NOVEL LC-MS METHOD FOR THE ANALYSIS OF BETA-HYDROXYBUTYRIC ACID (BHB) IN HEALTH FORMULATIONS

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The rise of nutraceutical health formulations has increased the need for more stringent analytical testing methods. Complex matrices present a new problem when determining concentration of compounds of interest. The presented method uses LC-MS analysis with a novel sample preparation method in the determination of beta-hydroxybutyric acid in health formulations. The use of an aqueous analytical column allows for separations of polar compounds after non-polar compounds are removed through C18 packed column filtration. The samples were analyzed through time-of-flight mass spectrometry and results show that this is an effective method for the presented samples with a range of expected concentrations of total BHB was from 11.80% to 38.92%. It was seen that all samples exhibited a less than 10% percent deviation from the expected concentrations of the nutraceutical health samples with the highest being 9.74 % for sample 9 and the lowest being sample 3 with a deviation of 0.08 % from expected values.

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CHAPTER 1

OVERVIEW OF CURRENT ANALYSIS METHODS FOR BETA-HYDROXYBUTYRIC ACID

1.1 Analysis of Gamma-Hydroxybutyric Acid

Hydroxybutyric acid is a group of organic compounds that are comprised of a carboxyl and carboxylic acid functionalization attached to a four-carbon chain. The rise in abuse and sexual assaults using strong neurotransmitters, such as gama-hydroxybutyric acid (GHB), created a need for reliable analysis methods for both law enforcement and prosecutors alike [1]. For a long time, the gold standard method of analysis for crime scene investigators was the functionalization of GHB into a more stable form for analysis by gas chromatography paired with flame ionization detection (GC-FID) or gas chromatography paired with mass spectrometry (GC-MS). The reason for the functionalization is because the molecules charge, small size, low volatility, and breakdown into precursors making analysis difficult [2]. This analysis was usually conducted on hair, urine, and other human specimens pertaining to toxicology reports and cases of foul play. As seen in Couper et al. GHB is functionalized using a derivatization agent of bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane creating the larger molecules that are more stable and easier to distinguish through mass spectrometry [3]. While this method is reliable, it is time intensive and only applicable to samples that have not degraded into other butyric acid derivatives before analysis. Another problem arises in the fact that β-Hydroxybutyric acid is naturally occurring in the human body and can interfere with the analysis as both GHB and BHB has the same molar mass of 104.10 g/mol. Therefore, this makes it almost impossible to distinguish between the two compounds using the GC/MS method of analysis when derivatized or in their native state in the body. The use of GC/MS analysis for

analysis of GHB was the most abundant method from 2001-2010 until LC/MS methods were developed as it has the ability to differentiate between GHB derivatives 1,4-butane-diol (1,4 BD) and gamma-butyrolactone (GBL) [4]. It was thought that methods used for the determination of GHB in blood and human serum could be used for BHB analysis, but the complex matrixes of heath formulations are more reluctant to be easily analyzed.

1.2 Analysis of Beta-Hydroxybutyric Acid

Beta-hydroxybutyric acid is an endogenous ketone that is used within the body for energy purposes when there are not enough carbohydrates available. It is also prevalent in diabetics and people with extensive liver damage caused by alcohol abuse [5]. There has been a significant increase in healthy and unhealthy people putting their bodies in a state of ketoacidosis and therefore creating a market for using BHB powders in their healthcare routines. Existing methods of analysis for BHB focus on derivatizing minimal amounts of the compounds found in hair, urine, and blood samples for forensic and law enforcement purposes exclusively. In the case of Mireault et al. they were able to use the same derivatization agent used in previous GHB methods to produce BHB-2TMS for analysis using GC-MS [6]. This method used a liquid-liquid extraction with methanol as the primary solvent followed by ethyl acetate. The problem with using this method for other types of analysis that are not in the forensic field of human serum is that these derivatization agents sometimes react to form isobaric compounds that interfere with analysis [7]. Current methods for forensic analysis do not account for BHB being an endogenous compound that will interfere with the measurement accuracy in human serums. This causes problems when conducting LC/MS analysis as these compounds could interfere with each other and give false positives and negatives [8]. FTIR

(Fourier transform infrared) and UV-Vis methods do not offer sufficient sensitivity to accurately determine concentrations in complex human serum samples.

