit of Detection Calculations for Blank Water Samples (n=12)

Acetone Peak Area	**n-Propanol Peak Area**	**Acetone/n-Propanol Peak Area Ratio**	**Calculated Acetone Concentration (mg/dL)**
585.8	474140	0.0012	0.117
556.9	493140	0.0011	0.104
660.3	474679	0.0014	0.137
475.7	460317	0.0010	0.092
367.2	449946	0.0008	0.064
751.4	475402	0.0016	0.161
856.0	461833	0.0019	0.195
339.8	491240	0.0007	0.049
209.5	512111	0.0004	0.013
629.3	491531	0.0013	0.123
1038.4	489477	0.0021	0.229
1031.3	478854	0.0022	0.233
Mean (mg/dL)	0.126		
Std. Dev. (mg/dL)	0.069		

From Table 8, the mean acetone concentration and the average standard deviation of the blank samples were calculated to be 0.126 ± 0.069 mg/dL. The limit of detection was calculated using the equation LOD = X + 3·SD where LOD stands for limit of detection, X for mean concentration of the blanks, and SD for standard deviation of the blanks. From this equation, the limit of detection was determined to be 0.333 mg/dL. In a similar fashion, the limit of quantitation was calculated using the equation LOQ = X + 10·SD, yielding a value of 0.816 mg/dL.

IV. G. Linear dynamic range

The linear dynamic range can be determined by calculating the limits of quantitation and linearity. The theoretical limit of quantitation was previously calculated

as 0.816 mg/dL. No experimentation was performed to calculate the limit of linearity. However, an administrative linear dynamic range can be established by taking into consideration known normal and toxic blood-acetone concentrations (1 and 33 mg/dL, respectively), acetone levels for persons experiencing ketoacidosis (10-70 mg/dL), and the determined acetone concentrations from previously analyzed cases (generally less than 30 mg/dL, one case at 230 mg/dL). For methods created with several target analytes, the normal and toxic concentrations of each analyte must be considered before an administrative linear dynamic range can be set. Figure 15 shows a calibration curve established using an administrative linear dynamic range up to 250.0 mg/dL. The corresponding data is given in Table 9.

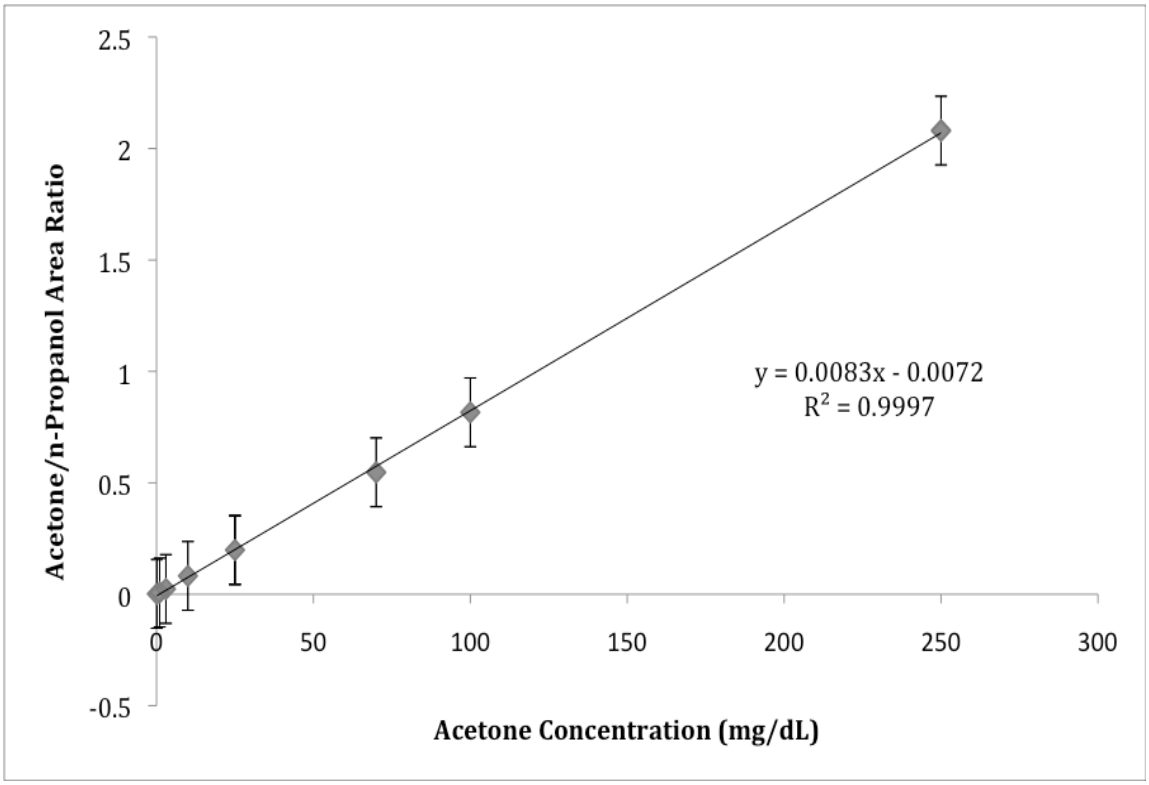

Figure 15. Linear Dynamic Range Curve (n=2). The acetone/n-propanol peak area ratio was plotted versus the acetone concentration and the best-fit line yielded a correlation coefficient of 0.9997.

