APPLICATION OF NMR ANALYSIS TO DETERMINE CONCENTRATION OF ETHANOL, ACIDS, AND SUGARS IN KOMBUCHA BEVERAGES

A Project Paper

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ABSTRACT

Kombucha is a popular, sweet and acidic tea beverage that is produced by a mixed fermentation with bacteria and yeast. Bacteria convert sugars (sucrose, dextrose, and fructose) into organic acids (such as acetic acid and lactic acid) by acid fermentation; and yeasts utilize sugars to produce very low concentrations of ethanol by alcohol fermentation. Kombucha is commonly sold refrigerated with the active cultures, which may continue to undergo fermentation after being bottled and cause changes in the composition, including alcohol concentration, of Kombucha over the shelf-life. The United States Department of the Treasury Alcohol and Tobacco Trade and Tax Bureau (TTB) requires that the alcohol content in Kombucha remain less than 0.5% by alcohol-by-volume (ABV) (0.4% alcohol-by-weight); otherwise, the beverage would be classified as an alcoholic beverage. The product would be subject to beverage regulations established by the Food and Drug Administration (FDA) if the alcohol content is less than 0.5%. In this study, we performed multiple experiments to measure the concentration of alcohol, sugars, and organic acids in commercial samples of refrigerated Kombucha via quantitative nuclear magnetic resonance (qNMR) spectroscopy. The experiments include comparison of ethanol content across several brands of commercial refrigerated Kombucha products, determination of the concentration of ethanol, acids, and sugars over the shelf-life of a refrigerated Kombucha product, and determination of the concentration of ethanol, acids, and sugars in refrigerated Kombucha at 22°C and at 4°C over three days. Quantitative NMR was performed by simple preparation of samples, integration of peaks in

spectra, and calculation of original mass of compounds in sample. The brand comparison tests revealed that many Kombucha beverages currently on the market contain 0.5% (v/v) or greater alcohol content, so these products should be labeled alcoholic, taxed accordingly, and subject to commercial alcoholic beverage regulations. The shelf-life test showed that, although significant differences in concentration of ethanol over time was not detected, the observed increase in ethanol production is likely to be the result of continual fermentation after bottling. The temperature comparison tests revealed that the change in concentrations of dextrose, sucrose, and citric acid were due to effects of time. In conclusion, this study shows that proton NMR is a powerful tool to quantify compounds in beverages under a variety of conditions, and is especially useful for beverages that contain ingredients that are federally regulated.

BIOGRAPHICAL SKETCH

Sandy Thai obtained a Bachelor of Science (B.S.) degree in Chemistry from the University of California, Los Angeles (UCLA), in 2017. Her interest in food science arose from a Science and Food course she took as an undergraduate, as the course introduced her to the application of chemistry to everyday foods. Right after graduation from UCLA, she entered the M.P.S. program in Food Science and Technology at Cornell. After completion of the M.P.S. program, she plans to intern at the USDA Agricultural Research Service in Albany, CA, for the summer and to enter the industry afterwards.

I would like to dedicate this work to my family: Grandpa & Nội, Ba và Mẹ, Kathy, Kimberly, Richard, and Julie.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Olga Padilla-Zakour, who provided me with direction, guidance, and encouragement on this project; and Kyle Kriner, who taught, explained, and assisted me immensely on this project. I would like to acknowledge Dr. Carmen Moraru for allowing me to prepare my NMR samples in her laboratory space; Dr. Ivan Keresztes and Anthony Condo at the Cornell NMR Facility; Kathleen O'Donnell-Cahill and Marcy Pontius from Wegmans Food Markets; and Erika Mudrak and Deniz Akdemir from the Cornell Statistical Counseling Unit. I would also like to thank all members of the Padilla-Zakour lab: Rohil Bhatnagar, Elizabeth Buerman, Julie Camacho Flinois, Junyi Chen, Nathan Feist, Kate Pinsley, Emile Punzalan, Dr. Shuang Qiu, and Lindsey Reardon, as well as Sarah Lincoln and Ashton Yoon.

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CHAPTER 1

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique that is commonly used to determine the molecular structure of a compound. In more recent years, NMR has been increasingly utilized for quantitative analysis of compounds (Avenoza, et al. 2006). Also known as quantitative NMR (qNMR), this method provides accurate and precise quantification of compounds. (Pauli, et al. 2012). Additionally, qNMR is preferred over traditional chromatography methods – like gas chromatography with flame ionization detection (GC-FID) (Ebersole, et al. 2017), headspace gas chromatography (HSGC) (Talebi, et al. 2017), or high-performance liquid chromatography (HPLC) (Neffe-Skocinska, et al. 2017) – due to its simple sample preparation, rapid data acquisition, and accurate quantification (Simmler, et al. 2013). One method of quantification by H-NMR, electronic reference to access in vivo concentrations (ERETIC), utilizes a calibration curve for a compound of known concentration to enable quantification of the target compound (Hill, et al. 2007). NMR is also more efficient than HPLC and GC, as it does not require extensive sample preparation or separation prior to analysis. In addition to rapid preparation of sample, NMR allows for simultaneous analysis of all compounds in a sample (Cazor, et al. 2006). HPLC and GC, however, require different columns, stationary phases, and mobile phases to analyze sugars and acids, so they must be analyzed separately.

Quantification by NMR spectroscopy of compounds is often used in the analysis of foods; therefore, qNMR can also be applied to a popular beverage known as Kombucha. Kombucha is a tea beverage that contains sugar and a symbiotic culture of bacteria and yeast (SCOBY), which undergoes mixed fermentation to produce acetic acid and lactic acid. The

beverage contains polyphenols, such as catechins and theaflavins, which are believed to contribute to its cancer-fighting properties (Jayabalan, et al. 2007). In a study from 2011, researchers extracted Kombucha into three different solvents and determined cytotoxic activity of the beverage through cell reduction, invasion, and destruction (Jayabalan, et al. 2011). From a more recent study, researchers observed the contribution of Kombucha to the destruction of pathogens that may have been transmitted to humans by different foods (Borkani, et al. 2016). In addition to limiting harmful activity, Kombucha is believed to promote health benefits within humans. An organic acid present in Kombucha, gluconic acid, contributes to the solubility of toxic chemicals (xenobiotics) so that the chemicals are easily removed from the body. Kombucha may also prevent diseases and disorders in organs due to its antioxidant properties against free radicals (Vina, et al. 2014).

The fermented beverage contains organic acids (such as acetic acid, lactic acid, succinic acid, citric acid, and gluconic acid); sugars (sucrose, dextrose, fructose); and ethanol, which is a by-product of the alcohol fermentation by yeast. Kombucha is generally considered non-alcoholic, due to the federal regulation limit of 0.5% (v/v) ethanol in the beverage by the Alcohol and Tobacco Tax and Trade Bureau (TTB) under the U.S. Department of the Treasury. Many Kombucha products have the potential to exceed this limit, often the result of fermentation that continues after bottling and during shipping and storage (Ebersole, et al. 2017). When Kombucha beverages do exceed 0.5% (v/v), they are no longer considered non-alcoholic by government standards. Furthermore, the excess in ethanol is problematic for children and minors, who could easily purchase the beverage and become intoxicated, as well as those who are sensitive to even low levels of alcohol. The sugar content of Kombucha has also been shown to exceed the value stated on the label, which is also a health issue for those seeking to consume less sugar. The

acids, some of which are added and some of which are by-products of fermentation impart flavor and acidity to Kombucha. The complex composition of Kombucha and the risks associated in not controlling the concentration of such compounds can be elucidated with the use of qNMR.

The objective of this study was to demonstrate the use of qNMR to determine the change in concentration of ethanol, acids, and sugars present in a commercial brand and flavor of Kombucha tea beverage under various conditions.

CHAPTER 2

TECHNICAL ABSTRACT

The purpose of this project was to apply quantitative ^{1}H-NMR to determine the concentration of ethanol, acids, and sugars in commercial Kombucha beverages under simulated real-life conditions. The experiments include determining the concentration of ethanol of several brands of Kombucha currently on grocery store shelves, and the concentration of ethanol, acids, and sugars over the shelf-life and at different temperatures. The temperature difference test was performed to simulate abuse by consumers, who may not refrigerate the beverage after opening. To determine the concentration of ethanol in beverages currently on the refrigerated store shelves, five brands and a total of 10 flavors were analyzed via qNMR. Six out of the 10 flavors analyzed contained greater than 0.4% by weight of ethanol, which is equivalent to the federal limit of 0.5% ABV. Significant differences were also found between different commercial brands, but these differences were not as apparent within brands. The pH of each was also recorded. We observed that the concentrations of ethanol of assorted brands appear across a range (0.01 – 2.08% ABW), with many exceeding the federal regulation limit. This variation in concentration may depend on the manufacturers' actions taken to slow or stop the fermentation reactions. Such Kombucha beverages should be labeled as alcoholic, taxed accordingly, and relocated to the alcoholic beverage section in grocery stores.

To determine the concentration of ethanol, acids, and sugars over the shelf-life of one commercial refrigerated Kombucha product, samples were analyzed monthly for four months via qNMR. The concentration of ethanol consistently exceeded 0.5% throughout the shelf-life; the increase above this limit may have occurred after bottling. Concentrations of sucrose decreased, while those of dextrose and fructose increased. The total concentration of sugars was calculated

to be 2.96% by qNMR, which is greater than the labeled total sugar content of 2.64%. Lactic, acetic, glucuronic, and citric acids were not found to significantly change in concentration over the duration of the shelf-life, while succinic acid displayed significant increases in concentration over time. The pH (3.33) and titratable acidity (0.62% expressed as acetic acid) of the refrigerated Kombucha sample was determined. Titratable acidity measures the total concentration of acids and expresses the value as a percentage of one acid. The concentrations of ethanol and succinic acid likely increase over the course of the shelf-life, as sucrose is consumed (or dissociates into fructose and dextrose) for alcohol and acid fermentations, respectively.

The concentration of ethanol, acids, and sugars under two different temperature treatments was also determined by qNMR. Over the short amount of time a consumer may consume their Kombucha product, they may either refrigerate the beverage or not. Temperature abuse refers to the failure to do so. Samples in triplicate were subject to either refrigerated or room temperature treatments, and quantification of ethanol, acids, and sugars was performed daily for a total of three days. There were not significant differences in concentration of ethanol and most acids and sugars over time nor under different storage temperatures. Time was shown to significantly affect the concentrations of dextrose, sucrose, and citric acid.

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a chemical, analytical technique that is commonly utilized for structure elucidation of a compound. In more recent years, it has been applied quantitatively to determine the concentration of a compound, either pure or within a complex matrix. The peak areas of an NMR spectrum correspond to the number of Hydrogens of

an analyte within the sample. Therefore, the peak area corresponds to the concentration of that compound and is useful in quantifying the target compound (Simmler, et al. 2014).

In this project, quantitative NMR is applied to determine the concentration of ethanol, acids (acetic, lactic, succinic, glucuronic, citric), and sugars (sucrose, dextrose, fructose). Ethanol content in commercial Kombucha beverages is regulated to 0.5% ABV. NMR can be used to verify that the concentration of ethanol in these beverages is frequently above this limit. The acids within Kombucha are a mix of added ingredients and fermentation by-products that contribute to the acidity and tart flavor (Jayabalan, et al. 2007). The concentrations were determined to observe the possibility of a trend in concentration, especially in relation to ethanol concentration, due to the ongoing fermentation reactions. Finally, sugars, which are also present in Kombucha and are fermentation substrates, were analyzed.

The three classes of compounds (alcohol, acids, and sugars) were analyzed in Kombucha subjected to a variety of real-life conditions. The ethanol concentration of several commercial Kombucha brands and flavors was determined to obtain a scope of the concentration range currently on the market shelves. Focusing on the shelf-life of one brand and flavor of refrigerated Kombucha, the concentration of alcohol, acids, and sugars over the shelf-life was determined. The concentrations were also determined under conditions that mimic temperature abuse, in which refrigerated Kombucha is stored at room temperature, by a consumer. The concentration of samples stored at room temperature were compared with samples stored at refrigeration temperatures.