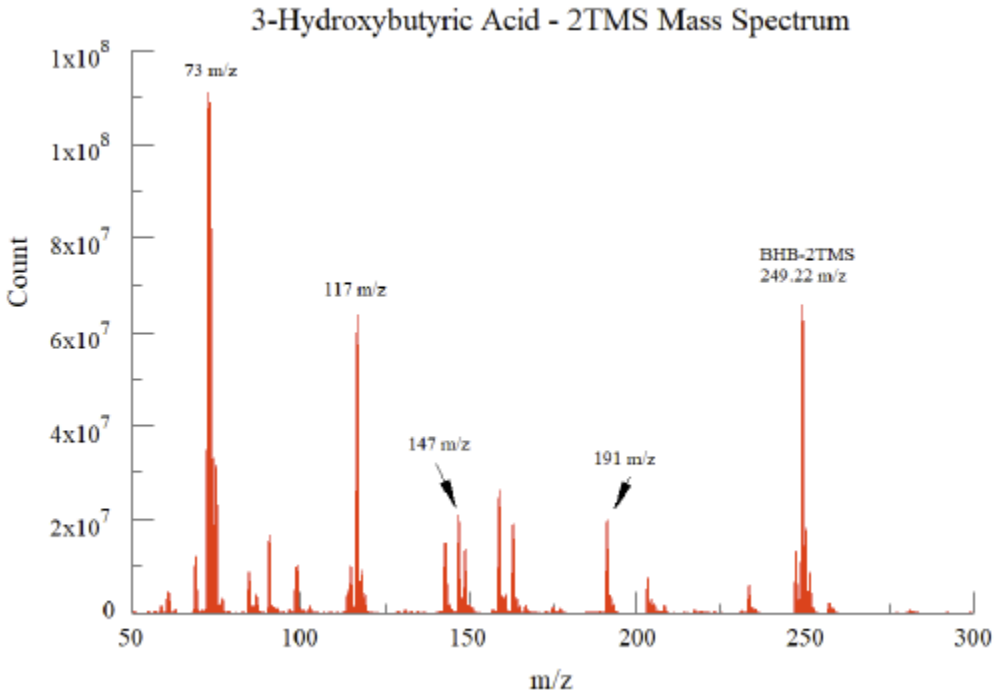

Figure 1.1: GC-MS analysis of derivatized BHB to form BHB-2TMS with respective peak at 249.22 m/z.

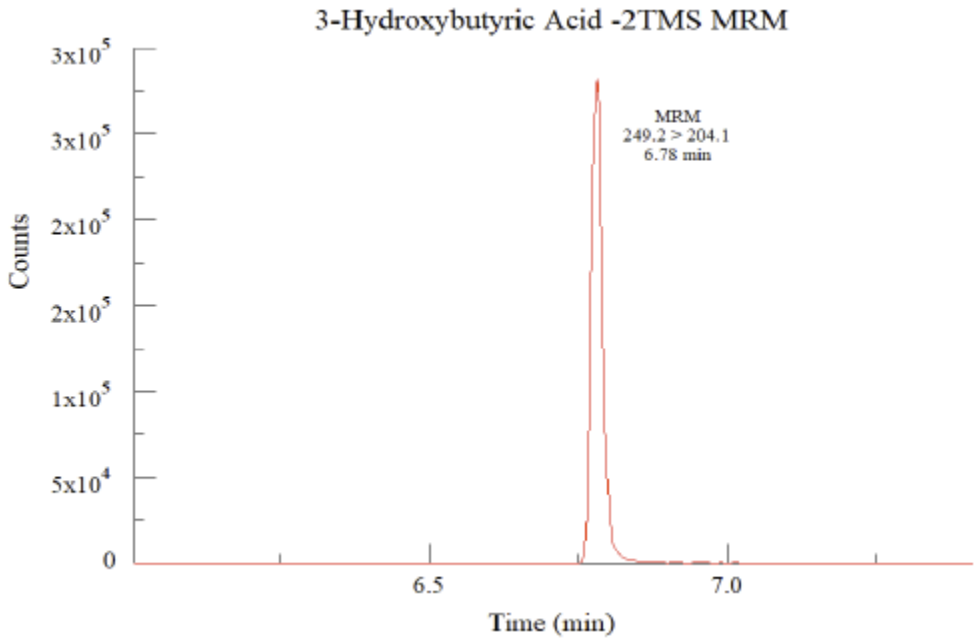

Figure 1.2: GC-MS analysis of derivatized BHB to form BHB-2TMS with respective multiple ion monitoring (MRM) peak at 249.22 > 204.1 m/z.

1.3 Challenges in Analysis of Nutraceutical Formulations

Aside from the lack of regulations associated with nutraceuticals and their presumed contents, the complex matrices of nutraceutical formations present new challenges for analytical chemists. The variability in the contents may include salts, lipids, carbohydrates, enzymes, proteins, nanoparticles, gels, and many other types of compounds making isolation and quantification [17]. Ionization methods such as electrospray ionization are susceptible to ion suppression with the presence of salt ions that reduce the molecular abundance of other ions [18]. This makes analysis of nutraceutical formulations difficult as many products include mineral salts or other forms to give the consumers their electrolytes. The wide range of compounds with differing concentrations lead to time-consuming method development for isolating compounds and removing incompatibles before running through GC-MS and LC-MS systems. In the case of β-hydroxybutyric acid (BHB), there has been minimal research done to quantify BHB in complex health formulations or any at all available through literature. The instability of testing through GC-MS and matrix effects that are not like urine or blood in literature make analysis of BHB in health formulations a novel, but complex problem [19].