Table 9. Linear Dynamic Range Data (n=2)

Sample	Average Calculated Acetone Concentration (mg/dL)	Percent Error
0.01 Calibrator	ND (not detected)	ND
0.10 Calibrator	0.201	101
1.0 Calibrator	0.950	5.02
3.0 Calibrator	2.96	1.27
10.0 Calibrator	11.7	17.3
25.0 Calibrator	25.3	1.20
70.0 Calibrator	67.3	3.80
100.0 Calibrator	99.5	0.467
250.0 Calibrator	251	0.321

IV. H. Carryover

Data and results from the carryover study are presented in Table 10.

Table 10. Carryover Assessment

Sample	Acetone Area	Calculated Acetone Concentration (mg/dL)
Blank	1761	1.01
2500 mg/dL	2668834	---
Blank	1726	1.01
2500 mg/dL	2670226	---
Blank	1638	0.993
2500 mg/dL	2681112	---
Blank	1715	1.01
2500 mg/dL	2687271	---
Blank	1724	1.01
2500 mg/dL	2683448	----

In Table 10, the spiked samples severely saturated the detector, making acetone-concentration calculations impossible. The calculated acetone concentration of the blank samples had a mean value of 1.01 ± 0.01 mg/dL.

IV. I. Stability study

Prior to starting the stability studies, all samples had been stored in the refrigerator. Results from the first analysis are provided in Table 11.

Table 11. Stability Study Data Time = 0 days

Case #	Average Calculated Acetone Concentration (mg/dL)	Standard Deviation	Percent Relative Standard Deviation
1179	24.15	0.596	2.47
1173	11.85	0.040	0.34
1182	44.33	0.685	1.54

The external laboratory reported the following acetone concentrations for cases 1179, 1173, and 1182, respectively: 23, 12, and 36 mg/dL. An unpaired t-test between the external laboratory reported concentrations versus concentrations calculated in this assay was performed using GraphPad Prism software, and the concentrations were not found to be statistically different.

After the initial analysis, separate 3 mL aliquots of each sample were stored at room temperature (23 °C), in the refrigerator (4 °C), and in the freezer (-11 °C). The samples were analyzed again after 7, 28, and 56 days. Results are summarized in Tables 12 through 14, where average calculated acetone concentration (mg/dL) was abbreviated as Conc, standard deviation as SD, and percent relative standard deviation as RSD.

Table 12. Stability Study Data Time = 7 days

	Case #1179			Case #1173			Case #1182		
Storage Location	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**
Freezer	25.56	1.08	4.24	12.17	0.022	0.177	42.31	0.785	1.86
Refrigerator	25.20	1.37	5.44	12.25	0.371	3.03	41.77	0.128	0.307
Room Temperature	25.10	1.04	4.16	10.56	2.05	25.1	43.55	0.676	1.55

Table 13. Stability Study Data Time = 28 days

	Case #1179			Case #1173			Case #1182		
Storage Location	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**
Freezer	26.35	0.496	1.88	13.23	0.454	3.43	42.88	0.933	2.18
Refrigerator	24.90	0.533	2.14	12.00	0.878	7.32	45.35	0.902	1.99
Room Temperature	26.05	0.801	3.08	9.55	0.015	0.160	43.72	1.14	2.61

Table 14. Stability Study Data Time = 56 days

	Case #1179			Case #1173			Case #1182		
Storage Location	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**	**Conc**	**SD**	**RSD**
Freezer	26.56	1.01	3.82	12.85	0.259	2.01	43.31	2.20	4.76
Refrigerator	25.47	0.886	3.48	13.21	0.587	4.45	44.38	0.843	1.90
Room Temperature	31.05	0.788	2.54	5.68	0.727	12.8	35.52	3.95	11.1

Data from Tables 11 through 14 were analyzed using GraphPad Prism software. From two-way ANOVA, temperature was found to account for 0.22% of the variation and time was found to account for 97.7% of the variation, though the relative standard deviation was quite small.

IV. J. Method validation

For the method validation, interday and interindividual variation was calculated. Table 15 presents the statistical results from the validation study.

Table 15. Statistics for Validation Study (n=33)

Batch Number	**Control Concentration (mg/dL)**		
	3.0	25.0	75.0
	Mean Concentration ± Standard Deviation (mg/dL)		
20090107	3.29 ± 0.01	25.1 ± 0.07	75.0 ± 0.30
20090108	2.93 ± 0.01	24.7 ± 0.07	74.5 ± 0.28
20090121	3.07 ± 0.01	24.9 ± 0.09	75.0 ± 0.28
20090209	3.06 ± 0.02	26.3 ± 0.10	79.8 ± 0.37
20090213	3.49 ± 0.01	24.4 ± 0.14	73.1 ± 0.51
20090302	4.40 ± 0.04	25.0± 0.17	----
20090305	2.72 ± 0.01	24.5 ± 0.08	75.1 ± 0.25
20090309	4.45 ± 0.03	24.9 ± 0.15	----
20090312	2.89 ± 0.01	24.4 ± 0.07	74.5 ± 0.28
20090316	4.02 ± 0.03	24.9 ± 0.17	----
20090317	2.88 ± 0.02	24.2 ± 0.08	74.1 ± 0.28
20090319	5.16 ± 0.03	25.2 ± 0.11	74.8 ± 0.42

