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QUANTIFICATION OF KETONE BODIES IN SALIVA USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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QUANTIFICATION OF KETONE BODIES IN SALIVA USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

by

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ABSTRACT

Type 1 Diabetes Mellitus is an autoimmune disease where pancreatic β-cells within the Islets of Langerhans are destroyed. A complication associated with type 1 diabetes mellitus is diabetic ketoacidosis, and if left untreated can result in coma or death. There are commercial methods for individuals to analyze ketone body concentrations within urine and blood, but these are invasive, expensive, and aren't always accurate. Therefore, saliva should be examined as a potential alternative to the current commercial methods on the market. This experiment aimed to quantify the amount of beta-hydroxybutyrate and acetoacetate, in the saliva, blood, and urine samples using GC-MS by inducing ketosis in consenting participants. The participants followed a ketogenic diet for four days, and their biological samples were obtained before and after. The blood samples were centrifuged to isolate the plasma and deproteinated. All sample matrices were evaporated, the ketone bodies were derivatized using BSTFA + 1% TMCS at 80°C for 1.5 h, and the headspace was analyzed. The participants followed an Atkins diet to induce ketosis and the amount of beta-hydroxybutyrate was able to be quantified for each participant. The urine samples had high concentrations of beta-hydroxybutyrate, which could be a result of the acidic urine increasing the derivatization efficiency. There was very little beta-hydroxybutyrate detected within saliva. Since there was detection, this could potentially be used as a method to quantify ketone bodies upon further method development. No acetoacetate was detected in any of the samples. Future work should further optimize the methodology to detect acetoacetate, and the addition of recovery and internal standards to the samples to ensure all the analyte is derivatized and to determine if there is any variation between runs.

Thesis supervisor: Dr. Kingsley Donkor

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1.0 INTRODUCTION

1.1 Type 1 Diabetes Mellitus

Diabetes mellitus is an epidemic in today's world with 600 million people worldwide being diagnosed as diabetic with 10% of those patients being diagnosed with type 1 diabetes mellitus (T1D) (Dholakia et al. 2016). T1D is an autoimmune disease where pancreatic β-cells within the Islets of Langerhans are destroyed, and the individual can no longer produce insulin. Unfortunately, the mechanism that triggers the autoimmune destruction of β-cells is unknown, but T1D pathogenesis is being hypothesized to be in three stages (Butalia et al. 2016; Pociot and Lernmark 2016). In the first stage, β-cell autoantibodies are present, but inactive (Pociot and Lernmark 2016). The second stage involves the autoantibodies attacking the β-cells once some sort of environmental trigger occurs, and the third stage involves the symptoms of T1D to be present (Pociot and Lernmark 2016). The pathogenesis of T1D seems to be a combination of many factors. For instance, there are many genetic loci that are attributed to increasing the chances of being diagnosed with T1D like the human leukocyte antigen class II molecules, DQ and DR, within the major histocompatibility complex locus, and more are being discovered still (Butalia et al. 2016; Pociot and Lernmark 2016). As well, certain environmental factors contribute to the increased risk of T1D like the mumps, rubella, microorganisms, and other factors which are still being studied (Butalia et al. 2016). Individuals with T1D have to monitor their blood glucose level every 4-8 h with the goal of being in a range of 4.0 to 7.0 mmol/L (Giugliano et al. 2008; Morales and Schneider 2014). If they are less than 3.9 mmol/L, they would have hypoglycemia and typically are treated with juice (Morales and Schneider 2014). Blood sugar levels consistently greater than 7.0 mmol/L while fasting refers to hyperglycemia and is treated with exogenously administered insulin (Giugliano et al. 2008).

There are many complications associated with T1D. For instance, those with T1D are prone to end-stage renal disease, blindness, foot and leg amputations, and cardiovascular diseases (Pociot and Lernmark 2016). Another complication of T1D is diabetic ketoacidosis. Essentially, it is a metabolic state in which the diabetic individual does not have enough insulin to stimulate glucose uptake by target cells, thus excreting glucose via urine, and increasing concomitantly the blood glucagon concentration which stimulates ketogenesis within the liver, and the excess of ketone bodies ends up acidifying the individuals blood (Laffel 1999; Mullins et al. 2011). By clinical definition, one is diagnosed with diabetic ketoacidosis when they have a blood glucose level greater than 11 mmol/L, a pH less than 7.3, ketonaemia, and ketonuria (Usher-Smith et al. 2011). One is considered in ketonaemia when blood ketone concentration is greater than 3 mM (Laffel 1999). The amount of ketone bodies can be as high as 25 mM in those suffering from T1D (Laffel 1999; Kanikarla-Marie and Jain 2016). In non-diabetic populations, ketoacidosis generally not a concern since these individuals have two feedback loops to maintain plasma ketone levels below 3 mM (Mullins et al. 2011).

Ketone bodies are ionized at physiological pH, so the hydrogen ions bind to the bicarbonate molecules, which tends to overwhelm the blood-buffering capacity and subsequently acidifies the blood (Laffel 1999; Mullins et al. 2011; Kanikarla-Marie and Jain 2016). This also leads to the loss of sodium ions, loss of ammonium, severe dehydration, and the loss of blood volume (Mullins et al. 2011). Those with T1D also struggle to clear ketone bodies through urine, whether or not they are suffering from ketoacidosis, and have decreased succinyl CoA-oxoacid transferase (SCOT) and 3-hydroxybutyrate dehydrogenase activities thus increasing ketone concentration within the blood (Kanikarla-Marie and Jain 2016; Sherwin et al.). If left untreated, this can result in coma or death and is the leading cause of death among children with T1D (Mullins et al. 2011;

Usher-Smith et al. 2011). This is concerning because between 10% and 70% of children recently diagnosed with T1D are in diabetic ketoacidosis, so it is crucial that the symptoms of diabetic ketoacidosis is acted upon and subsequently treated (Usher-Smith et al. 2011). Ketoacidosis is treated using insulin, potassium bicarbonate, and saline, which treat hyperglycemia, the lack of electrolytes within the individual and balance pH, and treat dehydration, respectively (Laffel 1999; Kanikarla-Marie and Jain 2016).

1.2 Ketogenic Diets and Ketone Bodies

Ketogenic diets (KD) have become very popular for those wishing to lose weight. KDs are a high-fat, moderate protein, and low carbohydrate intake diet that reduces glucose supply to decrease glycolysis and increase fatty acid breakdown through beta-oxidation (Branco et al. 2016; Roehl and Sewak 2017). Initially, acetyl-CoA is generated from fatty acid oxidation and can be used by the citric acid cycle to produce ATP. Using acetyl-CoA, the ketone bodies acetoacetate (AcAc), acetone, and beta-hydroxybutyrate (BHB) can be produced which serves as an energy source for the brain, the heart, the kidney cortex, and skeletal muscle (Laffel 1999; Roehl and Sewak 2017). The production of ketone bodies are necessary since they are water soluble, allowing energy sources to travel systemically, and can subsequently travel through the brain-blood barrier to enter neurons and generate ATP through their mitochondria (Mullins et al. 2011; Wibisono et al. 2015). There are four different types of KD which are the long-chain triglyceride KD (classic KD), modified Atkins diet, medium-chain triglyceride KD, and low glycemic index treatment (Branco et al. 2016; Roehl and Sewak 2017). The diets differ in the ratio of grams of fat consumed to grams of protein and carbohydrates consumed, percentage of calories consumed, and in the foods that are allowed to be consumed (Branco et al. 2016; Roehl and Sewak 2017). Originally,

KD was created in 1921 as a way to treat epilepsy, as it is believed to induce anticonvulsant effects in humans which affects neuronal firing and the spread of seizures (Branco et al. 2016). It has also been used as a therapeutic diet for those suffering from type II diabetes mellitus, obesity, and cancer (Wibisono et al. 2015; Branco et al. 2016; Roehl and Sewak 2017). The diet affects cancerous cells by reversing redox signaling pathways within tumors (Branco et al. 2016). Though it has been used therapeutically, there are potential short-term and long-term harms of the diet. In the short-term, participants may experience constipation, hypoglycemia, dehydration, lethargy, and acidosis (Branco et al. 2016). Long-term effects include hypercholesterolaemia, nephrolithiasis, and cardiomyopathy (Branco et al. 2016).

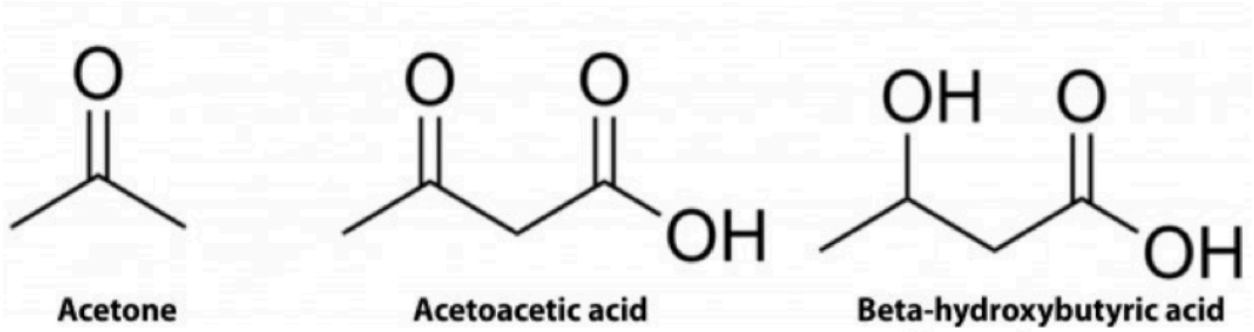


Figure 1. The ketone bodies that is produced within the human body.

Individuals on KDs usually go into ketosis, which refers to a metabolic state where ketone bodies are metabolized to produce ATP (Branco et al. 2016). Under normal physiological conditions, the ketone concentration within plasma is less than 0.2 mM (Garber et al. 1974; Courchesne-Loyer et al. 2013). Under ketosis, the concentration of BHB is around 1-2 mM and around 0.5 mM for AcAc after 3 days of fasting, and can reach as high as 8 mM, and between 1-2 mM for AcAc for obese individuals after 42 days of fasting (Owen et al. 1967; Owen et al. 1969; Garber et al. 1974). Further, it was found that the ketone bodies also can provide up to two-thirds

of the brain's energy requirement during ketosis (Owen et al. 1967; Courchesne-Loyer et al. 2013). Ketone bodies are generated in response to a change in the molar concentration of glucagon to insulin, as a result of a lower concentration of glucose circulating throughout the body (Mullins et al. 2011; Kanikarla-Marie and Jain 2016). Because the insulin content is so low, lipolysis is no longer inhibited, nor is hormone-sensitive lipase deactivated, allowing for fatty acids from adipose tissue to be metabolized (Laffel 1999; Kanikarla-Marie and Jain 2016). Therefore, the increase of cAMP, due to a high glucagon content, activates protein kinase A which signals for the enzyme adipose triglyceride lipase to begin hydrolysis of the triglycerides within adipose tissue (O'Neill et al. 2013; Steensels and Ersoy 2019). The newly formed diglycerides are then hydrolyzed by hormone-sensitive lipase to make monoglycerides, and glycerol (O'Neill et al. 2013; Steensels and Ersoy 2019). From here, the fatty acids are then released into circulation, and travels to various tissues through albumin (Laffel 1999; Steensels and Ersoy 2019). The fatty acid translocase/CD36, the most effective fatty acid transporter for increased fatty acid content, uptakes the fatty acids, and carnitine acyltransferase 1 then transports the fatty acids across the inner mitochondrial membrane, where beta-oxidation of fatty acids takes place to form acetyl CoA (Laffel 1999; Nickerson et al. 2009; Kanikarla-Marie and Jain 2016). Normally, under non-ketotic conditions, the acetyl CoA would enter the citric acid cycle, and condense with oxaloacetate (Laffel 1999). Since there is a lower content of glucose within the bloodstream, hepatic cells therefore utilize the oxaloacetate within gluconeogenesis, effectively slowing down the citric acid cycle (Laffel 1999). Further, under non-ketotic conditions, acetyl CoA could leave the inner mitochondria and be converted to malonyl CoA via acetyl CoA carboxylase, which is used for fatty acid biosynthesis (Laffel 1999). Though during ketosis, acetyl CoA carboxylase is inhibited by the increase in glucagon and lectin and decrease in insulin and adiponectin within the body, thus shunting these

pathways and using the acetyl CoA for the generation of ketone bodies (Laffel 1999; O'Neill et al. 2013). Acetyl CoA is converted to acetoacetyl CoA by 3-ketothiolase, followed by the formation of β-hydroxy β-methylglutaryl CoA through mitochondrial β-hydroxy β-methylglutaryl CoA synthase, and cleaved to form AcAc by β-hydroxy β-methylglutaryl CoA lyase (Laffel 1999; Kanikarla-Marie and Jain 2016). AcAc is reduced to BHB by 3-hydroxybutyrate dehydrogenase, which oxidizes an NADH as a result (Laffel 1999; Kanikarla-Marie and Jain 2016). Acetone is also produced from AcAc by spontaneous decarboxylation (Laffel 1999). BHB and AcAc are the two dominant ketone bodies that are used as energy sources within the body (Laffel 1999; Fujii et al. 2014; Kanikarla-Marie and Jain 2016). The ratio between the two ketone bodies is usually three BHB molecules to one AcAc molecule, which is a result from the $NADH/NAD^+$ ratio and activity of the 3-hydroxybutyrate dehydrogenase (Laffel 1999; Kanikarla-Marie and Jain 2016).

