Single Laboratory Validation of a GC-FID Method for Ethanol in Kombucha

With Supplementary Data:

Multi-Lab Study on Ethanol Content in Commercial Kombucha Samples and Certified Reference Materials

Prepared by: Blake Ebersole, NaturPro Scientific LLC

Study performed at:
Covance Laboratories Inc.
3301 Kinsman Blvd
Madison, WI 53704-2523

Study Investigators: Blake Ebersole, NaturPro Scientific LLC, Rich Schmidt, Covance Laboratories and Matt Eckert, Covance Laboratories.

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INTRODUCTION

Kombucha tea is a traditional beverage from Asia that has reached a rapid consumption rate in the U.S. The U.S. kombucha market has grown from humble beginnings just a few years ago, and is expected to reach $1.8B by 2020[1].

Historically, fermentation of kombucha allowed nature to run its course during fermentation, using relatively undefined mixture of microorganisms, including bacteria and yeast. The controls required to ensure product safety from large-scale commercial production of kombucha can be considered greater than those used for traditional methods of production. Regulatory experience and published data suggest that further research and effort should be dedicated to understanding product safety implications of uncontrolled fermentation processes.

In September 2015 FAQ were published by the U.S. Tax and Trade Bureau (TTB) addressing regulatory concerns regarding alcohol in kombucha[2]. This study was initiated in response to regulatory and compliance concerns raised regarding high ethanol content in kombucha products. Although published validated methods on the specific composition of kombucha are absent, several methods are commonly used to test kombucha products. Out of more than a dozen ways to test for ethanol, several methods are approved for beer, wine and vinegar products[3].The methods prescribed by AOAC for analysis of ethanol in alcoholic beverages like beer (AOAC 984.14) and wine (AOAC 983.13) use gas chromatography with flame ionization detector (GC-FID). GC-FID methods have been used by industry and regulators to measure ethanol in beverages for about 30 years[4]. Other AOAC official methods for ethanol in beer and wine are based on more traditional methods using distillation and densitometry, including AOAC 992.29 and 935.21, which is also listed by the United States Tax and Trade Bureau (TTB) as an acceptable method for analysis of alcoholic beverages[2,4]. More advanced technologies such as 1H qNMR may be used for analysis of ethanol in kombucha[5]. GC-FID methods are often considered more accurate and precise than many non-chromatographic methods due to greater specificity and sensitivity[6]. Based on the extensive history of testing ethanol in foodstuffs, it can be presumed that multiple methods will eventually be proven valid and fit for purpose for kombucha.

Kombucha is often marketed as a non-alcoholic beverage, which, according to TTB regulations, requires that it contain an alcohol content of less than 0.50% alcohol by volume (ABV). In 2010, kombucha was recalled in the U.S. due to test results above the legal limit[7]. Previous to our study, data from labs and manufacturers using GC, NMR, HPLC and traditional methods have reported that kombucha products contain alcohol levels in excess of 0.5% ABV[5,7,8,9,10,11,12,13,14,15,16,17.] Preliminary studies by our group suggested general agreement with previously reported results of alcohol levels above 1% ABV[8]. Further, general agreement in results was found when samples of the same manufacturing lots were sent to different laboratories, with the exception of one laboratory reporting inconsistent results that appeared to be generally lower than the others. These differences led to questions to be asked regarding accuracy of results.

The objective of this study was to examine the validity of a gas chromatography with flame ionization detection (GC-FID) method for quantifying ethanol content in kombucha, and determine whether the method met performance requirements set by AOAC International[18,19]. The Covance method “MP-ETME” commonly used for complex mixtures containing low levels of ethanol, including foods, beverages and botanical materials, was used in this study[20].

The commercial test sample selected was a ginger-flavored kombucha available in the U.S. which had been previously screened for alcohol content and suitability for such studies. Reference materials included pure

ethanol, 1-propanol as internal standard, Certified Reference Materials (ethanol-water solution and beer), and a control kombucha of similar composition to the commercial kombucha samples. Spiked reference materials were produced by adding a known amount of ethanol to either water or an ethanol-free control kombucha.

The study was initiated on March 2016 and completed in April 2016 by Covance Laboratories. A preliminary investigation in which multiple laboratories assessed the ethanol content in a number of commercial and spiked samples, as well as certified reference materials (CRM) was also performed[8].

CONCLUSIONS

Based on the analytical results obtained in this study, Covance method MP-ETME, which is also commonly used for foods, beverages and botanical materials, is appropriate for the quantification of ethanol in kombucha. The study method was found to be accurate and precise, meeting the Standard Method Performance Requirements (SMPR) established by the AOAC kombucha working group in 2015-2016. In this study, no indication of interferences from co-eluting peaks or other interferences was observed.

The limit of detection and limit of quantitation for the method were 0.015% ABV, below the typical ethanol content of commercial kombucha products based on preliminary analyses. Method linearity was shown between 0.1% and 2.8% ABV as represented by the correlation coefficient, r, of the calibration curve which was greater than 0.9996.

Using the MP-ETME method, the commercial kombucha sample was found to contain 1.61% alcohol by volume (ABV) with a relative standard deviation of +/- 0.06%.

Intermediate precision, measured by the relative standard deviation (RSD) across different days, instruments and technicians, was < 4%, meeting the SMPR.

Recovery for lab-spiked control kombucha samples ranged from 98.3 to 104.2% across spike levels of 0.13%, 1.3% and 3.3% ABV.

Recovery for lab-blinded Certified Reference Materials (CRM) was 101-104% for sealed CRM and 94-104% for both sealed and repackaged CRM, across spike levels of 0.1267%, 0.505% and 2.53% ABV.

EXPERIMENTAL DESIGN AND RESULTS

Results:
The study was conducted during March 2016. The method meets the draft AOAC SMPR (Version 4, December 9, 2015) for ethanol in kombucha. See figures. All data are presented in units of alcohol by volume (%ABV) under the definition in AOAC SMPR, unless otherwise specified.

Methodology
The Covance headspace GC-FID method (method MP-ETME, Version 1, effective date: April 9, 2010) was validated in this study according to AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.

Briefly, samples are heated and agitated in a 20-mL headspace vial. A portion of the headspace is injected into a gas chromatograph (GC) with a flame ionization detector (FID) on a DB-WAXetr GC column. Quantitation is performed using a 6-point calibration curve generated by a weighted (1/concentration) least squares linear regression analysis.

