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Determination of Ethanol in Kombucha Products: Single-Laboratory Validation, First Action 2016.12

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Kombucha is a fermented nonalcoholic beverage that has drawn government attention due to the possible presence of excess ethanol (≥0.5% alcohol by volume; ABV). A validated method that provides better precision and accuracy for measuring ethanol levels in kombucha is urgently needed by the kombucha industry. The current study validated a method for determining ethanol content in commercial kombucha products. The ethanol content in kombucha was measured using headspace GC with flame ionization detection. An ethanol standard curve ranging from 0.05 to 5.09% ABV was used, with correlation coefficients greater than 99.9%. The method detection limit was 0.003% ABV and the LOQ was 0.01% ABV. The RSD_r ranged from 1.62 to 2.21% and the Horwitz ratio ranged from 0.4 to 0.6. The average accuracy of the method was 98.2%. This method was validated following the guidelines for single-laboratory validation by AOAC INTERNATIONAL and meets the requirements set by AOAC SMPR 2016.001, "*Standard Method Performance Requirements* for Determination of Ethanol in Kombucha."

Kombucha is a traditional fermented drink that is prepared by fermenting sweetened green or black tea with the addition of "tea fungus," which is a symbiotic colony of bacteria and yeast (1, 2). This traditional Asian fermented beverage has gained significant popularity in the United States in recent years (1, 3). The U.S. market for kombucha products is expected to reach $1.8 billion in 2020 (1). Kombucha is usually marketed as a nonalcoholic beverage in the United States (1). To qualify as a nonalcoholic beverage in the United States, the products are required to contain an ethyl alcohol content of less than 0.50% alcohol by volume (ABV; 3). However, some kombucha products have been reported to have alcohol levels at or above 0.5% ABV (4–11). Another consideration for this type of beverage is the continuous fermentation of the product during transportation and storage, causing an increased ethanol level in the product at the time of purchase. Regulations regarding the alcohol content in kombucha are addressed by the U.S. Tax and Trade Bureau (3).

Even though some studies have been conducted on the beverage, there is no fully validated method for determining ethyl alcohol content in kombucha in the literature. Methods for determining the ethyl alcohol (ethanol) content in other beverages, such as beer, wine, and vinegar, have been published extensively in the literature (12–16). Existing methods have many drawbacks, including large RSD_r values, low accuracy, and not being suitable for kombucha products. The kombucha industry is in need of a fully validated method that can provide better precision and accuracy. GC with flame-ionization detection (FID) is one of the most common methods used, such as in beer ethanol determination (AOAC *Official Method*℠ **984.14**; 13) and wine ethanol determination (AOAC *Official Method* **983.13**; 14), and may be a great candidate for kombucha ethanol determination (17, 18).

To address the problem, AOAC INTERNATIONAL issued a call for methods that determine ethanol content in kombucha products. The candidate method needs to meet the *Standard Method Performance Requirements* (SMPRs®) established by the AOAC Stakeholder Panel on Strategic Food Analytical Methods (SMPR 2016.001; 19). The single-laboratory validation (SLV) requirements in the SMPRs are provided in Table 1.

This study provides a fully validated method for determining ethanol in kombucha products using headspace GC–FID. The validation of the method followed the SLV guidelines set out by AOAC (20) and by the SMPRs for the determination of ethanol in kombucha (19). This method was developed from a forensic method for measuring ethanol in human plasma (21). The method is suitable for ethanol determination in mixtures such as foods, beverages, and botanical materials.

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This method was approved by the AOAC Expert Review Panel for Kombucha as First Action.

The Expert Review Panel for Kombucha Methods invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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Table 1. SMPRs for the determination of ethanol in kombucha products

Parameter	Value, %
Analytical range	0.1–2.8 ABV
LOQ	≤0.05 ABV
Accuracy[a]	97–102
Repeatability, RSD_r	≤4
Reproducibility, RSD_R	≤6

[a] Mean spiked recovery over the range of the assay.