The ketone bodies, being water soluble, travel throughout the blood stream to serve as an energy source (Laffel 1999; Kanikarla-Marie and Jain 2016). The ketones enter organs and are transported into the cell by monocarboxylate transporter 1 and 2 (Puchalska and Crawford 2017). Subsequently, the ketones travel through to the inner mitochondria for ketolysis, but the mechanism is unclear (Puchalska and Crawford 2017).The abundant BHB molecules revert back to AcAc by 3-hydroxybutyrate dehydrogenase (Branco et al. 2016). From here, the AcAc molecules are used to regenerate acetoacetyl CoA through the enzyme SCOT and an acetyl group is cleaved from methylacetoacetyl CoA thiolase to regenerate acetyl CoA which enters the citric acid cycle (Laffel 1999; Branco et al. 2016; Kanikarla-Marie and Jain 2016). It should be noted that SCOT is the rate limiting step of ketolysis, and the highest expression of this enzyme is in the heart and the brain (Laffel 1999; Kanikarla-Marie and Jain 2016). Further, SCOT is downregulated

by high concentrations of AcAc, which is the reason for an increase in circulating ketone bodies during the initial phases of ketosis (Laffel 1999).

There are commercial methods for individuals to analyze ketone body concentrations. Commonly, urinary ketones are measured to screen individuals for diabetic ketoacidosis (Laffel 1999). The urine kit relies on the Legal reaction, in which AcAc reacts in presence of an alkaline buffer and nitroferricyanide to produce a purple color on a test strip (Laffel 1999; Brooke et al. 2016). This test does not react with BHB, nor does it measure the amount of ketones present (Laffel 1999; Brooke et al. 2016). Measuring AcAc through urine is a cheap method to determine if someone is in a state of ketoacidosis; although it can be perceived as intrusive and awkward for people and is less accurate than measuring blood ketone levels, which primarily focuses on BHB (Brooke et al. 2016). Using a blood ketone monitor, the amount of BHB is quantitatively determined (Brooke et al. 2016). It is believed that BHB levels within blood provide fewer false positives than urine kits that test for AcAc. Also there is a higher correlation between blood BHB concentrations and the clinical markers of diabetic ketoacidosis (Brooke et al. 2016). Though, measuring blood has its challenges since the meters have difficulties measuring concentrations greater than 5 mM, can be seen as invasive, and the blood strips and meter is expensive to purchase (Brooke et al. 2016). Thus, it would be in the best interest of the public to develop a less invasive and affordable way to analyze ketone bodies, especially BHB, within the body.

Saliva plays an important role within the body as it clears substances from the mouth, contains salivary amylase to break down carbohydrates, buffers pH, and protects, hydrates, and lubricates oral mucosal surfaces (Proctor 2016). Saliva consists mostly of water, but also contains hormones, microbes, enzymes, and other metabolites (Elmongy and Abdel-Rehim 2016; Viswanath et al. 2017). Most notably, saliva is isotonic to plasma (Aps and Martens 2005;

Elmongy and Abdel-Rehim 2016; Proctor 2016). The concept of using saliva as a diagnostic model is slowly gaining popularity within the literature, but there is very little research on the quantification of ketone bodies in the literature, not to mention quantifying the amount of BHB or AcAc within individuals undergoing ketosis (Elmongy and Abdel-Rehim 2016). Previously, it was noted that both saliva and blood contain BHB, and there is a positive correlation between them (Liu et al. 2015). The study done by Liu et al. also observed BHB within urine, but their subjects used were not in ketosis (2015). In another study, saliva was analyzed in diabetic patients and healthy volunteers for BHB, which was found to be around 25 ng/mL and 16.5 ng/mL, respectively, though the participants were also not in ketosis either (Tsutsui et al. 2012). AcAc was not explored in both experiments (Tsutsui et al. 2012; Liu et al. 2015).

The instrument technique of gas chromatography-mass spectrometry (GC-MS) involves producing and separating gas phase analytes, and detecting these analytes by first ionizing them using a mass spectrometer (Niwa 1995). This technique has been used in previous experiments to quantify ketone bodies within biological fluids (Paul et al. 2006; Hassan and Cooper 2009; Holm et al. 2010; Føreid and Gadeholt 2017), and it is a very powerful technique to detect analyze the analytes of interest. The gas chromatograph separates gaseous analytes which are carried through a stationary column by an inert gas (Niwa 1995). In the column, the analytes are separated based on their affinity with the column and vapor pressure (Niwa 1995). The stationary phase interacts with the analytes and separates them based on their affinity to the non-polar column (Niwa 1995); the more non-polar the analyte is, the longer it takes for it to elute through the column (Niwa 1995). If the analyte has a high vapor pressure, it will travel through the column quickly as well (Niwa 1995). Once it is separated, the analytes will be bombarded by electrons and ionized by an electron impact mass spectrometer, which then separates the charged ions through a mass analyzer by its

mass-to-charge ratio to obtain a mass spectrum (Niwa 1995). This allows for the investigators to determine what eluted from the gas chromatograph (Niwa 1995).

Therefore, this experiment looks to quantify the amount of BHB and AcAc, the two most common ketone bodies, found in the saliva, blood, and urine via GC-MS before and after inducing ketosis in consenting participants. Through quantification, the development of methods that are non-invasive and cheap to purchase to observe the ketone body content within individuals can be explored. Within this study, method development was conducted to try and optimize the detection of the ketone bodies. From there, nine participants followed a ketogenic diet for four days, where their blood, urine, and saliva were sampled before-the-diet and after-the-diet. The ketone bodies were then extracted, analyzed, and quantified for each individual participant.

2.0 METHODS

2.1 Standards and Materials

Lithium acetoacetate, (±)-sodium 3-hydroxybutyrate, and BSTFA + 1% TMCS were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). The acetonitrile was of HPLC grade. The trichloroacetic acid and Tris were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). The headspace vials were 20 mL and clear, while the caps were 18 mm magnetic screw caps. Both were purchased from Canadian Life Science (Peterborough, Ontario, Canada). They were previously never used before the experiment.

2.2 Ketogenic Diet

Participants were recruited to participate in a KD through posters and word of mouth around the Thompson Rivers University campus. The participants contacted the investigators and

arranged a meeting time to further discuss the experiment. The participants were given a numbered consent form upon arrival for them to read over and was signed if they consented to participating in the study. They were then given a numbered screening questionnaire to complete so the investigators could determine if they were healthy enough to participate in the study. Both the consent forms and questionnaires were numbered in effort to keep the participant's anonymous, and their name and corresponding number were recorded in a secure notebook. There were 10 participants originally, but Participant 1 removed themselves from the study prior to starting the diet.

The diet that the participants were following was a classic ketogenic diet that followed a 3:1 ratio of fats to protein and carbohydrates. The participants were encouraged to follow the meal plan that was created by the investigators (see Appendix), but they were able to eat other food as long as they received permission from the investigators and recorded it in a meal log. The diet was followed for four days. Because the participants have different eating habits, they were encouraged to eat until they were full as long as they were maintaining a 3:1 ratio of fats to proteins and carbohydrates.

2.3 Sampling

The participants fasted for a minimum of 8 h prior to sampling. The participants were instructed to urinate a minimum of 10 mL into a centrifuge tube. The tubes were sealed, wiped, and given to the investigators. The participants were to passively drool a minimum of 500 μL into a 1.5 mL Eppendorf tube. This was done by sitting down, and arching their back to a 45° angle, and further directing their head downward to allow for the saliva to pool in their mouth. They then had to manipulate their mouth to allow for the saliva to enter the Eppendorf tube. They had to

ensure that the saliva was not bubbly as it would not give an accurate representation of the amount currently in the tube itself. The participants then had 5 mL of their blood drawn into lithium heparin tubes. The blood was placed on ice after the initial collection. This was conducted before the participants started the diet and 4 days after following the ketogenic diet.

The urine was split into three 1.5 mL Eppendorf tubes as storage, and 500 μL of the sample was pipetted into a sterile Eppendorf tube for subsequent use within the experiment. The saliva was stored in its original container. The blood, after being drawn, was then centrifuged at 3000 rcf for 10 min at 4°C to separate the plasma. The plasma was then pipetted into 1.5 mL Eppendorf tubes as storage, and a 500 μL sample was taken to be used in the experiment. All fluids were then stored at -80°C until the samples were to be analyzed.

2.4 Sample Analysis

All of the sample tubes that were stored were dethawed on ice. The plasma was then deproteinated to remove potential protein interferences within the plasma. There was 500 μL of 2 M trichloroacetic acid that was added to the Eppendorf tube. It was then centrifuged at 13,000 rpm for 3 min at 4°C. The supernatant was moved, and 250 μL of 4 M Tris was then added. The solution was then centrifuged at 13,000 rpm for 15 min at 4°C. It was also brought back to pH 7 using KOH. Once the saliva had dethawed, the sample was centrifuged at 13,000 rpm for 10 min to pellet the mucins within the fluid. Afterwards, 200 μL of each fluid was pipetted into a new headspace vial, along with 10 μL of 70% ethanol. The solution was capped, mixed, and it sat for 1 h in order to sterilize the fluids. The vials had their caps removed, and the solutions inside were evaporated in an oven at 80°C. After the evaporation, 75 μL of BSTFA + 1% TMCS and 150 μL of acetonitrile were added to each headspace tube, were capped, and derivatized at 80°C for 1.5 h.

They were subsequently left at room temperature for three days afterwards. The samples were agitated at 250 rpm for 6 min at 80°C prior to analysis. The PAL headspace syringe, at 85°C, then penetrated through the vial cap and sampled 100 μL of headspace and injected it into the GC-MS.

2.5 Instrumental Conditions

Analysis was performed using an Agilent 7890B-GC coupled 5977A-MS (Agilent Technologies, Santa Clara, CA). The instrument operated using a PAL RSI 85 autosampler system equipped with a headspace function and an agitator with an oven. The column was an Agilent HP-5MS column (30 m x 250 μm x 0.250 μm) (Agilent Technologies, Santa Clara, CA). An agitator on the autosampler was heated to 80°C, and the syringe was heated to 85°C. The split was 2:1, and the split flow was 2 mL/min. The injector temperature was 250°C. The temperature program used was developed from a previous method (Hassan and Cooper 2009). The initial oven temperature was 60°C and was held at this temperature for 2 min. The temperature increased to 180°C at 20°C/min, and then increased to 250°C at 50°C/min which was then held for 1 min. The total run time was 10.4 min. The amount injected was 100 μL.

A scan on the mass spectrometer was used to detect the ions from 40-350 m/z. The ion chromatograms were extracted using selected ion monitoring to determine where the ketone bodies were eluting at. The ions 147 and 233 were used to identify the BHB, and 147 and 231 were used to identify AcAc; the 147 ion was used to determine the concentration of the ketone bodies. As well, the NIST database was used in elucidating the mass spectrum to help estimate the compound that eluted from the gas chromatograph (Linstrom and Mallard 2001). This software estimates the compounds that eluted from the instrument, giving a probability of accuracy, based on the mass spectrum of the peak at that specific point (Linstrom and Mallard 2001).

2.6 Preparation and Analysis of the Ketone Body Standards

A stock solution of 5000 ppm was made for BHB and AcAc, individually. The BHB stock solution was made up in a 50 mL volumetric flask with 18 MΩ water, while the AcAc solution was made up to 25 mL with 18 MΩ water. These stocks solutions were used to develop standards with concentrations of 50 ppm, 100 ppm, 250 ppm, 500 ppm, 1000 ppm, and 2000 ppm, made up to 10 mL with 18 MΩ water in a volumetric flask. The standards were mixed, and 225 μL of each solution was added to a new headspace vial. The solution was evaporated in an oven at 80°C. The solution then had 75 μL of BSTFA + 1% TMCS and 150 μL of acetonitrile added to each vial. They were derivatized in the oven for 1.5 h at 80°C and were run three days afterwards. Before analysis, the samples were agitated at 250 rpm for 6 min at 80°C. The PAL headspace syringe then was used to inject 100 μL of the standards headspace into the GC-MS.

3.0 RESULTS AND DISCUSSION

3.1 Detection and Calibration of the Ketone Bodies

The development of a method to detect the ketone bodies was investigated using the BHB standard. This standard was used for method development because of its prominence within the blood of individuals producing ketone bodies (Laffel 1999; Fujii et al. 2014). A mass of BHB was added into hexane, acetonitrile, water, and methanol. It was not soluble in the hexane since nothing seemed to be dissolved, but it was soluble in methanol, water, and acetonitrile; it was most soluble in methanol and water. Therefore, the BHB standard was dissolved in 18 MΩ water to create the stock solutions. The solution was then pipetted into a glass headspace vial, where the solution was evaporated to dryness using nitrogen gas. This was inefficient and extremely difficult to do, so the

headspace vials were transported to an oven which was set to 80°C to evaporate the solvent. The derivatizing agent was then pipetted into the vial, it was capped, and the vial was heated to promote the derivatization reaction. Upon analysis, there was no signal that was ascertained. Therefore, the stock solution would be acidified to ensure that all of the hydroxyl groups were protonated to help with the derivatization reaction. The same procedure was then conducted, but there were still no results that were ascertained. Acetonitrile was then added as a catalyst for the derivatization to take place, especially since BHB is soluble in it. There was a BHB signal detected with the GC-MS. The signal obtained had a mass-to-charge ratio at 147 and 231, and the NIST database predicted this molecule to be a trimethylsilyl beta-hydroxybutyrate molecule as result of the derivatization. The chromatogram in Figure 2 displays BHB found within the chromatogram and subsequent mass spectra.

