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Examination of the Varied and Changing Ethanol Content of Commercial Kombucha Products

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Abstract Kombucha is a fermented beverage made by mixing tea and sugar with bacteria and yeast. When kombucha products contain higher than 0.5% (*v*/*v*) alcohol, the legal limit for non-alcoholic drinks, they are classified as alcoholic beverages and are subject to relevant federal and state regulations. An efficient headspace gas chromatography technique utilizing an ionic liquid stationary phase is developed to accurately determine the ethanol content in 18 commercial kombucha samples. The range of ethanol in these products was 1.12–2.00% (*v*/*v*). The ethanol concentration of two batches of kombucha was analyzed over a period of 60 days under two different conditions. A significant increase in ethanol content of these samples was observed at 4 and 22 °C. The method accuracy was validated by analyzing 3 NIST ethanol-water standard reference solutions.

Keywords Kombucha · Ethanol analysis · Ionic liquid column · Headspace gas chromatography

Introduction

Kombucha is a traditional fermented beverage brewed from tea extract and supplemented with sugar and flavors (Jayabalan et al. 2014). A symbiotic culture of bacteria and yeast (SCOBY) form a surface mat or biofilm, which is known as tea fungus (Dufresne and Farnworth 2000). During inoculation with tea fungus, sucrose is converted into glucose and fructose to produce ethanol and carbon dioxide. While a substantial amount of fructose stays unfermented during this process, acetic acid and gluconic acid are produced from oxidation of ethanol and glucose, respectively (Dufresne and Farnworth 2000). Production of organic acids during the fermentation lowers the pH of kombucha (Sreeramulu et al. 2000). Therefore, the beverage tastes mildly acidic and mildly sweet, and the existence of residual carbon dioxide makes it slightly effervescent. An analysis of the chemical composition of kombucha indicates the presence of different metabolites (Dufresne and Farnworth 2000). A number of factors may affect the concentration of constituents in such beverages. The initial content of tea and sugar (Goh et al. 2012), the time course of fermentation (Jayabalan et al. 2010), incubation temperature (Jayabalan et al. 2008), and the nature of the kombucha culture (Greenwalt et al. 2000) all influence the concentration of components in this complex beverage.

While there have been investigations into the microbiological and "health-promoting" aspects of kombucha, there is a lack of crucial information on the actual concentrations of the key components of these beverages (Dufresne and Farnworth 2000; Vīna et al. 2014). Specifically, very few studies have been devoted to determination of alcohol content in kombucha beverages (Blanc 1996; Greenwalt et al. 1998; Reiss 1994; Sievers et al. 1995). Over the last few years, it has been found that some bottled kombucha products contain over 0.5% *v*/*v* alcohol (Nummer 2013). The US government's Alcohol and Tobacco Tax and Trade Bureau (TTB 2016) regulations stipulate that anything containing more than 0.5% alcohol by volume (ABV) is considered alcoholic. Consequently, these commercial products are required to comply with TTB labeling regulations as well as FDA labeling regulations.

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Utilizing an enzymatic method, Reiss (1994) measured 6.3 g/l ethanol in a kombucha beverage when 50 g/l sucrose was used for fermentation. The effect of incubation period on fermentation of kombucha was evaluated by Sievers et al. (1995). The ethanol content reached a maximum of 9.1 g/l after 24 days of incubation and then decreased to 0.7 g/l after 62 days. Greenwalt et al. (1998) found that by continuing fermentation beyond the desired endpoint, the alcohol concentration reached 14 g/l (approx. 1.8% ABV) and the solution became more acidic. These studies were part of a large, highly controlled, laboratory-scale investigation (Greenwalt et al. 1998; Reiss 1994; Sievers et al. 1995). However, the collective population of organisms and fermentation conditions in commercial bottled products can be different from such lab-scale trials. Besides, traditional analytical methods, such as enzymatry, may have inadequate accuracy and reproducibility given the stability of the enzyme substrate among other factors (Mason 1983). These approaches also can be time and labor intensive. Therefore, a rapid, accurate, and precise analytical method to measure alcohol content in large-scale commercial batches as well as individual consumer products appears to be necessary.

Headspace gas chromatography (HSGC) is an effective analysis technique for the determination of ethanol concentration in fermented beverages (Ibañez and Cifuentes 2001; Li et al. 2009; Liu et al. 2014). Headspace sampling is based on thermostatic partitioning of volatile compounds in a sealed vial between a liquid sample and the vapor phase (Cheng et al. 2010; Kolb and Ettre 2006). Using this approach, non-volatile constituents in the sample will not interfere with the determination of the volatile species of interest (Jeleń et al. 2017; Snow and Bullock 2010).

