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Article

FOOD CHEMICAL CONTAMINANTS

Determination of Ethanol Content in Kombucha Using Headspace Gas Chromatography with Mass Spectrometry Detection: Single-Laboratory Validation

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Abstract

Background: Kombucha is a fermented beverage made with tea, sugar, and a symbiotic colony of bacteria and yeast that is usually marketed as a non-alcoholic beverage. Products must contain <0.5% and <1.1% alcohol by volume in the United States and Canada respectively to be classified as non-alcoholic products. Prior studies have found that Kombucha beverages can become very acidic and may contain levels of alcohol above 1% which can be a potential health risk to children and the developing fetus during pregnancy.
Objective: Given the public safety concerns and legal requirements associated with the level of alcohol within Kombucha beverages, there is a need for accurate and reliable methods. Herein we describe the validation of a sensitive, rapid, and simple Headspace Gas Chromatographic method with mass spectrometric detection for determining ethanol in Kombucha.
Methods: Method performance characteristics measured included linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) as per AOAC International guideline Appendix K Part 1. Performance was evaluated against the AOAC Standard Method Performance Requirements 2016.001 for determination of ethanol in Kombucha.
Results: The linear dynamic range for this method was confirmed over the range of 0.025 to 2.47% ABV. The LOD and LOQ were determined to be 0.0002% and 0.002% ABV, respectively. With a spike recovery of 102% for accuracy and precision of $RSD_r \leq 4\%$ the method met the SMPR requirements within the analytical range.
Conclusions: The results of this validation study demonstrated the method is fit for the purpose of quantifying ethanol in Kombucha and is suitable for rapid and easy integration by laboratories to ensure that regulatory requirements are met.

Kombucha is a traditional fermented beverage with Asian heritage that has recently gained popularity in the North American market. Traditionally, black tea or green tea is brewed and sweetened with sucrose and fermented with the symbiotic colony of bacteria and yeast known more familiarly as a SCOBY (symbiotic colony of bacteria and yeast) (1). The primary fermentation time is between 7–10 days and if desired, a secondary fermentation can be performed with the addition of fruit juices, herbs or spices to impart different flavor profiles to the tea (1). Kombucha is described as a fermented beverage with a mildly sweet and acidic taste (2). Kombucha has become a popular beverage with global sales reaching $1.5 billion USD in

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2017, with projected growth estimates of global sales reaching $5.45 billion USD by 2025 (3, 4).

Kombucha is typically marketed as a non-alcoholic beverage. Under the United States Federal law, non-alcoholic beverages may not have more than 0.5% alcohol by volume (ABV) (5). However, several studies have reported Kombucha products containing alcohol levels more than 0.5% ABV (8–10). The United States Alcohol and Tobacco Tax and Trade Bureau (TTB) has emphasized that any Kombucha product that is at or above 0.5% ABV at any time during production, when bottled or after bottling, is subject to TTB regulations and all applicable state and local requirements for alcoholic beverages (9).

Regulatory considerations aside, there are health and safety concerns associated with the consumption of alcoholic beverages, particularly among at-risk populations including pregnant women, children, and people with significant renal, pulmonary, or liver disease (10). Acute alcohol intoxication is proportional to blood alcohol concentration and severe effects of ethanol toxicity occur at lower blood alcohol concentration in children and teenagers than in adults (11).Toxic reactions in children have been reported from doses as low as 0.6 g/kg (12). Children are also particularly susceptible of suffering severe hypoglycemia from ethanol ingestion (13). Ethanol is reported to be the most common human teratogen and can produce a variety of infant abnormalities (14). Though limited, there is evidence that suggests that even low levels of alcohol exposure are associated with fetal alcohol syndrome (15). As Kombucha products are typically sold as non-alcoholic beverages, the presence of any Kombucha with elevated alcohol content may result in significant health risks to at-risk populations that may consume the product. Kombucha beverages that do not accurately convey their alcohol contents could also pose risks to otherwise non-at-risk individuals who are unaware that *ad libitum* consumption of the beverage may result in decreased motor coordination, lethargy, and other impairments associated with ethanol intoxication.