1.4 Importance of Analytical Testing in Nutraceuticals and Beyond

Reliability between the consumer and the manufacturer is impertinent in providing a safe product and maintaining consistent business. The complexity of nutraceutical products makes it even more challenging for companies to ensure their products are safe and concentrations are as advertised. Periodic quality control and using validated methods with proper analytical standards is essential for labs to produce accurate results that are reliable [20]. An example of this is the regulatory problems that arise when a company labels a product

as gluten free but does not undertake the proper testing procedures. In Canada, Europe, and the United States the threshold for being certified gluten free is <20 ppm. Improper testing and lack of understanding of complex matrices may skew data leading to consumers unknowingly ingesting dangerous amounts of gluten that leads to long-term health problems [21]. Although the testing procedures for toxic impurities are well established in the pharmaceutical industry the same can not be said about nutraceuticals. Along with heavy metals and synthesis biproducts labelled compounds not accurately quantified can lead to unwanted problems with assuming only benefits will be claimed. This is seen when the concentration of Pyridoxine is greater than 500 mg per dose, photosensitivity and neurotoxicity can occur [22].

The importance of validated methods is true when law enforcement officials are determining cause of death or possible overdose. Unfamiliar sample matrices make determining drug concentrations seemingly impossible without proper methods to help sort out compounds that are not of interest. A small dose of a drug such as fentanyl to a nutraceutical powder will lead to the death of the consumer, but almost be impossible to distinguish from the matrix [23]. Creating new methods, whether it's for law enforcement or nutraceuticals, creates new pathways for reliability and safety by tackling possible complex matrices with well thought out isolation methods.

CHAPTER 2

LC-MS ANALYSIS OF BETA-HYDROXYBUTYRIC ACID

2.1 Introduction

In recent years there has been a significant increase in the use of nutraceutical products because of new health trends, promises of increasing life expectancy and new insights into general health. In 2020 the worldwide nutraceutical industry was estimated at $278.8 billion dollars with growth projected to exceed $441.7 billion dollars by 2026 [9]. Since many of these products are labelled as dietary supplements they do not need to adhere to the U.S Food and Drug Administration's strict regulations for pharmaceuticals [10]. With the increase in processed food, additives, unbalanced diets, there is an ever-increasing demand for products that deliver supplemental value without the potential costs associated with quality products [11]. Without proper regulation in place in the United States it is easy for consumers to be fooled into believing that the nutraceutical products are both safe and contain the listed compounds at the right concentrations. A substantial proportion (1 in 12) of Americans unknowingly take botanical supplements that could lead to kidney damage, contain carcinogens, or adulterated with unwanted chemistries [12]. Knowledge of unwanted compounds and false product claims have led consumers and producers alike to take stringent action to thoroughly understand the products they are consumers through analytical testing methods.

The development of novel methods for the analysis of nutraceutical products has been unreliable in many cases due to the complex matrices that many of these products come with [13]. In this case, the compound of interest is β-hydroxybutyric acid (BHB) and has become a

popular supplement for people who undertake a ketone diet in the absence of carbohydrates. BHB is added to supplemental powders and most often consumed with mixing with water, followed by ingestion [14]. Significant problems arise when trying to determine the actual concentration of BHB in mixtures that often include compounds such as Ascorbic acid (Vitamin C), Cholecalciferol (Vitamin D), d-Alpha Tocopherol (Vitamin E), Folic Acid, B Vitamins, artificial sweeteners, erythritol, and amino acids. BHB in this study is delivered in its salt form of Sodium, Magnesium, or Calcium. The complex nature and presence of compounds that have high boiling points do not make these nutraceutical health formulations suitable for GC-MS analysis [15]. The proposed method is one that isolates and dilutes BHB to a desired concentration while removing unwanted matrix compounds or minimizing their interference. An attempt was made to derivatize BHB for analysis through GC-MS with atmospheric pressure ionization but was unsuccessful due to issues with signal and the matrix causing problems with repeatability. It is also noted that sample preparation was extensive and required stringent attention as instrument maintenance became overwhelming.