Apparatus:
- Analytical balance
- J&W DB-WAXetrcolumn, 0.53 mm x 30 m, 2µm df
- Headspace vials and magnetic Teflon-lined caps, 20-mL
  - Screw-top vials (Restek, part # 23082)
  - Crimp top vials (Restek, part # 24685)
- Combi-PAL headspace autosampler
- Agilent 7890 GC system with flame ionization detector

Headspace conditions:

- Incubation temperature: 80°C
- Syringe temperature: 85°C
- Heating time: 15-20 minutes

Gas Chromatograph conditions

- Column: J&W DB-WAXetr
- Film thickness: 2 to 5 um
- Temperature: Initial 40°C for 10 minutes
- Rate: 25°C/minute to 240°C, hold 240°C for 1 minute
- Run Time: 20 minutes
- Detector: flame ionization
- Detector temperature: 250°C
- Injector temperature: 150°C
- Carrier gas: He, 7 mL/min

- Hydrogen flow: 40 mL/min
- Air flow: 400 mL/min
- Makeup flow: 40 mL/min
- Makeup gas: Nitrogen
- Injection volume: 2,000 uL

VALIDATION SAMPLE MATERIALS

Characterization

Commercial Kombucha: Kombucha is generally stated as a fermented beverage containing water, tea, sugar, yeast and bacteria, organic acids (such as acetic and gluconic acids), dissolved carbon dioxide and ethanol. Based on preliminary analysis of commercial kombucha products, the test sample was selected as a commercial kombucha, which was considered to be representative of many other kombucha products on the market based on preliminary chemical analysis, visual and organoleptic properties, and product labeling. The composition of the sample was evaluated in a nutritional analysis (see below). The sample was representative of kombucha products containing ethanol, solid matter, organic acids and carbon dioxide. Spices and flavors containing essential oils, like ginger root (one of the most popular kombucha flavors), have also been purported to interfere with analysis of volatiles. Therefore, a ginger-flavored kombucha was selected.

Test sample material:

The commercial kombucha sample used for the validation study was purchased from a grocery store in Carmel, Indiana on February 16, 2016. The sample was transported under cold conditions to the laboratory using validated coolers and temperature monitors.

Reference #	Identification	Exp. Date/Lot Number	Purity	Stability	Storage
1	Commercial Kombucha (Ginger flavor)	Exp. 3/25/16 Covance sample 4814442	“non-alcoholic”, ethanol content not labeled, product previously tested to contain >1.0% ethanol	Tested on or before expiration date (March 25, 2016)	In a chamber set to maintain 5 ±3 deg C. Labeled “Keep refrigerated. Do not shake”

Test sample stability

The commercial kombucha sample was kept under refrigerated temperatures in an unopened bottle with an intact manufacturer’s seal, prior to testing before “expiration/best by” date. All of these stability ensuring conditions were met. Preliminary studies on commercial spiked samples suggested that ethanol had acceptable recovery after spiking, handling and transportation (see Supplementary Data).

Control Kombucha: An ethanol-free reference sample of control kombucha was analyzed for nutritional composition. See below table.

Nutritional Composition (per 8 oz serving)	**Label:** Commercial Kombucha (Ref #1)	**Result:** Commercial Kombucha (Ref #1)	**Result:** Control Kombucha (Ref #3)
Ethanol	"This product contains a trace amount of ethanol."	1.61%	<0.015%
Specific gravity (g/mL) @ 20C	not listed	1.02%	1.00%
Moisture (%)	not listed	96.5	97.1
Calories	30	33	27
Calories from Fat	0	<2.4	<2.4
Cholesterol (mg/g)	0	<0.24	<0.24
Carbohydrates (g)	7	7.8	6.4
Total Sugar (g)	2	7.8	5.4
Sucrose (g)	not listed	0.5	5.4
Glucose (g)	not listed	2.8	<0.1%
Fructose (g)	not listed	4.5	<0.1%
Protein (g)	0	0.4	0.4
Vitamin C (mg/g)	not listed	<1.0	<1.0
Vitamin A (IU/g)	not listed	<1	<1
Calcium (mg)	not listed	1	20
Iron (mg)	not listed	<0.2	<0.2
Sodium (mg)	10	6	38
Acetic acid (mg)	15	608	1225
Citric acid (mg)	not listed	213	<118

Storage, Processing and Transportation Conditions

Commercial Kombucha:

1. Samples were purchased by NaturPro Scientific at grocery stores in Carmel, Indiana in February 2016.
2. All temperatures starting with the store shelf to lab storage were strictly controlled and recorded with time-stamped photographs of infrared thermometer readings of samples.
3. After purchase, samples were transferred from refrigerator shelf to PolarTech validated insulated carton shipper with 1.5" thick refrigerant packs, and temperature monitors TempTale 4, Sensitech)

4. The shippers were sealed immediately after purchase and transported overnight by FedEx to the laboratory in a box marked “Please refrigerate upon receipt”. The laboratory was notified of storage requirements before receiving.
5. The laboratory storage chamber was set to maintain 5±3° C.
6. During sample processing, the laboratory was requested to not permit samples to remain outside of refrigerated conditions for more than two hours.
7. During sample processing, kombucha materials were transferred based on weight, not volume, to account for dissolved gases.

Reference Standard Materials:

Standard preparation

See Reference Materials Table below. The internal standard 1-propanol (Reference #2) was used as an internal standard, since it is commonly used as an internal standard for analysis of ethanol in alcoholic beverages and blood alcohol analysis. 1-Propanol was spiked into the commercial kombucha sample (Ref #1), and separately into the standard solution (Lab-grade water spiked with Ref #4) at the same concentration. The area response ratio of the ethanol to the 1-propanol was used to normalize changes in injection volumes or detector response over time.

Before preparation, commercial and control kombucha reference samples (Refs #1 and #3) were allowed to warm to room temperature in ambient conditions before opening. The samples were then weighed and transferred to headspace vials or volumetric dilution glassware. Specific gravity was measured from a separate aliquot of the same sample using Covance method SPGP.

To prepare spiked samples, pure ethanol was transferred volumetrically by the lab into a pre-weighed amount of kombucha reference sample.

Certified Reference Materials (Ref# 7, #8 and #9) were tested by the lab, with the expected (certified) content blinded to the lab. Samples (intact glass ampoules) were received by NaturPro from the certifying agency (LGC, Cerilliant or NIST). Labels were removed from the ampoules and attached into the lab notebook. Unique identifier codes concealing the certifier code were assigned to the samples, to blind the laboratory to the expected amount.

Standard stability

Calibration standards prepared by the lab from dilutions of ethanol in water for calibration were used on the date of preparation only, based on standard laboratory procedure. Stability of other reference standards was based on manufacturer’s COA or label information, with the exception of non-lab spiked samples, which was not tested since all samples were kept sealed under refrigerated conditions, and were tested within one week of preparation.

Preliminary studies on control and commercial kombucha samples (Ref #5 spiked into Ref #3, spike volume blinded to the lab by NaturPro) showed that ethanol had acceptable recovery after spiking, transportation and resampling into another container (See Supplementary Data).

Reference Standard Materials:

The following reference materials were used in the study.