Given the regulatory legal and health implications associated with the alcohol content within fermented beverages available to the public, it is imperative that methods used to determine alcohol concentration in such beverages have undergone a rigorous validation study that ensures the method is accurate, precise, and fit for its intended purpose. Several methods for alcohol determination in various products have been previously described including enzymatic, densitometric, and gas chromatographic techniques that may be suitable for Kombucha (16–19). Headspace Gas Chromatography methods have been shown to be effective for the determination of ethanol in low concentrations in fermented beverages (6–8, 20–22). A significant advantage of such methods is the ability to minimize the interference of non-volatile constituents through utilizing the headspace injection (6, 22, 23). The Kombucha Brewers Association (KBI) recommends that a Headspace Gas Chromatography method coupled with a Flame Ionization Detector or Mass Spectrometer be used for alcohol testing for Kombucha (23).

An AOAC expert review panel had approved a Headspace Gas Chromatography coupled to a Flame Ionization Detector (GC–FID) method, which had undergone a single laboratory study, First Action status as an Official Method of Analysis (6, 7). In an effort to evaluate this method further, a collaborative laboratory study was initiated; however, it was found GC–FID instrumentation, equipped with headspace sampling systems, were few laboratories set up to perform this method (7). The multi-laboratory study was only able to recruit six laboratories despite seeking participation from labs throughout Canada and the United States (7). In contrast, many laboratories contacted as part of the multi-laboratory study reported that Headspace Gas Chromatography coupled with mass spectrometry (HS–GC–MS) detection instrumentation was the more common configuration and expressed interest in participating if the method being evaluated used Headspace GC–MS instrumentation instead of Headspace GC–FID instrumentation.

In this study a Headspace GC–MS method was developed and validated using AOAC International Guidelines (24) and evaluated against the AOAC Standard Method Performance Requirements (SMPR) 2016.001 published by AOAC for the determination of ethanol in Kombucha (25). The objective is to establish the suitability of the method for determining alcohol concentration within Kombucha products for regulatory compliance and to assist the BC Centre for Disease Control in evaluating whether Kombucha products locally presented a potential public health risk.

Experimental

Principle

This is a gas chromatography method utilizing a headspace auto-sampler and mass spectrometry detection for the determination of ethanol content in Kombucha beverages.

Apparatus and Equipment

(a) *GC system*.—Agilent 5975C series GC-MSD (Agilent, ON, Canada) equipped with aCTC Analytics CombiPal autosampler (CTC Analytics AG, Zwigen, Switzerland).
(b) *GC column*.—Agilent J&W DB-624UI (30 m × 0.25 mm, 1.4 μm film)
(c) *Analytical balance*.—Mettler Toledo AE 206 analytical range (±0.1mg; VWR International, AB, Canada).
(d) *Centrifuge*.—Eppendorf 5804 table top centrifuge (VWR International, AB, Canada).
(e) *Vortex mixer*.—Thermolyne Maxi Mix 1 (Thermo Scientific, NC, USA).
(f) *Conical tubes*.—Polypropylene, 50 mL.
(g) *Volumetric flasks*.—10 and 100 mL.
(h) *Beakers*.—2-L.
(i) *Graduated cylinders*.—10, 100, and 1000 mL.
(j) *Volumetric Pipettes*.—Eppendorf Series 100, 200, 1000, and 5000 μL.
(k) *GC headspace vials*.—20 mL with caps with PTFE septa.

Test Materials

For the precision study, three bottles stamped with the same lot number for eight different Kombucha products were purchased from the refrigerated section of a local grocery store. All bottles were immediately placed in a cooler with ice packs after purchase and transported to the laboratory. Once at the laboratory, the bottles were labeled and placed into a refrigerator at 4°C until ready to be opened for analysis. The flavors of the eight Kombucha samples were as follows:

(a) Kombucha: Tulsi Lavender
(b) Kombucha: Strawberry Lemonade
(c) Kombucha: Citrus Hops
(d) Kombucha: Mint and Chlorophyll
(e) Kombucha: Unflavored
(f) Kombucha: Ginger

(g) Kombucha: Lime Mint Coconut
(h) Kombucha: Tumeric Ginger

Reagents

ACS-grade 200 proof ethanol (>99.8%) was purchased from Greenfield Specialty Alcohols Inc. (ON, CAN). ACS grade propan-1-ol (>99.5%) was purchased from Sigma Aldrich (ON, CAN). Water was purified using a Barnstead Smart2Pure nanopure system (Thermo Scientific, MA, USA). Tea used for the accuracy study was prepared from tea bags purchased from a local grocery store.