2.2 Methods

2.2.1 Materials and Solutions Preparation

Methanol (LC/MS Grade, CAS 75-05-8), Formic Acid (LC/MS Grade, CAS 64-18-6), (R)-hydroxybutyric acid (99.9% analytical standard) was obtained from Sigma Aldrich. Hypersep SPE C18 filters (100mg), and 0.2 µm PTFE filters were obtained through Fisher. Ultrapure Water 18.3 Ω Resistivity was obtained from Aqua Solutions water purification system. Na hydroxybutyric acid, Mg hydroxybutyric acid and Ca hydroxybutyric were all obtained from Sigma Aldrich. ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm was purchased

from Waters Corporation. Analytical standards are prepared using Eppendorf Research plus pipettes to a concentration of 1.0 mg/mL using BHB salt standards. The calculations to determine the amount of BHB present in Na, Mg, and Ca BHB salts are seen in Table 2.1. This is necessary because the BHB present in health formulations is often in its salt form due to its instability when not present in serum or solutions [16]. The calibration curve for analysis was prepared in methanol at concentrations of 1,2,5,10, and 20 ppm. All mobile phases were sonicated for 15 minutes before purging through solvents lines connected from UPLC to mass spectrometer.

Table 2.1: List of calculations used to determine the amount of BHB present in BHB salts

Item	Value
Molecular Weight BHB-	103.0 g/mol
Molecular Weight Na+	23.0 g/mol
Molecular Weight Mg++	24.3 g/mol
Molecular Weight Ca++	40.0 g/mol
% Purity of Na-BHB	98.7%
% Purity of Mg-BHB + Ca-BHB	98.7%
Ratio Value Mg	2.0
Ratio Value Ca	1.0
% Na in Na-BHB	18.02%
%Mg in Mg-BHB	6.94%
%Ca in Ca-BHB	5.35%
Total BHB in Na-BHB	80.68%
Total BHB in Mg/Ca-BHB	86.41%

2.2.2 Sample Preparation

Health formulation powders were homogenized to ensure proper dispersion. If the samples are in pill form, they were crushed or grinded into a powder before analysis. A total of

9 different samples were analyzed in replicate and contained various flavors at differing concentrations of BHB as seen in Table 2.2. As the BHB salts are labelled as one of the highest concentrations of compounds in the health formulation a significant serial dilution is needed to lower the concentrations of analyte interferences not removed in the filtration process. A sample of 0.2 grams was weighted using Mettler Toledo ME140 Analytical balance into a 15 mL conical tube. The balance was verified before use by calibration weights. An aliquot of 10.0 mL methanol was pipetted into conical tube before being vortexed for a total of 5 minutes. The samples were then centrifuges at 3000 rpm for 10 minutes and it was observed that insoluble compounds and food dyes were successfully separated from solution. A 1.0 mL sample was pipetted from the supernatant layer into a 1 mL Hypersep SPE filter cartridge with a secondary 0.2 µm PTFE filter attached on the end. The purpose of the SPE cartridge is to retain non-polar, mid-polarity compounds and some organic molecules. The sample was slowly pushed through the filters with a pipette bulb into a 2 mL LC vial. The sample was further diluted 1:1000 by pipetting 5 µL of sample solution into 4995 µL of Methanol (LC/MS grade). Samples were then vortexed for a minute to ensure homogeneity.

2.2.3 Instrument Parameters

A Waters ACQUITY UPLC (Waters Corporation, Milford, MA, USA) and Waters Synapt G2-Si 4k HDMS Time-of-Flight mass spectrometer (Waters Corporation, Milford, MA, USA) was used in the determination of concentration of BHB in the samples. The analytical column installed to the UPLC system was a ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm. This column is a reversed phased C18 column that is compatible with 100% aqueous phases and extra low bleed. The mass spectrometer source was an Electrospray Ionization (ESI)

source set to negative ionization mode with a cone voltage of 30 volts with a capillary voltage of 3 kV. The scan time was 0.00-14:00 minutes, and the scanned mass was 103.0-103.08 m/z for 0.25 seconds per scan. The MassLynx 2.0 target enhancement feature was set to 103 Daltons. The UPLC mobile phase A was Water (18.3 mega ohm,0.1% Formic Acid) and mobile phase B was Methanol (0.1% Formic Acid). The initial flow rate was set to 0.6 mL/min with a starting mobile phase ratio of A 85% to B 15%. The flow gradient was as follows: 0.0-2.00 min A 85%-B 15%, 2.01-10.00 min A 50%-B 50%, 10.01-11.00 min A 85%- B 15%, 11.00-14.00 min A 85%- B 15%. The column sample injection was 2 µL and solvent lines were primed for 2 minutes before analysis. A calibration curve was prepared and analyzed with concentrations at 0,1,2,5,10, and 20 ppm.