In the present work, we report the development of a facile approach, based on headspace gas chromatography for rapid determination of ethanol in kombucha products. The effects of HSGC conditions on separation of ethanol from other volatile components also are discussed. In addition, changes in the ethanol content over the drinks' shelf life at two different temperatures were investigated.

Materials and Methods

Materials

Pure anhydrous 200 proof ethanol was purchased from Sigma-Aldrich (Milwaukee, WI). Deionized water was produced by Synergy 185 water purification system (Millipore, Billerica, MA). Kombucha bottled drinks with same expiration date were purchased at local grocery stores. Ethanol-water NIST standard reference solutions containing nominal mass fractions of 0.2% (SRM 2895), 2% (SRM 2897a), and 6% (SRM 2898a) were obtained from the National Institute of Standards and Technology (Gaithersburg, MD). Screw-thread vials (22.5 × 46 mm) were obtained from Supelco (Bellefonte, PA), and magnetic screw thread caps (18 mm) were purchased from Restek (Bellefonte, PA).

Apparatus and Conditions

The HSGC analyses were conducted on a GC-2010 plus model (Shimadzu Scientific Instruments, Kyoto, Japan) gas chromatograph equipped with a flame-ionization detector. An AOC-5000 plus model (Shimadzu Scientific Instruments, Kyoto, Japan) autosampler was employed to transfer sealed vials into an agitation unit, where the vials were incubated at 150 °C for 2 min. The oven was kept isothermally at 100 °C. The injection port was set at 200 °C and the FID was set at 250 °C. Helium was used as a carrier gas at a constant flow rate of 1 ml/min with a split ratio of 100:1. Total run time for retention of volatile components was under 3 min. The analyses were carried out on a 30 m × 0.25 mm ID × 0.2 μm film thickness of a Watercol 1910 (Electronic supplementary material) capillary column acquired from Supelco (Bellefonte, PA). This stationary phase was previously synthesized by our group (Huang et al. 2007). All samples were measured on a TLE204E Mettler Toledo balance (Columbus, OH). A G-560 Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY) was used when sample mixing was required.

Sample Preparation

All samples were filtered through a 0.22-μm PVDF membrane before measurement to remove suspended sediments and yeast strains from freshly opened kombucha product bottles. The vials were immediately covered to avoid evaporation of ethanol. Calibration curves were constructed using solutions of 12, 25, 50, 150, and 250 mg ethanol plus 4.988, 4.975, 4.950, 4.850, and 4.750 g water, respectively. After they were mixed, 500 mg of sample was pipetted to a 10-ml vial, and the sealed vials were analyzed. The standard addition samples were prepared by spiking 6, 12, and 25 mg ethanol to 500 mg kombucha. All samples were made and analyzed in quadruplicate.

Results and Discussion

GC Conditions

In order to obtain a rapid, efficient, and accurate ethanol measurement, optimum GC parameters were determined as indicated in the "Materials and Methods" section. An optimized split ratio of 100:1 and an isothermal oven temperature of 100 °C were selected for separation of ethanol from the other volatile species. The choice of an appropriate column is one of

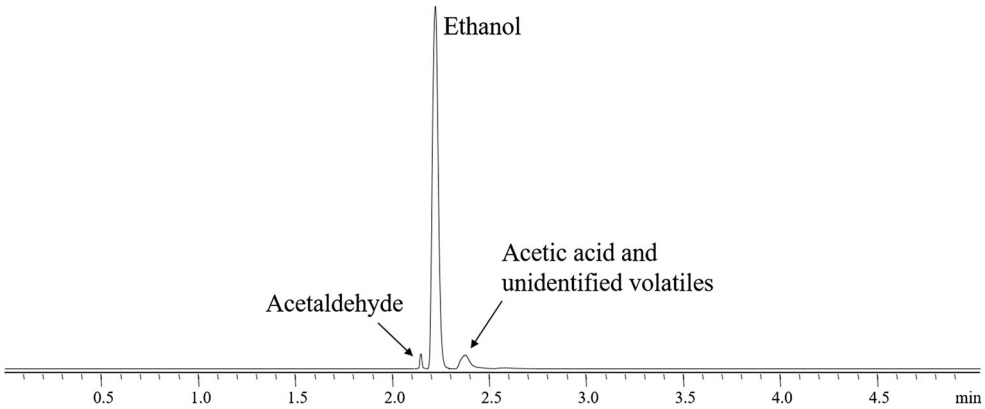

Fig. 1 A chromatogram of a typical analysis of ethanol in GT's "Kombucha Original" using HSGC-FID at 100 °C with a split ratio of 100:1. See the "Materials and Methods" section for method details