Standard Reference Materials

Standard reference materials (SRMs) of ethanol solution 2897a, nominal mass fraction 2% were obtained from the National Institute of Standard and Technology (NIST) (Gainsburg, Maryland).

Preparation of Calibration Solutions

(a) *Preparation of Propan-1-ol Intermediate Internal Standard Solution*.—A volumetric pipette was used to transfer 5 mL of propan-1-ol into a 100 mL volumetric flask. The flask was filled to the mark with water and inverted 10 times to mix.
(b) *Preparation of Propan-1-ol Standard Working Check Solution*.—A volumetric pipette was used to transfer 100 μL of the propan-1-ol intermediate internal standard solution into a 10 mL volumetric flask. The flask was filled to the mark with water and inverted 10 times to mix. The entire contents of the flask were transferred into a 20 mL headspace vial. The vial was capped and analyzed as per the Headspace GC-MS conditions described.
(c) *Preparation of Intermediate Ethanol Standard Stock Solution*.—A 100 mL volumetric flask was weighed on an analytical balance and its mass was recorded. A volumetric pipette was used to pipette 5 mL of the 200-proof ethanol solution into the volumetric flask and the added mass was recorded. The mass of ethanol in the flask was determined by calculating the difference between the two recorded masses. The volumetric flask was filled to the mark with water and inverted 10 times to mix. The concentration of this stock solution was determined using the appropriate equation in the calculations section below.
(d) *Preparation of Ethanol Standard Working Check Solution*.—A volumetric pipette was used to transfer 1 mL of the ethanol standard stock solution into a 10 mL volumetric flask. The flask was filled to the mark with water and inverted 10 times to mix. The entire contents of the flask were then transferred into a 20 mL headspace vial, capped, and analyzed as per the Headspace GC-MS conditions described.
(e) *Preparation of Ethanol External Calibration Curve Standard Working Solutions*.—A volumetric pipette was used to transfer 100 μL of the Propan-1-ol Intermediate Internal Standard Solution into each of 7 separate 10 mL volumetric flasks. In each separate 10 mL flask the following ethanol standard solutions were prepared by diluting an appropriate amount of Ethanol Standard Stock Solution with water at 0.2 mg/mL, 0.4 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 4.9 mg/mL, 9.8 mg/mL, and 19.5 mg/mL. The entire contents of each flask were transferred into separate labeled 20 mL headspace vials, capped, and analyze as per the Headspace GC-MS conditions described.

Preparation of Test Solutions

The Kombucha sample was allowed to reach room temperature prior to opening. Once opened, 20 mL of the Kombucha liquid was transferred into a 50-mL centrifuge tube. The tube was centrifuged at 5000 rpm for 5 min. Using a volumetric pipette, 5 mL of the supernatant was transferred into a 10 mL volumetric flask. Using a volumetric pipette, 100 μL of the 1-propanol intermediate internal standard solution was added to the flask. The flask was then filled to the mark with water, capped and inverted 10 times to mix. The entire contents of the flask was transferred into a 20 mL headspace vial, capped and analyzed as per the Headspace GC-MS conditions described.

Headspace GC-MS Operating Conditions

(a) *Headspace Injector Conditions*
  i. *Incubation temperature*.—70°C.
  ii. *Syringe temperature*.—70°C.
  iii. *Incubation time*.—300 s.
  iv. *Agitator speed*.—500 rpm
  v. *Injection volume*.—500 μL
  vi. *Split Ratio*.—10:1.
(b) *GC operating conditions*
  i. *Injector temperature*.—220°C.
  ii. *Carrier gas*.—Helium.
  iii. *Initial oven temperature*.—35°C.
  iv. *Oven gradient program*.—Initial 35°C. Hold for 4 min, then increase 45°C/min to 215°C, then hold for 2 min.
  v. *Flow rate*—1.4 mL/min (constant flow).
  vi. *Total run time*—10 min.
(c) *MS Conditions*
  i. *Source temperature*.—230°C.
  ii. *Quad Temperature*.—150°C.
  iii. *Acquisition mode*—Scan
  iv. *Scan settings*—a) Low Mass—20.0
    b) High Mass—100.0