AOAC Official Method 2016.12
Ethanol in Kombucha Products
Headspace Gas Chromatography with Flame-Ionization Detection
First Action 2016

A. Principle

This is a GC method utilizing a headspace autosampler and FID for the determination of ethanol in kombucha samples.

B. Apparatus

(a) *Chromatography system*.—Agilent 7890 GC system equipped with an FID and a Combi-PAL headspace autosampler (Agilent Technologies, Santa Clara, CA).

(b) *Headspace vials*.—Screw-top vials and crimp-top vials (Resteck, Bellefonte, PA).

(c) *Magnetic Teflon-lined caps*.—Restek.

(d) *Volumetric flasks*.

(e) *Micropipets*.

C. Headspace Conditions

(a) *Incubation temperature*.—80°C.

(b) *Syringe temperature*.—85°C.

(c) *Heating time*.—15–20 min.

D. GC Conditions

(a) *Column*.—J&W DB-WAXetr (0.53 mm × 30 m, 2 μm film).

(b) *Initial GC oven temperature*.—40°C.

(c) *Oven temperature gradient*.—Hold at 40°C for 10 min, increase 25°C/min until 240°C is reached, and hold at 240°C for 1 min.

(d) *Run time*.—20 min.

(e) *FID temperature*.—250°C.

(f) *Injector temperature*.—150°C.

(g) *Carrier gas*.—He at 7 mL/min.

(h) *Injection volume*.—200 μL.

E. Reagents

(a) *Ethanol*.—ACS reagent grade, >99.8% (Sigma-Aldrich, St. Louis, MO).

(b) *1-Propanol*.—ACS reagent grade, >99.5% (Sigma-Aldrich).

(c) *Water*.—ACS reagent (Sigma-Aldrich).

F. Standard Reference Materials

(a) *Propyl alcohol (1-propanol)*.—Purity 99.98% (Sigma-Aldrich).

(b) *Ethanol reference standard*.—Absolute 200 proof, purity 99.97% (Sigma-Aldrich).

(c) *Ethanol reference standard*.—Absolute 200 proof, purity 99.5% (Sigma-Aldrich).

(d) *Ethanol–water*.—Certified Reference Material, 100 mg/dL (0.1267% ethanol ABV at 20°C; Cerilliant Corp., Round Rock, TX).

Standard Reference Material, **F(a)**, was used as the internal standard. Standard Reference Material, **F(b)**, was used for preparing the standard stock solutions and standard curves. Standard Reference Materials, **F(c)** and **F(d)**, were used in the accuracy evaluation.

G. Sample Collection

A total of seven commercial kombucha products were obtained from a local market in Carmel, IN. The products were selected based on their high popularity, which was determined by a preliminary market survey conducted on nine food retailers in Carmel. The labeled alcohol level and the ingredients of the products were also considered during the product selection process to ensure the best coverage of the products in the market. An additional unflavored tea product, **G(h)**, formulated by KeVita, Inc. (Ventura, CA) to ensure that no ethanol was in the product, was used as the blank samples. All samples were sealed properly and stored in a (5 ± 3°C) refrigerator before analysis. Six samples, **G(a–f)**, were used in the precision evaluation. A seventh sample, **G(g)**, was used for the determination of the method LOD and LOQ, and the ethanol-free sample **G(h)**, was used in the accuracy determination.

(a) Elderberry-flavored kombucha (manufacturer 1).

(b) Berry-flavored kombucha (manufacturer 2).

(c) Raspberry-flavored kombucha (manufacturer 3).

(d) Unflavored kombucha (manufacturer 3).

(e) Ginger-lemon-flavored kombucha (manufacturer 4).

(f) Apple-flavored kombucha (manufacturer 4).

(g) Pineapple-peach-flavored kombucha (manufacturer 5).

(h) Ethanol-free unflavored tea (KeVita, Inc).

H. Standard and Sample Preparation

(a) *Ethanol stock solution*.—Mix 5 mL ethanol reference standard, **F(b)**, with 95 mL water.