In Table 15, Michael D. Jackson ran the following batches: 20090107, 20090108, 20090121, 20090305, 20090317, and 20090319. Susan L. Talbert ran the remaining batches. Results from batches ran by Susan L. Talbert were pooled for each control

concentration. Percent variation for each control was then calculated by dividing the standard deviation by the mean and multiplying by 100. The resultant percent variations were averaged to determine the interday variation. The final percent interday variation, or variation between days, was calculated as 6.93 percent.

Separate statistics for each experimenter were performed on the calculated acetone-control concentrations and the results are summarized in Table 16.

Table 16. Statistics for Interindividual Variation from Validation Study

Control Concentration (mg/dL)	Mean Concentration ± Standard Deviation (mg/dL)		
	Michael D. Jackson	**Susan L. Talbert**	**Pooled**
3.0	3.27 ± 0.79	3.81 ± 0.74	3.51 ± 0.74
25.0	24.7 ± 0.33	25.1 ± 0.66	24.9 ± 0.54
75.0	74.7 ± 0.45	76.5 ± 3.4	75.1 ± 1.79

The pooled mean concentrations and standard deviations in Table 16 were used to determine the corresponding percent variation for each control. Since the analyses were performed on separate days, the interday variation from either user was averaged, and then subtracted from this pooled interday and interindividual variation calculation, yielding an interindividual percent variation of 0.77%.

Post-mortem case samples known to contain acetone were also tested as part of the method validation. The following case samples were analyzed: 08M-256, 08M-271, 08M-587 08M-652, 08M-737, 08M-880, and 08M-903. Results are provided in Table 17.

Table 17. Postmortem Sample Analysis (n=2)

Case #	External Laboratory Reported Acetone Concentration (mg/dL)	Avg. Calculated Acetone Concentration ± Standard Deviation (mg/dL)
903	37	54.7 ± 0.28
880	6.8	8.31 ± 0.04
652	3.6	2.78 ± 0.03
737	230	400 ± 0.59
256	12	14.3 ± 0.04
587	0	0 ± 0.01
271	5.6	4.92 ± 1.3

For case 587, a calculated acetone concentration of 0.0099 mg/dL was obtained. Since the linear dynamic range has an administrative limit of quantitation at 1.0 mg/dL, the value was reported as 0 mg/dL. An unpaired t-test was performed using GraphPad Prism software between the external laboratory's reported acetone concentration and those calculated in our assay for each case. The concentration means were not found to be significantly different.

CHAPTER V

DISCUSSION

V. A. Method development

In terms of gas chromatography, resolution may be defined as the complete separation of components as demonstrated by the chromatogram. Resolution may be calculated using the retention times of the compounds as well as the width of the peak for each compound, as displayed in equation 2.1. A calculated resolution at or above 1.5 indicates that the compounds have been completely resolved while a resolution at 1.0 signifies a 10% overlap between peaks "a" and "b" (Levine, 2006). From Table 1, all compounds were shown to be fully separate from the closest neighboring compound, thereby proving this method to be selective.

Standard concentrations were selected based on previous case samples reviewed by the Portland lab. The concentrations of the controls were between the lowest and highest concentrations of the standards, but were not equivalent to any of the standard concentrations. From Figure 10, the calibrators from both Fisher Scientific and Sigma-Aldrich were statistically similar. Therefore, only standards and controls prepared using acetone from Fisher Scientific were used in subsequent experiments. The use of two columns served as a confirmatory tool in terms of identification via retention time.

Channel B was the confirmatory column, so only data from Channel A was analyzed in the remaining experiments.

The use of peak height as compared to peak area for developing the ratio of acetone to n-propanol was investigated. Levine states that the use of peak height ratios is only necessary "for the quantitation of a small peak in the presence of a larger, closely eluting peak" (2006). As demonstrated by the calculated resolution presented in Table 1, acetone and n-propanol were not shown to overlap but were cleanly separated using this method. Moreover, no significant difference in accuracy of calculated acetone concentration was noted and peak area ratios were used throughout the entire study.

Accuracy of the calculated control concentrations was demonstrated via percent error calculations. Table 2 shows that the percent error between the calculated acetone concentration and the expected concentration was less than 20% for all controls. This value is a general measure of quality control for quantitation in the field of forensic science (Levine, 2006).

Volatile chemicals often have a high vapor pressure, meaning that while they exist as a liquid at room temperature they evaporate rapidly in the open air and will form a concentrated headspace in a sealed container. The addition of salts to volatile chemical solutions has been found to increase the vapor pressure of some chemicals and thereby increase the sensitivity in detection during headspace analysis.