Determination

(a) *Retention time determination for propan-1-ol and ethanol peaks and system suitability tests*.—The propan-1-ol Standard Working Check Solution and the Ethanol Standard Working Check Solution were analyzed as per the GC-MS operating conditions. The identities of the propan-1-ol and ethanol peaks in each of the solutions were confirmed through their MS spectrums and their retention times were recorded. The chromatograms were visually examined to ensure no other peaks were present. Eight replicate injections of the 2.0 mg/mL ethanol working standard calibration solution were then made and analyzed as per the GC-MS operating conditions. A new cap was used for the vial following each injection. The peak area for the 1-propanol and ethanol peaks for each injection was determined and the RSD of the peak areas for all the injections was calculated. The system is considered suitable if the RSD of the peak areas for ethanol and 1-propanol was ≤4.0%.
(b) *External Calibration*.—Each of the ethanol external calibration curve standard working solutions was analyzed as per the GC-MS operating conditions. The ratio of the peak response of ethanol to the peak response of 1-propanol in each standard working solution was determined, recorded, and plotted. Simple linear regression was used to calculate the slope, intercept, and coefficient of determination (r^2)

value for the curve obtained from plotting the concentration of ethanol and the ratio of the ethanol peak to the 1-propanol peak in each standard.

(c) *Test sample analysis*.—Each prepared sample was analyzed as per the GC–MS operating conditions. The ratio of the peak area of ethanol to the peak area of propan-1-ol in each test solution was determined and used to determine the concentration of ethanol in the sample using the appropriate calculations below.

Calculations

The concentration of ethanol (mg/mL) in the Intermediate Ethanol Standard Stock Solution was determined using the following formula:

$$C_{stock} = \frac{(m_{total} - m_{flask})}{100}$$

where C_{stock} is the concentration of the ethanol stock solution in mg/mL, m_{total} is the mass of the volumetric flask with 5 mL of ethanol added, and m_{flask} is the mass of the volumetric flask itself.

The concentration of ethanol in the test solution vial in mg/mL was determined using the following formula:

$$C_{vial} = \left(\frac{P_0 - b}{a}\right)$$

where C_{vial} is the concentration of ethanol in the test solution vial in mg/mL, P_0 is the response ratio of the peak area of the ethanol peak to the peak area of the propanol peak determined for the vial, b is the y-intercept, and a is the slope of the calibration curve determined from the analysis of the calibration standards.

The concentration of ethanol in the sample in mg/mL was determined by the following formula:

$$C_{sample} = \frac{C_{vial} * 10}{5}$$

where C_{sample} is the concentration of ethanol in the test sample in mg/mL and C_{vial} is the concentration of ethanol in the test solution vial.

The concentration of ethanol in the test sample in %ABV was determined by the following formula:

$$C_{ABV} = \frac{C_{sample}}{789} * 100\%$$

where C_{ABV} is the concentration of ethanol in the test sample in %ABV, C_{sample} is the concentration of ethanol in the test sample in mg/mL, and the 789 is the specific gravity of ethanol in mg/mL at 20°C.

Single-Laboratory Validation (SLV) Parameters

This method was validated according to AOAC International single-laboratory validation guidelines for dietary supplements and botanicals (24).

(a) *Linearity*.—The linearity of the response of the ethanol and propan-1-ol ratio was assessed using a 7-point calibration curve prepared using the procedure described above. Two calibration curves were created per day on five separate days resulting in a total of ten calibration curves. Each curve was visually inspected and r^2 values were calculated to confirm linearity over the assessed range. An r^2 value of $\geq$99.5% was considered acceptable.

(b) *Method Detection Level and Limit of Quantification*.—The Method Detection Level (MDL) was determined as per the United States Environmental Protection Agency's (EPA) guidelines for determination of MDL. The MDL was determined using tea samples spiked with low levels of ethanol. Four tea bags obtained from a store and one liter of boiling water was added to a 2000 mL beaker. The contents of the beaker were stirred with a spoon and left to steep for 5 min. The tea bags were removed and the tea was left to cool to room temperature. The tea was then spiked to a concentration of approximately 0.01 mg/mL of ethanol. A total of nine separate aliquots of tea samples were prepared from this spiked tea and analyzed as per the analytical method for Kombucha. The MDL was defined as the standard deviation of the calculated concentration of the replicates multiplied by the t-statistic at a 99% confidence interval. The limit of quantification (LOQ) was defined as 10 times the standard deviation used to determine the MDL.