2.3 Results

The analytical standard was run for β-hydroxybutyric acid, and it was determined that the retention time was 3.88 minutes. The molar mass of BHB is 104.104 Da which correlates to the negative mode ionization of BHB at 103.043 m/z as seen in Figure 2.1. The calibration curve exhibited good linearity with an $R^2 > 0.991$ as seen in Figure 2.2 where the calibration curve is plotted with calibration points at 1,2,5,10,20 ppm, respectively. All calibrations were based on external standards with the response being the area under the curve.

The UPLC chromatogram in Figure 2.3 shows that BHB produces an appreciable peak with a retention time of 3.88 minutes. The presence of BHB at 3.88 minutes is further confirmed by doing an overlapping selected ion recording (SIR) at 103.04 m/z as seen in Figure 2.4. The change in the chromatogram is attributed to the concentration changes exhibited with the gradient from mobile phase A to mobile phase B.

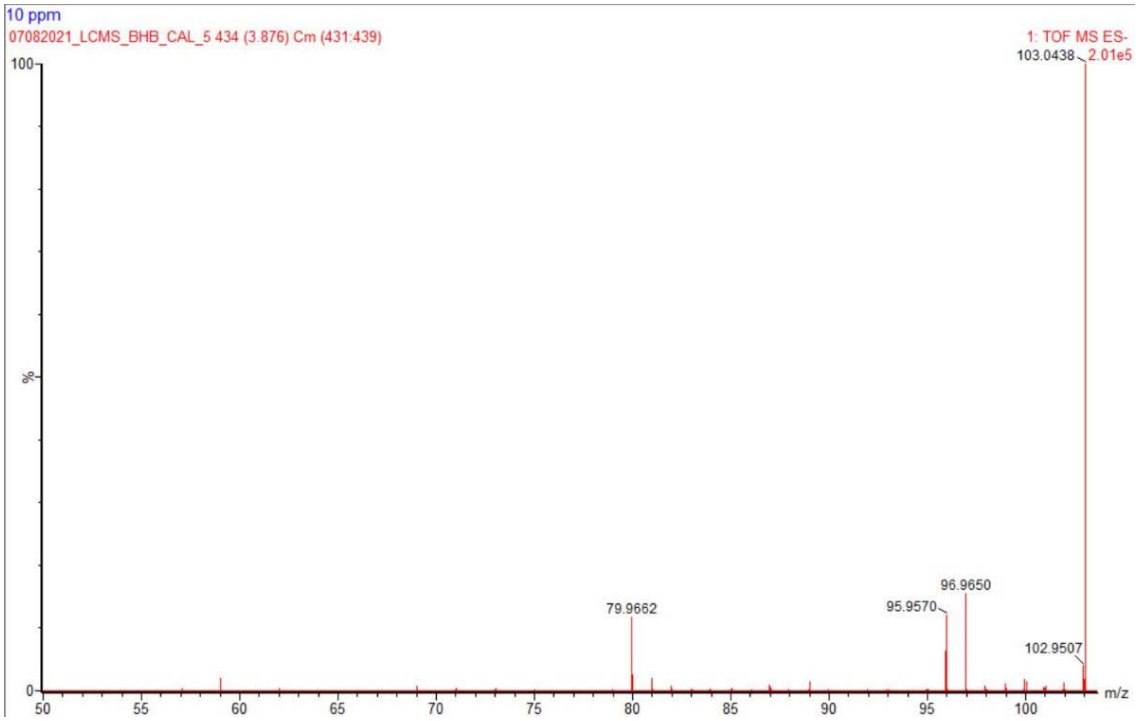

Figure 2.1: Mass spectrum of BHB using negative mode ionization with respective BHB peak at 103.04 m/z.