the key parameters in the GC separation. The Watercol 1910 column was selected since it provides more symmetric peak shapes and shorter retention times compared to other commercially available stationary phases. In addition, it is completely stable in the presence of water (Weatherly et al. 2014). This stationary phase has demonstrated promising results for separation of water and ethanol from many other matrices as well (Frink and Armstrong 2016a, b, c; Frink et al. 2014). Figure 1 gives an example chromatogram for the headspace analysis of GT's "Kombucha Original" obtained in under 3 min using the Watercol 1910 capillary GC column. Clearly, the headspace GC method can efficiently eliminate the interference from the coexisting volatile (e.g., acetic acid and acetaldehyde) and non-volatile species found in kombucha samples.

Headspace Conditions

The effect of headspace equilibration temperature as well as the equilibration time for partitioning of ethanol in kombucha samples was investigated. In general, higher temperatures are favorable provided they enhance the analyte amount in the vapor phase without degradation or excessive vaporization of interfering compounds (Frink et al. 2014). As shown in Fig. 2a, higher incubation temperatures resulted in the greater areas of the ethanol chromatographic peaks. Therefore, a temperature of 150 °C was optimal for this study. At a given temperature (150 °C), the effect of equilibrium time to the ethanol mass transfer from GT's Kombucha Original sample was investigated. As depicted in Fig. 2b, the ethanol peak area increases with the incubation time period up to 2 min, after which there is no significant change because there is complete vaporization. Since a short equilibrium time is desired for an efficient analysis, we selected 2 min as the equilibrium time in all following experiments.

Sample Analysis

A calibration curve was made using different concentrations of ethanol in water as standard solutions (see the "Materials and Methods" section). A regression coefficient (r^2) equal to 0.995 with a line equation of y = 4.4E + 08× + 3.2E + 05 was obtained for the linear relationship between the peak area and the ethanol concentration (Electronic supplementary material). The limit of detection (LOD) and limit of quantification (LOQ) for this method are determined to be 4.7 and 14.4 ppm respectively. The alcohol content of 18 commercial kombucha beverages was measured using the indicated calibration plot, and the results are given in Fig. 3. The range of ethanol was 1.12–2.00% (*v*/*v*), all of which exceeded the US regulatory limit for non-alcoholic beverages. The determined alcohol concentration in different kombucha beverages differ due to different manufacturing procedures including variation of microbial

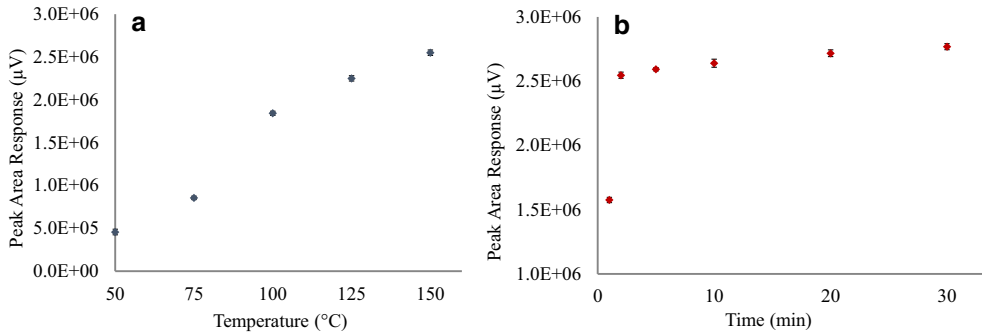

Fig. 2 **a** The effect of the equilibration temperatures on measured ethanol from a sample of GT's Kombucha Original. **b** The effect of equilibrium time at 150 °C on the amount of ethanol detected in the vapor phase from a GT's Kombucha Original sample