(c) *Precision*—Precision of the method was assessed through analysis of the Kombucha test samples described above. For all Kombucha products evaluated in the precision study, data from a total of 12 replicates were collected over 3 separate days and analyzed. On the first day of the precision study, one bottle of each Kombucha product was analyzed as per the described analytical method in quadruplicate. For each of the Kombucha products means, the within-day, between-day, and total standard deviations were calculated for the determined %ABV. The relative standard deviation (RSDr, %) and Horwitz ratio (HorRat) values were determined and used to evaluate the precision of the method. As per AOAC SLV guidelines, a HorRat value from 0.5 to 2.0 was considered acceptable (25). Additionally, as per the SMPR 2016.001 for determination of ethanol in Kombucha, a repeatability (RSD_r) of $\leq$ 4% was considered acceptable for all samples in the range from 0.1 to 2.0% ABV (25).

(d) *Accuracy*—Accuracy of the method was assessed using spike recovery studies and the analysis of SRMs. As part of the accuracy study, tea spiked at 0.5% ABV was prepared from teabags purchased from a grocery store and brewed as per the manufacturer's instructions. Four tea bags and 1 L of boiling water was added to a 2000 mL beaker. The content was stirred with a spoon and left to steep for 5 min. The tea bags were removed and the tea was left to cool to room temperature. Using a graduated cylinder 5 mL of 200-proof ethanol was added to a 1000 mL volumetric flask sitting on a tared analytical balance. The mass of ethanol added was then determined by difference to be 3.9923 g. Tea was then added to fill the 1000 mL flask to the mark, capped, and mixed well. The spike recovery study was then performed on the in-house tea samples spiked with ethanol described in the test materials section above. Accuracy was also evaluated through the analysis of NIST SRM 2897a (2% ethanol nominal mass fraction) obtained from NIST. A total of four separate NIST SRM 2897a vials were analyzed as per the described analytical method. For both tea and NIST standard samples, the recovery was

then determined by comparing expected %ABV versus determined %ABV.

Results and Discussion

Method Validation Results

Chromatograms showing one of the standards prepared in the study and a sample prepared for the precision study are shown in Figures 1 and 2, respectively. The peaks of propan-1-ol and ethanol, confirmed using their mass spectrums, were separated from all other peaks in the samples.

Linearity

The seven-point calibration curves used on each day of the validation was linear upon visual inspection. The coefficient of determination was calculated and confirmed as acceptable with r^2values greater than 99.5% for all calibration curves on each day. A representative standard curve prepared during this study is shown in Figure 3. All curves prepared over the 5 day period were linear and had r^2>99.9% indicating linearity over the evaluated range.

Repeatability and Intermediate Precision

Precision of the method was assessed through the calculation of relative standard deviation (RSDr, %) and Horwitz ratio (HorRat) values calculated using data from 12 replicates obtained over 3 separate days for each Kombucha market sample described above. On the first day of the precision study, one bottle of each Kombucha product was analyzed as per the described analytical method in quadruplicate. On the second day of the precision study, a second bottle from each of the Kombucha products was prepared and analyzed as per the described analytical method in quadruplicate. It was discovered that despite coming from bottles stamped with the same lot number, several of the Kombucha products had determined %ABV values that differed significantly between the bottles. The differences in the mean %ABV between these bottles were verified using Student t-tests (p-value <0.01, data not shown). As the between bottle variances were much greater than the observed within day variances in these samples, it was determined that the between bottle variances were due to inherent differences between the bottles and these results were not attributed to the method's performance.

On the third day of the precision study, the third bottle of each Kombucha product was prepared and analyzed in quadruplicate. For those samples identified on the second day of the precision study as having statistically significantly high bottle-to-bottle variance, two additional aliquots from each bottle was collected in container, capped, and frozen in a -20°C freezer. For all other Kombucha products that did not show statistically significant (as determined using Student's t-tests) bottle-to-bottle variance, data collected from these three days were compiled and analyzed.

On the fourth day of the precision study, one of the frozen aliquots from each of the Kombucha products showing high bottle-to-bottle variance was prepared and analyzed as per the described analytical method. On the fifth day of the precision study, the final frozen aliquots from each of the Kombucha products showing high bottle-to-bottle variance was prepared and analyzed as per the described analytical method.