MATERIALS AND METHODS

Chemicals

Sodium-3-trimethylsilylpropionate (TMSP, D, 98%) and deuterium oxide (D_2O, D, 99.9%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The internal standard of TMSP in D_2O, was prepared to a concentration of approximately 0.02 % (w/w).

Sample preparation

A plastic transfer pipette was used to add one drop of Kombucha to an amber vial. Approximately 600 – 800 µL of the standard TMSP in D_2O was added via 1000 µL micropipette. The contents were vortexed, and transferred to clean and dry 500 MHz NMR tubes via plastic transfer pipette. The tubes were sonicated to remove the presence of gaseous bubbles in the sample, and wiped dry with a Kimwipe. The samples were then submitted for NMR analysis.

NMR Spectroscopy

Samples were submitted on a Bruker 500 MHz Avance NMR spectrometer with autosampler (Billerica, MA, USA). The ^{1}H-NMR spectra were obtained at 500 MHz with a 30-second relaxation delay, 90-degree excitation pulse angle, and 128 scans for acids and sugars and 8 scans for ethanol. The spectra were processed with the MestReNova computer software (v. 11.0, Mestrelab Research S.L.). Apodization of the Fourier Transform spectrum was set to exponential with 0.3 Hz spacing. Manual phasing and baseline correction was performed to achieve a consistent baseline. The TMSP in D_2O standard was set as the reference peak and calibrated to a chemical shift of 0 ppm. Subsequent compounds were integrated relative to this

internal standard peak. Peaks of ethanol, acids, and sugars were identified by comparison with NMR spectra of standard compounds.

Quantitative NMR Analysis

The integrated peak areas, number of Hydrogen atoms per mole, purity, molecular weight, and mass of the internal standard values were used to determine the mass of target compound within the sample (Bharti, et al. 2012):

$$\frac{Area_{ETHANOL}}{Area_{TMSP}} \times \frac{H-atoms_{TMSP}/mol}{H-atoms_{ETHANOL}/mol} \times \frac{Purity_{TMSP}}{Purity_{ETHANOL}} \times \frac{MW_{ETHANOL}}{MW_{TMSP}} \times mass_{TMSP} = mass_{ETHANOL} \quad (1)$$

The mass of TMSP internal standard is calculated by multiplying the concentration of the prepared internal standard mixture by the mass of internal standard mixture added:

The mass of desired compound is finally divided by the total sample mass of Kombucha and

$$mass_{TMSP} = (mass\ of\ \frac{TMSP}{D_2O}\ added)(\frac{mass_{TMSP}}{mass_{TMSP}+mass_{D_2O}}) \quad (2)$$

standard to obtain the percent concentration of the desired compound within the sample:

Comparison across brands

$$\%\ ETHANOL = \frac{mass_{ETHANOL}}{mass_{KOMBUCHA} + mass_{TMSP}} \quad (3)$$

A total of 10 flavors from five brands of commercial refrigerated Kombucha were analyzed via NMR spectroscopy within one week of purchase. Each sample was analyzed in duplicate for ethanol.

Shelf-life testing

A top-selling Kombucha brand and flavor at a local grocery store in Ithaca, NY, was analyzed for this experiment. The bottled beverage was stored at 4 °C. Every month, samples in triplicate, each one from a different bottle of Kombucha, were submitted for NMR analysis of the acids, sugars, and ethanol present.

Refrigerated vs. room temperature

Each day for three days, samples in triplicate for each treatment temperature of 22°C and 4 °C were analyzed for quantification of the acids, sugars, and ethanol over time.

Titratable acidity

The titratable acidity of the samples was measured in triplicate with Mettler Toledo G20 Compact Titrator with Rondolino and overhead stirrer accessories (Columbus, OH, USA).

Determination of pH

The pH of the samples was measured in duplicate electronically with Orion 3 Star Benchtop pH Meter by ThermoFisher Scientific™ (Waltham, MA, USA).

Comparison of labeled sugar content with calculated sugar content

The concentration of sucrose in the sample was analyzed via qNMR and compared to the amount declared on the label.

Statistical Analysis

Analysis of variance (ANOVA) was performed to determine the presence of significant differences between samples of the same brand and samples of different brands. Where significance was detected with ANOVA, Tukey's Honest Significant Difference (HSD) post-hoc test was performed to assess pairwise differences between samples within the experiment. These tests were performed for the brand comparison and shelf-life studies. For the short-term shelf life study, a mixed effect linear model with ethanol, acids, and sugars as the response variable was performed. Fixed effects of treatment crossed with time (treated as categorical variable), treatment alone, and time alone with bottle variation as random effects were the parameters for this statistical method. The JMP 14.0.1 software (SAS Institute, Inc. 1989-2018, Cary, NC, U.S.A.) was used.

RESULTS AND DISCUSSION

The analysis via quantitative NMR required generating NMR spectra of the target compounds. These spectra display peaks that are characteristic of the compound, which makes them useful tools in determining the compounds present in our sample. Compounds such as ethanol (Figure 1), sucrose (Figure 2), fructose (Figure 3), dextrose (Figure 4), lactic acid (Figure 5), acetic acid (Figure 6), succinic acid (Figure 7), citric acid (Figure 8), and glucuronic acids (Figure 9) were identified by comparing the NMR spectra.

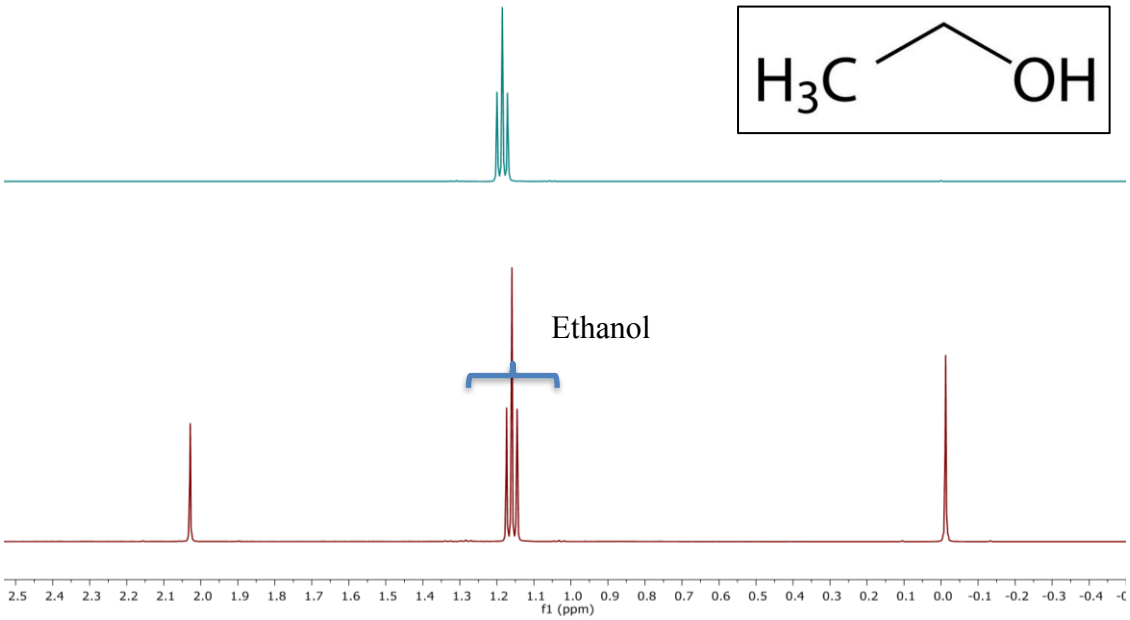

Figure 1. NMR Spectra of ethanol standard (top) and refrigerated Kombucha (bottom). Triplet peak at 1.10-1.20 ppm corresponds to 3 H.

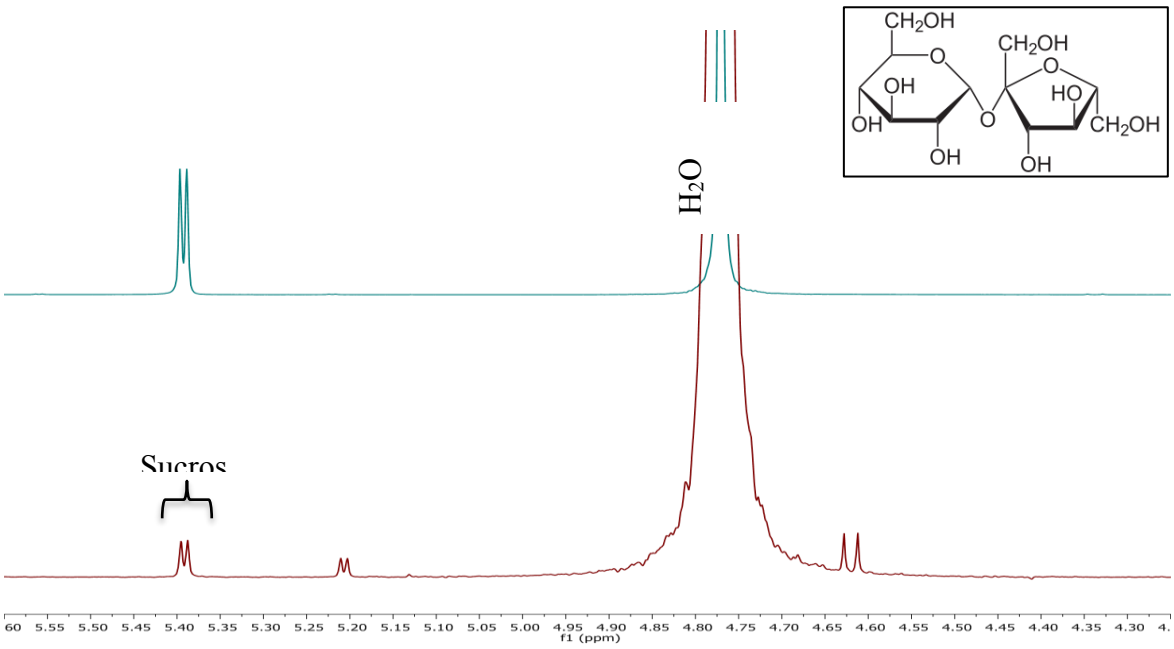

Figure 2. NMR Spectra of sucrose standard (top) and refrigerated Kombucha (bottom). Doublet peak at 5.4 ppm corresponds to 1 H.

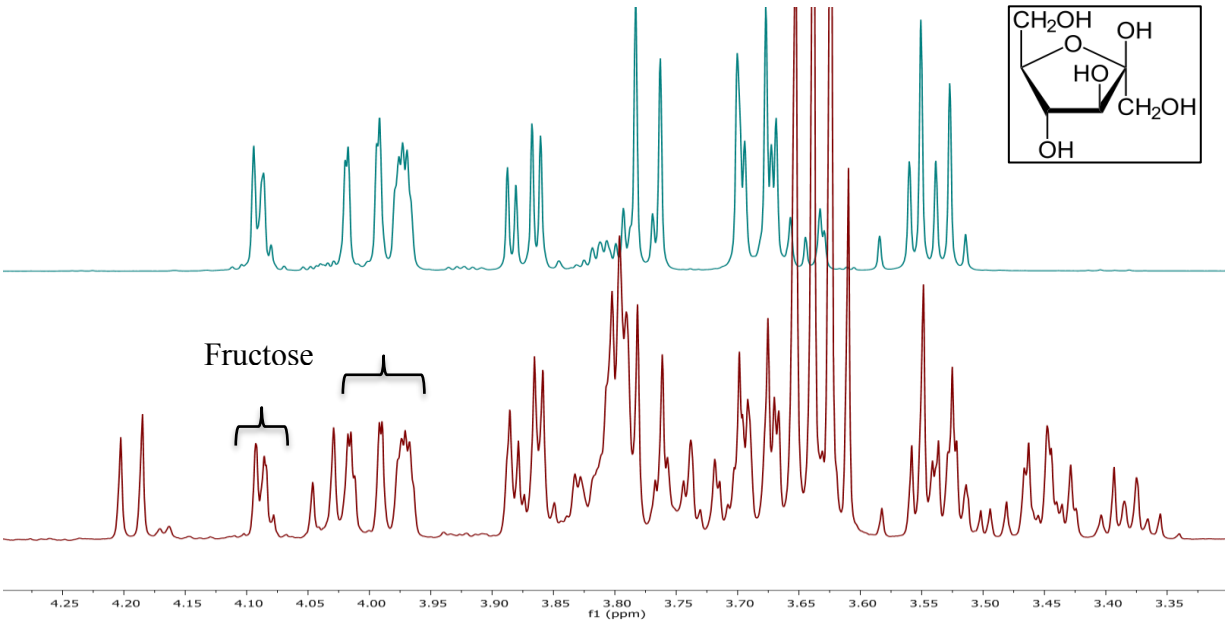

Figure 3. NMR Spectra of fructose standard (top) and refrigerated Kombucha (bottom). Multiplet splitting peaks at 3.95-4.10 ppm is believed to correspond to 2 H.