From the salt study (section IV. C.), the addition of salt was found to be unnecessary for acetone-containing samples. Salts decreased the accuracy in calculated acetone concentrations because they exhibited a greater effect on n-propanol than acetone. According to Kolb and Ettre, polar compounds are more affected by salts than

nonpolar compounds (2006). Acetone and n-propanol are both polar, though acetone is slightly more polar than n-propanol, with polarity indices of 5.1 and 4.0, respectively. In terms of the extent to which compounds of varying degrees of polarity are affected by salts, the authors simply state, "The effect is not the same for every analyte" (Kolb & Ettre, 2006). For this study, salts appear to have a greater effect on n-propanol than on acetone. The addition of salt to acetone-containing samples was deemed unnecessary, as it did not result in greater sensitivity in detecting acetone.

Internal standard concentration was not found to affect calculated acetone concentrations. Decreasing the internal standard concentration led to an increase in the ratio of analyte to internal standard. That is, the analyte peak appeared larger than the internal standard peak as the internal standard concentration decreased. An optimal internal standard concentration should be small enough to allow peak detection at low concentrations and concurrently high enough to provide a comparable internal standard peak intensity to the acetone peak at high concentrations. For the 0.05 percent internal standard concentration, the acetone peak is clearly discernable at an acetone concentration of 1.0 mg/dL and is only twice as large as the n-propanol peak at an acetone concentration of 250.0 mg/dL. Therefore, the internal solution concentration was maintained at 0.05 percent n-propanol in water.

Sample size was increased to see if a larger volume resulted in better detection of acetone at low concentrations, then decreased to determine the lowest volume that still produced sufficient detection of low acetone levels. Sample volume was not found to have a great effect on calculated acetone concentrations, indicating that as long as enough total acetone is present in a sample, the calculated concentrations will be accurate.

The truncated acetone peak in Figure 14 illustrates that, as a result of the increased total acetone in the larger sample, a large sample size saturates the detector. Furthermore, the acetone peak is more than five times the size of the internal standard peak. The analyte peak should not completely overwhelm the internal standard peak, and therefore a smaller analyte/internal standard ratio is desirable.

At the same time, a low sample size resulted in decreased peak areas, which made it difficult to detect the acetone peak at low acetone concentrations. The acetone peak at 1.0 mg/dL is easily detectable at a sample volume of 0.25 mL and the acetone peak does not overwhelm the internal standard peak at the highest acetone standard concentration, as seen in Table 6. In terms of calibration curve linearity, all of the tested sample volumes do meet the Portland Metropolitan Forensic Laboratory's lower correlation coefficient limit of 0.99. However, the calibration curve created using standards with a sample volume of 0.25 mL had the correlation coefficient closest to 1.0. Therefore, of the sample sizes considered, 0.25 mL is the optimal sample size. Since quantitation samples are generally run in duplicate, only a total sample volume of 0.50 mL is required for this acetone quantitation method.

The instrument's limit of detection was calculated to be 0.333 mg/dL. The external laboratory reports a limit of detection for acetone of 1 mg/dL. Though the instrument may detect acetone as low as 0.333 mg/dL, quantitations should not be reported at concentrations below the limit of quantitation. The limit of quantitation for this method represents the lowest acetone concentration that can be accurately and precisely measured (Levine, 2006). The limit of quantitation was calculated to be 0.816 mg/dL.

Percent error calculations were performed on the calculated acetone concentrations in the dynamic range study. A small percent error signifies that the calculated acetone concentration is close to the expected acetone concentration. Percent error of less than 20 percent was chosen as the acceptance criteria. This value serves as a general measure in evaluating quality control for quantitation in the field of forensic science (Levine, 2006). Because accuracy was not within 20 percent at acetone concentrations below 1mg/dL and samples are rarely presented at concentrations greater than 250.0 mg/dL, the linear dynamic range was administratively set from 1.0 to 250.0 mg/dL. If a sample has a calculated acetone concentration below 1.0 mg/dL, it should be reported as 0 mg/dL, or simply as negative for acetone unless sample size is modified such that the quantitative ratio falls between the cutoff and high calibrator. Samples with acetone concentrations above 250.0 mg/dL may be extrapolated from the calibration curve. However, many laboratories require that samples determined to have concentrations above the highest tested standard be diluted and reanalyzed.

No carryover was found to exist between substantially spiked and blank blood samples. Since the normal level of acetone in the blood is around 1 mg/dL (Ashley et al., 1994), it can be assumed that the acetone present in the blank blood samples was endogenous and was not a result of carryover from the spiked samples. Moreover, the samples were run in the sequence shown in Table 10. A blank sample was run first and the standard deviation was only 0.007 mg/dL. From one-way ANOVA, the mean concentrations from the ten blank blood samples were not found to be significantly different. Tukey's multiple comparison post-test confirmed that none of the calculated acetone concentrations from the blank blood samples were statistically different at 95%

confidence. Furthermore, the headspace autosampler heated samples to 200 °C before analysis so any variability in room temperature can be negated.