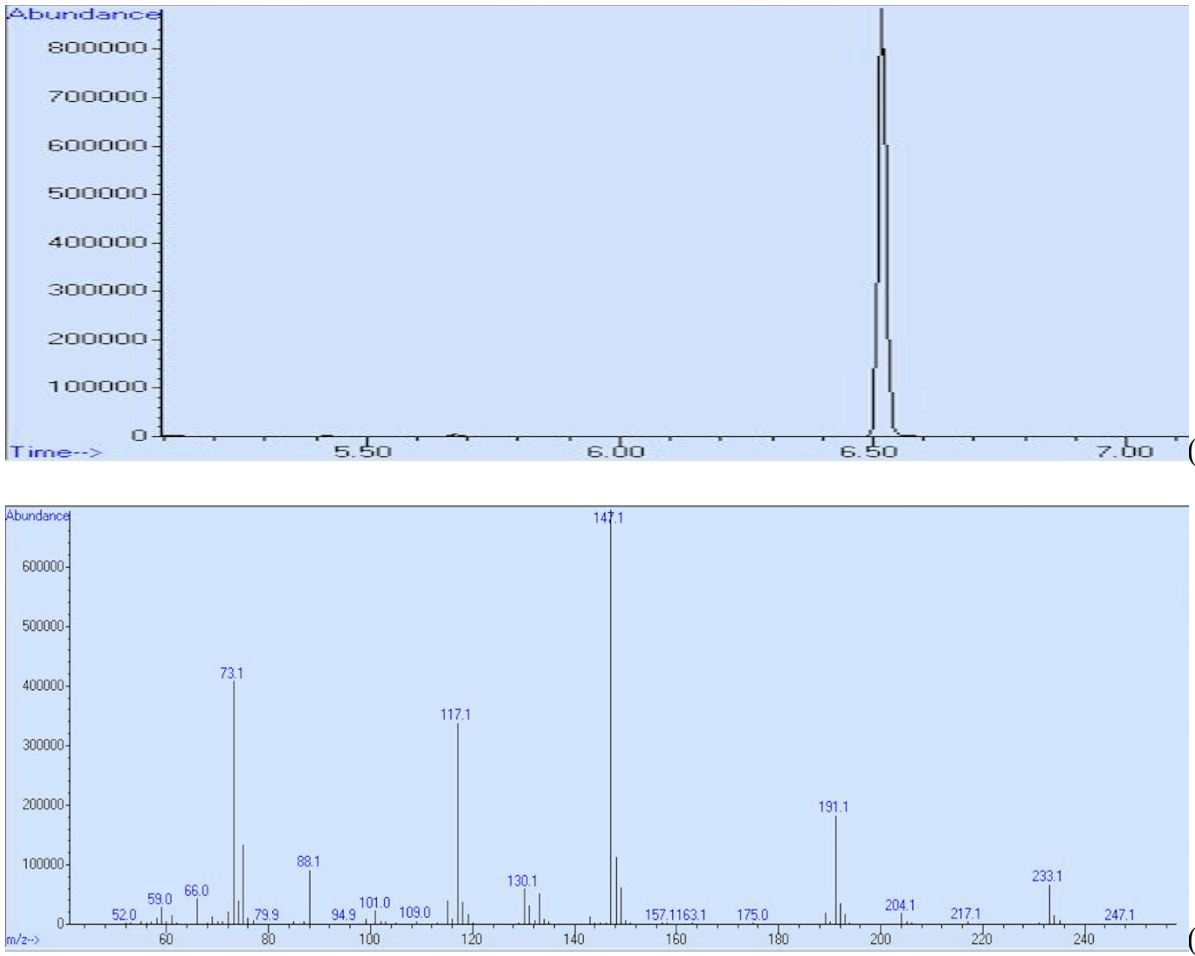


Figure 2. The chromatogram (a) of 5000 ppm derivatized BHB with its corresponding mass spectrum (b). The BHB signal is found at 6.521 min.

Next, we determined how much acetonitrile to be added to help the derivatization occur. In each reaction, 225 µL of 5000 ppm BHB stock solution was added to a headspace vial and 75 µL of BSTFA + 1% TMCS was added; the amount of acetonitrile added was 75, 150, and 225 µL to each reaction afterwards. All were derivatized for 25 min at 80°C, and it was found that the 150 µL solution produced the highest signal for BHB. The optimal time of the derivatization was determined afterwards. A sample that was produced during the method development for the optimal amount of acetonitrile added was analyzed one month following to its initial derivatization was found to have an extremely large peak area. Because of this, it was assumed that the amount

of time the initial derivatization in the oven was for was irrelevant as the derivatization seemed to occur for longer than anticipated. A solution was then derivatized in an oven for 1.5 h at 80°C to aid in as much derivatization as possible and was then kept at room temperature and analyzed every day. The solution seemed to have the same peak area on the third and fourth day of analysis. Therefore, the solutions were to be derivatized for 1.5 h at 80°C and then placed in a room temperature environment for the next three days before they were analyzed by the GC-MS.

The next standard that was used was AcAc. This standard was difficult to use since its chromatograms had many impurities, and the peak area was not as high as it was for BHB. A 2000 ppm stock solution was made for method development nonetheless, and the peak obtained was very small, especially in comparison to the BHB peak area. This standard had two derivatizations occur instead of one. AcAc has one hydroxyl group, but based on the mass spectra and the NIST database, it seemed like there were two derivatizations occurring. The carboxylic acid remained the same, but the lone carbonyl tautomerized and was subsequently converted to a hydroxyl group which was also derivatized.

The AcAc standard solution was not acidified to see if it had any effect on the end result. This produced a chromatogram that had a greater peak area than the one previously seen with the acidified AcAc. Therefore, the experimental procedure for BHB was reexamined since both ketone bodies were predicted to be within the biological fluids. The BHB sample solution was not acidified, derivatized, and subject to analysis; the results showed that it still had a distinct signal, but the peak area was not as high as it was for the acidified BHB. In an effort to detect quality ketone body signals, the solutions were not acidified to aid in detecting AcAc among the biological samples. Figure 3 includes a chromatogram and mass spectrum for AcAc.

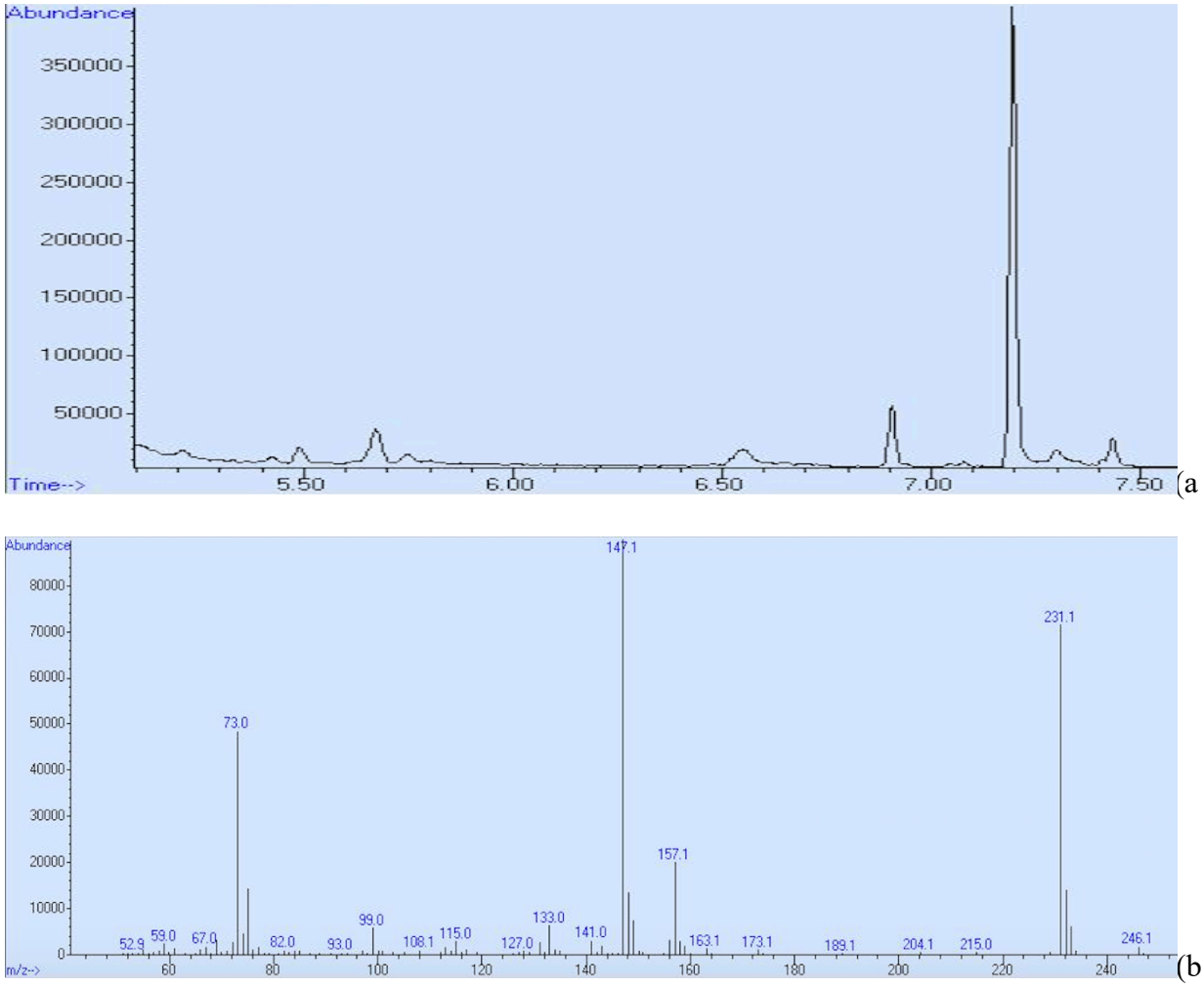


Figure 3. The chromatogram (a) of 5000 ppm derivatized AcAc with its corresponding mass spectrum (b). The AcAc signal is found at 7.197 min.

A calibration curve was produced for both analytes separately. This was done by preparing standards of increasing concentrations and obtaining the peak areas of each signal and plotting it on a graph. The concentrations used were 50 ppm, 100 ppm, 250 ppm, 500 ppm, 1000 ppm, 2000 ppm, and 5000 ppm. The range of concentrations was very wide in an effort to account for the variation in ketone bodies produced by each individual. These solutions were analyzed in triplicate, save for the 100 ppm standard of BHB which was analyzed in duplicate; this is because the run

had an error which stopped the run before the BHB signal was analyzed. The derivatized ketone bodies that the mass spectrometer detected are found in Figure 2.

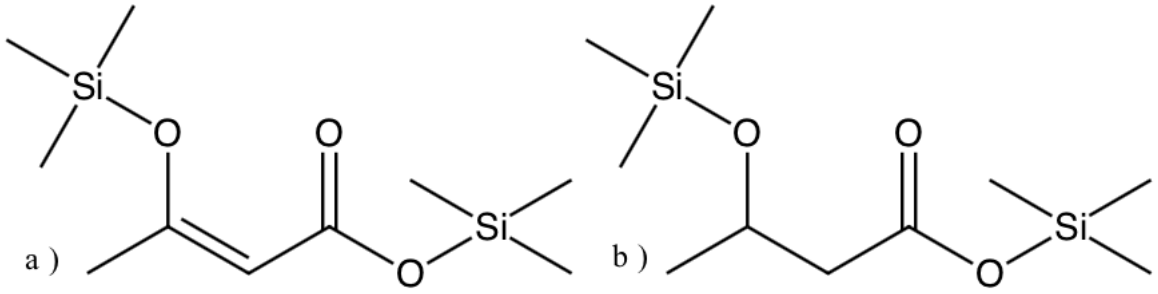


Figure 4. The derivatized acetoacetate (a) and beta-hydroxybutyrate (b) that were to be analyzed by the GC-MS.

For the BHB standard curve, there was a trend of linearity based on the coefficient of variation (R^2=0.9742). The limit of detection (LOD) for the BHB standard curve was found using the standard deviation of the lowest concentration standard used divided by the slope of the curve and multiplied by 3. Likewise, the limit of quantification (LOQ) was found in the same manner but was multiplied by 10 instead of 3. The LOD and the LOQ was found to be 26.48 ppm and 88.28 ppm, respectively. Other papers have determined the LOD to be 0.833 ppm (Holm et al. 2010), and 10 ppm (Paul et al. 2006). The closest to the LOD obtained in this experiment is by Paul et al., but that result is much smaller than the results experimentally determined. Because of this, the method should be further optimized since this method isn't able to detect low levels of ketone bodies as effectively as the other methods do. One way to do this could be to run many trials, and then average the spectra together to lower the signal-to-noise ratio.