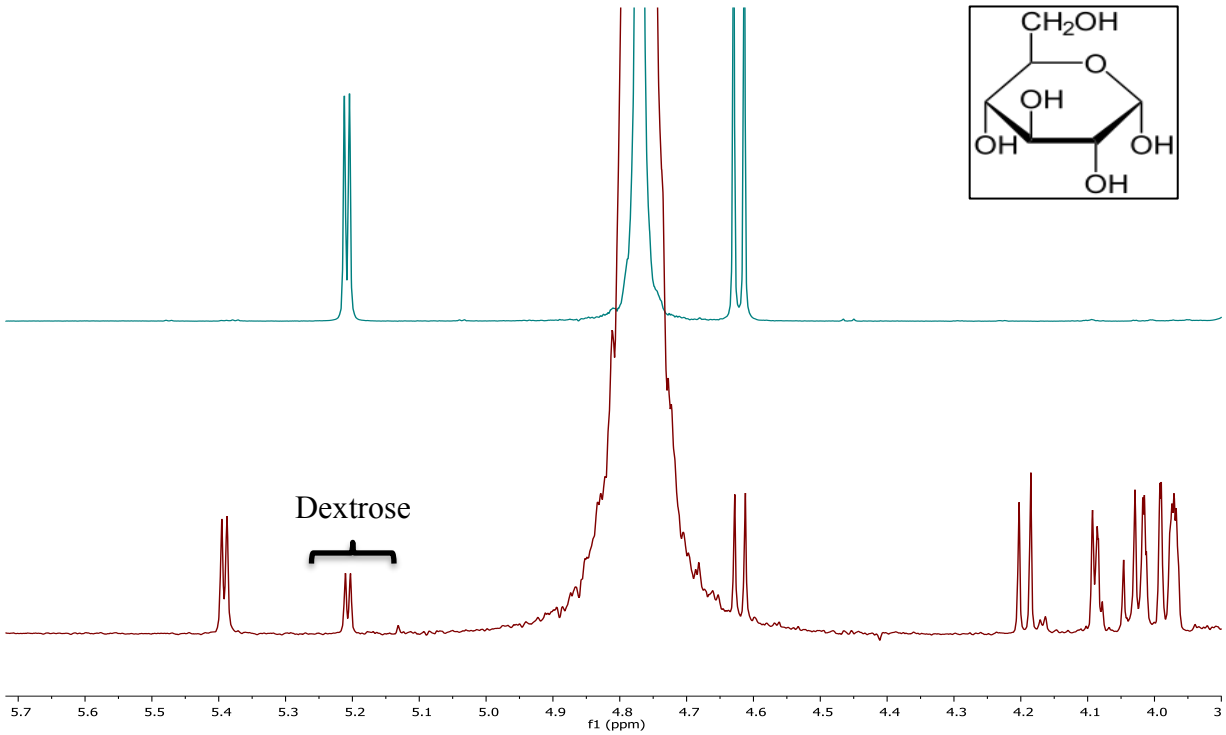

Figure 4. NMR Spectra of dextrose standard (top) and refrigerated Kombucha (bottom). Doublet peak at 5.2 ppm corresponds to 1 H.

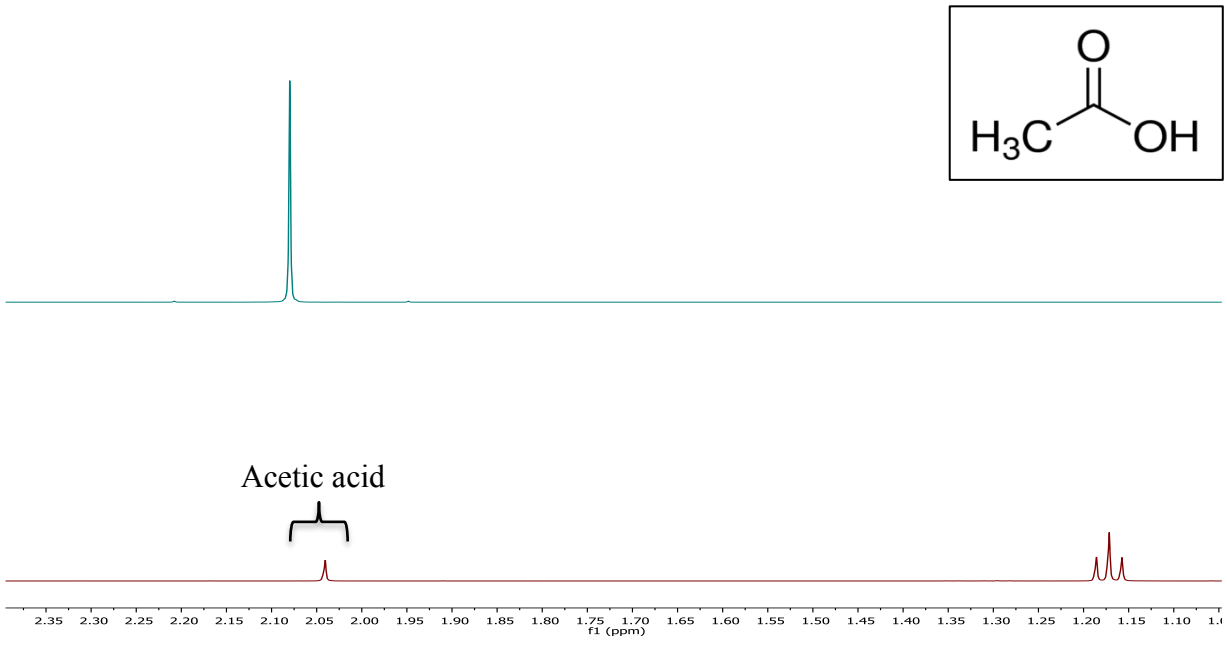

Figure 5. NMR Spectra of acetic standard (top) and refrigerated Kombucha (bottom). Singlet peak at 2.04 ppm corresponds to 3 H.

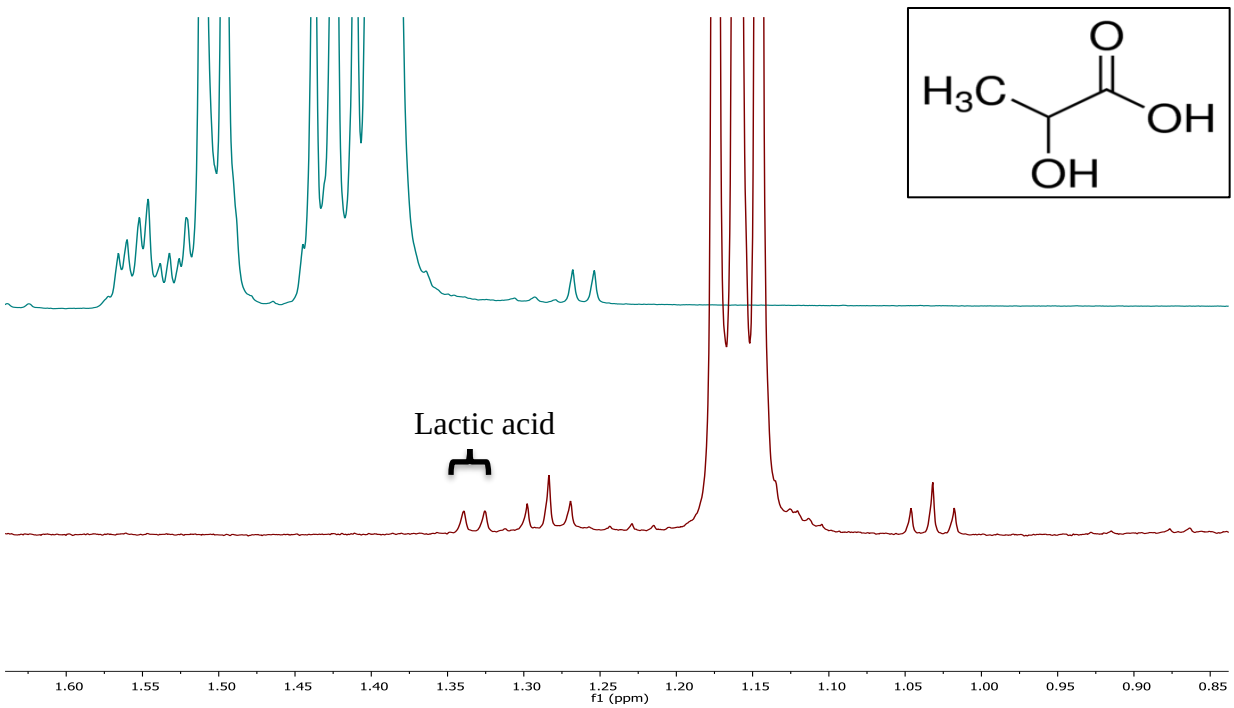

Figure 6. NMR Spectra of lactic acid standard (top) and refrigerated Kombucha (bottom). Doublet peak at 1.33-1.35 ppm corresponds to 3 H.

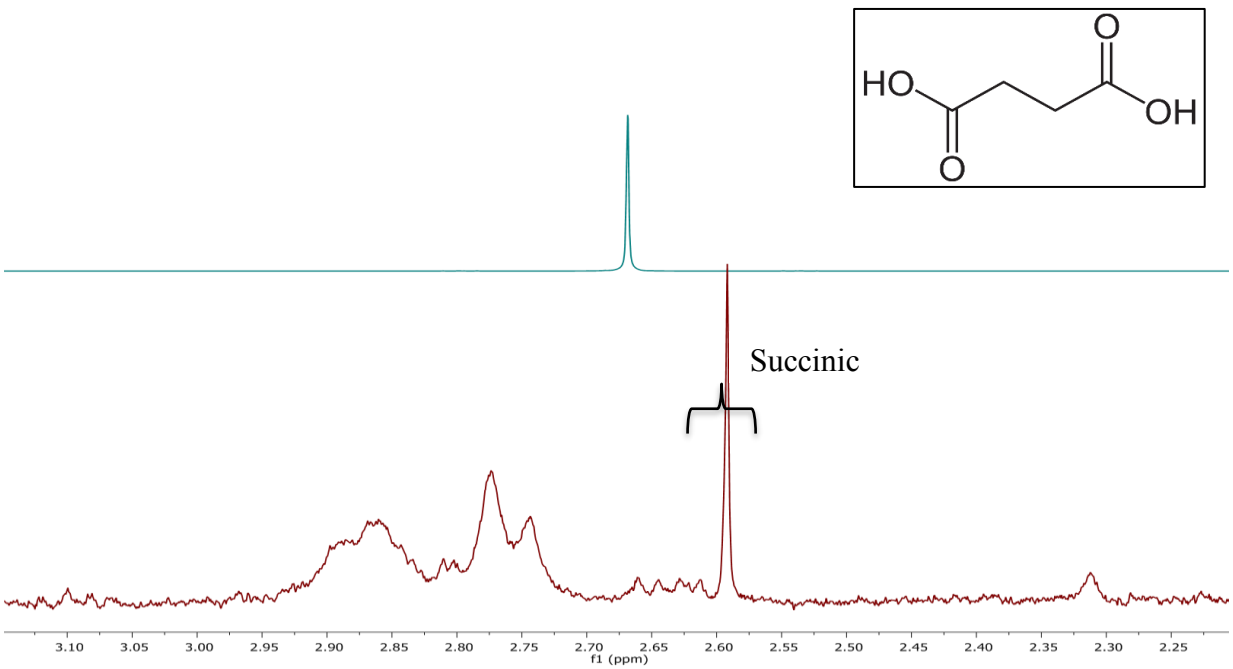

Figure 7. NMR Spectra of succinic acid standard (top) and refrigerated Kombucha (bottom). Singlet peak at 2.60 ppm corresponds to 4 H.