For the stability study, all samples were stored in microcentrifuge tubes. Two microcentrifuge tubes were used for each case at each temperature with a total volume of about 3 mL. All of the samples had some degree of coagulation, or clotting. Therefore, the AutoPipettor could not be utilized when preparing samples for analysis. Instead, the samples had to be manually pipetted. In order to compare storage temperature to time for each case sample, two-way ANOVA of the data was performed using GraphPad Prism software. Temperature was only found to account for 0.22 percent of variation (not significant) while time accounted for 97.7 percent variation (extremely significant). From Bonferroni post-tests, only case 1182 at time 56 days had mean concentrations that were significantly different (for both the freezer vs. room and refrigerator vs. room tests).

Though GraphPad Prism software determined that time but not temperature plays a significant role in acetone concentration variation, no overall trend was apparent in the data. The calculated acetone concentration increased for some cases but decreased for others, even at the same storage temperature. An increase in calculated acetone concentrations could be due to microorganism activity. Acetone is one of many metabolic byproducts produced by microorganism activity in postmortem blood samples, though the main product is ethanol. Glucose, fatty acids, and amino acids may all be converted to acetone by microorganisms (Levine, 2006). Numerous species of microbes are capable of producing volatiles, but the yeast *Candida albicans* has been identified as one of the highest contributors. Adding a preservative to postmortem samples may prevent microorganism growth and thereby reduce the amount of volatiles produced

postmortem (Lewis, Johnson, Angier, & Vu, 2004). The Portland Metropolitan Lab has attempted to decrease or prevent postmortem volatile production by adding 0.5% sodium fluoride to blood samples.

A decrease in calculated acetone concentrations may be due to simple evaporation. With numerous analyses, the sample in the storage container is depleted, and a larger space between the top of the sample and the lid is created. Since acetone is a volatile chemical, it has the tendency to diffuse out of the sample into the air space. Loss of acetone can subsequently occur when the storage container is opened to aliquot the sample for analysis.

The calculated acetone concentration from each case at time 0 was compared to the reported concentrations from the external laboratory. The concentrations were not found to be significantly different. For each case, the blood collection site given on the sample vials at the Portland Metropolitan Forensic Laboratory was the same as that provided on the external laboratory case report. Moreover, all samples (from both the stability study and the postmortem study) analyzed by the external lab and those present at the lab were stored in gray-stopper vials. A summary of the collection information for the stability study blood samples is given in Table 18.

Table 18. Stability Study Blood Sample Collection Information

Case #	Origin	Collection Date
1173	Femoral	11/28/08
1179	Femoral	Not given
1182	Femoral	12/02/08

V. B. Method validation

From the validation experiment, interday variation was found to be 6.93% while interindividual variation was found to be 0.77%. Both types of variation serve as a measure of precision. Before averaging the percent variations from each control concentration for both variation studies, the low control at 3.0 mg/dL exhibited the greatest percent variation. Therefore, precision is greater at high acetone concentrations than it is at low acetone concentrations.

In order to calculate the interindividual variation, the interday variation had to be subtracted from a preliminary interindividual variation because the analyses were performed on different days. For that reason, when two different operators perform analyses on different days, the variation in calculated acetone concentrations would be greater than if the same operator performed analyses on different days or if two operators performed analyses on the same day. Though both experimenters follow the same general procedure for sample preparation, different people do things in a slightly different fashion. A greater degree of variation should therefore be expected between individuals as compared to the same individual running the same experiment on different days.

The pooled mean and standard deviation for each control from either analyst was compared to further evaluate accuracy and precision between individuals. The average calculated means for the low and high controls from the batches ran by Michael D. Jackson were slightly more accurate than those ran by Susan L. Talbert. The standard deviations for either operator at the low control concentration were more consistent than the standard deviations at the high control concentration.

Acetone is normally present in the blood at a concentration around 1 mg/dL (Ashley, 1994) while concentrations above 33 mg/dL are toxic (Burns et al., 1998). Of the postmortem cases analyzed, all calculated acetone concentrations were above the normal level. Only cases 903 and 737 had concentrations above the toxic level. The calculated acetone concentration for case 737 was 400 mg/dL; more than ten times the toxic level.

In controlled diabetics, acetone concentrations in blood are usually under 3 mg/dL (Jones, 1993) and the calculated acetone concentration for case 652 was 2.57 mg/dL. People on ketogenic diets or experiencing ketoacidosis may have acetone blood concentrations ranging from 10 to 70 mg/dL (Baselt, 2000). The blood sample from case 903, with a calculated acetone concentration of 53.7 mg/dL, could have been drawn from an individual on a ketogenic diet or in diabetic ketoacidosis. The remaining two cases, 880 and 271, had only slightly elevated levels of acetone, which can be caused by diet and/or exercise.

The acetone quantitation method developed during this study was similar to the method used by the external lab, which was GC/FID with n-propanol as the internal standard. However, the external lab calculated acetone concentrations using a historic curve rather than running standards with every batch. Historic calibration curves are effective as long as positive and negative control samples are included with each batch to ensure that the "calibration has not changed between batches" (Levine, 2006). The external lab ran samples in singlet, so no mean or standard deviation data was available (personal communication, April 21, 2009). This led to a decrease in statistical power

when performing comparisons between acetone concentrations calculated using the developed method and those reported by the external lab.