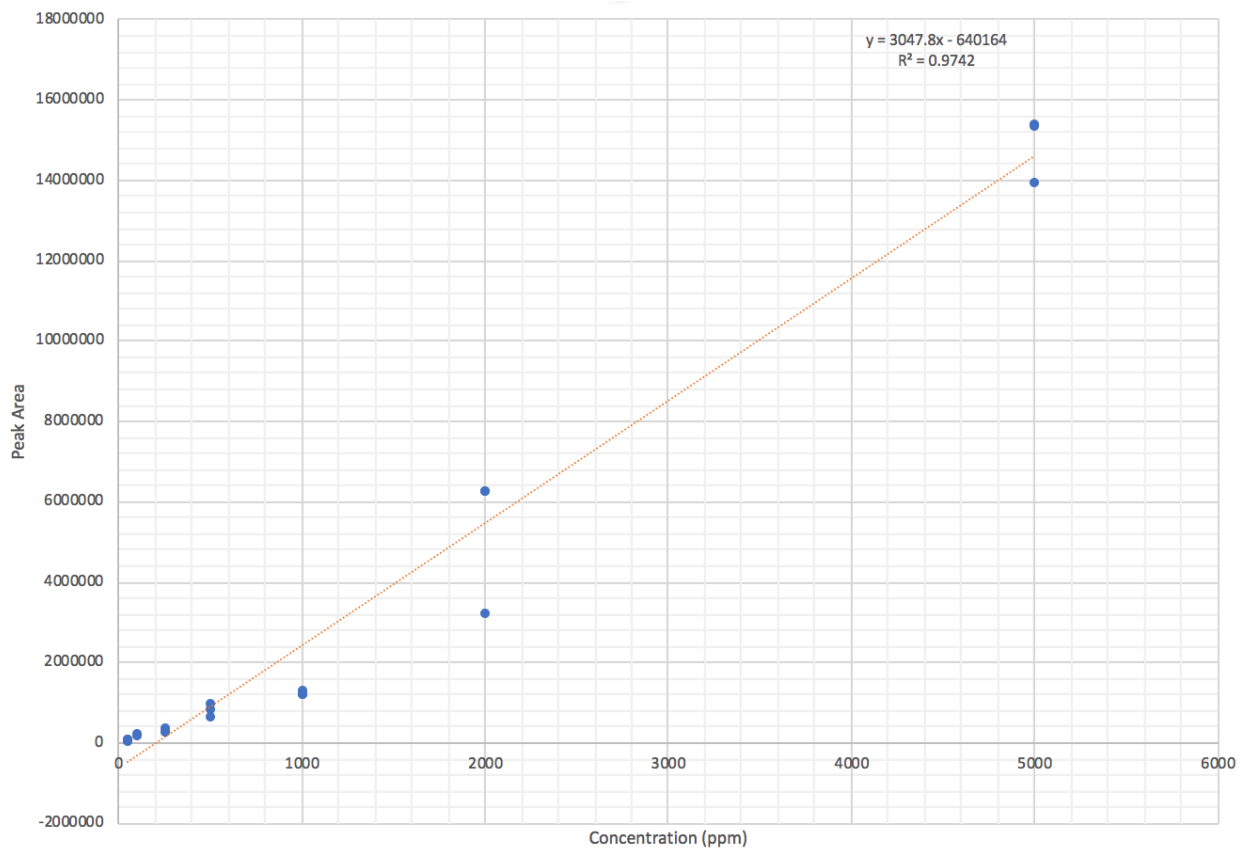


Figure 5. The beta-hydroxybutyrate standard curve using the GC-MS.

For the AcAc standard curve, the GC-MS was not able to detect the ketone body standards below 1000 ppm. It also produced unreliable signals for standards that were greater than 500 ppm as the 2000 ppm standard seemed to have a peak area consistently larger than 5000 ppm. Because the chromatogram had many foreign peaks as well, it was suspected that the standard was contaminated. Upon obtaining a second batch of AcAc, it also seemed to have foreign peaks and difficulties detecting the AcAc ketone body. Thus, there was no standard curve generated for AcAc.

3.2 The Qualitative Results of the Diet

The participants were to follow a ketogenic diet in an effort to produce ketone bodies within their body. The participants were to eat 3 g of fats for every 1 g of protein and carbohydrates. Each participant documented what they ate, and the time they ate the food at. They were encouraged to eat food until they were full, and not eat a specific amount of calories.

The first meal that was recorded was breakfast. It was defined as a substantial meal that was ingested before 10:30 am. Because each participant had different eating habits, this meal was not forced upon the participants. The food that was consumed by the participants were recorded and displayed in Table 1. It was the least consumed meal as there were two participants (2 and 4) who exclusively did not eat breakfast; Participant 5 and 6 each had breakfast only on one day, and Participants 7 and 8 seemed to have only tea in the morning save for the Day 2 breakfast for Participant 7. For those who did not eat breakfast, their body would be in a fasting state which would increase the amount of triglycerides that were eventually oxidized to produce ketone bodies (Mullins et al. 2011; Kanikarla-Marie and Jain 2016). As for those who ate a breakfast, they mainly ate some form of eggs with or without bacon which are both higher in protein and fat content. This is fine since biochemical pathways will prefer to use triglycerides as an energy source before proteins (Kanikarla-Marie and Jain 2016). Though the protein content is high, the amount of carbohydrates ingested were very low. This is beneficial since the amount of glucose circulating within the blood is minimized. Therefore, it seemed like the participants were following the ketogenic diet during breakfast.

Table 1. The breakfast consumed by each participant during the course of the diet.

	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8	Participant 9	Participant 10
Breakfast Day 1	N/A	Bacon and Eggs	N/A	Omelette	N/A	English tea and 2 cheese strings	N/A	Scrambled eggs with spinach, avocado, and pepperoni stick	N/A
Breakfast Day 2	N/A	Bacon and Eggs	N/A	N/A	Bacon and peanut butter	English tea with some almond milk, omelette cooked in butter with pepper, onions, hams, and mozzarella cheese	Mint green tea	Scrambled eggs	Red pepper slices, black coffee
Breakfast Day 3	N/A	2 egg omelette with onions, green peppers, bacon, cheese, and chicken	N/A	N/A	N/A	English tea with some almond milk	Mint green tea	Greek yogurt and strawberries	Bacon and eggs
Breakfast Day 4	N/A	Bacon and Eggs, berries, and whip cream	N/A	N/A	N/A	N/A	Mint green tea	Greek yogurt and strawberries	Bacon and eggs and black coffee.

The second meal that was recorded was lunch. It was defined as a substantial meal that was ingested after 10:30 am and before 4:00 pm since each participant would have different eating habits and schedules. The food that was consumed by the participants at lunch were recorded and displayed in Table 2. Lunch was eaten by all of the participants, but Participant 3 only consumed lunch 2 out of the 4 days. It's worth considering that this participant was also not consuming breakfast; there is a chance that this participant was producing many ketone bodies as a result of this fast. The meals that were eaten seem to consist of a lot of vegetables, mainly in the form of a salad. There was also a noticeable amount of bacon and pork chops consumed. The vegetables contain many vitamins that the participants would be ingesting during the course of the diet; they themselves have little protein, fat, and carbohydrates. Pork chops have a high protein content, but

there still is a substantial amount of fat which is still relevant for the purpose of the diet. Bacon has more fat then protein, which is extremely beneficial for the purpose of this diet. There does not seem to be any particular meal with a high content of carbohydrates which would therefore hinder the results of this experiment. Thus, it seemed like the participants were following the ketogenic diet during lunch.

Table 2. The lunch consumed by each participant during the course of the diet.

	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8	Participant 9	Participant 10
Lunch Day 1	Black Coffee, Beef Jerky, Cucumber	Chicken breasts with hot sauce	Scrambled eggs cooked in butted with cheese and ketchup	Lettuce wrap burger	Celery with peanut butter, red bell pepper, pepperoni sticks, bacon	2 eggs with siracha, bacon with mustard	4 eggs with bacon	N/A	Red pepper and cucumber
Lunch Day 2	Pork Chops (cooked in butter), broccoli, bell peppers, zucchini, beans (called vegetable medley)	N/A	Chicken, bacon, lettuce, cheese salad	N/A	Pepperoni sticks, chicken sausage, Brie cheese, celery sticks with peanut butter, and 1 red bell pepper	N/A	Omelette with ham	Brie cheese, pepperoni stick, some red pepper, and strawberries	Keto Avocado, Bacon, and goat-cheese salad with ranch dressing
Lunch Day 3	Pork chops with vegetable medley	Raspberries with whipping cream	Scrambled eggs, butter, shredded cheese, and strawberries	Green salad with Chinese-styled chicken and beef	Zucchini boats	Bacon with mustard and an apple	Bacon, apple, and dark chocolate	Cheddar cheese, chia tea with heavy cream, ½ green pepper	Keto pancakes with raspberries
Lunch Day 4	Cucumber and cheese	N/A	Pepperoni sticks and pork chop	Chicken Caesar salad	Spinach and goat cheese salad with olive oil	Tea with Steak and parmesan cheese chips	Steak and dark chocolate	Salad with cheddar cheese, feta, eggs, bacon, and Caesar dressing	Keto pancakes with raspberries and strawberries

The third meal that was recorded was dinner. It was defined as a substantial meal that was ingested after 4:00 pm and before 8:30 pm since each participant would have different eating habits and schedules. The food that was consumed by the participants at dinner were recorded and displayed in Table 3. Every participant ate dinner for the duration of the ketogenic diet, except for Participant 9 who did not eat dinner on Day 4. There seemed to be a large amount of protein eaten

during this time; the protein includes burgers and chicken mainly, but other options like pork chops and steak. The burgers all seem to have been eaten with a lettuce bun, save for Participant 8 on Day 2. This is beneficial since the bread is a source of carbohydrates which would have potentially tamper the results of the experiment. As for Participant 8, the participant seemed to still consume a high enough content of fats in comparison to the amount of carbohydrates eaten, so the effect of glucose within the bloodstream may be minimized. Chicken has a higher content of protein in it, but there still is a fat content making it suitable for the diet. Other than the previously mentioned dinner, there does not seem to be any particular meal with a high content of carbohydrates which would therefore hinder the results of this experiment. The tacos consumed by Participants 7 and 8 slightly raise a flag because the taco shell contains carbohydrates, but there should be enough fat within the meal that minimizes the effects of the carbohydrates. Thus, it seemed like the participants were following the ketogenic diet during dinner.

Table 3. The dinner consumed by each participant during the course of the diet.

	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8	Participant 9	Participant 10
Dinner Day 1	Pork Chops, green Beans, bell peppers, broccoli, zucchini	2 beef patties, lettuce wrap, onion, hot sauce	2 Burgers with cheese, pickles, bacon, onions, mayo, mustard, and lettuce as a bun	Green salad and chicken wings	Hard-boiled egg. A salad with bacon, avocado, and goat cheese.	3 tacos with lettuce as a bun, ground beef, onion, red and green peppers, cheese, and hot sauce	3 tacos with lettuce as a bun, ground beef, onion, red and green peppers, cheese, and hot sauce	Spinach salad with bacon, goat cheese, and avocado	Bacon and eggs
Dinner Day 2	Pork chops, bacon, vegetable medley, cheese	Rotisserie chicken	2 Burgers with cheese, pickles, bacon, onions, mayo, mustard, and lettuce as a bun	Chicken burger (with lettuce as the bun), Green salad	Bacon and avocado salad with spinach and goat cheese, and 2 pepperoni sticks	Burger with lettuce bun, cheddar cheese, tomato, pickles, bacon, special sauce, served with a side Caesar salad with bacon and no croutons	Cheeseburger and Caesar salad without croutons	Zucchini boats with pepperoni sticks, parmesan, and goat cheese	Pork chops with green beans and pickles
Dinner Day 3	Chicken and avocado salad (with cucumbers, bell peppers, kale, lettuce)	Chicken breasts and bacon	Caesar salad (bacon cheese, lettuce, grilled chicken, and Caesar dressing)	Burgers with lettuce bun	Cucumber, green bell pepper with cream cheese, peanut butter, bacon, cheese, and bacon-cheese balls.	Steak and left-overs from the tacos from Dinner Day 1	Jerk chicken legs, pepperoni, and cheese string. Later was steak	Zucchini boats and a pepperoni stick	Pork chops with green beans
Dinner Day 4	Avocado Salad (with cheese, cucumber, lettuce, bacon, olive oil)	Chicken breasts	Caesar salad (bacon cheese, lettuce, grilled chicken, and Caesar dressing)	Southern Chicken and Beef salad	2 beef burgers in iceberg lettuce with Brie, garlic aioli, and onion	Jerk chicken leg, one hot Italian sausage, veggie stir fry	chicken leg, one hot Italian sausage, veggie stir fry	N/A	Keto Avocado, Bacon, and goat-cheese salad with ranch dressing

The last meal that was recorded was snacks that would occur throughout the day. To be a snack, it had to be eaten at a separate time from when the three major meals were consumed and in a lesser amount. Snacks also included beverages that were had throughout the day that weren't designated as a breakfast drink or water. The food that was consumed by the participants as snacks were recorded and displayed in Table 4. Majority of the participants had snacks throughout the day. The snacks seem to be potentially worrisome in regard to the carbohydrates being consumed. For instance, Participants 7 and 8 both consumed beer and carrots which are extremely high in carbohydrates. Their dinners also had a higher amount of carbohydrates in comparison to the other

participants. Because of this, they may have ingested more carbohydrates than fats, and compromised their results for the experiment. Of course, this would have to be determined based on the analysis of their biological fluids. Pepperoni sticks were the most common snack that was ingested. This food contains an extremely high concentration of fat in it, a moderate amount of protein, and essentially no carbohydrates. This was an ideal food to snack on during the duration of the diet. Participants 7 and 8 did eat these, but it is unclear if they ate enough for it to make the carbohydrates they consumed to follow the 3:1 ketogenic diet ratio. In general, it seemed like the snack foods had very little carbohydrates in it.

Table 4. The snacks consumed by each participant during the course of the diet.