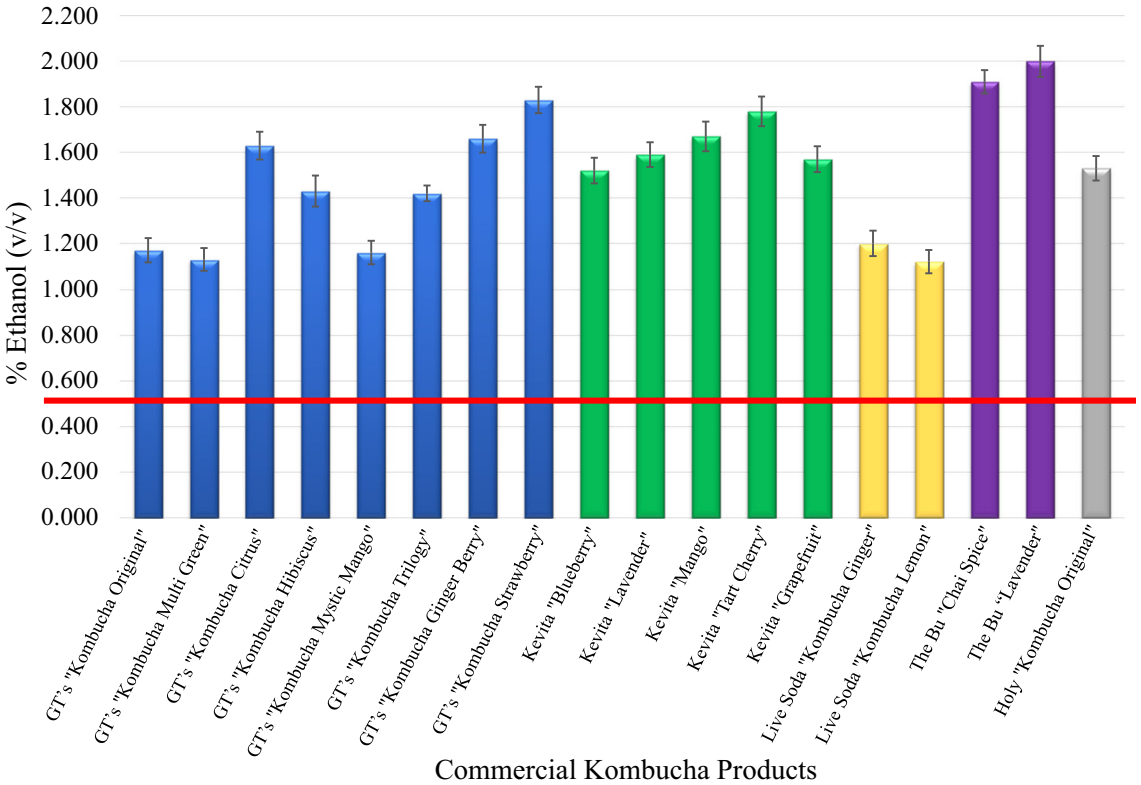

Fig. 3 Determination of ethanol in commercial kombucha samples. The different *colors* in the graph represent different manufacturing products. The *red line* at 0.5% ethanol (by volume) represents the highest amount of ethanol that can be contained in commercial products to be considered as nonalcoholic beverage by US Alcohol and Tobacco Tax and Trade Bureau (TTB 2016). For chromatographic conditions, see the "Materials and Methods" section

composition of tea fungus, sugar concentration, and fermentation conditions (Reva et al. 2015). In addition, to rule out the possibilities that ethanol was adsorbed by the sediment that was removed by filtration, a kombucha sample was directly injected to the gas chromatogram (Electronic supplementary material). Also, a new calibration curve was constructed using the direct injection of ethanol-water solutions (Electronic supplementary material). The amount of ethanol measured by direct injection was in agreement with the HSGC results.

Method Precision and Accuracy

The precision of this method was determined by evaluating relative standard deviation (RSD) of multiple injections. It can be observed from the data in Table 1 that the RSDs were all <5%, indicating a precise method for analysis of all kombucha samples. Standard addition was also employed (see the "Materials and Methods" section) to determine the ethanol content in five of the samples. It was found that standard

Table 1 Comparison of the measured amount of ethanol in five different kombucha products using external calibration and standard addition methods

Product	Alcohol by weight (ABW%)		Alcohol by volume (ABV%)	
	External calibration[a]	Standard addition[a]	External calibration	Standard addition
GT's "Kombucha Original"	0.94 ± 0.04	0.93 ± 0.04	1.17 ± 0.05	1.16 ± 0.05
GT's "Synergy Ginger Berry"	1.33 ± 0.05	1.32 ± 0.05	1.66 ± 0.06	1.65 ± 0.06
Kevita "Lavender"	1.28 ± 0.04	1.29 ± 0.04	1.59 ± 0.05	1.60 ± 0.05
The Bu "Lavender"	1.60 ± 0.04	1.61 ± 0.04	2.00 ± 0.05	2.01 ± 0.05
Holy "Kombucha Original"	1.23 ± 0.04	1.25 ± 0.04	1.53 ± 0.05	1.56 ± 0.05

[a] See the "Materials and Methods" section for a complete description of procedures.