The SMPR 2016.001 requires the method to have an analytical range from 0.1 to 2.0%ABV. A total of six samples possessing %ABV values within these parameters were used to evaluate the precision of the method against the AOAC SMPR 2016.001. The RSD_r values for these samples ranged from 1.9% to 3.8%

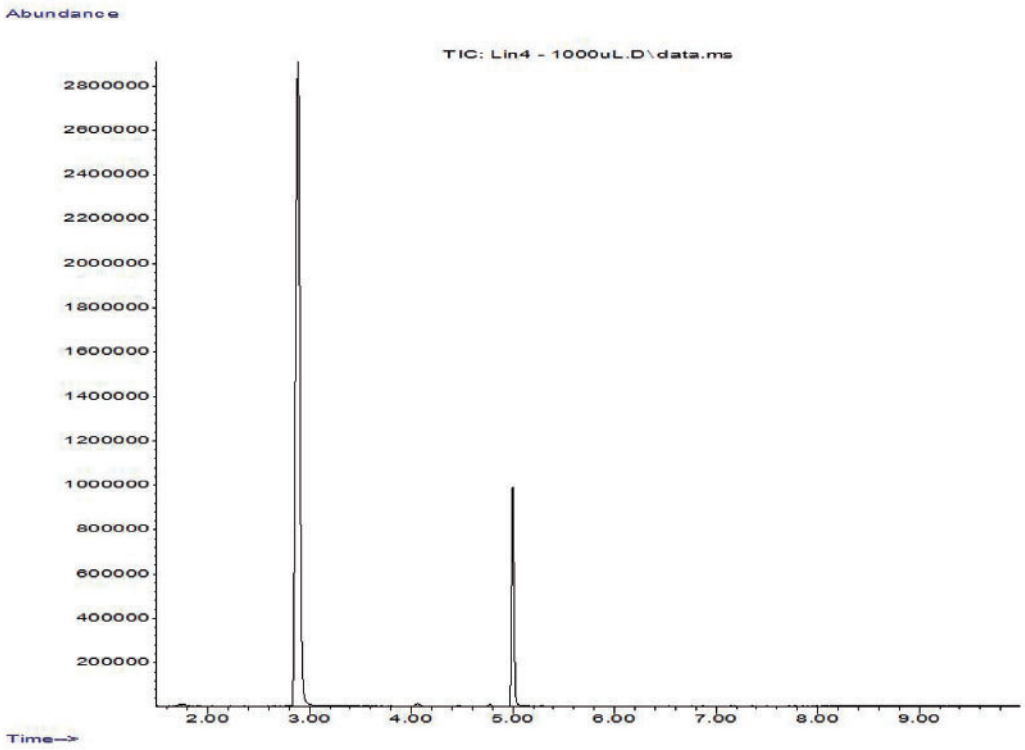

Figure 1. Chromatogram obtained from analysis of a standard solution.

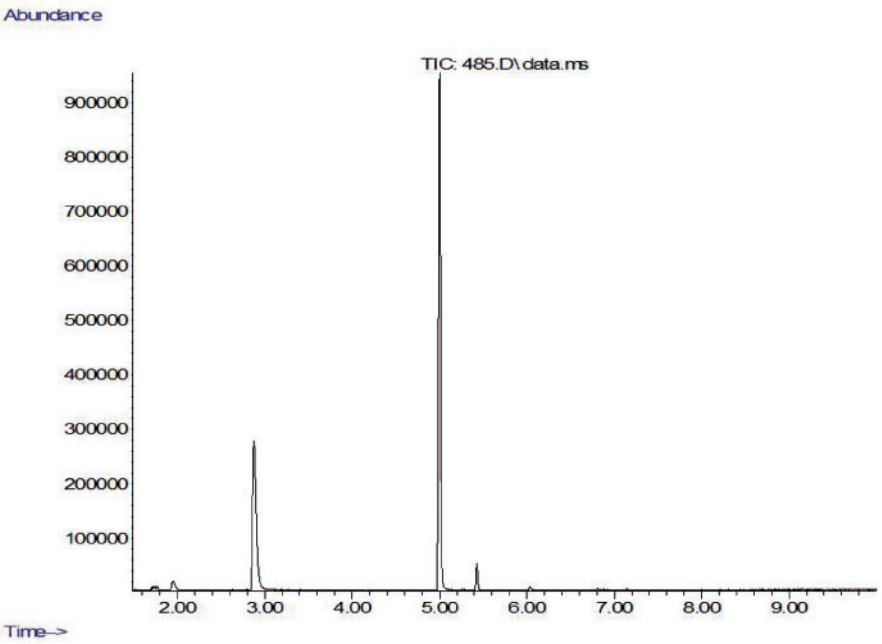

Figure 2. Chromatogram obtained from analysis of a Kombucha sample.