	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8	Participant 9	Participant 10
Snacks Day 1	Pepperoni Sticks	Beef jerky	N/A	N/A	Coffee, pepperoni sticks, and bacon	Parmesan cheese chips, strawberries, salami, cheese string, some dark chocolate, and tea.	Carrots, tea, cheese strings, pepperoni sticks, apple, dark chocolate	Red pepper, pepperoni sticks, and a cup of halotop ice cream	Pepperoni sticks
Snacks Day 2	Cucumber, pepperoni, cheese, pork rinds	Almonds and beef jerky	3 eggs cooked in butter with shredded cheese and pepper	Raspberries, strawberries, and blueberries	Coffee	Strawberries, carrots, cheese strings, hot pepperoni sticks, pickles, double gin and tonic	Dark chocolate, apple, pepperoni sticks, and cheese strings	Haltotop ice cream, Brie cheese	Pepperoni sticks and cucumber
Snacks Day 3	Pepperoni sticks, cheese	Almonds and beef jerky	N/A	N/A	Coffee, bacon-cheese balls, celery sticks with peanut butter, red bell pepper with cream cheese	3 beers, strawberries, dark chocolate	4 beers	Peanut butter and halotop ice cream.	Halotop ice cream, pepperoni sticks
Snacks Day 4	Pepperoni sticks, cheese	N/A	Pepperoni sticks, strawberries, peanut butter, ketogenic pancakes	N/A	Coffee, Bacon-cheese balls, celery stick with cream cheese, and 3 pepperoni sticks	Tea with almond milk, hot salami, cheese strings, strawberries	Berries	Brie cheese, cheddar cheese, strawberries, and pepperoni sticks, peanut butter	Salad with avocado, cheese, beans, tomatoes, and corn

In general, the participants overall ate minimal carbohydrates, but they did consume a large amount of protein and fat. The targeted ratio of fat to protein and carbohydrates was supposed to be 3:1. Based on the food that was ingested, it seems like the amount of fats consumed was essentially the same compared to the amount of fats, and that the carbohydrates consumed were much lower. It seemed like the participants ended up following the Atkins diet eating a ratio closer to 60% fats, 30 % protein, and 10% carbohydrates, as opposed to the classical diet (Kang et al. 2007). Even though the diet has changed, blood ketosis is still probable as previous studies have recorded patients having BHB levels greater than 3 mM within 3 days of following the diet (Kang

et al. 2007). But it is worth noting that the amount of food and the way it was cooked was not recorded, so there is a chance that there are more carbohydrates within the meals than was written by the participants. There is also a chance that the participants did not write down all of the food they ate, and they cheated on the diet. Though difficult to prove, it is worth considering when observing the results from the analysis.

3.3 The Quantification of Ketone Bodies

3.3.1 Quantification in Blood

Blood is one of the most accurate ways to determine the concentration of ketone bodies within the individual as ketone bodies circulate through the body via blood (Laffel 1999; Kanikarla-Marie and Jain 2016; Brooke et al. 2016). The sample was then brought back to physiological pH because acetoacetate was better able to be derivatized and detected at pH 7 compared to below pH 2. The samples were analyzed in duplicate on the GC-MS, and the results are displayed in Table 5. Based on the results, it seems like Participant 2, 4, 5, and 8 seem to have increased the amount of BHB within their blood. Each trial of Participant 2's blood was able to detect BHB, which strengthens the argument that there was an increase in the levels of BHB circulating their body. Both Participants 4 and 8 did not have a BHB signal detected before the diet occurred, which could mean that the amount of BHB within their system was below the LOD and LOQ of the instrument, or that there was variation within the instrument during the different runs. Participants 6, 7, 9, and 10 seem to have a lower amount or similar amount of BHB in the after-the-diet compared to the before-the-diet samples. This could result from the participants not following the diet that was prescribed to them. By eating carbohydrates, their body would not have shunted glycolysis and the citric acid cycle, which would have resulted in little amounts of ketone

bodies produced. Another reason why BHB may have been detected could be a result of the participants fasting longer than 12 h as the concentration of BHB increases over time (Marinou et al. 2011). Statistics were not conducted since not every participant was able to generate average values of BHB before and after the diet.

Table 5. The experimentally determined amounts of beta-hydroxybutyrate in the blood samples.

Participant Number	Trial Number	BHB Before Diet (ppm)	Retention Time (min)	Average BHB Before Diet (ppm)	BHB After Diet (ppm)	Retention Time (min)	Average BHB After Diet (ppm)
2	1	245.46	6.520	274.96	390.89	6.521	398.09
2	2	304.48	6.519		405.29	6.520	
3	1	0	N/A	N/A	0	N/A	N/A
3	2	248.90	6.519		268.83	6.520	
4	1	0	N/A	N/A	240.48	6.522	245.45
4	2	0	N/A		250.43	6.520	
5	1	0	N/A	N/A	0	N/A	N/A
5	2	246.69	6.519		339.99	6.520	
6	1	0	N/A	N/A	0	N/A	N/A
6	2	247.04	6.520		258.12	6.520	
7	1	250.64	6.519	261.75	247.34	6.518	247.05
7	2	272.86	6.520		246.75	6.5117	
8	1	0	N/A	N/A	240.24	6.52	242.22
8	2	0	N/A		244.21	6.518	
9	1	0	N/A	N/A	0	N/A	N/A
9	2	266.06	6.521		283.55	6.646	
10	1	0	N/A	N/A	239.60	6.519	254.09
10	2	257.57	6.519		268.58	6.518	

Interestingly enough, it seems like the results agreed with the results that were expected within the blood. By converting the concentrations in ppm to mM, the amount of BHB within the blood samples seem to agree with the results of previously found by Owen et al. (1967), Owen et al. (1969), Galvin et al. (1968) and Garber et al. (1974) being in between around 1-2 mM after 4 days. There is variation between individuals as everyone has a slightly different metabolism and ate slightly different foods. It is also worth acknowledging that Participant 2 had a concentration

of 3.82 mM of BHB which refers to potential ketoacidosis arising based on the symptom ketonaemia (Laffel 1999; Usher-Smith et al. 2011). It is unknown if that was the situation since the blood glucose and blood pH was not measured, but it is possible.

Within the blood, there was no AcAc signals detected in any of the samples. It is possible that there was AcAc within the blood samples, but the concentration was below the LOD. In the future, certified reference material should be utilized during method development to help quantify the AcAc from within the blood.

There are many runs where there is no signal detected. It does not necessarily mean that there were no ketone bodies present within the fluid, but that it may have been below the LOD. As well, there was no internal standard used which would have been used to determine if there was any variation between runs. It does seem like there was variation between runs since some samples have BHB detected in one trial, but not the other trial. It is unknown what could have caused that variation between runs to occur.

3.3.2 Quantification in Urine

The participants also had their urine collected before and after conducting the diet. The samples were analyzed in duplicate on the GC-MS, and the results are displayed in Table 6. There was no AcAc detected in the urine. This is somewhat surprising since it is measured in commercial methods currently (Brooke et al. 2016). A possible reason could be that the urine had an acidic pH and was not adjusted to pH 7. Because of this, there needs to be further method development conducted to ensure that AcAc is observed within urine. As well, there was an extremely high amount of BHB recorded within urine. The amount of BHB was negligible for all of the participants before the diet, but they almost all seemed to have higher BHB levels within their

urine than in their blood. Statistics were not conducted since not every participant was able to generate average values of BHB before and after the diet.

Table 6. The experimentally determined amounts of beta-hydroxybutyrate in the urine samples.

Participant Number	Trial Number	BHB Before Diet (ppm)	Retention Time (min)	Average BHB Before Diet (ppm)	BHB After Diet (ppm)	Retention Time (min)	Average BHB After Diet (ppm)
2	1	0	N/A	N/A	7461.37	6.521	9572.11
2	2	0	N/A		11682.85	6.520	
3	1	0	N/A	N/A	871.75	6.520	663.56
3	2	0	N/A		455.37	6.521	
4	1	0	N/A	N/A	294.94	6.523	369.18
4	2	0	N/A		443.423	6.521	
5	1	0	N/A	N/A	276.17	6.522	278.74
5	2	0	N/A		281.31	6.523	
6	1	0	N/A	N/A	1133.22	6.520	1042.82
6	2	0	N/A		952.41	6.519	
7	1	0	N/A	N/A	324.80	6.520	349.49
7	2	0	N/A		374.19	6.520	
8	1	0	N/A	N/A	269.51	6.523	275.54
8	2	0	N/A		281.56	6.524	
9	1	0	N/A	N/A	350.65	6.520	366.65
9	2	0	N/A		382.65	6.520	
10	1	0	N/A	N/A	1103.14	6.519	1553.35
10	2	0	N/A		2003.56	6.520	

In a study conducted by Liu et al., they observed participants biological fluids after fasting a minimum of 9 h and found 0.12 μM of BHB in urine compared to 68.8 μM in plasma (2015). Based on this piece of data, it seems like BHB is at a higher concentration within the bloodstream than it is within urine. But in the past, as ketosis occurs for a longer period of time, the amount of AcAc in urine decreases and the amount of BHB excreted increases (Galvin et al. 1968). Though, it is difficult to compare how much AcAc and BHB were analyzed since it was in units of μmol/min

(Galvin et al. 1968). Nonetheless, it is still believed that the amount of BHB detected is positively skewed since the urine is acidic. Having acidic urine would likely derivatize more BHB within the vial, thus a higher concentration was seen by experiment. To know for sure, the experiment would have to be conducted again, except there would have to be a control for the pH of urine.

3.3.3 Quantification in Saliva

Saliva was also collected and analyzed to see how much ketone bodies were present. Saliva is becoming a more popular diagnostic model to use as it is less invasive and isotonic to plasma (Aps and Martens 2005; Elmongy and Abdel-Rehim 2016; Proctor 2016). The concept of quantifying ketone bodies within saliva in ketotic participants is rather novel. The samples were analyzed in duplicate on the GC-MS, and the results are displayed in Table 7. There was no AcAc detected in the saliva. It was expected that it would have very little amounts of AcAc since saliva is isotonic to blood which contains a small amount of AcAc in it (Liu et al. 2015). As for BHB, there was very little signal that was detected overall. There were 4 signals that were obtained from the before-the-diet samples. The concentration of BHB in these samples are very similar to the results obtained in these participants bloods samples. There were only two signals obtained in the after-the-diet samples by Participant 3 and 4. These signals were smaller than the signal detected in the before-the-diet sample for these participants. Theoretically, it should be higher since the ketogenic diet would have occurred, increasing the amount of ketone bodies within the individuals. Statistics were not conducted since not every participant was able to generate average values of BHB before and after the diet.

Table 7. The experimentally determined amounts of beta-hydroxybutyrate in the saliva samples.

Participant Number	Trial Number	BHB Before Diet (ppm)	Retention Time (min)	Average BHB Before Diet (ppm)	BHB After Diet (ppm)	Retention Time (min)	Average BHB After Diet (ppm)
2	1	253.70	6.520	249.59	0	N/A	N/A
2	2	245.48	6.520		0	N/A	
3	1	253.22	6.521	N/A	0	N/A	N/A
3	2	0	N/A		243.84	6.519	
4	1	244.44	6.518	N/A	240.48	6.522	N/A
4	2	0	N/A		0	N/A	

In a study conducted by Liu et al., they previously observed participants biological fluids after fasting a minimum of 9 h and found 1.74 μM of BHB in saliva compared to 68.8 μM in plasma (2015). Based on this result, it would be expected that there is some BHB detected, but not as much as in the blood. There are two potential reasons why the amount of signal detect was very low. The first could be that the amount of BHB within the sample was below the LOD, which is logical considering the amount of BHB in the plasma is almost 40x larger than that observed in the saliva (Liu et al. 2015). The other reason is that there was not a lot of BHB that was derivatized within the sample, so the signal was very low as a result. Likewise, the samples that did produce a signal could have had a very effective derivatization by chance. A way to improve upon this would be to add a recovery standard to determine how effective the derivatization was to ensure that all of the potential analyte was derivatized and detected.

4.0 CONCLUSIONS

In conclusion, participants underwent a ketogenic diet to induce ketosis; samples of their blood, urine, and saliva were collected before and after the diet to determine if BHB and AcAc could be quantified through the GC-MS. The conditions were moderately optimized to obtain the best possible signals for the BHB and AcAc ketone bodies for the time being. A calibration curve for BHB was produced ($R^2 = 0.9742$) and was used to quantify the amount of ketone bodies present in the biological samples. For the participants whose blood samples were able to be quantified, it seems like they were in ketosis and the amount of BHB seems within range of what was expected. The urine samples had a high concentration of BHB, which could be a result of the acidic urine which would help in the derivatization step, so it could be analyzed. There was very little signal detected within saliva, but the fact that signal was detected means that this could potentially act as a method to quantify ketone bodies upon more method development.

5.0 FUTURE WORK

Future work for this research would look to optimize the detection of AcAc from biological fluids. Possible ideas include trying to separate this ketone body from the BHB and using a different derivatizing agent to make it volatile. Future work should also consider using two recovery standards to know how effective the derivatization is, and if any analyte is lost during the matrix evaporation step. Adding an internal standard would be useful to determine if there is any variation between runs. Another idea for future work would be to control the pH across all biological fluids since it seems like urine produced a higher signal because the acidity of the matrix favored the derivatization reaction to occur. Lastly, optimizing a better method to extract, derivatize, and analyze the BHB from saliva. Possible ideas include acidifying the pH of a sample to protonate the analyte in an effort to do liquid-liquid microextraction with hexane. Then, concentrating the solution and derivatizing it. This may be effective for detecting BHB, so the future work on AcAc will dictate if the method would change to detect the amount of AcAc in the biological fluid. Potentially, this method could be used on all biological fluids as well.

6.0 LITERATURE CITED

Aps JKM, Martens LC. 2005. Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci. Int. 150:119–131. doi:10.1016/j.forsciint.2004.10.026.

Branco AF, Ferreira A, Simões RF, Magalhães-Novais S, Zehowski C, Cope E, Silva AM, Pereira D, Sardão VA, Cunha-Oliveira T. 2016. Ketogenic diets: From cancer to mitochondrial diseases and beyond. Eur. J. Clin. Invest. doi:10.1111/eci.12591.

Brooke J, Stiell M, Ojo O. 2016. Evaluation of the Accuracy of Capillary Hydroxybutyrate Measurement Compared with Other Measurements in the Diagnosis of Diabetic Ketoacidosis : A Systematic Review. :1–9. doi:10.3390/ijerph13090837.

Butalia S, Kaplan GG, Khokhar B, Rabi DM. 2016. Environmental Risk Factors and Type 1 Diabetes: Past, Present, and Future. Can. J. Diabetes 40:586–593. doi:10.1016/j.jcjd.2016.05.002.