Both a t-test and an F-test were performed on the postmortem sample data using GraphPad Prism software. Neither the mean nor variance from the calculated acetone concentrations was statistically different from values reported by the external lab. The calculated concentration from case 737 was 400 mg/dL. While this value is within the known saturation level, it had to be extrapolated from the calibration curve. Generally, a protocol exists requiring that samples beyond the range of calibration standards be diluted and subsequently reanalyzed.

Samples analyzed by the external lab were drawn from the same part of the body as the samples analyzed using this method (except for case 737, which they reported as unknown). However, the case samples were not returned after analysis at the external lab. Therefore, the blood samples analyzed using the new method, while they did come from the same subject, may have been more or less viscous than the previously analyzed samples. Samples with lower viscosity contain more water, which can lead to a higher calculated acetone concentration since acetone is hydrophilic and is therefore distributed to the water portion of the blood. Coagulation can also affect the calculated acetone concentration, and all of the samples were at least slightly coagulated.

V. C. Conclusion

A precise, accurate, selective, and sensitive method for acetone quantitation in blood using headspace gas chromatography with flame ionization was both developed and validated. Precision was determined using standard deviation, accuracy by percent

error, selectivity by chromatographic resolution of components, and sensitivity by the limits of detection and quantitation.

The final method used standards prepared from Fisher Scientific acetone at concentrations of 1.0, 10.0, 50.0, 100.0, and 250.0 mg/dL. Positive controls were prepared in a similar fashion at concentrations of 3.0, 25.0, and 70.0 mg/dL. For sample analysis, a 0.25 mL aliquot of blood and 1.5 mL of 0.05 percent n-propanol in water internal standard solution were combined in a headspace vial. No salt was added to the vial. Samples were run in duplicate and the acetone concentration was determined by interpolation from a calibration curve created using standards included in the same batch as the samples. If the correlation coefficient of the calibration curve was at or above 0.99 and the standard concentrations were within 20 percent of the expected value, the calculated acetone concentrations were assumed to be accurate.

Though the limit of detection was 0.333 mg/dL, any calculated concentrations below the administrative limit of quantitation at 1.0 mg/dL were reported as negative. Based on the administrative limit of linearity, samples with calculated acetone concentrations above 250.0 mg/dL should be diluted and reanalyzed. Carryover from samples of high acetone concentration to samples of lower acetone concentration was not observed. The method can be used on different days and/or by different experimenters while expecting calculated-concentration variations of 6.93 and 8.59 percent, respectively. Samples may be stored at any temperature, though the acetone concentration will fluctuate over time.

REFERENCES

(2009). *Rtx®-BAC1 (Fused Silica)*. Retrieved October 21, 2008, from Restek Chromatography Products Web site: http://www.restek.com/restek/prod/57.asp

Amalfitano, G., Bessard, J., Vincent, F., Eysseric, H., & Bessard, G. (1996). Gas chromatographic quantitation of dextropropoxyphene and norpropoxypheine in urine after solid-phase extraction. *Journal of Analytical Toxicology*, *20*(7), 547-554.

American Society of Crime Laboratory Directors. (2009). *About ASCLD*. Retrieved June 10, 2009, from American Society of Crime Laboratory Directors Web site: http://www.ascld.org/content/about-ascld

Ashley, D. L. (1994). Blood concentration of volatile organic compounds in a nonoccupationally exposed U.S. population and in groups with suspected exposure. *Clinical Chemistry*, *40*, 1401-1404.

Barua, R., Chi, L., Fitzpatrick, R., Gillard, D., & Kostyniak, P. J. (2008). Determination of Volatile Organic Compounds in Biological Samples Using Headspace Solid-Phase Microextraction and Gas Chromatography: Toluene and Styrene. *Journal of Analytical Toxicology*. *32*(5), 379-386.

Baselt, R. C. (2000). *Disposition of Toxic Drugs and Chemicals in Man* (5th ed.). Foster City, CA: Chemical Toxicology Institute.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). *Biochemistry* (5th ed.). New York, NY: W. H. Freeman and Co.

Bowen, S. E., Daniel, J., & Balster R. L. (1999). Deaths associated with inhalant abuse in Virginia from 1987 to 1996. *Drug and Alcohol Dependence. 53*(3), 239-245.

Buchinger, S. (2006). *Metabolome analysis of Corynebacterium glutamicum*. Retrieved May 24, 2009, from Cologne University Bioinformatics Center Web site: http://www.cubic.uni-koeln.de/research/horstmann/metabolom.html

Chang, R. (2000). *Physical Chemistry for the Chemical and Biological Sciences* (3rd ed.). Sausalito, CA: University Science Books.

Deng, C., Zhang, W., Zhang, J., & Zhang, X. (2004). Rapid determination of acetone in human plasma by gas chromatography-mass spectrometry and solid-phase microextraction with on-fiber derivatization. *Journal of Chromatography B*. *805(2)*, 235-240.

Dietz, D. D., Leininger, J. R., Rauckman, E. J., Thompson, M. B., Chapin, R. E., Morrissey, R. L., & Levine, B. S. (1991). Toxicity studies of acetone administered in the drinking water of rodents. *Fundamental and Applied Toxicology*. *17*(2), 347-360.