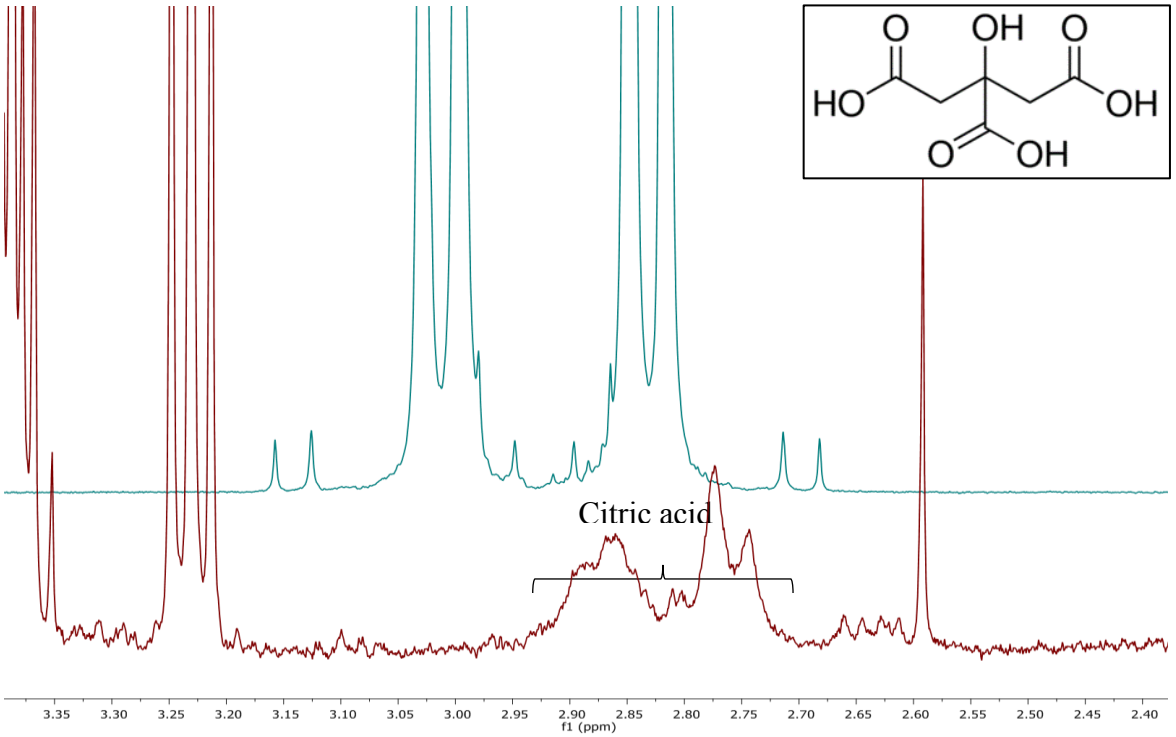

Figure 8. NMR Spectra of citric acid standard (top) and refrigerated Kombucha (bottom). Multiplet peaks at 2.70-2.90 ppm corresponds to 4 H.

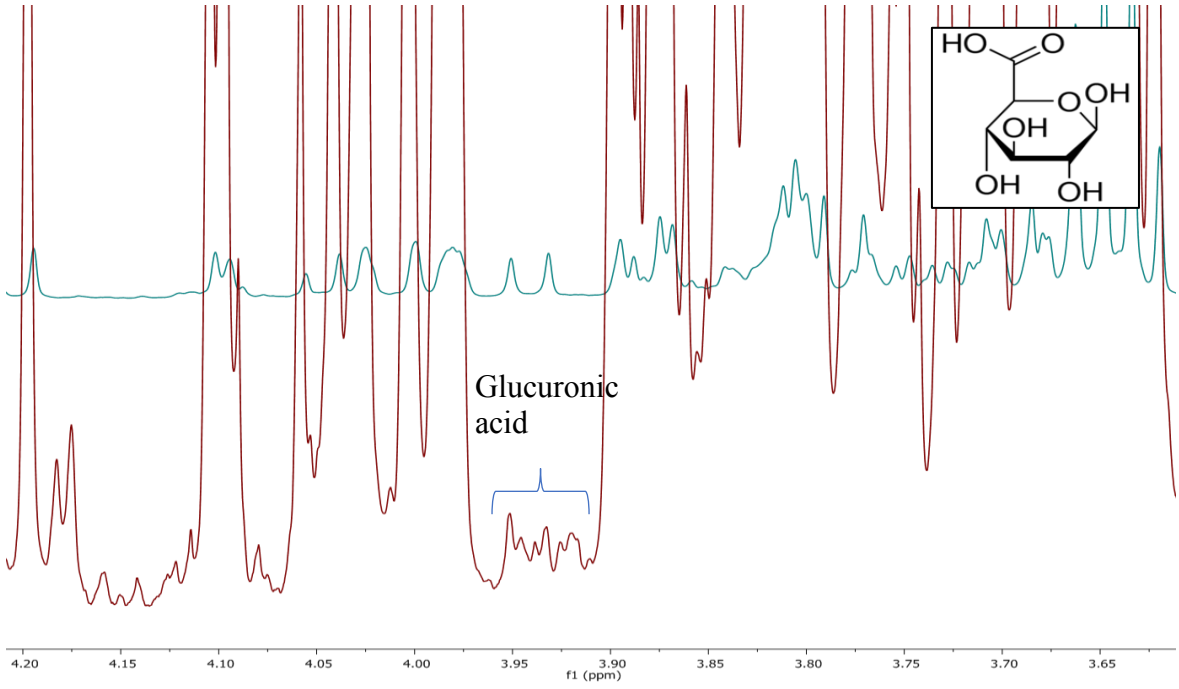

Figure 9. NMR Spectra of glucuronic acid standard (top) and refrigerated Kombucha (bottom). Doublet peak embedded at 3.90 - 3.95 ppm corresponds to 1 H.

Comparison across brands

Once the compounds in the Kombucha sample were identified, analyses were performed to quantify these compounds. The comparison of ethanol concentration across several commercial refrigerated Kombucha brands was performed not only to observe the wide range of ethanol concentration currently on the market, but also to demonstrate that many products do not adhere to the TTB federal regulations of containing less than 0.5% ABV. Furthermore, this experiment also demonstrates the utility of ^{1}H-NMR in a legal situation, as well as the sensitivity of ^{1}H-NMR in an analytical setting to detect compounds of very low concentrations. The analysis of 10 flavors from five brands certainly revealed a wide spectrum of concentrations of ethanol currently on supermarket shelves (Figure 10). The values ranged from approximately 0.007% to 2.08% by weight. ANOVA revealed $p < 0.0001$, which indicates the presence of a significant relationship between brand/flavor and concentration of ethanol. Tukey's HSD analysis determined a significant difference in concentration between brands, although overlap

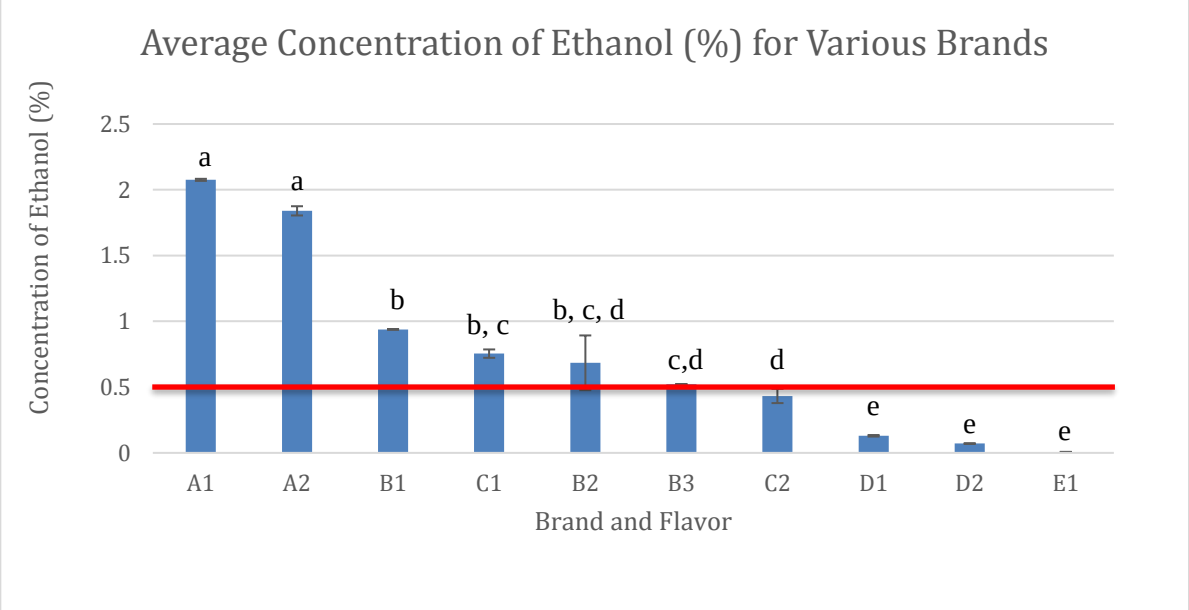

Figure 10. Bar chart displays the average concentration of ethanol (% w/w) for commercial Kombucha brands A, B, C, D, E and flavors 1, 2, 3. Data are expressed as mean ± standard deviation of n=2 samples

between brands B and C was present.

Of the 10 flavors analyzed, the concentration of ethanol in 6 of the flavors exceeded 0.5%. This implies that Kombucha manufacturers may need to analyze the concentration of ethanol in their own products prior to bottling in experiments like those conducted in this paper. The commercial beverages should thus be labeled alcoholic beverages, relocated in the grocery store, and taxed accordingly, such that vulnerable consumers, like children and pregnant women, are aware of the risks associated with consumption of the product. Otherwise, they may be subject to lawsuits for claiming the beverage as non-alcoholic when it is.

Shelf-life testing

Over the duration of the shelf-life of a brand and flavor of commercial refrigerated Kombucha, ^{1}H-NMR analysis was performed to quantify ethanol, fructose, dextrose, sucrose, lactic acid, acetic acid, succinic acid, glucuronic acid, and citric acid. The concentration of ethanol consistently remained above 0.5%, but significant difference in concentration was only found between time 0 and times 1, 2, 3, and 4 (Figure 11a). Between the first and second time points, the concentration of ethanol increased significantly. This data indicates that there may not necessarily be a trend of increasing alcohol concentration over time. However, because the values exceeded the federal regulation limit, it is likely that increase in ethanol content did occur after bottling as yeast populations continue to grow, unless the microorganisms are specifically controlled for (Teoh, et al. 2004). The yeast continues to ferment the sugars into alcohol, which is the reason for the decrease in concentration of sucrose. Decrease in ethanol concentration would be attributed to consumption by acetic acid bacteria to produce acetic acid; however, acetic acid concentration did not appear to increase over time, so it is less likely that ethanol concentration decreased (Chakravorty, et al. 2016). Ethanol fermentation may have exhibited a decrease and then plateaued due to the acidity or absence of sugar in tea medium (Chen & Liu,

2000). Additional work may include characterization of the yeast and bacteria within the SCOBY, to fully understand the fermentation mechanisms and sources of acids present Additionally, Kombucha SCOBY are typically reused to make more SCOBY (Greenwalt, et al. 2000), which can result in contamination by microbial pathogens due to a contaminated SCOBY that is reused over time. It is possible that this contaminated SCOBY induces uncontrolled alcohol fermentation, which would result in uncontrolled levels of ethanol production. Kombucha manufacturers, in keeping with the law, should not bottle the beverage if the concentration of ethanol exceeds 0.5%.