Reference #	Identification	Lot Number	Purity	Stability	Storage
2	1-Propanol, Sigma #34871	SHBF0634V	99.98%	July 2018	Not specified
3	Control Kombucha (ethanol-free, non-carbonated)	01206-1, Covance sample 4814443	<0.015% ethanol. Total acids 1.15%, Brix 3.0, pH 3.0 as per manufacturer.	Not specified	In a chamber set to maintain 5 ±3 deg C.
4	Ethanol (Reference standard, absolute (200 proof), Sigma-Aldrich # 459836)	SHBG7349V	99.97%	Not specified	Closed original container, room temp
5	Ethanol (Reference standard, absolute (200 proof), Sigma-Aldrich # 459836)	SHBG4976V	>99.5%	Not specified	Closed original container, room temp
6	Ethanol-water Certified Reference Material, NIST # 2894	Not applicable	0.10084% ±0.00083% certified mass fraction	valid until 30 April 2023	Refrigerate (do not freeze)
7	Ethanol-water Certified Reference Material, Cerilliant E-031	FN06181501,	100 mg/dL, (0.1267% ABV @ 20C)	exp June 2020	Refrigerate (do not freeze)
8	Beer Certified Reference Material, LGC BCR-651	000149, 000150, 000189, 000191	0.505 +/- 0.006 % ABV	valid until April 1, 2017	Approx 4 deg C. Room temp before opening. Do not freeze.
9	Ethanol-water Certified Reference Material, NIST 2897a	Not applicable	2% nominal mass fraction (2.53% +/- 0.057%)	Exp April 2025	Refrigerate (do not freeze)

Calculations

A calibration curve was generated based on the response ratio of ethanol and the internal standard. The level of ethanol in the matrix was determined by calculating the response ratio of ethanol to the internal standard, then back-calculating from the calibration curve the concentration of the alcohol in the headspace that was analyzed. Then, dividing that result by the sample mass and multiplying by the final volume gave the result in the sample as provided.

Analyte (µg/g) = C x V / m

Analyte (% ABV) = Analyte (µg/g) x SG(E) / SG(K) / 10,000

where:

C = concentration from calibration curve (ug/mL)
V = final volume (mL)
m = sample mass (g)
SG(E) = specific gravity of ethanol (0.789 @ 20°C)
SG(K) = specific gravity of kombucha (1.02 @ 20°C)

References certified by mass fraction were converted to % ABV using the specific gravity of ethanol at 20°C of 0.789 g/mL.

Intermediate precision (RSD(R)) was taken as the standard deviation from 12 data points of commercial kombucha taken over two days on different instruments by different technicians.

System Suitability

Quality Assurance

Blanks were injected after the standards at the beginning of the sequence to assess analyte carry-over within the instrument. Recovery samples were prepared on at least 10% of all samples in a batch by spiking a sample with a known volume of stock standard. Duplicates were run at the discretion of the analyst.

This work was performed in compliance with Covance standard operating procedures (SOPs) and general documentation requirements of ISO 17025. Although method validations do not fall under the scope of Good Laboratory Practice (GLPs), GLP's were used as guidance where necessary and practical. In many cases, this study sought to exceed testing validation requirements under GLP's and AOAC guidances.

Acceptance criteria

90-110% recovery was required for this study, based on the limits specified in the AOAC SMPR. The minimum requirements for routine use of the method include three standard points with concentrations bracketing the expected sample concentration, and correlation coefficient greater than or equal to 0.99. In this validation, calibration curves with at least six different concentrations (excluding the blank) were analyzed at the beginning of each validation analysis run except on Day 1, when calibration samples were evenly interspersed throughout the run.

Validation Procedure and Results

Validation procedures followed AOAC guidelines for single laboratory validation. Two different technicians on two different instruments on two different days evaluated the same sets of samples for precision, accuracy and reliability, as well as repeatability and reproducibility in a separate lab.

Specific Gravity

Covance method SPGP was used based on the weight of a known volume of sample[21].

Identity of Ethanol

The identity of the ethanol was confirmed. The retention time of reference standards agreed with method requirements, with ethanol eluting at approximately 8.5 minutes and 1-propanol eluting at approximately 12.3 minutes.

Specificity

Specificity was found to be acceptable based on the following.

- An injection of 1-propanol as internal standard (Ref #2) was run with every ethanol spike. Relative retention times and AUC between ethanol and 1-propanol remained stable during the study.
- Routine blanks were run on each day and after calibration runs to ensure no carryover.
- An ethanol-free control kombucha (Ref #3) was also analyzed for absence of ethanol, and lack of interference. On one run, three replicates of the control kombucha were analyzed by GC-FID and found to contain no detectable ethanol.

No interfering peaks were found in any of the analyses (See Figure 1, Chromatograms)

Limit of Detection (LOD)

The LOD was defined as the concentration of the lowest working standard with a signal-to-noise ratio equal to or exceeding 10:1. For the purpose of this study, the LOD and LOQ were considered to be equivalent at the lowest working standard of 0.0150% ABV. Therefore, the LOD was determined to be 0.0150% ABV.

Limit of Quantitation (LOQ)

The LOQ for this method was previously determined to be 10 ppm across various matrices. Since determining a LOD of 10 ppm required a relatively higher sample mass, and the analytical range of interest for kombucha was 0.1-3% ABV, the LOQ was considered to be 0.0150% ABV for this study, the same as the LOD.

Linearity

In this validation, calibration curves with nine different concentrations of ethanol (Reference #4) in aqueous (purified water) standards were analyzed at the beginning of each analysis run, with the exception of Day 1. On

Day 1, a single set of standards were interspersed evenly throughout the analytical run to control for potential replicate error. A minimum of two standard points at each concentration were analyzed for every run. See Figure 2. The required range of 0.1% to 2.8% corresponded to an "on instrument" range of 31.865 to 887.20 µL/mL. Nine concentrations were prepared for the standard curve covering an actual range of 0.005% to 5.09 % ABV. The linearity of both the interspersed and consecutive standards in the nine-point curve had an acceptable linear regression on each day ($r > 0.9996$). The method was acceptable since the standard curve had a correlation coefficient (r) of greater than or equal to 0.995, and the individual back-calculated standard concentrations were within ±15% (±20% for LOQ) of nominal.

Precision

Precision was determined by analyzing six replicates of one lot of commercial kombucha (Ref #1) over a minimum of two days, including one day with a second analyst on a different instrument using a different type of headspace vial (12 total replicates), intended to represent a measure of intermediate precision.

Intermediate precision (RSD(R)) was taken as the standard deviation from all 12 data points of commercial kombucha taken over two days. An alternative calculation for total standard deviation, taken as the square root of the sum of each day's standard deviations divided by the number of samples, gave a similar result. See Figure 3.

The method was considered acceptable because both the intra-day precision and intermediate precision between days had a relative standard deviation (RSD) meeting the repeatability requirement of <4% established by the AOAC SMPR's

Accuracy

Accuracy was determined by testing duplicates at each of three spike levels of pure ethanol (Reference #4 spiked into control kombucha (Reference #3) over three days (totaling 18 total replicates). The spike levels were 0.13, 1.3, and 3.3% ABV.

Percent recovery ranged from 98.3 to 104.2%. The accuracy was considered acceptable since the means of each spike level were between 90% to 110%. This method is accurate for the quantification of ethanol in kombucha at concentrations between 0.13 to 3.3% ABV. See Figure 4.

Change of vial type

Aside from different operators and instruments from Day 1 to Day 2, the only other difference in experimental conditions was the type of headspace vial used. The headspace vial is important in order to ensure efficient extraction into the headspace with no leakage.