Courchesne-Loyer A, Fortier M, Tremblay-Mercier J, Chouinard-Watkins R, Roy M, Nugent S, Castellano CA, Cunnane SC. 2013. Stimulation of mild, sustained ketonemia by medium-chain triacylglycerols in healthy humans: Estimated potential contribution to brain energy metabolism. Nutrition 29:635–640. doi:10.1016/j.nut.2012.09.009.

Dholakia S, Mittal S, Quiroga I, Gilbert J, Sharples EJ, Ploeg RJ, Friend PJ. 2016. Pancreas Transplantation: Past, Present, Future. Am. J. Med. 129:667–673. doi:10.1016/j.amjmed.2016.02.011.

Elmongy H, Abdel-Rehim M. 2016. Saliva as an alternative specimen to plasma for drug bioanalysis. A review. TrAC - Trends Anal. Chem. 83:70–79. doi:10.1016/j.trac.2016.07.010.

Føreid S, Gadeholt G. 2017. Beta-hydroxybutyrate and pyroglutamate can be included in a rapid GC-MS screening method for differential diagnosis of metabolic acidosis. Scand. J. Clin. Lab. Invest. 77:149–152. doi:10.1080/00365513.2016.1278261.

Fujii S, Maeda T, Noge I, Kitagawa Y, Todoroki K, Inoue K, Min JZ, Toyo'oka T. 2014. Determination of acetone in saliva by reversed-phase liquid chromatography with fluorescence detection and the monitoring of diabetes mellitus patients with ketoacidosis. Clin. Chim. Acta 430:140–144. doi:10.1016/j.cca.2014.01.006.

Galvin RD, Harris JA, Johnson RE. 1968. Urinary Excretion of Beta-Hydroxybutyrate and Acetoacetate During Experimental Ketosis. Q. J. Exp. Physiol. Cogn. Med. Sci. 53:181–193. doi:10.1113/expphysiol.1968.sp001958.

Garber AJ, Menzel PH, Boden G, Owen OE. 1974. Hepatic ketogenesis and gluconeogenesis in humans. J. Clin. Invest. 54:981–989. doi:10.1172/JCI107839.

Giugliano D, Ceriello A, Esposito K. 2008. Glucose metabolism and hyperglycemia. Am. J. Clin. Nutr. 87:217S–222S. doi:10.1093/ajcn/87.1.217S.

Hassan HM a, Cooper G a a. 2009. Determination of -Hydroxybutyrate in Blood and Urine Using Gas Chromatography--Mass Spectrometry. J. Anal. Toxicol. 33:502–507. doi:10.1093/jat/33.8.502.

Holm KMD, Linnet K, Rasmussen BS, Pedersen AJ. 2010. Determination of Ketone Bodies in Blood by Headspace Gas Chromatography-Mass Spectrometry. J. Anal. Toxicol. 34:549–554. doi:10.1093/jat/34.9.549.

Kang HC, Lee HS, You SJ, Kang DC, Ko TS, Kim HD. 2007. Use of a modified Atkins diet in intractable childhood epilepsy. Epilepsia 48:182–186. doi:10.1111/j.1528-1167.2006.00910.x.

Kanikarla-Marie P, Jain SK. 2016. Hyperketonemia and ketosis increase the risk of complications in type 1 diabetes. Free Radic. Biol. Med. 95:268–277. doi:10.1016/j.freeradbiomed.2016.03.020.

Laffel L. 1999. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes. Metab. Res. Rev. 15:412–26.

Linstrom PJPJ, Mallard WGG. 2001. NIST Chemistry webbook; NIST standard reference database No. 69 . NIST Chem. Webb.:20899. doi:10.18434/T4D303.

Liu SL, Oyama T, Miyoshi Y, Sheu SY, Mita M, Ide T, Lindner W, Hamase K, Lee JA. 2015. Establishment of a two-dimensional chiral HPLC system for the simultaneous detection of lactate and 3-hydroxybutyrate enantiomers in human clinical samples. J. Pharm. Biomed. Anal. 116:80–85. doi:10.1016/j.jpba.2015.05.036.

Marinou K, Adiels M, Hodson L, Frayn KN, Karpe F, Fielding BA. 2011. Young women partition fatty acids towards ketone body production rather than VLDL-TAG synthesis, compared with young men. Br. J. Nutr. 105:857–865. doi:10.1017/S0007114510004472.

Morales J, Schneider D. 2014. Hypoglycemia. Am. J. Med. 127:S17–S24. doi:10.1016/j.amjmed.2014.07.004.

Mullins G, Hallam CL, Broom I. 2011. Ketosis, ketoacidosis and very-low-calorie diets: putting the record straight. Nutr. Bull. 36:397–402. doi:10.1111/j.1467-3010.2011.01916.x.

Nickerson JG, Alkhateeb H, Benton CR, Lally J, Nickerson J, Han XX, Wilson MH, Jain SS, Snook LA, Glatz JFC, et al. 2009. Greater transport efficiencies of the membrane fatty acid transporters FAT/CD36 and FATP4 compared with FABPpm and FATP1 and differential effects on fatty acid esterification and oxidation in rat skeletal muscle. J. Biol. Chem. 284:16522–16530. doi:10.1074/jbc.M109.004788.

Niwa T. 1995. Basic theory of mass spectrometry. Clin. Chim. Acta. 241–242:15–71. doi:10.1016/S0009-8981(00)89104-4.

O'Neill HM, Holloway GP, Steinberg GR. 2013. AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. Mol. Cell. Endocrinol. 366:135–151. doi:10.1016/j.mce.2012.06.019.

Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF. 1967. Brain metabolism during fasting. J. Clin. Invest. 46:1589–1595. doi:10.1172/JCI105650.

Owen OE, Wahren J, Jr GFC, Owen OE, Felig P, Morgan AP, Wahren J, Cahill GF. 1969. Liver and kidney metabolism during prolonged starvation Find the latest version : Liver and Kidney Metabolism during Prolonged Starvation. 48:574–583. doi:10.1172/JCI106016.

Paul R, Tsanaclis L, Kingston R, Berry A, Guwy A. 2006. GC-MS-MS determination of gamma-hydroxybutyrate in blood and urine. J. Anal. Toxicol. 30:375–379. doi:10.1093/jat/30.6.375.

Pociot F, Lernmark Å. 2016. Genetic risk factors for type 1 diabetes. Lancet 387:2331–2339. doi:10.1016/S0140-6736(16)30582-7.

Proctor GB. 2016. The physiology of salivary secretion. Periodontol. 2000 70:11–25. doi:10.1111/prd.12116.

Puchalska P, Crawford PA. 2017. Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics. Cell Metab. 25:262–284. doi:10.1016/j.cmet.2016.12.022.

Roehl K, Sewak SL. 2017. Practice Paper of the Academy of Nutrition and Dietetics: Classic and Modified Ketogenic Diets for Treatment of Epilepsy. J. Acad. Nutr. Diet. doi:10.1016/j.jand.2017.06.006.

Sherwin RS, Hendler RG, Felig P, Haven N. Effect of Diabetes Mellitus and Insulin on the Turnover and Metabolic Response to Ketones in Man. 25.

Steensels S, Ersoy BA. 2019. Fatty acid activation in thermogenic adipose tissue. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1864:79–90. doi:10.1016/j.bbalip.2018.05.008.

Tsutsui H, Mochizuki T, Maeda T, Noge I, Kitagawa Y, Min JZ, Todoroki K, Inoue K, Toyo T. 2012. Simultaneous determination of DL-lactic acid and DL-3-hydroxybutyric acid enantiomers in saliva of diabetes mellitus patients by high-throughput LC-ESI-MS/MS. Anal. Bioanal. Chem. 404:1925–1934. doi:10.1007/s00216-012-6320-0.

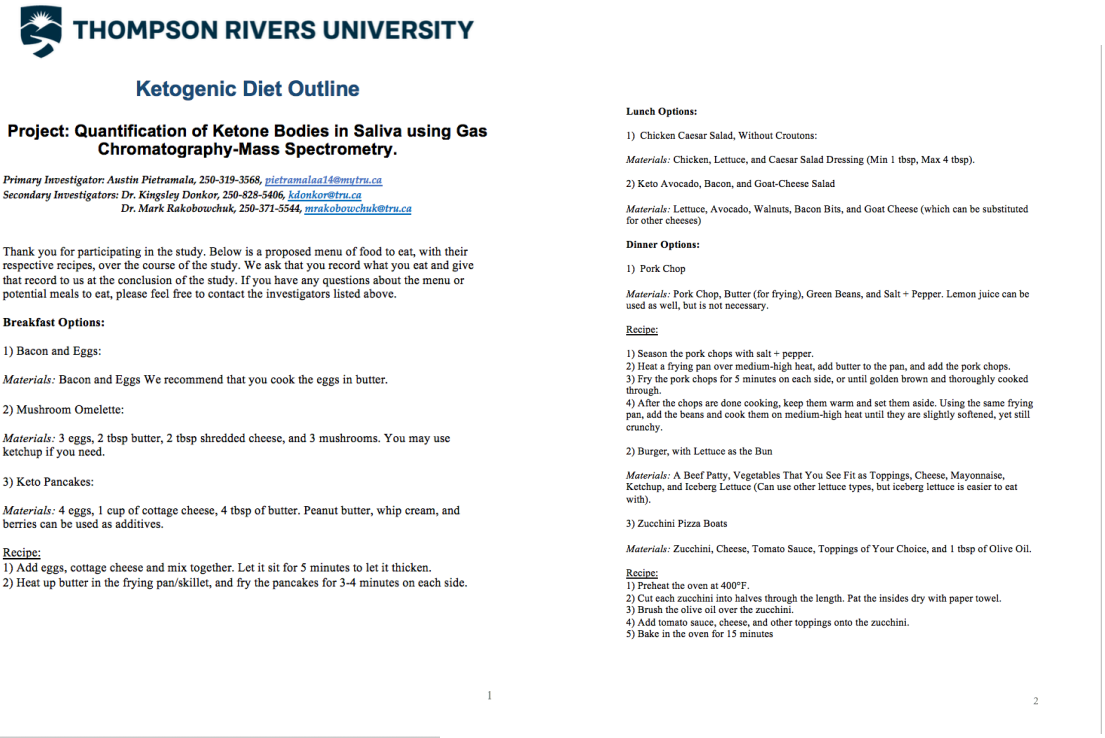
Usher-Smith JA, Thompson MJ, Sharp SJ, Walter FM. 2011. Factors associated with the presence of diabetic ketoacidosis at diagnosis of diabetes in children and young adults: a systematic review. BMJ 343:d4092–d4092. doi:10.1136/bmj.d4092.

Viswanath B, Choi CS, Lee K, Kim S. 2017. Recent trends in the development of diagnostic tools for diabetes mellitus using patient saliva. TrAC - Trends Anal. Chem. 89:60–67. doi:10.1016/j.trac.2017.01.011.

Wibisono C, Rowe N, Beavis E, Kepreotes H, Mackie FE, Lawson JA, Cardamone M. 2015. Ten-year single-center experience of the ketogenic diet: Factors influencing efficacy, tolerability, and compliance. J. Pediatr. 166:1030–1036.e1. doi:10.1016/j.jpeds.2014.12.018.

7.0 APPENDICES

The Ketogenic Diet Outline (3 Figures below)



THOMPSON RIVERS UNIVERSITY

Ketogenic Diet Outline

Project: Quantification of Ketone Bodies in Saliva using Gas Chromatography-Mass Spectrometry.

Primary Investigator: Austin Pietramala, 250-319-3568, pietramalaa14@mytru.ca
Secondary Investigators: Dr. Kingsley Donkor, 250-828-5406, kdonkor@tru.ca
Dr. Mark Rakobowchuk, 250-371-5544, mrakobowchuk@tru.ca

Thank you for participating in the study. Below is a proposed menu of food to eat, with their respective recipes, over the course of the study. We ask that you record what you eat and give that record to us at the conclusion of the study. If you have any questions about the menu or potential meals to eat, please feel free to contact the investigators listed above.

Breakfast Options:

1) Bacon and Eggs:

Materials: Bacon and Eggs We recommend that you cook the eggs in butter.

2) Mushroom Omelette:

Materials: 3 eggs, 2 tbsp butter, 2 tbsp shredded cheese, and 3 mushrooms. You may use ketchup if you need.

3) Keto Pancakes:

Materials: 4 eggs, 1 cup of cottage cheese, 4 tbsp of butter. Peanut butter, whip cream, and berries can be used as additives.

Recipe:
1) Add eggs, cottage cheese and mix together. Let it sit for 5 minutes to let it thicken.
2) Heat up butter in the frying pan/skillet, and fry the pancakes for 3-4 minutes on each side.

1

Lunch Options:

1) Chicken Caesar Salad, Without Croutons:

Materials: Chicken, Lettuce, and Caesar Salad Dressing (Min 1 tbsp, Max 4 tbsp).

2) Keto Avocado, Bacon, and Goat-Cheese Salad

Materials: Lettuce, Avocado, Walnuts, Bacon Bits, and Goat Cheese (which can be substituted for other cheeses)

Dinner Options:

1) Pork Chop

Materials: Pork Chop, Butter (for frying), Green Beans, and Salt + Pepper. Lemon juice can be used as well, but is not necessary.

Recipe:

1) Season the pork chops with salt + pepper.
2) Heat a frying pan over medium-high heat, add butter to the pan, and add the pork chops.
3) Fry the pork chops for 5 minutes on each side, or until golden brown and thoroughly cooked through.
4) After the chops are done cooking, keep them warm and set them aside. Using the same frying pan, add the beans and cook them on medium-high heat until they are slightly softened, yet still crunchy.