Fructose, dextrose, and sucrose were also analyzed via ^{1}H-NMR analysis. Analysis of variance revealed a correlation between time (months) and concentration of the specific sugar. Sucrose clearly decreased in concentration over time, as there was significance in the difference of concentration from the first time point to the last (Figure 11b). The plots for dextrose (Figure 11c) and fructose (Figure 11d) do not explicitly display a trend in concentration over time. However, ANOVA tests for dextrose ($p = 0.0038$) and fructose ($p = 0.0008$) indicated there is a significant difference between the respective concentrations over time. Tukey's HSD was subsequently performed, and showed that the trend in concentration of fructose and dextrose is not immediately clear but subtly increased over time. The decrease in concentration of sucrose can be explained by hydrolysis by an invertase enzyme, which converts sucrose into dextrose and fructose and thus increases the concentration of the dextrose and fructose compounds. Then, these monosaccharide sugar sources are utilized by the bacteria and yeast to perform the mixed fermentation reactions, causing the concentration of those sugars to ultimately decrease. This trend may become more apparent as fermentation proceeds over time (Kallel, et al. 2012).

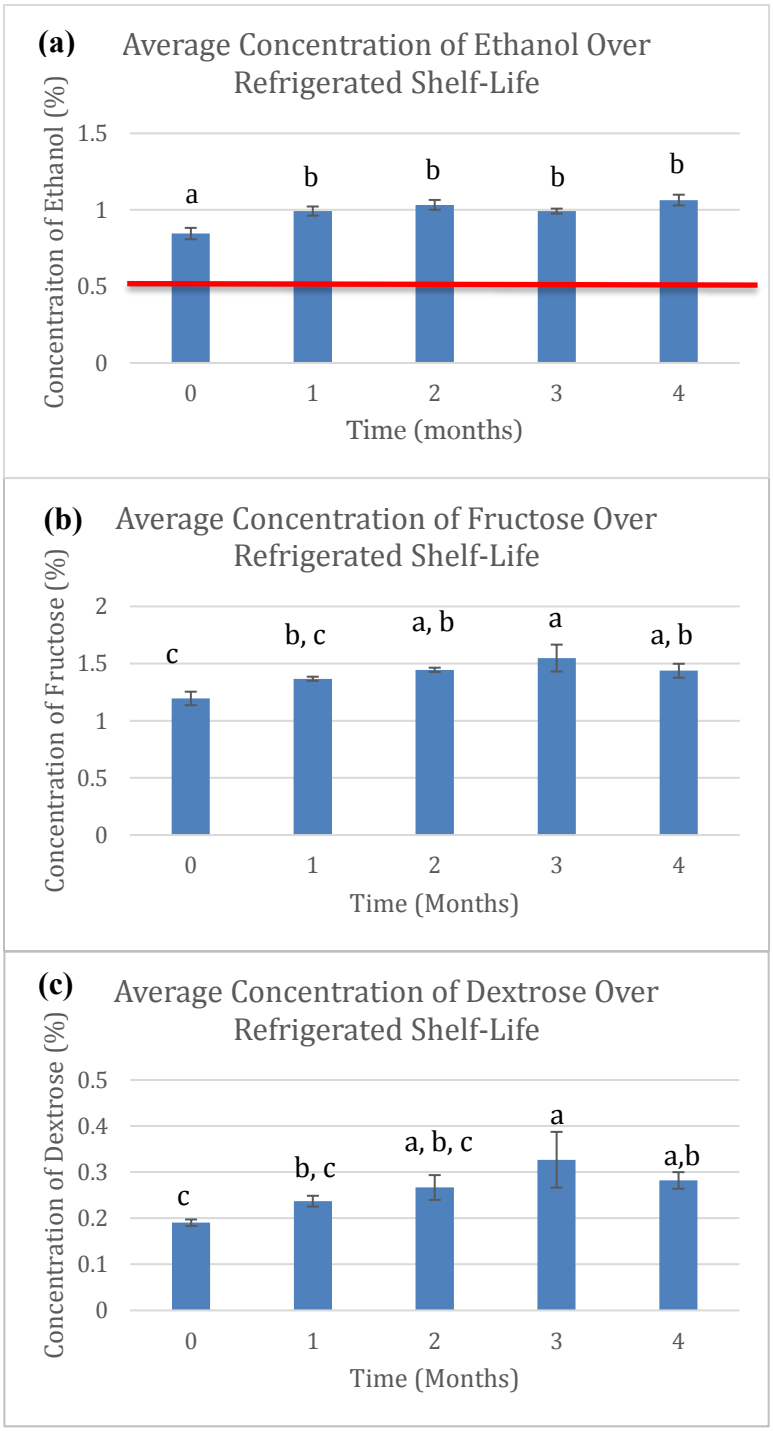

Figure 11. Bar chart displaying the average monthly concentration (% w/w) of fermentation substrates and products (a) ethanol, (b) fructose, (c) dextrose of one commercial Kombucha sample over four months for n=3 samples. Tukey's HSD Test: Levels not connected by same letter are significantly different.

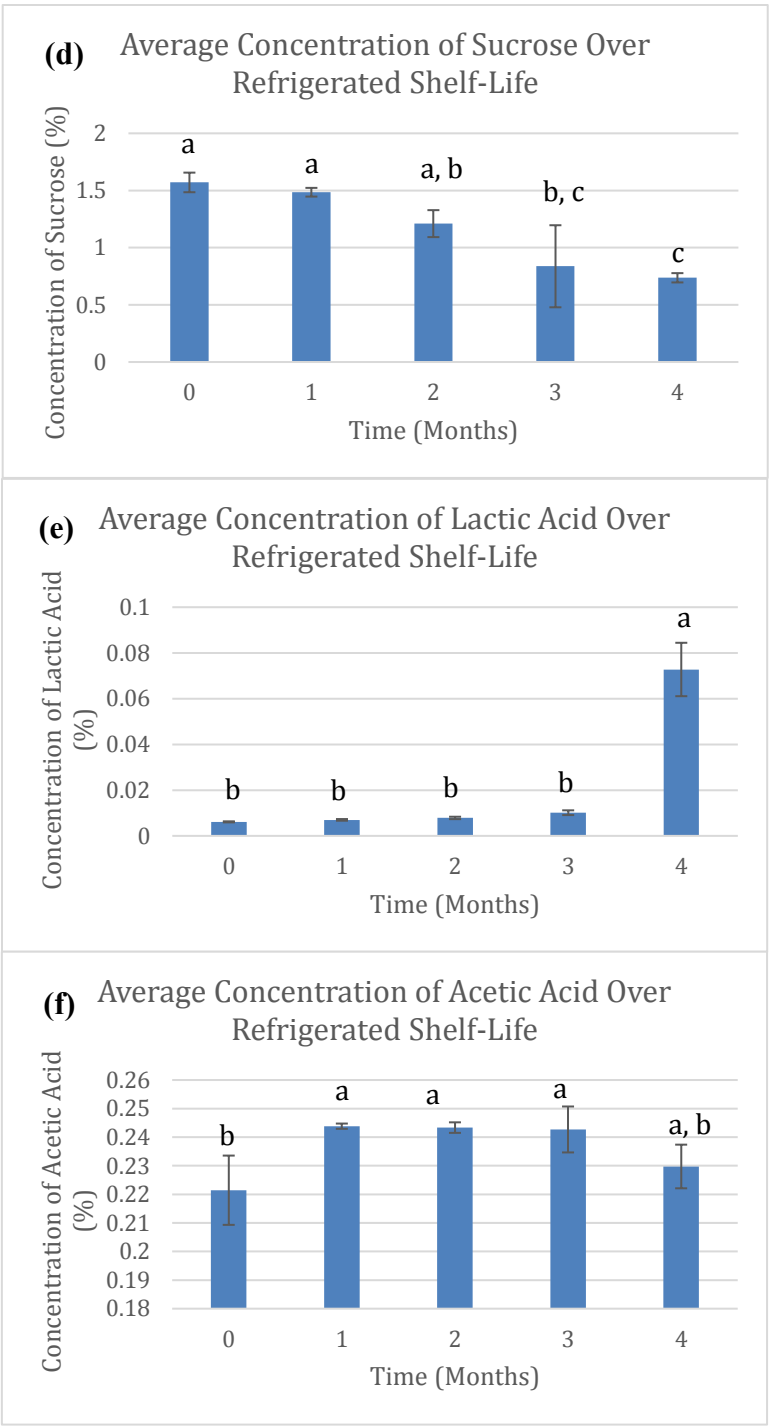

Figure 11 (continued). Bar chart displaying the average monthly concentration (% w/w) of fermentation substrates and products (d) sucrose, (e) lactic acid, and (f) acetic acid of one commercial Kombucha sample over four months for n=3 samples. Tukey's HSD Test: Levels not connected by same letter are significantly different.

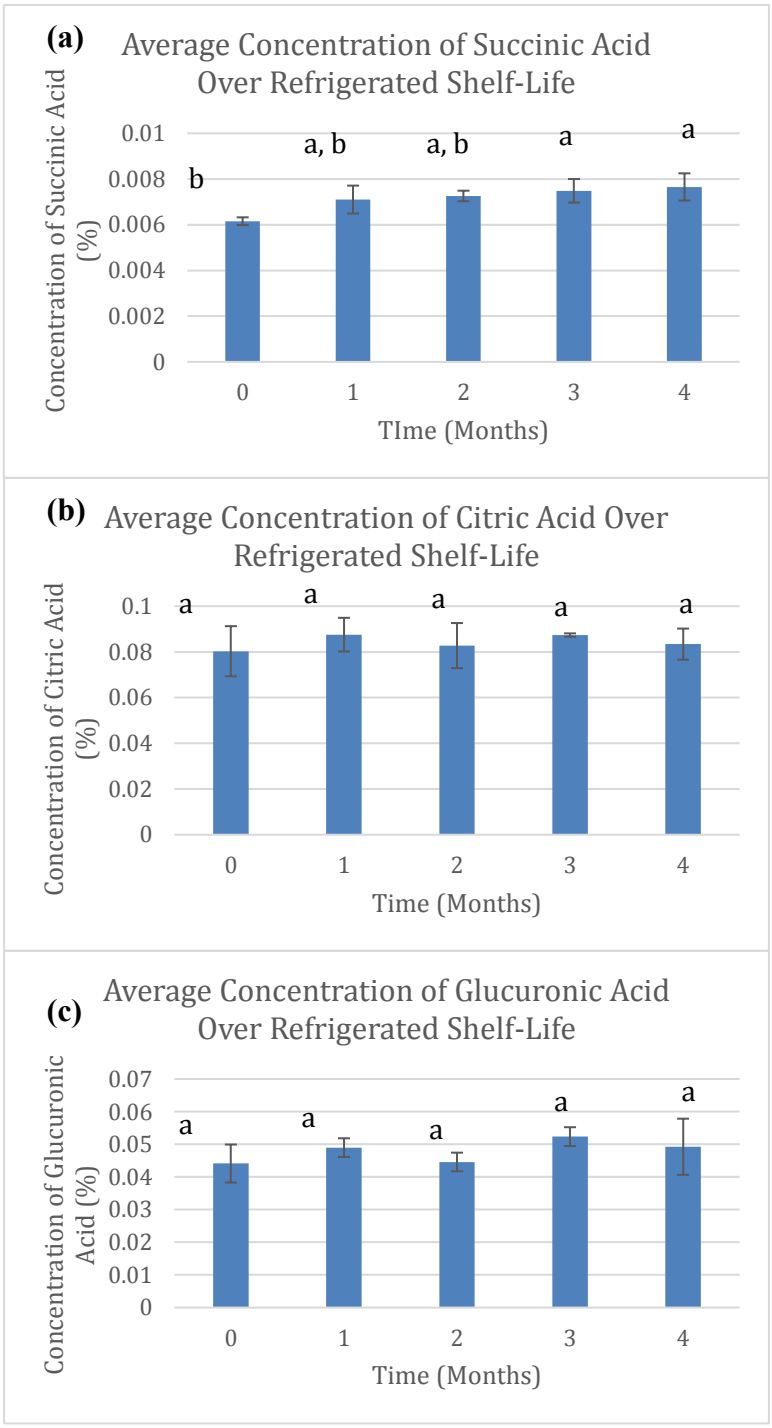

Figure 12. Bar chart displaying the average monthly concentration (% w/w) of acids (a) succinic acid, (b) citric acid, and (c) glucuronic acid of one commercial Kombucha sample over four months for n=3 samples. Tukey's HSD Test: Levels not connected by same letter are significantly different.