Screw cap vials (used during routine testing) were used on Day 1, and crimp cap vials were used on Day 2. The purpose of using different vials was to determine whether the type of headspace vial led to any differences in results. No discernible differences in inter-day means were found, although the precision from using the crimp cap was lower than the precision using the screw cap.

Certified Reference Material (CRM) evaluation

Although it was not required by the SLV protocol, testing of NIST-certified reference materials (ethanol-water, Ref #6, 0.127% ABV) was initiated by the lab, and performed on Day 1 and Day 2. Percent recovery ranged from 97.1 to 99.2%. See Figure 5.

Additional testing of CRM's (Reference #7, #8 and #9) was initiated by NaturPro Scientific (NP) to enable proper lab blinding of expected concentrations (See Figure 6). NP sent five samples of each reference via priority overnight shipping according to the following plan: one unopened glass ampoule, and four additional 1mL vials (amber glass with screw cap/foil seal). Empty vials were labeled in sequential order, and coded in order to blind the lab to the certified concentration of ethanol. The resampling procedure was added to determine the potential impact of an additional handling step on method accuracy. After results were reported, the certified purity of the samples were reported by NP to the lab to contain 0.13% ABV (ethanol-water, Cerilliant), 0.505% ABV (beer, LGC) and 2.53% ABV (ethanol-water, NIST). References certified by mass fraction were converted to % ABV using the specific gravity of ethanol at 20 degrees Celsius of 0.789 g/mL.

Recovery of ethanol from all spiked samples was found to be acceptable. For the unopened ampoules, recovery was 101, 104, and 102% for Ref #7, #8 and #9, respectively. Including only the resampled CRM's, the recovery and %RSD was 91% and 5% for Ref #8 and 94% and 4% for Ref #9. Including all CRM (unopened plus resampled) the recovery and %RSD was 94% and 7% for Ref #8 and 95% and 5% for Ref #9. These were all considered acceptable since average recovery was between 90 and 110% and % RSD values were roughly similar to those established by AOAC Method Performance Requirements

Statistical evaluation

Quantitation was performed using a 6-point calibration curve generated by a weighted (1/concentration) least squares linear regression analysis. Other statistical tools used include percent recovery, standard deviation (SD), intraday percent relative SD (Repeatability RSD, (RSD(r)), and intermediate precision relative SD (RSD(R)).

Control of bias

All samples were treated in a similar manner during purchasing, storage, transportation and processing to minimize assay bias. All samples were stored and processed according to instructions.

NaturPro Scientific LLC is an independent scientific consultancy who performed the following: study design, purchasing reference materials, data analysis and interpretation. Covance Laboratories is an independent laboratory commissioned by NaturPro to conduct the validation. Covance performed study design, analysis of materials, and data analysis and interpretations. No restrictions on data publication or other conflicts of interest exist. This study had financial support from KeVita Inc. Neither Covance nor NaturPro has financial interest or ownership of KeVita or vice versa. KeVita had no influence on study execution, analysis or reporting.

Acknowledgments

We acknowledge KeVita Inc. for their support and the donation of standardized kombucha materials used for control reference samples.

APPENDIX A

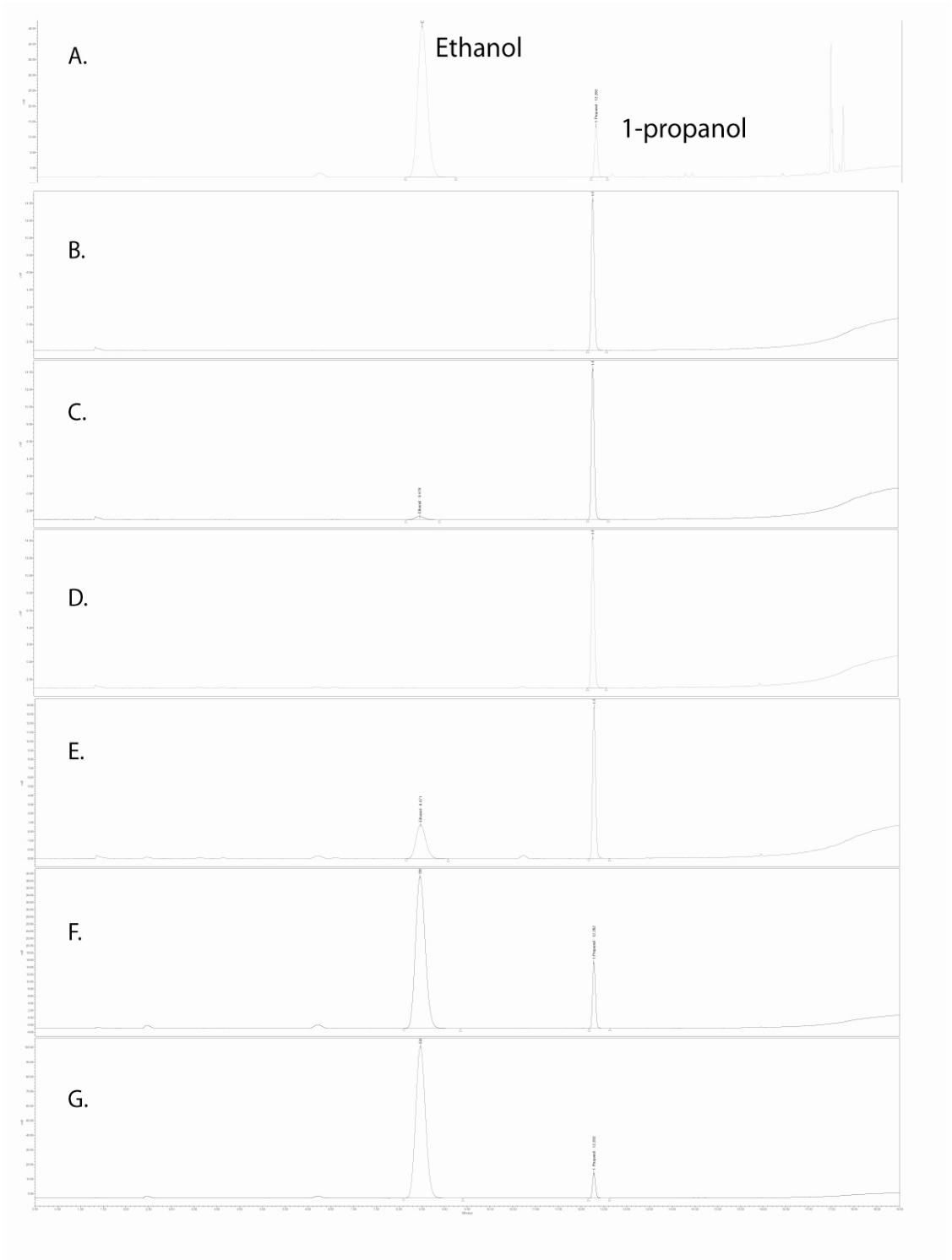
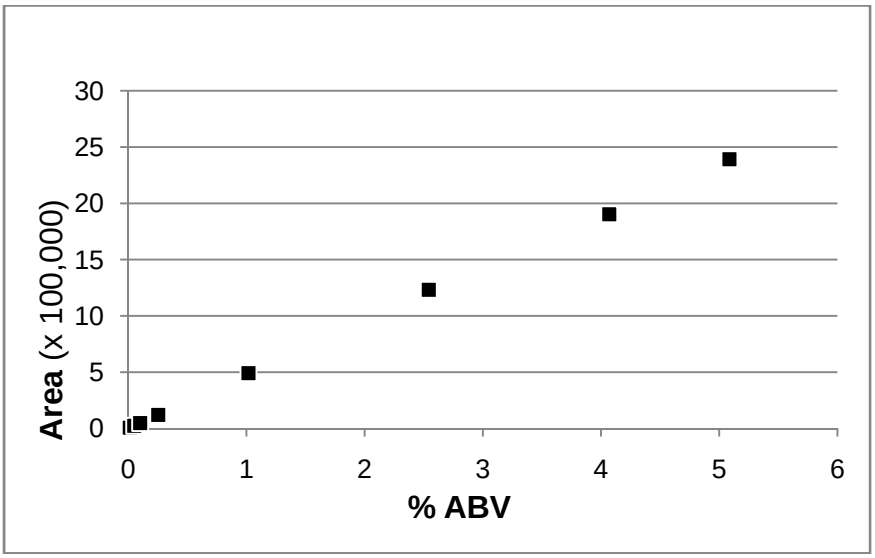