(b) *Internal standard stock solution*.—Mix 5 mL 1-propanol, **F(a)**, with 95 mL water.

(c) *Ethanol calibration solution*.—Dilute the ethanol stock solution, **H(a)**, with water to reach final concentrations of 0.05, 0.10, 0.25, 0.25, 1.002, 2.54, 4.07, and 5.09% ABV ethanol standard solution with 1% internal standard stock solution, **H(b)**. Transfer a 10 mL portion of the individual ethanol standard solution into a 20 mL headspace vial.

(d) *Sample preparation*.—Weigh 0.01–0.02 g sample, **G(a–h)**, into a volumetric flask. Add a sufficient amount of internal standard stock solution, **H(b)**, to the vial to reach a final concentration of 1% 1-propanol by volume before diluting to 10 mL with water. Transfer 10 mL of the sample solution into a 20 mL headspace vial.

I. Analysis

(a) *GC–FID system*.—Set up the GC–FID system according to the conditions listed in **C** and **D**.

(b) *Analysis*.—Make single injections of each sample and standard solution. Measure chromatographic peak response (area).

(c) *Identification*.—Identify ethanol and 1-propanol peak in the sample solution by comparison with the retention time of the ethanol standard solution.

J. SLV Parameters

(a) *Selectivity and specificity*.—Chromatographs of the samples and the ethanol standard were evaluated to determine the selectivity and specificity of the method. Blank sample, **G(h)**, demonstrated no interfering matrix effects in the analysis of ethanol.

(b) *Linearity*.—Seven-point calibration curves were prepared from the ethanol standard solutions (0.05–5.09% ABV) on separate days in triplicate. Calibration curves were built based on the ratio of the ethanol signal response to the internal standard (1-propanol) signal response, and linearity was visually confirmed. Linear regression was then used to determine the correlation coefficient (r) of the curves. Linearity was considered acceptable if all curves had r^2 values >0.999.

(c) *LOD and LOQ*.—The LOD of the method was determined using method detection limit (MDL) guidelines from the U.S. Environmental Protection Agency. A preliminary study was conducted to determine the ethanol level in the kombucha samples. One sample, **G(g)**, was found to contain the lowest amount of ethanol (approximately 0.05% ABV). Thus, four replicates of this sample were analyzed on 3 different days. The MDL was calculated based on the formula given in **K**. The LOQ of the method was calculated as 10× the SD determined for the MDL.

(d) *Precision*.—Four replicates of six samples, **G(a–f)**, were analyzed over 3 different days. Statistical analysis was performed to determine within-day, between-day, and overall precision of the method. The Horwitz Ratio (HorRat) was calculated using the calculation in **K**.

(e) *Recovery*.—Recovery of the method was evaluated first through a spike recovery study. The ethanol-free sample, **G(h)**, was spiked with the ethanol reference standard, **F(c)**, at three different levels: 0.13, 1.30, and 3.30% ABV on 3 different days in duplicate. Recovery was also determined by analyzing the certified ethanol reference standard, **F(d)**, in duplicate on 2 days.

K. Calculations

The concentration of ethanol in the injected sample solution was calculated as

$$AC = \frac{(y - \varepsilon)}{\beta}$$

where AC = the ethanol concentration in the injected sample solution (µg/mL); y = the ratio of the peak area of ethanol to the peak area of 1-propanol in the solution; ε = the intercept of the calibration curve; and β = the slope of the calibration curve.

The concentration of ethanol in the original sample, measured in micrograms per milliliter, was calculated as

$$AM = \frac{AC * VV}{SM}$$

where AM = the concentration of ethanol in the original sample (µg/mL); AC = the concentration of ethanol in the injected sample solution (µg/mL); VV = the volume of sample solution in the headspace vial (mL); and SM = the mass of the sample (g).