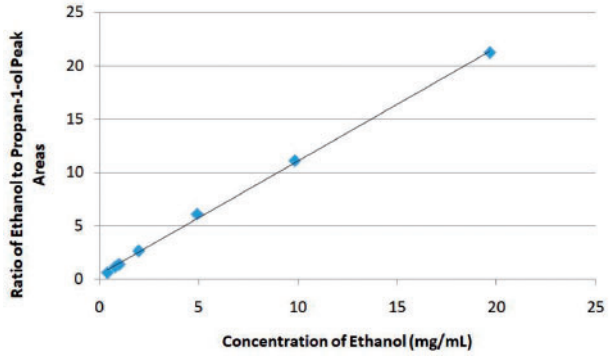

Figure 3. Representative standard curve prepared during the study. Visual inspection of the curve shows a linear relationship. The calculated r^2 for this curve is 99.9%.

which meet the Kombucha SMPR 2016.001 requirements of an RSD_r of <4% (see Table 1).

Two additional Kombucha samples possessing %ABV below the analytical range requested in the SMPR were also tested in this study. These two samples, turmeric ginger and strawberry lemonade, had RSD_r values 4.9% and 8.4%, respectively. As these two samples were outside the range of the acceptable criteria stated in the SMPR 2016.001, the criterion for acceptable precision was used from Appendix K of the AOAC Official Methods of Analysis: Guidelines for Dietary Supplements and Botanicals (24). The acceptable precision from this guideline states that the $HorRat_r$ should be between 0.5 and 2.0. All Kombucha samples evaluated, including the two samples with %ABV, 001% had $HorRat_r$ values ranging from 0.5–1.2 which fall into the acceptable range in Appendix K. As such, the precision of this method was determined to be acceptable, meeting both the AOAC SMPR 2016.001for determination of ethanol in Kombucha and Appendix K requirements.

Table 1. Intermediate precision results for ethanol quantification from Kombucha samples from SLV study

Sample	Mean Ethanol Concentration (%ABV)	% RSDr	HorRat
Tulsi Lavender[a]	1.63	1.9	0.5
Strawberry Lemonade[b]	0.03	8.4	1.2
Citrus Hops[a]	0.22	2.7	0.5
Mint and Chlorophyll[b]	0.16	2.2	0.5
Unflavored[a]	1.23	3.8	1.0
Ginger[a]	1.41	2.0	0.5
Lime Mint coconut[a]	0.14	2.6	0.5
Turmeric Ginger[b]	0.07	4.9	0.8

[a]Results from these samples are derived from data obtained from aliquots obtained from only the third bottle of product analyzed as samples demonstrated high bottle-to-bottle variance.
[b]Results from these samples are derived from data obtained from analysis of all three bottles stamped with the same lot number.

Accuracy

The accuracy of the method was evaluated first through a spike recovery study and then through the analysis of NIST SRM 2897a (2% ethanol nominal mass fraction). The results of the analysis of the accuracy samples are summarized in Table 2. Mean recovery values of 102% were obtained for both and are within the acceptable ranges as specified by SMPR 2016.001 guidelines.

Limits of detection and quantification

The MDL and LOQ were determined using the EPA's method detection limit procedure. The MDL for this method was determined to be 0.0002% ABV and the LOQ was determined to be

Table 2. Recovery results for quantification of ethanol

Sample	Expected %ABV	Mean measured %ABV	Recovery %
Tea spiked with ethanol	0.51	0.52	102
NIST SRM 2897a	2.55	2.60	102

0.002% ABV. The MDL was defined as the standard deviation of the calculated concentration of the replicates multiplied by the t-statistic at a 99% confidence interval.

Conclusions

With the growing popularity of fermented beverages in the marketplace, it is imperative to have a robust, versatile method to analyze ethanol in beverages with this matrix. As demonstrated by the results of this study, products with >0.5% ABV are present on the marketplace. An optimized Headspace GC–MS method for the quantitation of ethanol in Kombucha was validated according to AOAC guidelines for *Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*. This method is simple, rapid, and can be integrated by commercial laboratories and industries to determine the ethanol content within Kombucha products and ensure regulatory requirements are met.

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Conflict of interest

The authors declare that they have no conflict of interest.

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