The concentration of lactic acid did not significantly change throughout the shelf-life of the Kombucha beverage (Figure 11e). The concentration of lactic acid at time 4 is significantly different than the preceding concentration values (ANOVA $p = 0.0001$); this may be due to batch variation, as the bottles for this time point were retrieved from a different box of 12 bottles. There is large bottle to bottle variation, but box to box variation may be even greater, especially as the SCOBY originate from a different source. We would have expected an increase in concentration of lactic acid, since fermentation is ongoing in the bottle, as reported in literature (Jayabalan, et al. 2007).

Succinic acid data show significance in the increase in concentration over the shelf-life of the product (Figure 12a), with ANOVA $p = 0.0207$, but Tukey's test did not reveal significant differences between samples. The compound, like lactic acid, is a by-product of acid fermentation of glucose by bacteria. Succinic acid is one acid in Kombucha with a concentration that tends to vary within the fermented beverage (Greenwalt, et al. 2000). Acetic acid (Figure 11f), citric acid (Figure 12b) and glucuronic acid (Figure 12c), data did not exhibit any notable trends in the concentration over time. Tukey's test results also did not indicate significant differences between samples analyzed at different time points. Contamination of the SCOBY can introduce microorganisms that compete with glucuronic acid-producing bacteria for nutrients, such that glucuronic acid concentration does not increase (Nguyen, et al. 2015). Citric acid may be an acidulant added in certain amounts to the Kombucha beverage, which may explain the stability in concentration (Jayabalan, et al. 2014). Characterization of the SCOBY should be performed to determine the presence of acetic acid bacteria and to further understand the trends observed in the concentration.

Refrigerated vs. room temperature

In the temperature comparison tests, glucuronic acid was not analyzed due to the inability to distinguish peaks in the NMR spectrum. The concentrations of the compounds did not differ significantly between the two temperature treatments. The concentration of ethanol over three days did not differ significantly when stored at room temperature and refrigeration temperature (Figure 13). Fixed effect tests did not yield effects of treatment, time, or time/treatment interactions on the concentration of ethanol ($p > F$). Additionally, about 30% of variability was attributed to the difference between bottles, while about 70% was attributed to the individual samples. We would have expected the room temperature concentrations to be greater than those at refrigerated temperatures. Higher temperatures increase the reaction rate of fermentation, such that greater amounts of alcohol would be produced in the same amount of time under warmer temperatures than colder temperatures. However, in a study of Kombucha beverages containing milk of different fat contents, the effects of temperature on the rate of fermentation are less significant than the factor of time (Milanovic, et al. 2008). This may be more apparent, as the time frame for the tests was only three days, which may not be an adequate amount of time to observe the obvious effects of temperature. The concentration did remain above the TTB federal limit of 0.5% throughout the period of analysis. The concentrations exceeded the limit most likely due to the ongoing yeast fermentation in the bottle.

The plots for the sugars (Figure 14) also do not display significant differences in concentration between the two temperature treatments. However, the fixed effect tests for

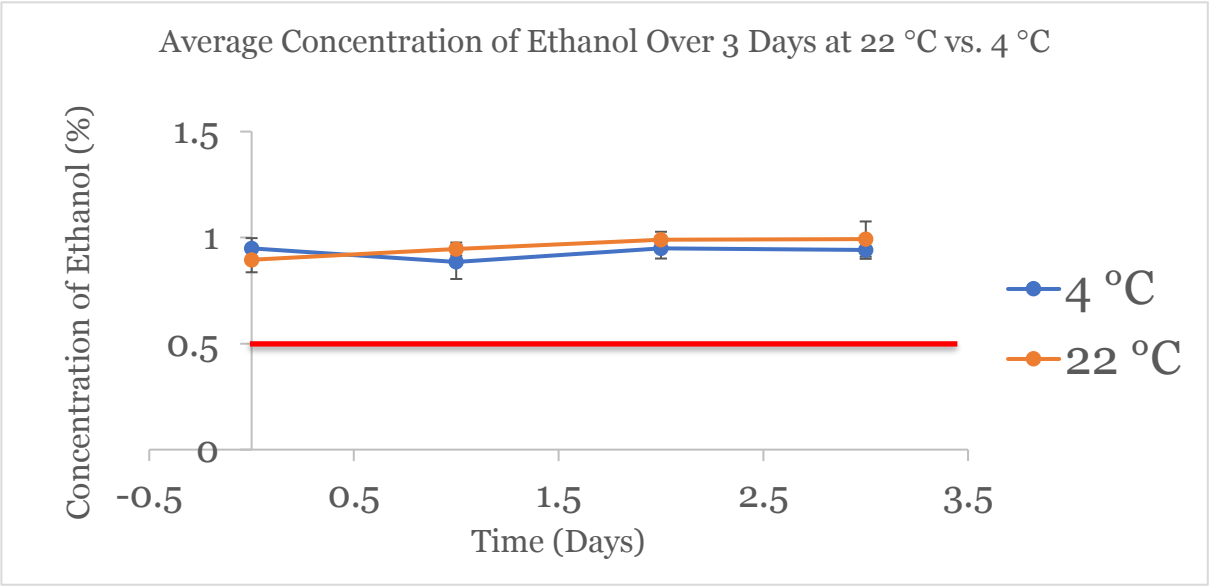

Figure 13. Scatterplot of average daily concentration of ethanol (% w/w) over three days. Data are expressed as mean ± standard deviation of n=3 samples.

dextrose (P = 0.0153) and sucrose (P = 0.0053) showed that the concentrations of these compounds were affected by time. Tukey's HSD post-hoc test was performed to verify the presence of significant differences in sucrose and dextrose concentrations between samples, regardless of the temperature treatment. As previously mentioned, these sugars are substrates for the fermentation products. As fermentation proceeds over time, sucrose was found to decrease, as it is hydrolyzed into dextrose and fructose. However, these monosaccharide sugars are used as starting materials in fermentation reactions, ultimately leading to a decrease in the concentration of dextrose (Kallel, et al. 2012). It is also worth noting that dextrose is more readily used as a substrate for fermentation than fructose, unless isomerization of the fructose molecule into dextrose occurs (Kallel, et al. 2012). The fixed effect test for citric acid also produced a significant p-value (0.0381), which indicates that time did affect the concentration of citric acid. By Tukey's test, we observe that citric acid concentration tended to decrease over time, although

this was not the case for the shelf-life experiments. Citric acid is not a by-product of fermentation, so the observed increase in concentration may be the result of bottle variation (Jayabalan, et al. 2014). It is also possible that error was introduced during sample preparation, which imparts a large amount of error to the analysis (Bharti & Roy, 2012). The titratable acidity of one brand and flavor of refrigerated Kombucha beverage was calculated to be 0.62% by acetic acid. The average pH was determined to be 3.33.

All other compounds analyzed (fructose, lactic acid, acetic acid, and succinic acid) did not result in significant p-values to perform Tukey's test (Figures 14c, 15a, 15b, 15c). Therefore, a difference in the concentrations of the samples over time, at different temperatures, or of both factors is not said to be detected. From the long-term shelf-life tests, we did not see changes in lactic acid and acetic acid over time. The duration of the experiment of only three days may not have been enough time to observe significant changes in the composition of Kombucha due to control factors applied by manufacturers. The inconsistency in data of fructose and succinic acid in this experiment compared to the shelf-life tests may be due to error introduced during sample preparation, processing NMR spectra, or performing calculations.

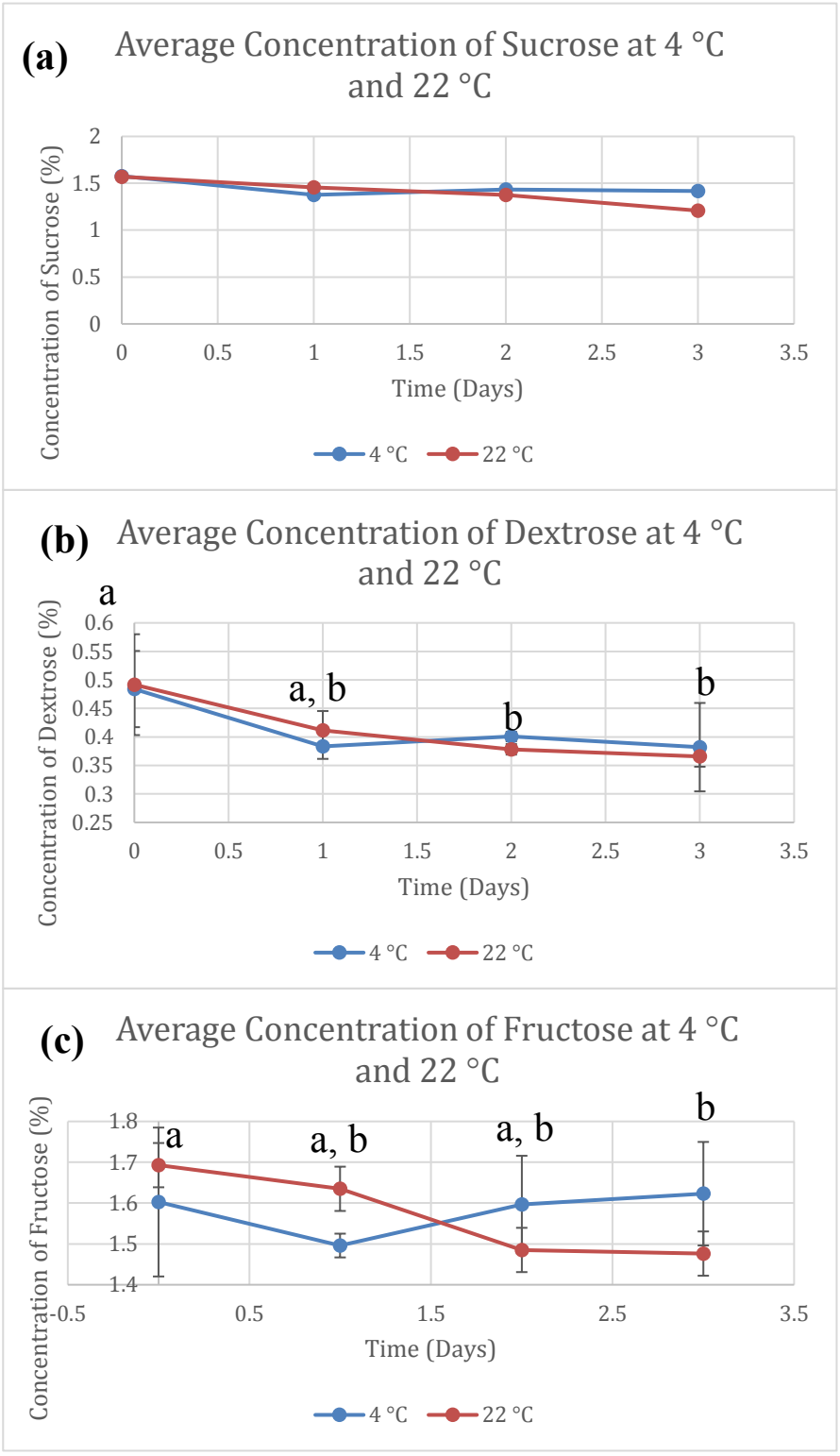

Figure 14. Plots of concentration of sugars (a) sucrose, (b) dextrose, and (c) fructose in commercial Kombucha stored at refrigeration and room temperatures.