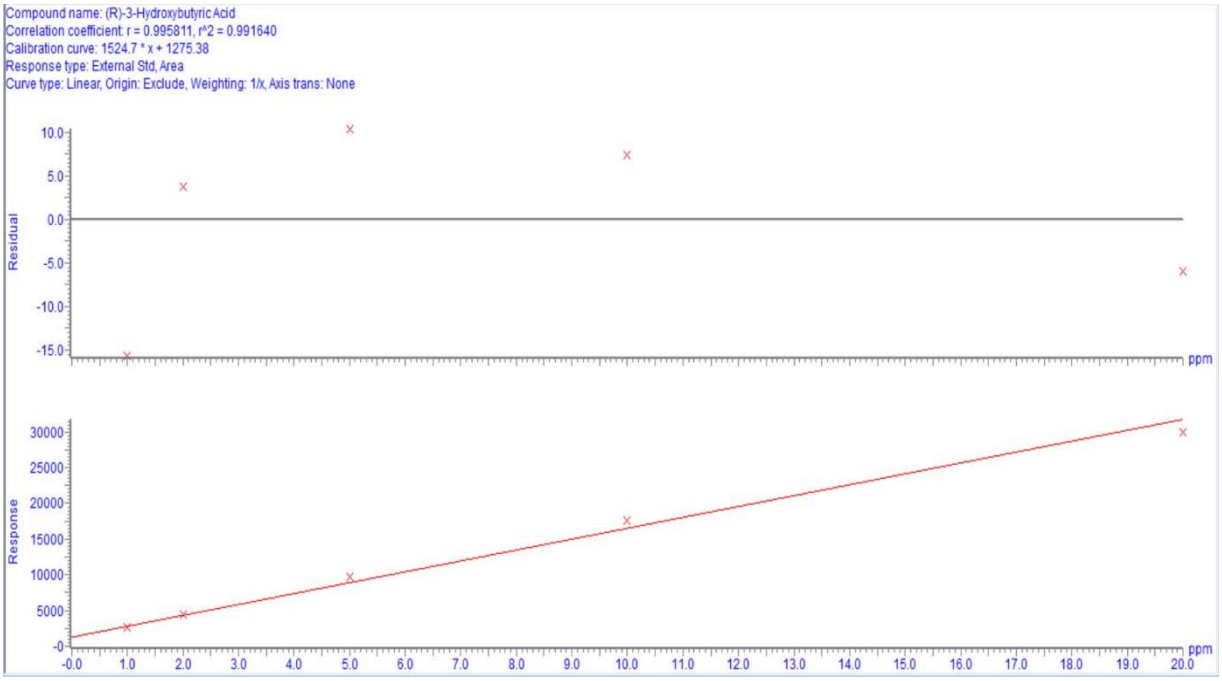

Figure 2.2: Calibration curve for BHB exhibiting good linearity with a $R^2 > 0.991$.

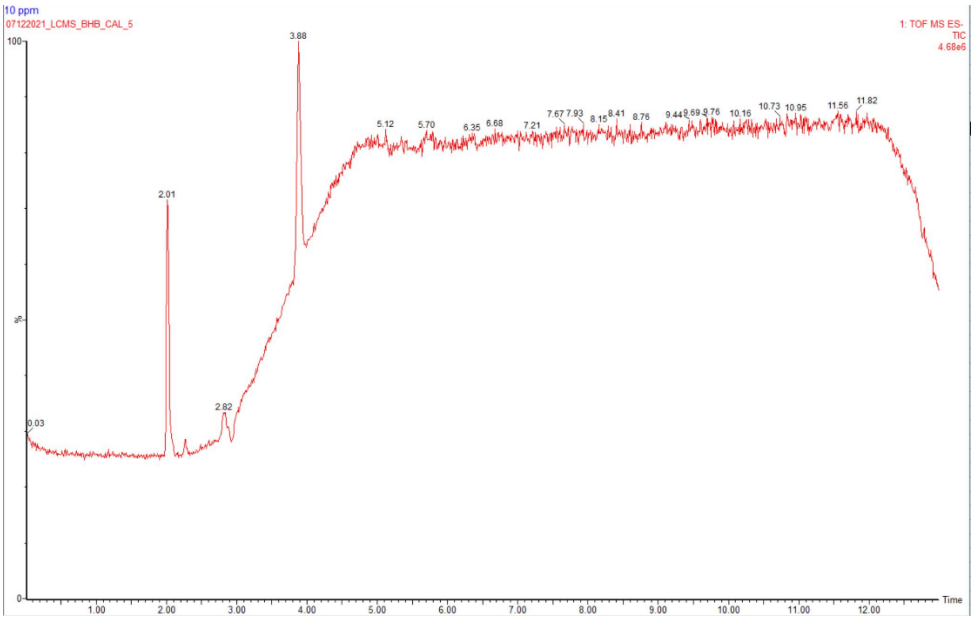

Figure 2.3: UPLC Chromatogram for BHB standard at 10 ppm with a retention time of 3.88 minutes.