The concentration of ethanol in the original sample, measured in % ABV, was calculated as

$$AV = \frac{AM * GK}{GE * 10000}$$

where AV = the concentration of ethanol in the original sample (% ABV); AM = the concentration of ethanol in the sample (µg/mL); GE = the specific gravity of ethanol (0.789 g/mL at 20°C); and GK = the specific gravity of kombucha (1.02 g/mL at 20°C).

The HorRat was calculated as

$$HorRat = \frac{RSD_r}{PRSD_r}$$

where $PRSD_r$ = the predicted RSD_r. The $PRSD_r$ value was $C^{-0.15}$, where C = the concentration of the analyte expressed as a mass fraction.

The MDL of the method was calculated as

$$MDL = s * t_{(0.01,\ n-1)}$$

where s = the sample SD of the concentration determined for the replicates; and $t_{(0.01,n-1)}$ = the t statistic value at $\alpha = 0.01$ and $n - 1$ degrees of freedom.

Results and Discussion

Selectivity and Specificity

Resolution was sufficient between the analyte peaks and other peaks in the samples, and all analyte peaks were consistent, with no splits, shoulders, or other indications of interference by coeluting compounds (Figure 1). There were no interfering peaks observed at the retention times of ethanol and the internal standard in any of the spiked or blank samples evaluated.

Linearity

An extended calibration range of 0.05–5.09% ABV was used for linearity demonstration. The correlation coefficient (r) for each day was 1.0000, 1.0000, and 0.9997, with an average of 0.9998. All the prepared standard curves appeared linear and had r^2 values >0.999. The coverage of the calibration curve