Figure 1: Chromatograms of reference materials. In spiked samples, the ethanol spike corresponded to the internal propanol standard. The retention time of reference standards agreed with method requirements, with ethanol eluting at approximately 8.5 minutes and 1-propanol eluting at 12.3 minutes. The identity of the ethanol was confirmed by the corresponding retention time and relative retention time to 1-propanol. A. Commercial kombucha sample (Ref #1); B. Water blank with 1-propanol internal standard (Ref #2); C. Ethanol standard (Ref #4) at LOQ of 0.01272%; D. Control kombucha with no detectable ethanol (Ref #3). Three replicates were analyzed and found to contain no detectable ethanol; E. Spiked control kombucha at 0.127% ABV (Ref #4 in Ref #3); F. Spiked control kombucha at 1.27% ABV; G. Spiked control kombucha at 3.30% ABV.



Day	Correlation Coefficient (r)
1	**0.999999**
2	**0.999967**
3	**0.999722**

Figure 2: Linearity. On three separate days, nine-point calibration curves from 0.005% to 5.09% ABV were prepared with serial dilutions of pure ethanol (Ref #4) in purified water. The correlation coefficient (r) based on linear regression analysis was greater than 0.9996 on each day.

	Results (%ABV)
Replicate	**Day 1**
1	1.5860
2	1.5885
3	1.5937
4	1.6154
5	1.5967
6	1.6102
Mean	1.60
SD	0.119
RSD(r) (%)	0.743
	Day 2
1	1.6830
2	1.6048
3	1.4589
4	1.6444
5	1.6345
6	1.7181
Mean	1.62
SD	0.0900
RSD(r) (%)	5.542
Overall Mean	1.61
Overall SD	0.0626
Intermediate Precision RSD(R) (%)	**3.888**

Figure 3: Precision. Using the MP-ETME method, the commercial kombucha sample was found to contain 1.61% alcohol by volume (ABV) with a relative standard deviation of +/- 0.06%. Intermediate precision, measured by the relative standard deviation

(RSD) across different days, instruments and technicians, was < 4%. Abbreviations: SD: Standard deviation; RSD: Relative standard deviation; RSD(r): Repeatability (same-day %RSD); RSD(R) Intermediate precision (interday %RSD)

	Results		
Day	0.13%	1.3%	3.3%
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	100.0	102.5
	96.2	104.2	103.4
Mean	99.6	100.4	100.4
RSD(R) (%)	**2.33**	**1.84**	**2.03**

Figure 4: Accuracy. Recovery for lab-spiked control kombucha samples (Ref #4 into Ref #3) ranged from 98.3 to 104.2% across spike levels of 0.13%, 1.3% and 3.3% ABV. Intermediate precision for each concentration ranged from 1.84 to 2.33%.

Day	Percent Recovery
1	98.0
	99.2
2	98.5
	97.1

Figure 5: Recovery of Certified Reference Material. A lab-initiated analysis of NIST CRM of 0.127% ABV ethanol in water (Ref #6) reported acceptable recovery within the 90-110% range.

Product	**Composition**	**Lot#**	**Exp**	**Certified Concentration %ABV**	**Lab Result**	**Percent Recovery**
Certified Reference Material (Cerilliant E-031) 1.2mL Ampoule **(Ref #7)**	Ethanol-water	FN06181501 (Internal code ending in 06181501)	June 2020	**0.1267%** +/- 0.0011%	0.131, 0.127, 0.129, 0.127, 0.126% ABV	**101%**
Certified Reference Material (LGC BCR-651) 10mL Ampoule **(Ref #8)**	Beer	000149, 000150, 000189 (Internal code B1-B5)	April 1, 2017	**0.505%** +/-0.006%	0.526*, 0.455, 0.490, 0.439, 0.463% ABV	**104%***
Certified Reference Material (NIST 2897a) 10mL Ampoule **(Ref #9)**	Ethanol-water	Not specified (Internal code E1-E5)	April 30, 2025	**2.53%** +/-0.057%	2.59*, 2.34, 2.50, 2.29, 2.34% ABV	**102%***

Figure 6: Recovery of Certified Reference Material. Recovery of ethanol from all spiked samples was found to be within acceptable limits of 90-110%. Recovery for lab-blinded Certified Reference Materials (CRM) was 101-104% for sealed CRM and 94-104% for both sealed and resampled CRM, across spike levels of 0.1267%, 0.505% and 2.53% ABV. Ref #8 recovery was 94% (range 87-104%) and Ref #9 recovery was 95% (range 90-102%) including sealed and resampled CRM. Labs were blinded to expected concentration. First data point in each set was recovery of unopened ampoule. References certified by mass fraction were converted to % ABV using the specific gravity of ethanol at 20 degrees Celsius of 0.789 g/mL.

APPENDIX B

PROTOCOL DEVIATION

Protocol	Actual Procedure
EXPERIMENTAL DESIGN. **Linearity.** The Linearity section of the protocol requires that a standard curve with at least six different concentrations be analyzed at the beginning of each validation analysis run.	The linearity determination on Day 1 was interspersed throughout the analytical run, and not run simultaneously to control for replicate bias.

This deviation supports the integrity or quality of the study.

DISPOSITION OF TEST SAMPLES

Remaining unused test samples and reference standards may be kept as retained samples under proper storage conditions by Covance or NaturPro.

RECORD RETENTION

The raw data, including documentation, study protocol, final report, and study correspondence, resulting from this study will be retained in the Covance archives for at least 1 year from the date of report finalization.