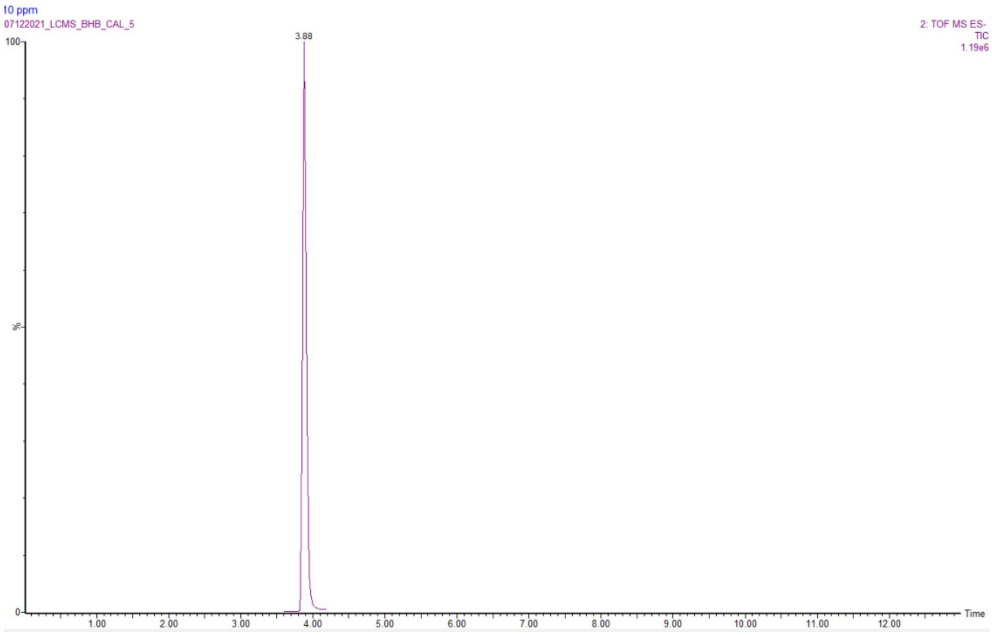

Figure 2.4: UPLC Chromatogram for BHB standard using SIR mode at 103.04 m/z with a retention time of 3.88 minutes.

A total of 18 samples were run with 9 different unique samples in replicate. The range of expected concentrations of total BHB was from 11.80% to 38.92%. It was seen that all sampled exhibited a less than 10% percent deviation from the expected concentrations of the

nutraceutical health samples with the highest being 9.74 % for sample 9 and the lowest being sample 3 with a deviation of 0.08 % from expected values. The acceptable limits for quantification of BHB in quality control standards was +/-30% deviation. The samples showed symmetrical peak shapes and there were no prevalent matrix effects due to sample cleanup and serial dilutions. Sample matrix blanks showed no presence of BHB and were ran in a replicate of 7. All samples are listed were their measured and expected values in Table 2.2.

Table 2.2: List of samples, their respective expected BHB concentrations, the measured values with average concentrations, and the percent deviation from the expected values.

Sample	Expected Concentration % BHB	Measured Concentration % BHB	Average % BHB	Deviation from Expected %
1	38.70	33.15	35.33	8.71
1R		37.5		
2	38.70	40.2	37.78	2.37
2R		35.35		
3	36.25	36.02	36.22	0.08
3R		36.41		
4	36.47	40.56	37.04	1.45
4R		33.51		
5	38.70	37.42	36.09	6.74
5R		34.76		
6	11.80	10.28	10.68	9.49
6R		11.07		
7	38.92	38.24	39.16	6.16
7R		40.08		
8	38.70	40.43	40.98	5.56
8R		41.52		
9	11.80	10.28	10.65	9.74
9R		11.02		

2.4 Conclusions

In conclusion, it can be said that a novel LC-MS method for the analysis of BHB in health formulations was developed and tested with acceptable accuracy. Limitations that are prevalent in analysis using GC-MS were overcome with proper sample clean-up, dilutions, centrifuging and instrument settings. Sample preparation methods that were once used for GHB analysis in human serums and urine are now obsolete with the presence of LC-MS instrumentation. This method exhibited good linearity with a calibration range from 1-20 ppm and acceptable sensitivity. Confirmatory analysis shows the presence of BHB in health formulation samples with great certainty as per analytical standards and matrix blanks.

2.5 Future Work

The limitations of this method are presumed to be matrix specific. Future work will include the analysis of BHB in diverse types of health formulations other than the ones specified and tested in this method. These other formulations could be in the form of liquids or solids and changes may have to be made to account for differences in the matrixes. This method could also be applied to other exogenous ketone bodies.

REFERENCES

[1] Rd, S. O.; Gibson.; Gamma-hydroxybutyric acid. **2005.** *New England J. of Med.* (353)15: 1632-1633.

[2] Sabucedo, A. J.; Furton, K. G.; Extraction less GC/MS analysis of γ-hydroxybutyrate and γ-butyrolactone with trifluoroacetic anhydride and heptofluoro-1-butanol from aqueous samples. **2004.** *J. Sep. Sci.* (27):703-709.

[3] Couper, F.J.; Logan, B.K.; Determination of γ-hydroxybutyrate in biological specimens by Gas Chromatography-Mass Spectrometry. **2000.** *J. Of Anal. Tox.* 24(1):1-7.