Federal Bureau of Investigation. (2009). *Standards for forensic DNA testing laboratories*. Retrieved June 10, 2009, from Federal Bureau of Investigation Web site: http://www.fbi.gov/hq/lab/codis/forensic.htm

Felby, S., Nielsen, E. (1994). Determination of ketone bodies in postmortem blood by head-space gas chromatography. *Forensic Science International*. *64(2-3)*, 83-88.

Gluodenis, T. (2007). *Blood. sweat, and tears -- GC/MS in forensic toxicology*. Retrieved February 27, 2009, from Laboratory News Web site: http://www.labnews.co.uk/feature_archive.php/2079/5/blood,-sweat-and-tears---gc/ms-in-forensic-toxicology

Haggard, H. W., Greenberg, L. A., & Turner, J. M. (1944). Physiological principles governing action of acetone together with determination of toxicity. *Journal of Industrial Hygiene and Toxicology*, *26*(5), 133-151.

International Organization of Standardization/International Electrotechnical Commission. (2005). *ISO/IEC 17025 - General requirements for the competence of testing and calibration laboratories* (2nd ed.). Geneva, Switzerland: ISO.

James, S. H. & Nordby, J. J. (Eds.). (2005). *Forensic Science: An Introduction to Scientific and Investigative Techniques* (2nd ed.). New York: CRC Press.

Jones, A. W., Sagarduy, A., Ericsson, E., & Arnqvist, H. J. (1993). Concentrations of acetone in venous blood samples from drunk drivers, type-I diabetic outpatients, and healthy blood donors. *Journal of Analytical Toxicology*. *17*(3), 182-185.

Kalapos, M. P. (2003). On the mammalian acetone metabolism: from chemistry to clinical applications. *Biochimica et Biophysica Acta*. *1621*(2), 122-139.

Kalapos, M. P. (2007). Possible mechanism for the effect of ketogenic diet in cases of uncontrolled seizures: The reconsideration of acetone theory. *Medical Hypotheses*. *68(6)*, 1382-1388.

Kalra, A., Tugcu, N., Cramer, S. M., & Garde, S. (2001). Salting-in and salting-out of hydrophobic solutes in aqueous salt solutions. *Journal of Physical Chemistry B. 105*(27), 6380-6386.

Kolb, B., & Ettre, L. S. (2006). *Static Headspace-Gas Chromatography: Theory and Practice.* Hoboken, NJ: John Wiley & Sons, Inc.

Koplow, L. (2006, June 24). Scientific defenses that may be available in Arizona DUI cases. Message posted to http://duiblog.arizonaduicenter.com/tags/defense/

Legacy Laboratory Services. (2007). *2007-2008 Guide to Laboratory Services*. Portland, OR: Legacy Health System.

Levine, B. (Ed.). (2006). *Principles of Forensic Toxicology* (2nd ed.). Washington, DC: American Association for Clinical Chemistry.

Lewis, R. J., Johnson, R. D., Angier, M. K., & Vu, N. T. (2004). Ethanol formation in unadulterated postmortem tissues. *Forensic Science International*. 146, 17-24.

Likhodii, S. S., Serbanescu, I., Cortez, M. A., Murphy, P., Carter Snead III, O., & McIntyre Burnham, W. (2003). Anticonvulsant properties of acetone, a brain ketone elevated by the ketogenic diet. *Annals of Neurology*. *54(2)*, 219-226.

Linde Group. (2008). *Gas Chromatography*. Retrieved June 1, 2009, from Linde Gases Division Web site: http://hiq.linde-gas.com/international/web/lg/spg/like35lgspg.nsf/docbyalias/anal_gaschrom

Martinez, M. A., & Ballesteros, S. (2006). An unusual case of drug-facilitated sexual assault using aromatic solvents. *Journal of Analytical Toxicology*, *30*(7), 449-453.

McCarver-May, D. G., & Durisin, L. (1997). An accurate, automated, simultaneous gas chromatographic headspace measurement of whole blood ethanol and acetaldehyde for human in vivo studies. *Journal of Analytical Toxicology*, *21*(2), 134-141.

Miekisch, W., Schubert, J. K., Vagts, D. A., & Geiger, K. (2001). Analysis of Volatile Disease Markers in Blood. *Clinical Chemistry*. *47*, 1053-1060.

Moeller, M. R., Steinmeyer, S., & Kraemer, T. (1998). Determination of drugs of abuse in blood. *Journal of Chromatography B*, *713,* 91-109.

Morris-Kukoski, C., Jagerdeo, E., Schaff, J. E., & LeBeau, M. A. (2007). Ethanol analysis from biological samples by dual rail robotic autosampler. *Journal of Chromatography B*, *850*(1-2), 230-235.

National Institute on Drug Abuse. (2009). NIDA infofacts: inhalants. Retrieved June 25, 2009, from NIDA Web site: http://www.nida.nih.gov/infofacts/inhalants.html

O'Neil, M. J., Smith, A., Heckelman, P. E., & Obenchain, Jr., J. R. (Eds.). (2001). Merck Index . Whitehouse Station, NJ: Merck & Co.