Supplementary Data

Multi-Lab Study on Ethanol Content in Commercial Kombucha Samples and Certified Reference Materials

Introduction

A multi-lab study was performed prior to and after single-lab method validation of a GC-FID method commonly used for food, beverages and drugs. The study, conducted between December 2015 and April 2016, tested various types of materials (commercial kombucha products, 'placebo' control kombucha reference materials spiked with ethanol, and certified reference materials (CRM).

The study was initiated in response to regulatory compliance concerns raised by researchers, the kombucha industry and regulators regarding observed alcohol levels above the legal limit, in addition to differences in lab results for ethanol content[5,7,22,23,24,25,26,27,28,29,30,31]. Although published validated methods specifically on kombucha are absent, several methods have been used for kombucha based on gas chromatography (GC) and other methods such as densitometry, near-infrared spectrometry and distillation. GC methods are often considered more specific and precise than non-chromatographic methods, although it is possible that multiple methods may be valid and fit for purpose.

Kombucha tea products contain a number of constituents common with other fermented products, but there are some differences in composition. Many kombucha products claim to contain living micro-organisms called SCOBY (symbiotic community of bacteria and yeast) which appears as solids, part settled sediment and part floating mass (known as the 'mushroom'). In these products, ethanol, carbon dioxide and organic acids are produced and consumed by the SCOBY during fermentation that can continue after bottling. Other ingredients typically found in kombucha include tea (*Camellia sinensis* L.), sugars, plant extracts and flavors. The composition of kombucha and purported changes during its shelf life and handling have been suggested as reasons for differences in ethanol test results.

Conclusions:

In this study, 16 preparations of commercial and control kombucha were tested at a total of four laboratories using different methods, totaling 45 test samples and 84 analytical runs. Results reported by Lab #1 using GC-FID method MP-ETME on duplicates of commercial samples indicated a high precision. Acceptable recovery of spiked 'control' kombucha samples at Lab #1 and Lab #2 indicated accuracy of GC-FID methodologies at different labs. See data in Table 2 in Supplementary Data.

Same-sample results from Anton-Paar Alcoholizer (NIR), distillation and GC-FID suggested a general agreement of traditional methods with GC-FID. NIR and distillation could become valid screening or field tests for kombucha products, especially those containing higher amounts of alcohol.

Testing of Certified Reference Materials found recovery for the method used by Lab #1 and Lab #2 to be generally within the AOAC SMPR requirements of 90-110%. Recovery of ethanol in spiked samples was also determined to be within acceptable ranges for the GC-FID methods used by Lab #1 and Lab #2.

Objectives:

NaturPro Scientific, LLC (NPS) independently conducted this study, mainly as a preliminary survey of analytical laboratories offering ethanol testing for kombucha. This study was done in advance of laboratory selection for single laboratory validation (SLV) for the quantification of ethanol in kombucha. The following were key objectives of the preliminary study:

- To determine whether acceptable precision on same-sample duplicates was reported by labs
- To determine if ethanol concentrations above the legal limit of 0.50% ABV in commercial products previously reported by kombucha manufacturers and labs could be independently replicated.
- To determine whether further method optimization was required before performing validation; to determine an appropriate analytical range for the validation.
- To determine whether there was general agreement in results on the same samples tested by different labs and methods.

After an initial round of testing done in December 2015 to January 2016, spiking studies were conducted in March 2016 to determine whether laboratories were able to accurately recover known amounts of pure ethanol that was added to both commercial kombucha and ethanol-free control kombucha materials at concentrations across the analytical range of kombucha products, generally 0.2 to 3%.

Methods:

Preliminary study

Analytical methods for ethanol quantification were reviewed. GC-FID was considered to be generally robust and appropriate based on its use in official methods for alcohol in beverages published by AOAC. GC-MS was also selected due to its ability to measure ethanol at trace levels. Traditional methods based on distillation/densitometric and near-infrared (NIR) were also considered.

Laboratory selection for testing of commercial materials was based on an initial survey of laboratories offering kombucha ethanol testing. Four labs were selected, three of which use GC-based methods reflecting a range of instrumental conditions and reference standards, with the remaining lab using NIR and distillation methods.

Analysis of 45 test and control samples representing 84 analytical runs was conducted by selected labs using multiple methods. With the exception of the spiked samples, all samples were purchased and sent in unopened bottles with original seals. All samples were tested before their expiration date. All samples were purchased, transported and stored in cold conditions using validated cold-transport shippers (Polartech). A temperature monitor (TempTale 4, Sensitech) recorded changes in temperature during shipping. All samples were sent overnight and received by the lab the following day. Temperatures of samples did not exceed general limits set for cold storage of refrigerated/perishable samples. A list of samples is found in Table 2 (Supp).

The GC-FID method used by Laboratory #1 (Covance, Madison, WI) is detailed in the single lab validation report previous to this section.

The GC-FID method used by Laboratory #2 (ETS Laboratories, St. Helena, CA) is commonly used for testing alcohol in beer, wine and vinegar. The laboratory references AOAC 983.13, Final Action 1988, JAOAC 66, 1152 (1983). The reference standard cited is 99.5% ACS reagent grade ethanol (Sigma).

The GC-MS method used by Laboratory #3 is referenced to EPA methods for a panel of hazardous waste contaminants at trace levels, including ethanol. The laboratory references the following methods in a self-published method: EPA Headspace Method 5030B, EPA Method 624 Purgeables, Part 136, Title 40 and EPA Method 8260B, SW-846[32].The reference standards cited are "NSI Environmental Solutions in methanol C-350 (200ug.mL and W-0017 (1000ug/mL of Ethanol diluted 1:5 or 200 ug/mL)". Although EPA methods are not commonly used to test for ethanol in beverages, this laboratory was selected based on a public recommendation by a kombucha industry organization[33].

The methodologies used by Laboratory #4 (Brewing and Distilling Analytical Services, Lexington, KY) were based on traditional distillation and densitometry methods, in addition to the Anton-Paar Alcolizer based on near-infrared spectroscopy. Both methods are commonly used for testing alcohol in beer and wine.

Lab #	**Instrument**	**Method Reference**	**Instructions to Lab**
1	GC-FID Headspace and GC-MS Headspace	"MP-ETME"[20] "RESO" (GC-MS)	Refrigerate samples immediately upon opening, return TempTale and cooler. Use specified method.
2	GC-FID Headspace	AOAC 983.13 (GC-FID)	Refrigerate samples immediately upon opening, return TempTale and cooler. Use specified method
3	GC-MS Headspace	ANACHEM-32013 (EPA 624 Part 136, Title 40, EPA 8260B, SW-846)[32]	Refrigerate samples immediately upon opening, return TempTale and cooler. Use [industry organization] approved method.
4	Distillation and NIR	Anton Paar DMA5000/NIR-Alcolyzer; Anton Paar DMA 5000 density meter	Refrigerate samples immediately upon opening, return TempTale and cooler. Use specified method.