[4] Dahl, S. R.; Olsen, M. K.; Strand, D. H.; Determination of gamma-hydroxybutyrate (GHB), beta-hydroxybutyrate (BHB), pregabalin, 1,4-butane-diol (1,4BD) and gamma butyrolactone (GBL) in whole blood and urine samples by UPLC-MSMS. **2012.** *J. of Chromatography.* (37)42: 885-886.

[5] Iten, P. X.; Meier, M.; Beta-hydroxybutyric acid – an indicator for an alcoholic ketoacidosis as cause of death in deceased alcohol abusers. **2000.** *J. Forensic Sci.* 45(3): 624-632.

[6] Mireault, C.; Lamarche, M.; Vaillancourt, L.; Gosselin, M.; Desharnais, B.; Mireault, P.; Beta-Hydroxybutyric acid (BHB) Quantification by GC-MS. **2014.** *Inter. Association Forensic Tox.* (2014) 1.

[7] Sorenson, L.K.; Rittig, N.F.; Holmquist, E.F.; Jorgenson, K.A.; Jorgenson, J. O.; Moller, N.; Johannsen,M.; Simultaneous determination of B-hydroxybutyric acid and B-hydroxy-B methyl butyrate in human whole blood using hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry. **2013.** *Clin. Biochem.* 46(18):1877-1883.

[8] Desharnais, B.; Lajoie, M.J.; Laquerre, J.; Savard, S.; Mireault, P.; Skinner, C.D.; A Tool for Automatic Correction of Endogenous Concentrations: Applications to BHB Analysis by LC-MS-MS and GC-MS. **2019.** *J. Of Anal. Tox.* 43(7):512-519.

[9] GIA. Nutraceuticals – Global Market Trajectory & Analytics. July 2021. *Global Industry Analysts Inc.* **2021.**

[10] Goldman, P.; Herbal Medicines today and the roots of modern pharmacology. **2001.** *Annals of Internal Medicine.* 135(8):594-600.

[11] Perez-Cueto, F. J.; Verbecke, W.; de Barcellos, M. D.; Kehagia, O.; Chryssochoidis, G.; Scholderer, J.; Grunert, K. G.; Food Related lifestyles and their association to obesity in five European countries. **2010.** *Appetite.* 54(1):156-162.

[12] Starr, R. R.; Too Little, Too Late: Ineffective Regulation of Dietary Supplements in the United States. **2015.** *Am. J. Public Health. 1*05(3):478-485.

[13] Bernal, J.; Mendiola, J. A.; Ibanez, E.; Cifuentes, A.; Advanced analysis of nutraceuticals. **2011.** *J. of Pharm. And Biomedical Analysis.* 55(4):758-774.

[14] Stubbs, B. J.; et al.; On the Metabolism of Exogenous Ketones in Humans. **2017.** *Front. Physiol.* 8(848): 1-13.

[15] Thomas, R. J.; Lee, E.; Truong, T.; Porter, N.; The applicability of Field Portable GC-MS for the rapid sampling and measurement of high-boiling-point-semi volatile organic compounds in environmental samples. **2016.** *Special Issues.* 14(3): 20-26.

[16] Fritzsche, I.; Buhrdel, P.; Melcher, R.; Bohme, H.J.; Stability of Ketone bodies in serum in dependance on storage time and storage temperature. **2001.** *Clinical Laboratory*. 47(7-8):399-403.

[17] Helal, N.A.; Eassa, H.A.; Amer, A.M.; Eltokhy, M. A.; Edafiogho, I.; Nounou, M. I.; Nutraceuticals Novel formulations: The Good, the bad, the unknown and Patents Involved. **2019.** *Recent Pat. Drug Delivery Formulations.* 13(2):105-156.

[18] Flick, T. G.; Cassou, C, A.; Chang, T.M.; Williams, E. R.; Solution Additive that Desalt Protein Ions in Native Mass Spectrometry. **2012.** *Anal. Chem.* 84(17):7511-7517.

[19] Bairros, A. V.; Ugalde, G.A.; Yonamine, M.; Simple and Fast Determination for Gamma-Hydroxybutyrate (GHB) in Urine Samples by LLE and GC-MS. **2021.** *Nota Tecnica.* 44(2):211-216.

[20] Otles, S.; Cagindi, O.; Safety considerations of nutraceuticals and functional foods. **2012.** *Novel Technologies in Food Sci.* 121-136.

[21] Bagchi, D. Nutraceuticals and Functional Food Regulations in the United States and around the world. 3rd Ed. Academic Press. **2019.**

[22] Telessy, I.G. Chapter 24 – Nutraceuticals. 1st Ed. Academic Press. **2019.**

[23] O'Connor, S. Fentanyl: China's Deadly Export to the United States. Staff Research Report. U.S Economical Security Review Commission. **2017.**