Oregon State Police Forensic Services Division. (2008). Blood alcohol procedures manual (Revision 12). Unpublished manuscript.

Ramsey, J. D., & Flanagan R. J. (1982). Detection and identification of volatile organic compounds in blood by headspace gas chromatography as an aid to the diagnosis of solvent abuse. *Journal of Chromatography*, *240*(2), 423-444.

Ramu, A., Rosenbaum, J., & Blaschke, T. F. (1978). Disoposition of acetone following acute acetone intoxication. *Western Journal of Medicine*, *129*(5), 429-432.

Restek Corporation. (1999). *GC analysis of commonly abused inhalants in blood using RTX-BAC1 and RTX-BAC2 columns* (Restek Clinical/Forensic Applications Note No. 59548). Bellafonte, PA: Resktek Corporation.

Saferstein, R. (2007). *Criminalistics: An Introduction to Forensic Science* (9th ed.). Upper Saddle River, New Jersey: Pearson Education, Inc.

Singer, P. S., & Jones, G. R. (2006). An unusual autoerotic fatality associated with chloroform inhalation. *Journal of Analytical Toxicology*, *30*(3), 216-218.

Skoog, D. A., Holler, F. J., & Crouch, S. R. (2007). *Principles of Instrumental Analysis* (6th ed). Belmont, CA: Thomson Brooks/Cole.

Streete, P. J., Ruprah, M., Ramsey, J.D., & Flanagan, N.J. (1992). Detection and identification of volatile substances by headspace capillary gas chromatography to aid the diagnosis of acute poisoning. *Analyst*. *117*(7), 1111-1127.

Sulway, M. J., & Malins, J. M. (1970). Acetone in diabetic ketoacidosis. *Lancet*, *1*, 736-740.

United States Public Health Service. (1994). *Toxicological Profile: Acetone*. Retrieved November 8, 2008, from Agency for Toxic Substances and Disease Registry Web site: http://www.atsdr.cdc.gov/toxprofiles/tp21-c1.pdf

Utah Public Health Laboratories Bureau of Forensic Toxicology. (2008). *Volatiles in biological specimens by dual headspace gas chromatography*. Retrieved June 11, 2009, from Utah Department of Health Web site: http://health.utah.gov/lab/toxicology/M-001_VolatilesByHSGC_Rev04.pdf

Verstraete, A. G. (2004). Detection times of drugs of abuse in blood, urine, and oral fluid. *Therapeutic Drug Monitoring. 26,* 200-205.

Virginia Department of Forensic Science. (2009). *Toxicology Procedures Manual*. Retrieved June 11, 2009, from Virginia Department of Forensic Science Web site: http://www.dfs.virginia.gov/manuals/toxicology/procedures/220-D100%20Toxicology%20Procedures%20Manual.pdf

Wise, R. A. (1996). Neurobiology of addiction. *Current Opinion in Neurobiology. 6*(2), 243-251.

Zhu, J. Y., & Chai, X. S. (2006). Some recent developments in headspace gas chromatography. *Current Analytical Chemistry*, *1*, 79-83.

Zuba, D., Parczewski A., & Reichenbacher, M. (2002). Optimization of solid-phase microextraction conditions for gas chromatographic determination of ethanol and other volatile compounds in blood. *Journal of Chromatography B. 773*(1), 75-82.

VITA

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Master of Science

Thesis: QUANTITATIVE ANALYSIS OF VOLATILES IN BLOOD VIA HEADSPACE GAS CHROMATOGRAPHY/ FLAME IONIZATION DETECTION

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Pages in Study: 60 Candidate for the Degree of Master of Science

Major Field: Forensic Toxicology

Scope and Method of Study: The purpose of this research project was to develop and validate a method for the quantitation of acetone in blood using Headspace Gas Chromatography with Flame Ionization Detection. The following items were included in the development: preparation of standards and positive controls, testing the necessity of using salts, establishment of a suitable internal standard concentration, determination of an appropriate sample size, limit of detection calculation, establishing the linear dynamic range, testing for carryover effects, method validation including case samples, and checking the stability of samples containing acetone.

Findings and Conclusions: The blood-acetone quantitation method was successfully developed and validated. Standards and controls were accurate and precise and yielded linear results. The addition of salt to sample vials was deemed unnecessary. An internal standard concentration of 0.05% (v/v) n-propanol in water and a sample size of 0.25 mL were found to be suitable. The limit of detection was calculated to be 0.333 mg/dL while the limit of quantitation was calculated to be 0.816 mg/dL. However, quantitations were inaccurate below 1.0 mg/dL and blood-acetone concentrations above 250.0 mg/dL are not generally encountered. Therefore, an administrative linear dynamic range was set from 1.0 to 250.0 mg/dL. No carryover between samples occurred. Interday and interindividual variations were 6.93 and 0.77%, respectively. When checking the stability of samples containing acetone, time accounted for 97.7% variation while temperature only accounted for 0.22% variation.

ADVISER'S APPROVAL: Dr. Jarrad Wagner