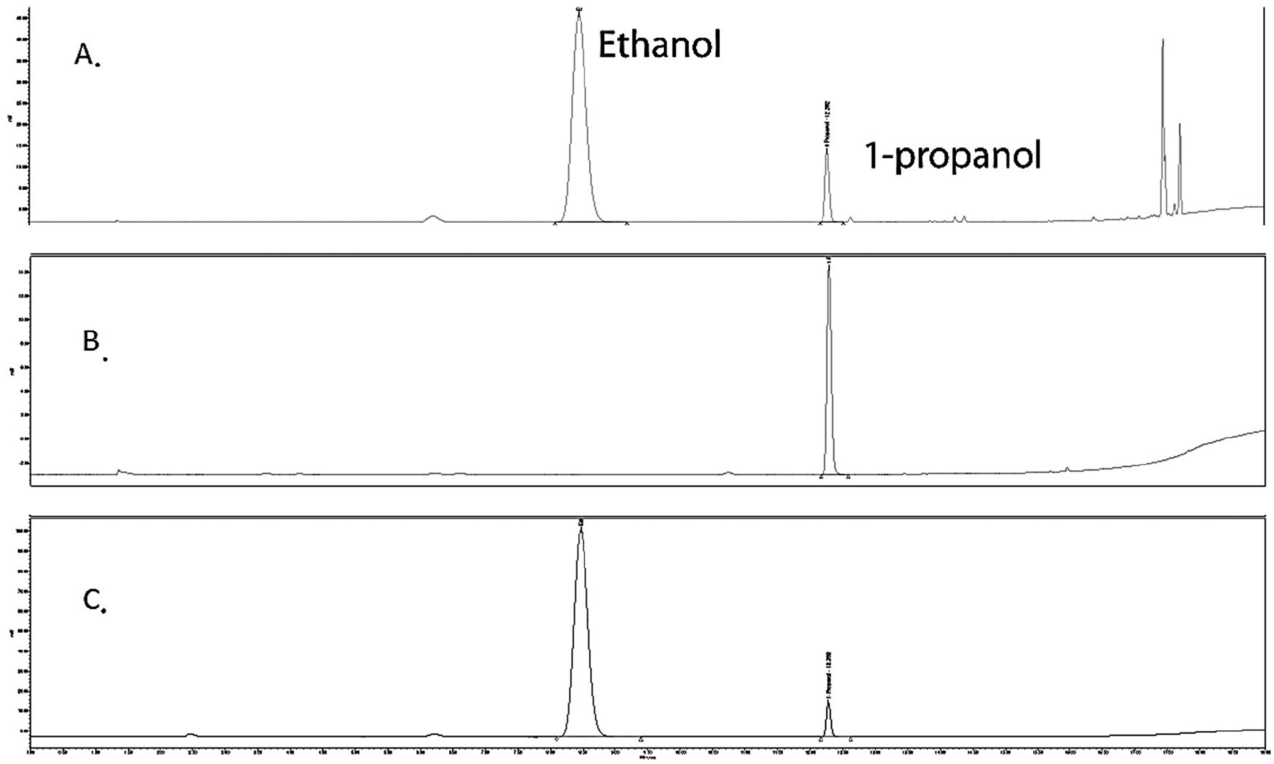

Figure 1. Gas chromatograms of commercial kombucha products and ethanol references. (A) Representative commercial kombucha sample; (B) blank sample, G(h); (C) blank sample, G(h), spiked with ethanol standard solution at 3.30% ABV.

included the analytical range of 0.1–2.8% ABV required by SMPR 2016.001 for kombucha products.

LOD and LOQ

The results from the 12 independent analyses showed that the MDL was 0.003% ABV and that the LOQ of the method was 0.01% ABV, which is lower than the LOQ value of ≤0.05% ABV specified in SMPR 2016.001 (Table 1).

Precision

Results of the precision evaluation for the six samples are summarized in Table 2.

The overall RSD_r values ranged from 1.62 to 2.21%, which are within the AOAC range for the sample concentration (20) and the SMPR limit of ≤4% (Table 1). The HorRat values, which ranged from 0.4 to 0.6 for all the samples, are within the AOAC guideline of 0.5–2.0 (20).

Table 2. Precision determinations for ethanol in kombucha beverages

Kombucha sample	Mean, % ABV	RSD_r, %	HorRat
Elderberry-flavored	2.18	2.14	0.6
Berry-flavored	0.11	2.21	0.4
Raspberry-flavored	2.22	1.62	0.5
Unflavored	1.56	1.67	0.5
Ginger-lemon-flavored	1.21	1.80	0.5
Apple-flavored	1.30	2.18	0.6

Accuracy

Results of the spike recovery study are summarized in Table 3. The mean recovery for each of the three levels tested was found to be 99.6, 100.4, and 100.4%. The lowest recovery (96.2%) was found in the low-level ethanol-spiked kombucha sample on day 3. Table 4 shows the accuracy of the method for analyzing the certified ethanol reference standard, **F(d)**. The average recovery over 2 days was 98.2% ABV. Overall, the results from the recovery assessments are within AOAC guidelines and meet the requirements of AOAC SMPR 2016.001 for the determination of ethanol in kombucha, which states that recovery should be 97–102% over the range of the assay (Table 1).

Conclusions

The method, validated following AOAC *Guidelines for Single Laboratory Validation of Chemical Methods for Dietary*

Table 3. Spike recovery of ethanol using matrix at three different levels[a]

Day	Low, %	Medium, %	High, %
1	98.3	99.7	99.9
	99.9	99.5	99.1
2	99.7	99.5	98.4
	100.4	99.6	99.2
3	103.2	100	102.5
	96.2	104.2	103.4
Mean	99.6	100.4	100.4

[a] Low = 0.13% ABV; medium = 1.30% ABV; and high = 3.3% ABV.

Table 4. GC-FID analysis of the certified ethanol reference standard results

Day	Accuracy, %
1	98.0
	99.2
2	98.5
	97.1
Mean	98.2

Supplements and Botanicals (20), demonstrated acceptable performance for the determination of ethanol content in kombucha products using GC–FID. The SMPRs approved by the AOAC Stakeholder Panel on Strategic Food Analytical Methods have been met, thereby supporting the First Action status of the method. This method will serve as an improved tool for industry, government, and academia in their respective efforts in investigating and ensuring the safety and quality of kombucha.

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