Table 2 Reported and calculated values of ethanol concentration in NIST standard reference samples

NIST standard reference material	Certified mass fraction of ethanol[a] (%)	Measured ethanol content[b] (%)
2895	0.1701% ± 0.0014%	0.171 ± 0.004
2897a	2.001% ± 0.045%	2.02 ± 0.06
2898a	6.01% ± 0.13%	6.10 ± 0.09

[a] The certified mass fraction values are based on results obtained from the gravimetric preparation of the solutions and from the analytical results determined using gas chromatography and titrimetry

[b] Calculated values were extrapolated from the calibration curves (see the "Materials and Methods" section)

addition produced similar values to those calculated using the external calibration curve (Table 1).

The validation of method accuracy was assessed by analyzing three NIST standard reference materials (SRM 2895, SRM 2897a, SRM 2898a). The results are given in Table 2. In all cases, the ethanol content found via this procedure was identical to the NIST values within experimental error.

Effect of Storage Period on Alcohol Content

The effect of storage time was investigated for two sets of kombucha samples (eight each) with same expiration date and from the same batch. As demonstrated in Fig. 4, the alcohol content of kombucha bottles kept at room temperature (22 °C) increased significantly after 7 days. The amount of ethanol in these bottles reached at maximum level of 1.57% ABV after 14 days, followed by a slight decline after 21 days. The ethanol concentration of bottles stored at 4 °C started climbing gradually for 14 days. Subsequently, their alcohol content remained nearly unchanged (Fig. 4a). Aging analysis of kombucha samples found that development of carbon dioxide produced from "secondary fermentation" inhibited further conversion of ethanol to acetic acid in closed containers (Nummer 2013). However, refrigerating the beverage comparatively reduced the alcohol production. The increase in ethanol content of kombucha beverages might be associated with hydrolysis of ethyl esters in the mixture. It is also possible that high concentration of sugar and active yeast in kombucha beverages leads to further fermentation and increases in the concentration of ethanol while the products sit on shelves. Furthermore, the ethanol analysis of an opened kombucha bottle showed a similar trend to the refrigerated unopened bottles (Fig. 4b). The ethanol content in an opened bottle reached a peak at 1.37% (*v*/*v*) after 14 days and leveled out after that.

Conclusions

Headspace gas chromatography technique is an effective methodology for determining the ethanol content in complex commercial kombucha products. The Watercol 1910 ionic liquid column effectively separated ethanol from other volatile species in kombucha drinks and showed no degradation or change after 1200 injections. The ethanol concentration in all commercial kombucha products examined was higher than the federal limit of 0.5% ABV. Moreover, the ethanol concentration of commercial kombucha products changes with time. Longer storage times resulted in formation of higher ethanol concentrations in both sample products at 4 °C and at room temperature. The developed HSGC method is simple, rapid, and accurate. Thus, it can be employed in food industries and regulatory agencies to monitor the alcohol content in kombucha products.

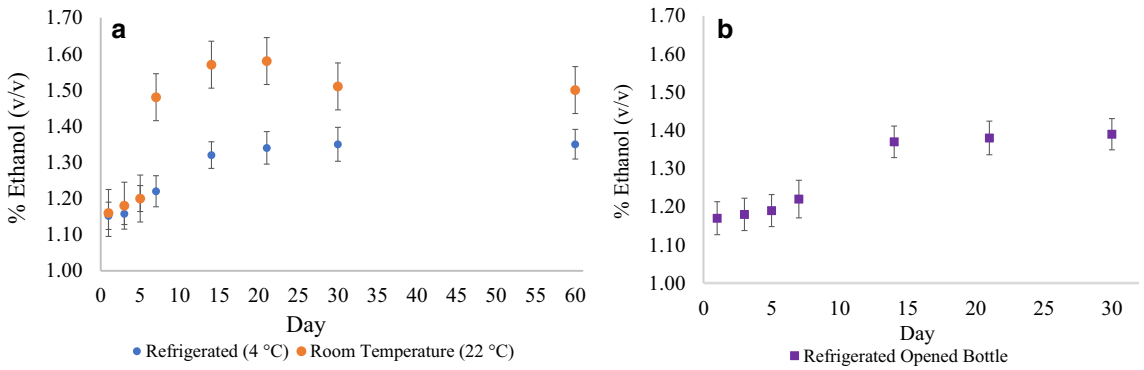

Fig. 4 **a** The effect of storage period on ethanol content of new unopened GT's Kombucha Original samples. **b** The effect of storage period on ethanol concentration of opened but refrigerated GT's Kombucha Original sample. See the "Materials and Methods" section for method details

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Compliance with Ethical Standards

Conflict of Interest Mohsen Talebi declares that he has no conflict of interest. Lilian A. Frink declares that she has no conflict of interest. Rahul A. Patil declares that he has no conflict of interest. Daniel W. Armstrong declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent Not applicable.

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