Data Analysis
Appropriate statistical analyses were conducted with Microsoft Excel to include mean and relative standard deviation. All labs reported results in % alcohol by volume (ABV) except for Lab #1 reporting in ABW. For this lab, ABW was converted to ABV assuming a specific gravity of 1.00 g/mL across all products, based on measured range of specific gravity of 1.00-1.02 g/mL at 20° Celsius measured for various kombucha products. With respect to the overall findings, small potential variations in specific gravity did not impact overall results.

Data Management
All reports of raw data are on file and available on request.

Materials selection:
Materials were selected from an initial survey of six retail locations in Carmel, Indiana. Multiple flavors from multiple manufacturers were selected.

Commercial Kombucha Handling Precautions:

1. Samples purchased by NaturPro at grocery stores in Carmel, Indiana in December 2015 to March 2016
2. All temperatures starting with the store shelf to lab storage were recorded with time-stamped photographs of infrared thermometer readings of samples
3. After purchase, samples were transferred from refrigerator shelf to PolarTech validated insulated carton shipper with 1.5" thick refrigerant packs, and temperature monitors TempTale 4, Sensitech)
4. The shippers were sealed immediately after purchase and transported overnight by FedEx to the laboratory in a box marked "Please refrigerate upon receipt". Labs were notified of storage requirements before receiving.
5. The laboratory storage chamber was set to maintain 5±3deg C.
6. During sample processing, the laboratory was requested to not permit samples to remain outside of refrigerated conditions for more than two hours.
7. During sample processing, kombucha materials were transferred based on weight, not volume, to account for dissolved gases.

Samples were shipped via FedEx priority overnight shipping with instructions to refrigerate samples (do not freeze).

Reference Materials:

The following reference samples were used in the study:

Reference #	Identification	Lot Number	Purity	Stability	Storage
2	1-Propanol, Sigma #34871	SHBF0634V	99.98%	July 2018	Not specified
3	Control Kombucha (ethanol-free, non-carbonated)	01206-1, Covance sample 4814443	<0.015% ethanol. Total acids 1.15%, Brix 3.0, pH 3.0 as per manufacturer.	Not specified	In a chamber set to maintain 5 ±3 deg C.
4	Ethanol (Reference standard, 200 proof, Sigma-Aldrich # 459836)	SHBG7349V	99.97%	Not specified	Closed original container, room temp
5	Ethanol (Reference standard, 200 proof, Sigma-Aldrich # 459836)	SHBG4976V	>99.5%	Not specified	Closed original container, room temp
6	Ethanol-water Certified Reference Material, NIST # 2894	—	0.10084% ±0.00083% certified mass fraction	valid until 30 April 2023	Refrigerate (do not freeze)

7	Ethanol-water Certified Reference Material, Cerilliant #E-031	FN06181501,	100 mg/dL, (0.1267% ABV @ 20C)	exp June 2020	Refrigerate (do not freeze)
8	Beer Certified Reference Material, LGC # BCR-651	000149, 000150, 000189, 000191	0.505 +/- 0.006 % ABV	valid until April 1, 2017	Approx 4 deg C. Room temp before opening. Do not freeze.
9	Ethanol-water Certified Reference Material, NIST 2897a	—	2% nominal mass fraction (2.53% +/-		Refrigerate (do not freeze)

Spiked Reference Materials

A composite of three lots of ginger-flavored kombucha was prepared. Three unopened bottles of the same product used for the SLV that expired within the same two days (March 25-26, 2016) were removed from refrigeration. The bottles were inverted gently 10 times to disperse solids, allowed to settle for a few seconds, and then opened. The compositing procedure was intended to correct for intermediate manufacturing and storage differences that might be expected to cause differences among same-product composition. Equal amounts of each bottle were slowly poured into a 1000mL glass beaker which was gently swirled to mix. Then, three aliquots of kombucha from the beaker was slowly drawn into a 100mL volumetric pipet (Wilmad) to avoid any gas bubbles, and slowly dispensed into separate beakers. Spiked samples of 0, 250 and 500 µL of ethanol (200 proof, Sigma (Ref #4) was slowly dispensed into the separate beakers using a Scilogix Micropette certified accurate within the nearest 0.23%. These samples were gently swirled for about ten seconds, and then each were poured into separate 15mL glass vials with screw cap foil seals. The samples were then immediately stored in the refrigerator.

Then, a control kombucha (Ref #3) confirmed ethanol-free (<0.015% ABV) (donated by KeVita Inc.) was spiked with 0, 500 and 1500 µL of ethanol per 100mL kombucha, using the same procedure just described.

Certified Reference Material analysis

Testing of CRM's (Reference #7, #8 and #9) was performed in April 2016. For Reference #7, five unopened glass vials containing 0.1267 +/- 0.0011% ABV (certified by Cerilliant) were sent to each lab.

For References #8 and #9, five samples of each reference (except four samples for Lab #2) were prepared according to the following plan: one unopened glass ampoule, and an additional ampoule opened at room temperature and immediately poured into four 1mL amber glass vials and sealed with standard screw caps. All samples were relabeled in sequential order with codes (E1-E5, B1-B5) to conceal the certified expected value of ethanol. All vials were filled approximately 4mm from the lip of the vial, to allow for some headspace. The transfer into new vials, the relatively small (~1mL) sample size, and the allowance of headspace in the vial were deliberately added to determine whether transfer to another container before shipping resulted in recoveries different than for the unopened ampoule.

Samples were shipped via FedEx priority overnight with instructions: "Refrigerate samples (do not freeze)".

Results

Certified Reference Material (CRM) Evaluation

Testing of Certified Reference Materials found method recovery from Lab #1 to be within the requirements of 90-110%.

Additional testing of CRM's (Reference #7, 8 and 9) at three labs, including the validation lab, was initiated by NaturPro (See Table 1 (Supp)). Five samples of each reference material were sent to Lab #1 and #3, and four to Lab #2 via priority overnight shipping according to the following plan: one unopened glass ampoule, and four (or three for Lab #2) additional 1mL vials. All samples were relabeled in sequential order and coded to conceal the certified concentration of ethanol. Resampling into vials was performed to determine if an additional sample transfer step impacted the accuracy of the method.

After results were reported, the certified purity of the samples was reported by NaturPro to the labs to contain 0.13% ABV (ethanol-water, Cerilliant), 0.505% ABV (beer, LGC) and 2.53% ABV (ethanol-water, NIST). References certified by mass fraction were converted to % ABV using the specific gravity of ethanol at 20° Celsius of 0.789 g/mL.

All recovery values for the CRMs were found to be within acceptable ranges. For Lab #1, the recovery from unopened ampoules was 101, 104, and 102% for Ref # 7, 8 and 9, respectively. The recovery and %RSD of only resampled CRMs was 91% and 5% for Ref #8 and 94% and 4% for Ref #9. Recovery and %RSD of all CRM (unopened and resampled) was 94% and 7% for Ref #8 and 95% and 5% for Ref #9. These were all considered acceptable since average recovery was between 90 and 110%, and % RSD values were roughly similar to those established by AOAC Method Performance Requirements.