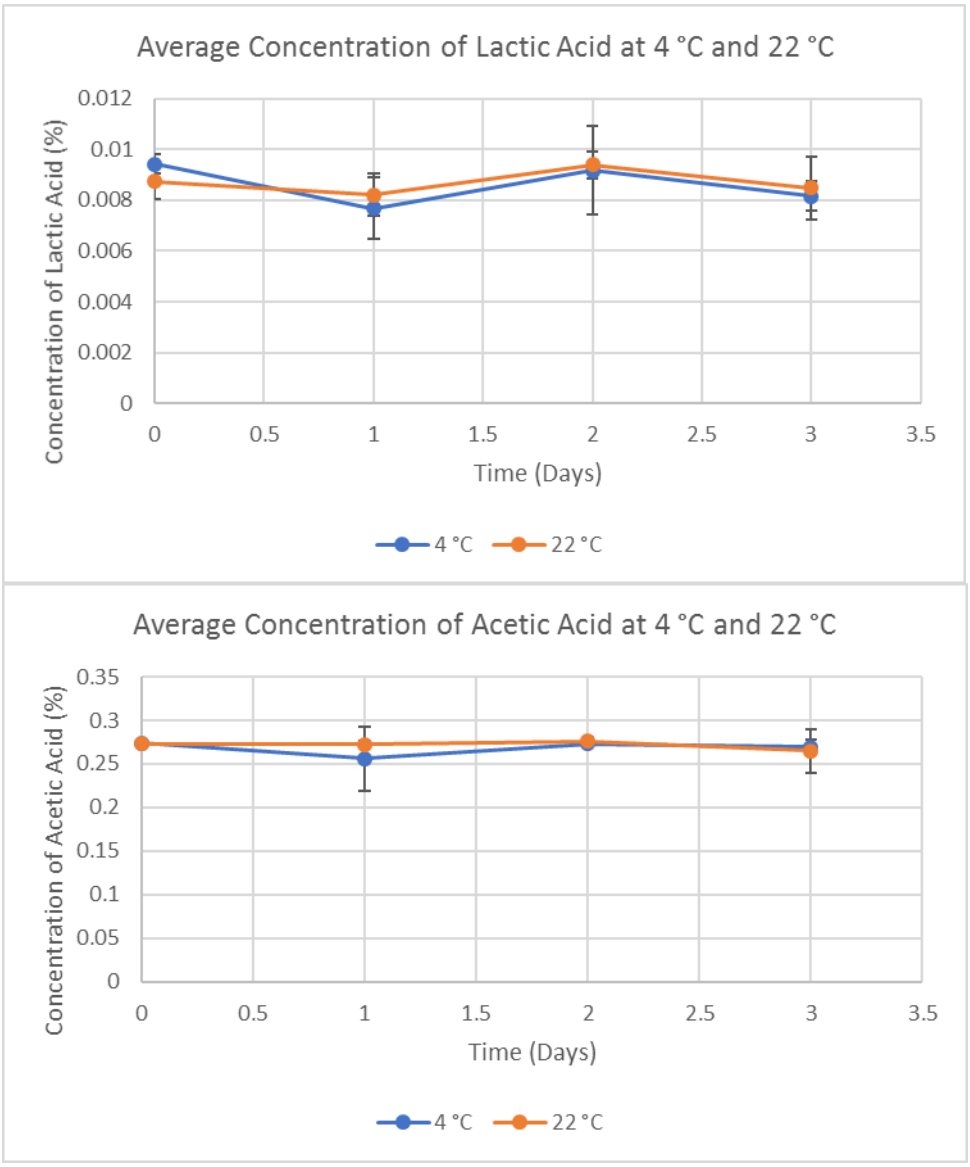

Figure 15. Plots of average concentration of acids (a) lactic, and (b) acetic, (c) succinic, and (d) glucuronic over three days. Data points are expressed as mean ± standard deviation for n=3 samples.

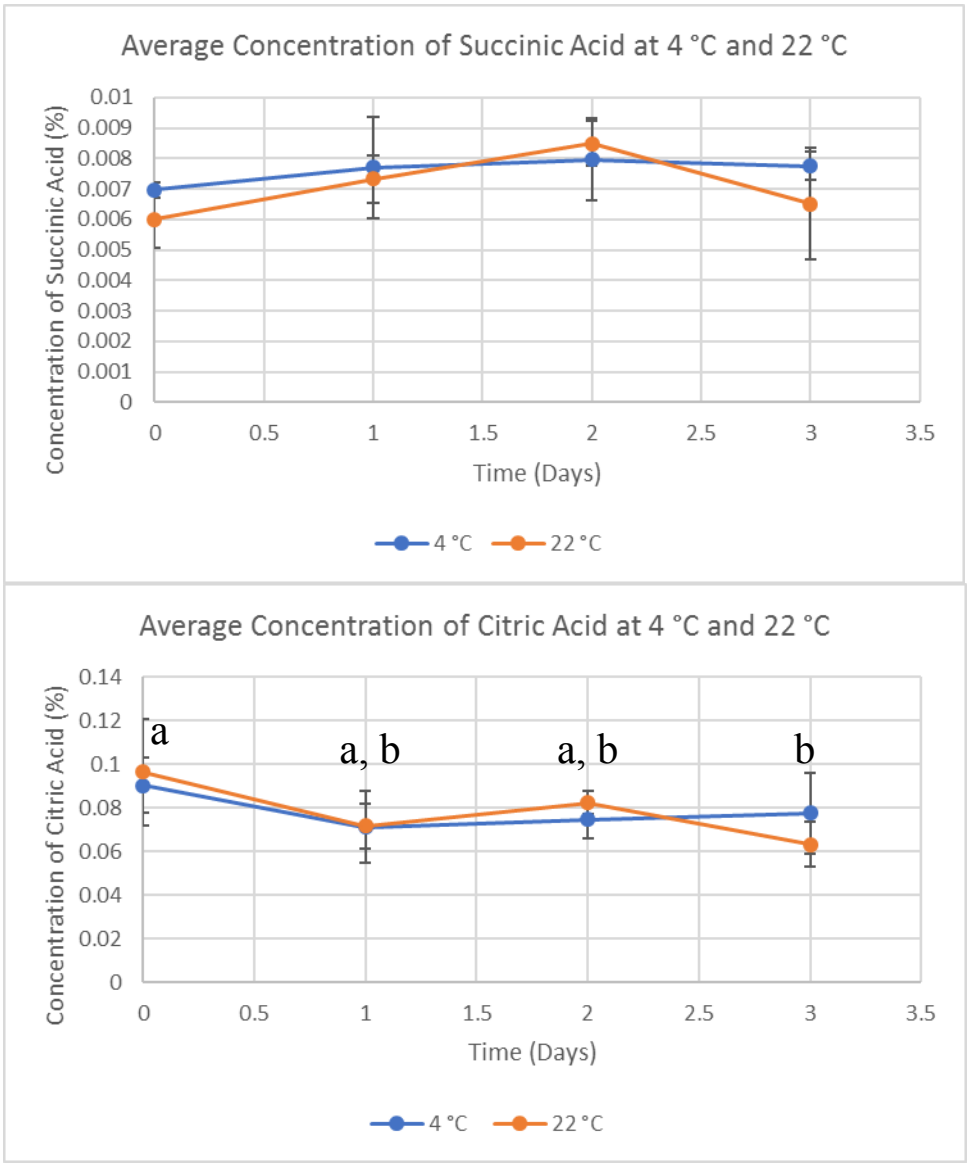

Figure 15 (continued). Plots of average concentration of acids (c) succinic and (d) glucuronic over three days. Data points are expressed as mean ± standard deviation for n=3 samples.

CONCLUSION

Proton NMR is a rapid, accurate, and simple chemical analytical technique to quantify ethanol, acids, and sugars in refrigerated Kombucha beverages. This instrument is especially important in determining the concentration of ethanol, which is federally regulated to a maximum concentration of 0.5% ABV, as there are legal implications for Kombucha manufacturers. Six out of the 10 commercial Kombucha samples analyzed exceeded this concentration, which indicates that many of the products currently on grocery store shelves should be labeled alcoholic and taxed accordingly. The concentration of ethanol consistently remained above 0.5% over the course of the shelf-life as well as over three days, both at room and refrigeration temperatures. It is likely that increase in ethanol production occurred after bottling, as companies are unlikely to bottle Kombucha with an alcohol level above 0.5%. This increase is due to the yeast that continues to ferment sugars into ethanol, perhaps because the reused yeast is not controlled in the rate of ethanol production.

Over the shelf-life, sucrose was found to decrease as dextrose and fructose levels increased. Succinic acid, a by-product of fermentation, increased steadily while the remaining acids analyzed – lactic, acetic, glucuronic, and citric – did not exhibit significant changes in concentration over the shelf-life. The temperature abuse test did not reveal meaningful results regarding the effects of time, temperature, or time and temperature interactions on the concentration of most of the compounds analyzed. The concentrations of dextrose, sucrose, and citric acid were affected by the time.

Proton NMR is a method that requires minimum sample preparation and is useful for accurate quantification that can be utilized in the Kombucha industry to maintain ethanol and sugar label claims.

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CHAPTER 3

CONCLUSIONS AND RECOMMENDATIONS

This work demonstrates the utility of proton NMR in the quantification of ethanol, acids, and sugars in Kombucha beverages. The concentration of ethanol and sugars is of concern in the Kombucha industry, as companies are exceeding the federal regulation limit of ethanol and the and the labeled amount of sugar. Kombucha beverages that exceed 0.5% alcohol concentration should be labeled alcoholic, taxed accordingly, and not consumed by minors or pregnant women.

Comparison of alcohol content between commercially bottled Kombucha with Kombucha sold on tap or home-brewed Kombucha can also be performed by qNMR. The different methods of brewing Kombucha may be of interest to some companies to improve the manufacturing of Kombucha, specifically to slow down or completely halt alcohol fermentation reactions.

It may be of interest to compare proton NMR with more common methods of quantification of compounds. Currently, industries enlist the aid of laboratories equipped with high-performance liquid chromatography (HPLC) or gas chromatography (GC) with mass spectrometry (MS) or flame ionization detection (FID) to quantify ethanol, acids, and sugars. Comparison of costs and of accuracy of quantification would provide an adequate overview of the available techniques that scientists and industry professionals may find useful in the decision to choose one method.

Proton NMR can be applied to quantify preservatives in beverages, such as juice. Food preservatives, like sodium benzoate and potassium sorbate, are federally regulated to be present no more than 0.1% by weight in food and beverages. An analysis of various beverages that contain preservatives can be performed to assess the regulatory compliance of products currently on grocery store shelves.

Finally, use of Direct Analysis in Real Time with Mass Spectrometry (DART-MS) to identify additional compounds in Kombucha can be performed. The mass spectrum displayed the

presence of an aldehyde compound, which prompts investigation as to its origins within the complex world of Kombucha.

Analysis of the microbiological life within Kombucha may assist in accounting for the bottle to bottle variation that existed in the samples. Each SCOBY is different, and may result in different fermentation production capacities, producing varying quantities of ethanol, acids, and sugars. There may be standardization of commercial Kombucha fermentation to control for these anticipated differences.

Shortcomings and Recommendations

For future experiments based on this project, it is recommended that the brand comparison test consider the entire shelf-life of a commercial product of Kombucha. The purpose of the experiment was to determine the concentration of ethanol in Kombucha products as they sit on the shelves. However, it may be interesting to obtain information regarding the production date and point in shelf-life of the different products, as this may account for the wide range in ethanol concentrations.

The shelf-life analysis was performed monthly, which may not have been enough samples. Biweekly instead of monthly analysis would provide more data and perhaps a more accurate picture of the trend in concentration over time.

As for qNMR specifically, there is error that could have been introduced at various steps, such as systematic error during sample preparation, inherent error associated with quantification of the compounds, or subjective error introduced by manual integration of peaks. Peaks may have included interference from other compounds.

APPENDIX

Table A1. Raw data of concentration of ethanol and pH, with average and standard deviation for n=2 samples, for a variety of commercial brands and flavors of Kombucha.