2) Burger, with Lettuce as the Bun

Materials: A Beef Patty, Vegetables That You See Fit as Toppings, Cheese, Mayonnaise, Ketchup, and Iceberg Lettuce (Can use other lettuce types, but iceberg lettuce is easier to eat with).

3) Zucchini Pizza Boats

Materials: Zucchini, Cheese, Tomato Sauce, Toppings of Your Choice, and 1 tbsp of Olive Oil.

Recipe:
1) Preheat the oven at 400°F.
2) Cut each zucchini into halves through the length. Pat the insides dry with paper towel.
3) Brush the olive oil over the zucchini.
4) Add tomato sauce, cheese, and other toppings onto the zucchini.
5) Bake in the oven for 15 minutes

2

Snack Options

Possible choices for you to snack on throughout the day:

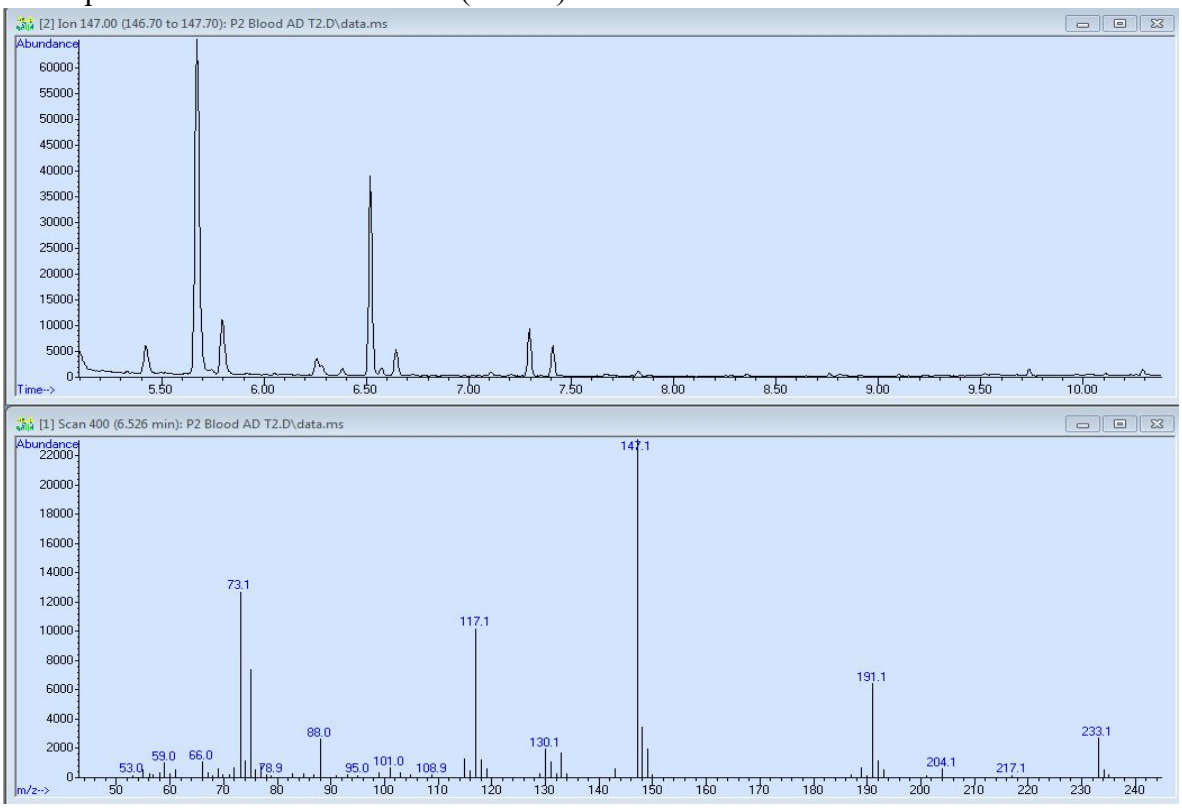
- Pork Grinds
- Pickles
- Celery (Can have with peanut butter and/or cream cheese)
- Cucumber
- Peppers (red, yellow, green)
- Avocado
- Olives
- Kale (kale chips)
- Raspberries
- Strawberries
- Pepperoni Sticks
- Beef Jerky
- Halotop Ice Cream
- Whipping Cream
- Mustard
- Coffee (served black)
- Tea
- Macadamia Nuts
- Almonds
- Peanuts

The list of snacks above is not exhaustive. If you wish to eat a certain snack, beverage, or different meal, contact either Mr. Austin Pietramala or Dr. Mark Rakobowchuk to determine if the food you wish to eat is compatible with the diet. As well, please refrain from eating "sugary foods" like donuts, chocolate bars, and certain fruit bananas and grapes.

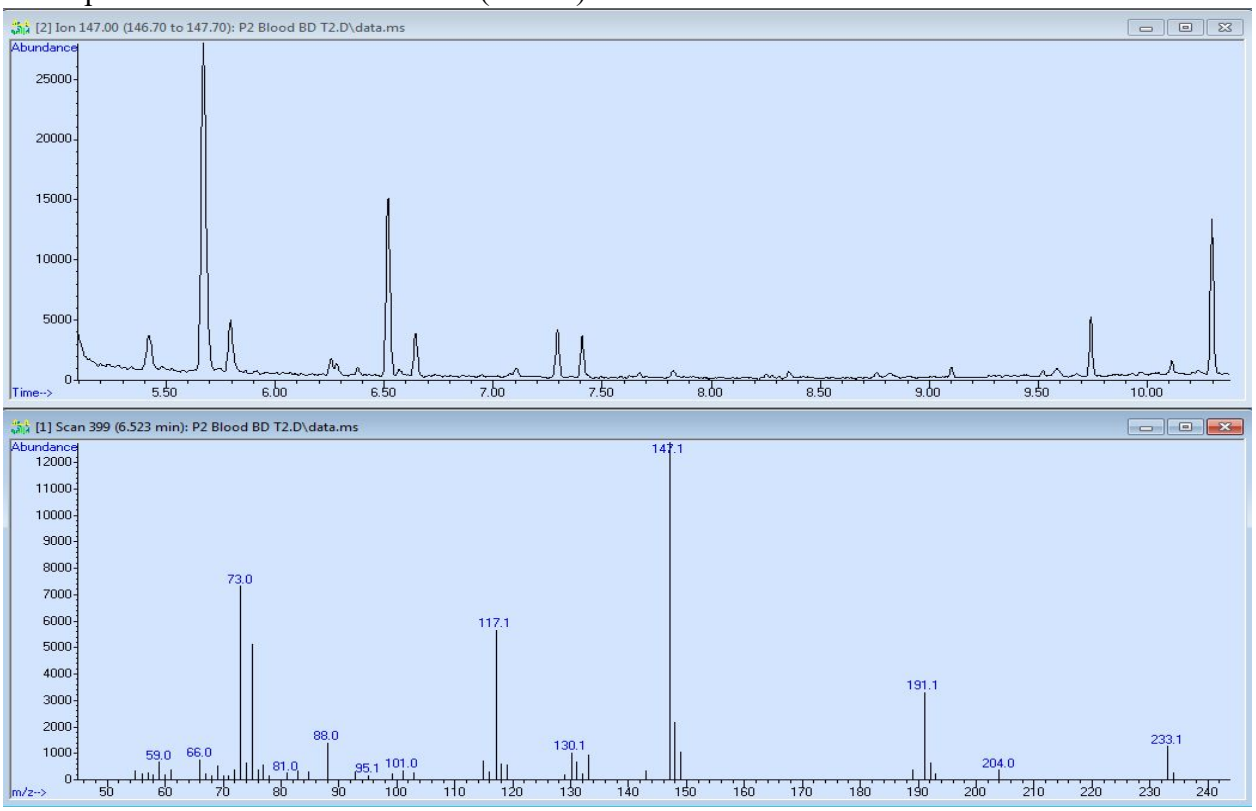
Please be advised that the ketogenic diet can be awkward to get used to at first and you may experience nausea, low blood sugar levels, tiredness, and constipation. If you experience any symptoms of low blood sugar, nausea, or any other side effects, you will be directed to call Dr. Rakobowchuk who will instruct you to consume a glass of fruit juice. Dr. Rakobowchuk will then keep in touch with you for the next few hours. If symptoms do not subside, you will be asked to resume your normal diet.

3

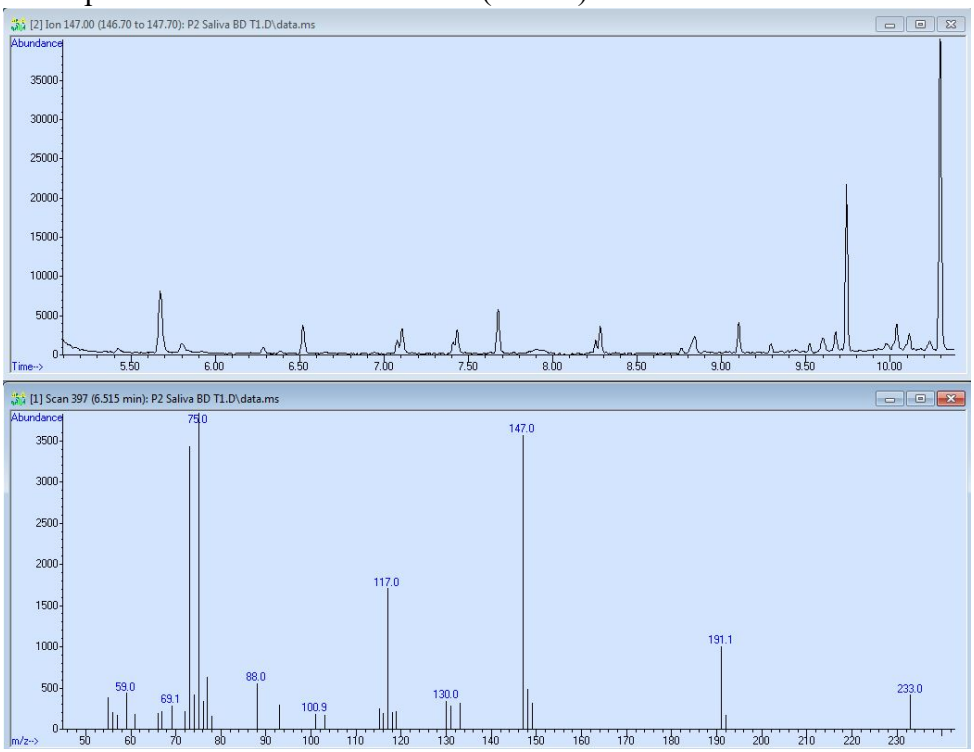
Participant 2 Blood After-The-Diet (Trial 2)



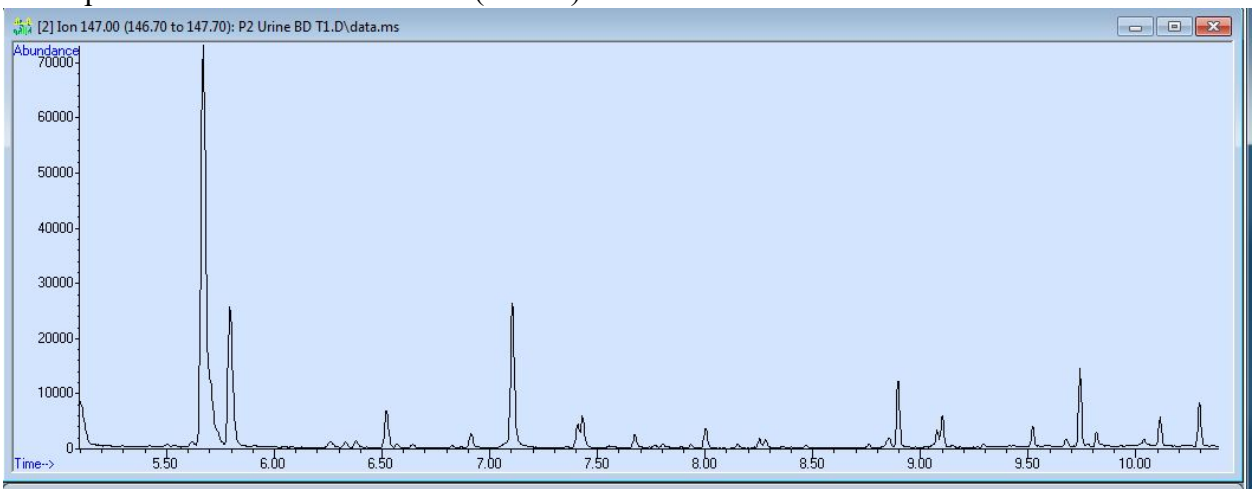
Participant 2 Blood Before-The-Diet (Trial 2)



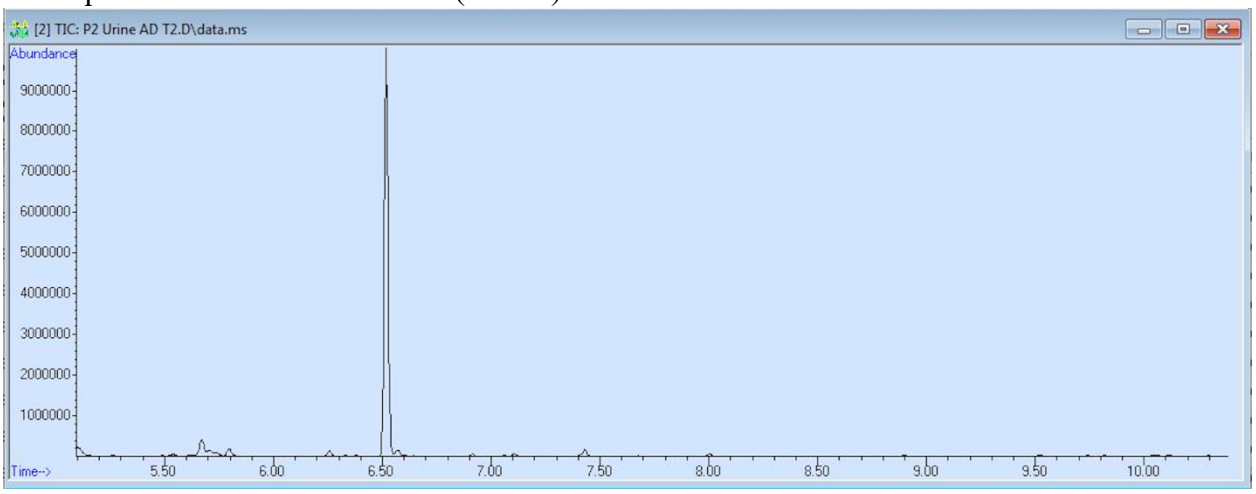
Participant 2 Saliva Before-The-Diet (Trial 1)



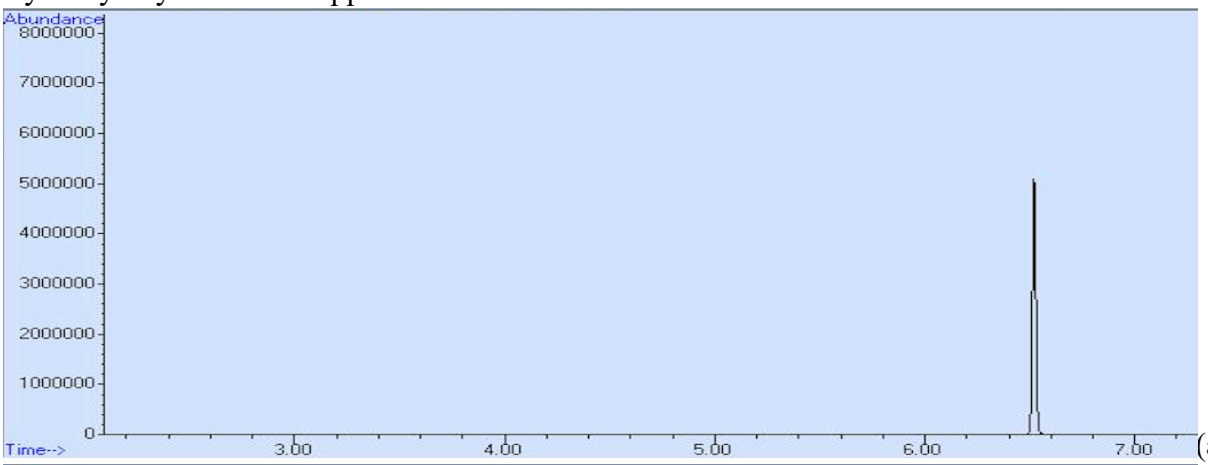
Participant 2 Urine Before-The-Diet (Trial 1)



Participant 2 Urine After-The-Diet (Trial 1)



Comparison Between Acidified Beta-Hydroxybutyrate (a) and Non-Acidified Beta-Hydroxybutyrate at 5000 ppm Each



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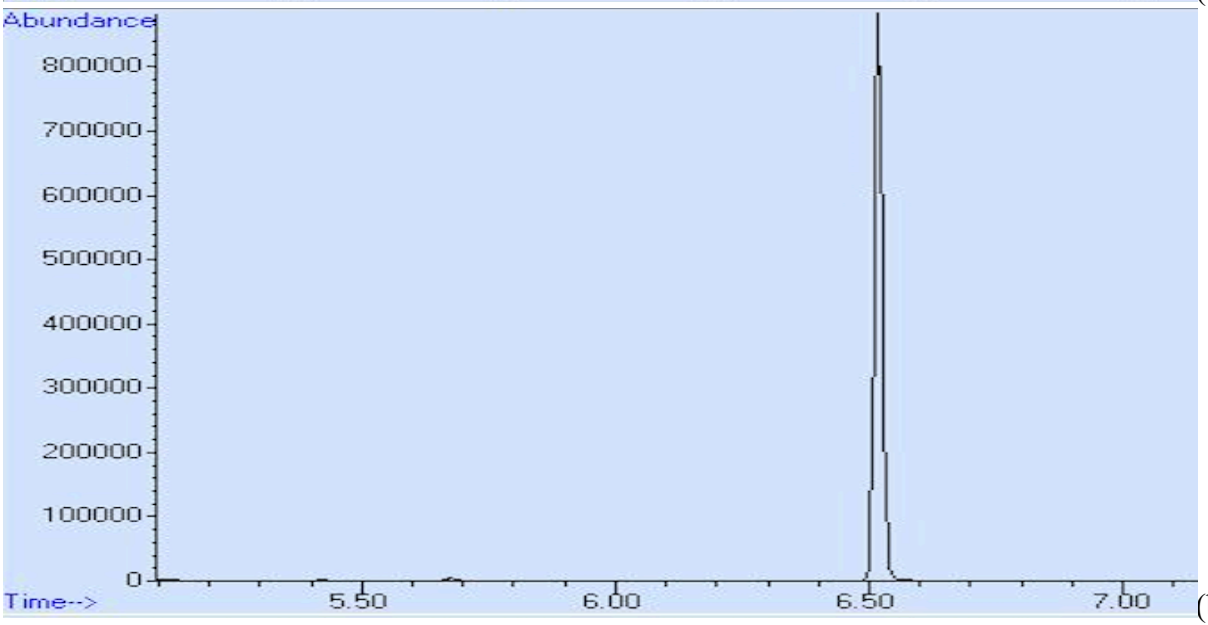

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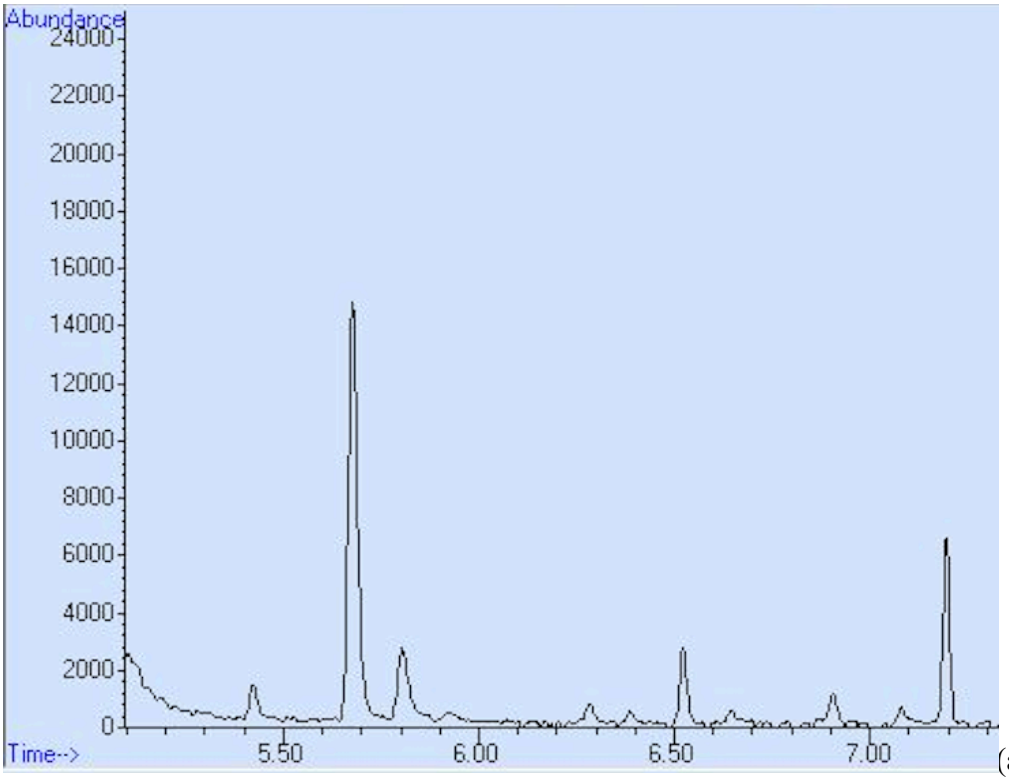
Comparison Between Acidified Acetoacetate(a) and Non-Acidified Acetoacetate at 1600 ppm Each



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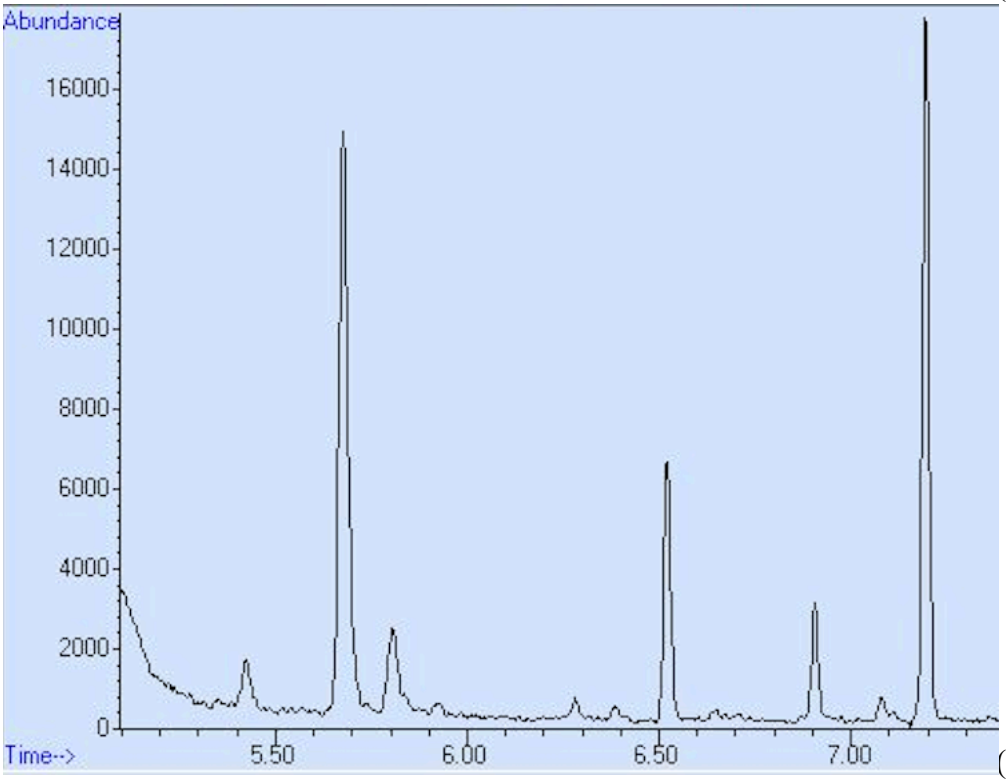

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Ethics Approval

Truromeo

From: do-not-reply-TRU@researchservicesoffice.com
Sent: Thursday, November 08, 2018 1:35 PM
To: Kingsley Donkor; Pietramala Austin(Primary Investigator); Mark Rakobowchuk
Cc: Truromeo; do-not-reply-TRU@researchservicesoffice.com
Subject: REB Approval (COA)

THOMPSON RIVERS UNIVERSITY

November 08, 2018

Mr. Austin Pietramala
Faculty of Science
Thompson Rivers University

File Number: 101956
Approval Date: November 08, 2018
Expiry Date: November 07, 2019

Dear Mr. Austin Pietramala,

The Research Ethics Board has reviewed your application titled 'Quantification of Ketone Bodies in Saliva using Gas Chromatography-Mass Spectrometry'. Your application has been approved. You may begin the proposed research. This REB approval, dated November 08, 2018, is valid for one year less a day: November 07, 2019.

Throughout the duration of this REB approval, all requests for modifications, renewals and serious adverse event reports are submitted via the Research Portal. To continue your proposed research beyond November 07, 2019, you must submit a Renewal Form before November 07, 2019. If your research ends before November 07, 2019, please submit a Final Report Form to close out REB approval monitoring efforts.

If you have any questions about the REB review & approval process, please contact the Research Ethics Office via 250.852.7122. If you encounter any issues when working in the Research Portal, please contact the Research Office at 250.371.5586.

Sincerely,
Joyce O'Mahony
Chair, Research Ethics Board

1

Biosafety Approval

Truromeo

From: do-not-reply-TRU@researchservicesoffice.com
Sent: Wednesday, November 21, 2018 9:04 AM
To: Kingsley Donkor; Pietramala Austin(Primary Investigator); Mark Rakobowchuk
Cc: Truromeo; do-not-reply-TRU@researchservicesoffice.com
Subject: Your Biohazardous Materials Application has been approved.

THOMPSON RIVERS UNIVERSITY

November 21, 2018

Mr. Austin Pietramala
Faculty of Science
Thompson Rivers University

File Number: 101957
Approval Date: November 21, 2018
Expiry Date: November 20, 2019

Dear Mr. Austin Pietramala,

The Biosafety Committee has reviewed your application titled' Quantification of Ketone Bodies in Saliva using Gas Chromatography-Mass Spectrometry'. Your application has been approved. You may begin the proposed research. This BSC approval, dated November 21, 2018, is valid for one year: November 20, 2019. Please post a copy of this letter in your work area prior to the start of your research.

Throughout the duration of this BSC approval, all requests for modifications, renewals and serious adverse event reports are submitted via the Research Portal. To continue your proposed research beyond November 20, 2019, you must submit a Renewal Form before November 20, 2019. If your research ends beforeNovember 20, 2019, please submit a Final Report Form to close out BSC approval monitoring efforts.

If you have any questions about the BSC review & approval process, please contact the Research Ethics Office via 250.852.7122. If you encounter any issues when working in the Research Portal, please contact the Research Office at 250.371.5586.

Sincerely,

Jonathan Van Hamme
Chair, Biosafety Committee

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