Flavor	Ethanol (%)	Average	SD	pH	Average	Standard Deviation
A1	2.07002846	2.075559358	0.007821871	3.63	3.64	0.014142136
A1	2.081090256			3.65		
A2	1.864682427	1.839919091	0.035020645	3.28	3.28	0
A2	1.815155755			3.28		
B1	0.939234258	0.938415966	0.001157239	3.11	3.125	0.021213203
B1	0.937597675			3.14		
B2	0.831935008	0.684834804	0.208031103	3.78	3.79	0.014142136
B2	0.537734601			3.8		
B3	0.520837257	0.52158043	0.001051005	3.33	3.345	0.021213203
B3	0.522323603			3.36		
C1	0.732076727	0.754557621	0.031792785	3.31	3.275	0.049497475
C1	0.777038515			3.24		
C2	0.471235504	0.432810171	0.054341627	3.27	3.24	0.042426407
C2	0.394384838			3.21		
D1	0.13403148	0.129890976	0.005855556	3	3.02	0.028284271
D1	0.125750472			3.04		
D2	0.071100436	0.07083848	0.000370462	3.49	3.5	0.014142136
D2	0.070576524			3.51		
E1	0.006952448	0.007215561	0.000372097	3.36	3.36	0
E1	0.007478673	2.075559358	0.007821871	3.36	3.64	0.014142136

Table A2. Average concentration of ethanol and pH of commercial refrigerated Kombucha samples. Data are expressed as mean ± standard deviation of n=2 samples.

Brand and Flavor Code	Mean Concentration ± SD*	Mean pH ± SD
A1	2.08 ± 0.01^{a}	3.64 ± 0.01
A2	1.84 ± 0.04^{a}	3.28 ± 0.00
B1	0.94 ± 0.00^{b}	3.13 ± 0.02
B2	$0.68 \pm 0.21^{b, c}$	3.79 ± 0.01
B3	$0.52 \pm 0.00^{b, c, d}$	3.35 ± 0.02
C1	$0.75 \pm 0.03^{c, d}$	3.28 ± 0.05
C2	0.43 ± 0.05^{d}	3.24 ± 0.04
D1	0.13 ± 0.01^{e}	3.02 ± 0.03
D2	0.07 ± 0.00^{e}	3.50 ± 0.01
E1	0.01 ± 0.00^{e}	3.36 ± 0.00

* Levels not connected by the same letter are significantly different.

Table A3. Raw data of concentration of ethanol, fructose, dextrose, sucrose, lactic acid, acetic acid, succinic acid, glucuronic acid. Each compound was measured in triplicate (A, B. C) for each month (0, 1, 2, 3, 4).

Bottle ID	Time	EtOH	Fructose	Dextrose	Sucrose	Lactic Acid	Acetic Acid	Succinic Acid	Glucuronic Acid	Citric Acid
A0	0	0.860573	1.256448	0.198156	1.665461	0.00638639	0.232532821	0.006338961	0.049549858	0.087174
B0	0	0.80286	1.138658	0.18456	1.49869	0.00602847	0.20851149	0.006003234	0.037977941	0.067626
C0	0	0.872465	1.190309	0.188605	1.54795	0.00596457	0.223274046	0.006128589	0.044817936	0.086041
A1	1	1.017105	1.356259	0.230148	1.464422	0.00731302	0.244903704	0.006511909	0.049959122	0.079017
B1	1	1.000677	1.357093	0.230483	1.461265	0.00660022	0.243271916	0.007062434	0.051230204	0.092394
C1	1	0.959109	1.388166	0.250583	1.528924	0.00707729	0.243352193	0.007731624	0.045758616	0.091126
A2	2	0.997021	1.432881	0.29525	1.325642	0.00792208	0.245474527	0.006993102	0.041499932	0.071686
B2	2	1.041741	1.466623	0.263099	1.215768	0.00728734	0.242390937	0.007414198	0.047116683	0.085957
C2	2	1.059671	1.437245	0.241848	1.089969	0.00833093	0.242181614	0.007371943	0.045162944	0.090618
A3	3	1.00739	1.441244	0.283215	1.059643	0.01129755	0.239882588	0.008028454	0.049178764	0.088211
B3	3	0.993442	1.529537	0.301593	1.029295	0.00980635	0.236452965	0.007427673	0.05307103	0.086798
C3	3	0.9745	1.674139	0.395972	0.423489	0.00930196	0.251767037	0.007004045	0.054813272	0.087017
A4	4	1.023622	1.506286	0.268755	0.700534	0.0787001	0.22100386	0.007350975	0.040283142	0.076268
B4	4	1.083463	1.390962	0.302557	0.728663	0.08028304	0.233132924	0.008340058	0.057435003	0.089852
C4	4	1.085241	1.414993	0.274679	0.780896	0.05930604	0.235102879	0.007270844	0.050014739	0.084049

Table A4. Raw data of concentration of ethanol, fructose, dextrose, sucrose, lactic, acetic, succinic, and citric acids in refrigerated Kombucha for temperature comparison study.

		Refrigerated			**Shelf-storage**			
Ethanol	Time	a	b	c	Time	a	b	c
	0	0.962406	0.895697	0.98969	0	0.906072	0.948369	0.831903
	1	0.950862	0.909119	0.795601	1	0.936522	0.980193	0.920114
	2	0.952865	0.899298	0.994081	2	0.970304	1.033309	0.964428
	3	0.92139	0.913618	0.9888	3	1.029246	1.05195	0.89784
Fructose	0	1.629518	1.56707	1.611158	0	1.901178	1.561929	1.615655
	1	1.587893	1.609376	1.290806	1	1.660961	1.603526	1.640392
	2	1.580175	1.617152	1.592468	2	1.41439	1.622894	1.418319
	3	1.609985	1.644062	1.61496	3	1.443938	1.616159	1.368855
Dextrose	0	0.49299	0.413044	0.545846	0	0.468963	0.416773	0.58922
	1	0.384241	0.405269	0.361142	1	0.450215	0.386564	0.397031
	2	0.410118	0.397755	0.394238	2	0.381323	0.385488	0.367836
	3	0.293228	0.436274	0.416618	3	0.359857	0.385927	0.351372
Sucrose	0	1.571757	1.506325	1.65089	0	1.676403	1.416798	1.617337
	1	1.474805	1.520015	1.134009	1	1.519935	1.379026	1.472517
	2	1.461574	1.484539	1.357518	2	1.379753	1.376375	1.373186
	3	1.348763	1.499217	1.400718	3	1.285868	1.191218	1.146991
Lactic acid	0	0.009816	0.009078	0.00936	0	0.009497	0.008237	0.008475
	1	0.008953	0.006509	0.007587	1	0.007658	0.009172	0.007821
	2	0.01114	0.008637	0.007758	2	0.008926	0.009286	0.009942
	3	0.007661	0.008035	0.008809	3	0.009501	0.007122	0.008804
Acetic Acid	0	0.277861	0.271377	0.272995	0	0.274862	0.274102	0.270004
	1	0.27641	0.277706	0.21384	1	0.276484	0.27085	0.27058
	2	0.270582	0.273094	0.276052	2	0.272663	0.282212	0.273506
	3	0.259025	0.2739	0.27525	3	0.281965	0.276581	0.235397
Succinic Acid	0	0.006711	0.007004	0.007183	0	0.004934	0.006498	0.006555
	1	0.008942	0.008373	0.005802	1	0.007924	0.006452	0.007595
	2	0.009445	0.006823	0.007627	2	0.008382	0.009267	0.0078
	3	0.007532	0.007446	0.008306	3	0.007389	0.007761	0.004397
Citric Acid	0	0.082875	0.08348	0.086268	0	0.124401	0.078792	0.086268
	1	0.082748	0.07843	0.073628	1	0.080947	0.060661	0.073628
	2	0.077271	0.081425	0.088272	2	0.079525	0.079414	0.088272
	3	0.072704	0.098091	0.054748	3	0.074385	0.060703	0.054748

Table A5. Average monthly concentration of ethanol, sugars, and acids for commercial refrigerated Kombucha beverage. Data are expressed as mean ± standard deviation of n=3 samples.

	Compound								
Time	**Ethanol**	**Fructose**	**Dextrose**	**Sucrose**	**Lactic Acid**	**Acetic Acid**	**Succinic Acid**	**Glucuronic Acid**	**Citric Acid**
0	0.85 ± 0.04^{a}	1.20 ± 0.06^{c}	0.19 ± 0.01^{c}	1.57 ± 0.09^{a}	0.0061 ± 0.0002^{b}	0.22 ± 0.01^{b}	0.0062 ± 0.0002^{b}	0.044 ± 0.006^{a}	0.080 ± 0.011^{a}
1	0.99 ± 0.03^{b}	$1.37 \pm 0.02^{b, c}$	$0.24 \pm 0.01^{b, c}$	1.48 ± 0.04^{a}	0.0070 ± 0.0004^{b}	0.24 ± 0.00^{a}	$0.0071 \pm 0.0006^{a, b}$	0.049 ± 0.003^{a}	0.088 ± 0.007^{a}
2	1.03 ± 0.03^{b}	$1.45 \pm 0.02^{a, b}$	$0.27 \pm 0.03^{a, b, c}$	$1.21 \pm 0.12^{a, b}$	0.0078 ± 0.0005^{b}	0.24 ± 0.00^{a}	$0.0073 \pm 0.0002^{a, b}$	0.045 ± 0.003^{a}	0.083 ± 0.010^{a}
3	0.99 ± 0.02^{b}	1.55 ± 0.12^{a}	0.33 ± 0.06^{a}	$0.84 \pm 0.36^{b, c}$	0.010 ± 0.001^{b}	0.24 ± 0.01^{a}	0.0075 ± 0.0005^{a}	0.052 ± 0.003^{a}	0.087 ± 0.001^{a}
4	1.06 ± 0.04^{b}	$1.44 \pm 0.06^{a, b}$	$0.28 \pm 0.02^{a, b}$	0.77 ± 0.04^{c}	0.07 ± 0.01^{a}	$0.23 \pm 0.01^{a, b}$	0.0077 ± 0.0006^{a}	0.049 ± 0.009^{a}	0.083 ± 0.007^{a}

*Levels within the same column not connected by the same letter are significantly different.

Table A2. Average daily concentration of ethanol, sugars, and acids for commercial Kombucha stored at 4 °C. Data are expressed as mean ± standard deviation of n=3 samples.

	Compound							
Time	**Ethanol**	**Fructose**	**Dextrose**	**Sucrose**	**Lactic Acid**	**Acetic Acid**	**Succinic Acid**	**Citric Acid**
0	0.95 ± 0.05	1.60 ± 0.03	0.48 ± 0.07	1.58 ± 0.07	0.0094 ± 0.0004	0.274 ± 0.003	0.0070 ± 0.0002	0.09 ± 0.01
1	0.89 ± 0.08	1.50 ± 0.18	0.38 ± 0.02	1.38 ± 0.21	0.0077 ± 0.0012	0.256 ± 0.036	0.0077 ± 0.0017	0.07 ± 0.02
2	0.95 ± 0.05	1.60 ± 0.02	0.40 ± 0.01	1.43 ± 0.07	0.0092 ± 0.0018	0.273 ± 0.003	0.0080 ± 0.0013	0.07 ± 0.01
3	0.94 ± 0.04	1.62 ± 0.02	0.38 ± 0.08	1.42 ± 0.08	0.0082 ± 0.0006	0.269 ± 0.009	0.0078 ± 0.0004	0.08 ± 0.02

Table A7. Average daily concentration of ethanol, sugars, and acids for commercial Kombucha stored at 22 °C. Data are expressed as mean ± standard deviation of n=3 samples.

	Compound							
Time	**Ethanol**	**Fructose**	**Dextrose**	**Sucrose**	**Lactic Acid**	**Acetic Acid**	**Succinic Acid**	**Citric Acid**
0	0.90 ± 0.06	1.69 ± 0.18	0.49 ± 0.09	1.57 ± 0.14	0.0087 ± 0.0007	0.273 ± 0.003	0.0060 ± 0.0009	0.10 ± 0.02
1	0.95 ± 0.03	1.63 ± 0.03	0.41 ± 0.03	1.46 ± 0.07	0.0082 ± 0.0008	0.273 ± 0.003	0.0073 ± 0.0008	0.07 ± 0.01
2	0.99 ± 0.04	1.49 ± 0.12	0.38 ± 0.01	1.38 ± 0.00	0.0094 ± 0.0005	0.276 ± 0.005	0.0085 ± 0.0007	0.08 ± 0.01
3	0.99 ± 0.08	1.48 ± 0.13	0.37 ± 0.02	1.21 ± 0.07	0.0085 ± 0.0012	0.265 ± 0.025	0.0065 ± 0.0018	0.06 ± 0.01

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