Lab #2 reported 0.11, 0.11, 0.11, 0.11, and 0.11% on unopened ampoules of Ref #7, certified by Cerilliant to contain 0.127% ABV. Due to the reporting of the same replicated value to two significant figures, no statistical analysis was done. The recovery and %RSD for unopened and resampled together, versus resampled vials only were similar. Including all of the same CRM (unopened plus resampled) the recovery and %RSD was 93% and 2% for Ref #8 and 98% and 2% for Ref #9. These were all considered acceptable since average recovery was between 90 and 110%, and % RSD values were below those established by AOAC SMPR. While precision and accuracy for the CRM samples were within acceptable limits, it can be stated that some loss due to resampling into vials has the potential to occur. Extra effort should be taken to minimize the number of times samples are transferred to different containers before analysis. See Table 1 (Supp.) for data.

Lab #3 reported an average recovery for Ref #7 of 96% with %RSD of 8%, while Ref #8 recovery was 145% with %RSD of 44%. Ref #9 recovery was 81%, also with %RSD of 44%.

Product	Composition	Lot#	Expiry Date	Certified %ABV	Lab #1 (GC-FID) (Validated method)	Lab #2 (GC-FID)	Lab #3 (GC-MS)
Certified Reference Material (Cerilliant E-031) Ampoule	Ethanol-water	FN06181501	June 2020	**0.1267%** +/- 0.0011%	0.131, 0.127, 0.129, 0.127, 0.126%	0.11, 0.11, 0.11, 0.11, 0.11%	0.120, 0.140, 0.120, 0.115, 0.115%
Certified Reference Material (LGC BCR-651) Ampoule	Beer	000149, 000150, 000189, 000191	April 1, 2017	**0.505%** +/-0.006%	0.526, 0.455, 0.490, 0.439, 0.463%	0.48, 0.47, 0.47, 0.46 %	0.496, 0.290, 0.915, 1.000, 0.950%
Certified Reference Material (NIST 2897a) Ampoule	Ethanol-water	Not specified	April 30, 2025	**2.53%** +/-0.057%	2.59, 2.34, 2.50, 2.29, 2.34%	2.53, 2.45, 2.53, 2.49%	0.920, 1.280, 2.410, 2.860, 2.810%

Table 1 (Supp). Recovery of Certified Reference Material with labs blinded to certified concentration.

Commercial Sample Evaluation and Spiking Studies

Laboratory selection for SLV

84 analytical runs were performed on 16 commercial and control kombucha materials across four laboratories. See Table 2 in Supplementary Data (below). Results reported by Lab #1 using GC-FID method MP-ETME on duplicates of commercial samples indicated a high precision. Acceptable recovery of spiked 'control' kombucha samples indicated accuracy of the method. Based on these initial indicators of reliability, the lab's routine use of this method, and previous validations conducted with this method on food and beverage matrices, this method and lab was selected to undergo validation of the method without further optimization required.

Estimated content of commercial samples

Several samples of commercial kombucha samples tested contained greater than 1% alcohol by volume (ABV), consistent with previous laboratory and manufacturer reports. The range of ethanol content of commercial kombucha samples reported using method MP-ETME was 0.114% to 1.75% ABV (n=26). All samples were confirmed to meet all storage requirements, specifically cold chain control throughout the sampling process. All samples were tested before the expiration date marked on the bottle.

Inter-lab agreement on same-lot samples

Lab #1 running GC-FID and GC-MS, and Lab #2 running GC-FID reported generally consistent results on the same-lot samples. On control kombucha spike recovery samples, Lab #1 and #2, both using GC-FID, reported acceptable recovery. Lab #3 recovered levels lower than the expected amount in spiked reference samples, with some imprecision observed among consecutively processed and analyzed duplicate samples. After the original test of samples on March 1, inter-laboratory results were disclosed to the lab, in advance of a retest of retained samples.

		Sample submission date	Mar 1	Jan 11	Dec 15	Jan 11	Dec 15	Mar 1	Mar 23	Mar 1	Jan 11	Dec 15	Jan 11	Jan 11
Product	**Expiry/Best by**	**Expected Value from Spike**	**Lab #1 (GC-FID)**	**Lab #1 (GC-FID)**	**Lab #1 (GC-FID)**	Lab #1 (GC-MS)	Lab #1 (GC-MS)	Lab #2 (GC-FID	Lab #3 (GC-MS) retest	Lab #3 (GC-MS)	Lab #3 (GC-MS)	Lab #3 (GC-MS)	Lab #4 NIR Alcoholizer	Lab #4 Distillation
Commercial kombucha	1/17/16	NA		**1.545, 1.545%**	**1.45, 1.46%**	1.60, 1.61%	1.42%, 1.38				0.545%	1.690%		
Commercial kombucha	1/20/16	NA		**1.41, 1.37%**	**1.34, 1.36%**	1.50, 1.47%	1.41%, 1.45%					1.510%		
Commercial kombucha	1/17/16	NA		**1.39, 1.44%**		1.51%, 1.50%					0.570%		1.36%	1.27%
Commercial kombucha	1/31/16	NA									0.510%		1.51%	
Commercial kombucha	5/23/16	NA		**1.67, 1.75%**										
Commercial kombucha	4/30/16	NA			**1.23, 1.21%**		1.21, 1.19%					1.590%		
Commercial kombucha	2/1/16	NA		**0.114, 0.128%**							0.040%		0.11%	0.08%
Commercial kombucha	3/27/16	NA			**0.263, 0.253%**		0.275, 0.271%					0.255%		
Commercial kombucha	4/20/16	NA		**0.234, 0.232%**	**0.236, 0.232%**		0.251, 0.244%				0.080%	0.225%		
Commercial kombucha	3/25/16	NA	**1.61%**					1.44%						
Commercial kombucha	3/25-3/26**	1.432%*	**1.432%**					1.37%	0.290%	0.540%				
Commercial kombucha (spiked)	3/25-3/26**	1.68%*	**1.658%**					1.61%	0.320, 0.300%	0.340, 0.610%				
Commercial kombucha (spiked)	3/25-3/26**	1.93%*	**1.922%**					1.86%	0.400, 0.360%	0.820, 0.620%				
Control (ethanol-free kombucha, Ref #3)	NA	0%	**<0.015 %**					<0.05%	<0.05%	<0.05%				
Control (ethanol-free kombucha, Ref #3) and pure ethanol (Ref #5)	NA	0.500%	**0.692%**						0.290%	0.300%				
Control (ethanol-free kombucha, Ref #3) and pure ethanol (Ref #5)	NA	1.500%	**1.47%**					1.43%	0.370, 0.380%	0.390, 0.430%				

Table 2 (Supp). Analysis and Recovery of Ethanol from Commercial and Reference Materials
Analysis of 16 commercial and control kombucha materials across four labs resulted in 84 data points, including duplicates run at the discretion of the laboratory. *Expected (calculated) recovery based on spike addition of 0.250 or 0.500% to the unspiked sample (1.432%). **Samples taken from a mixture of three bottles of the same product (100mL from each) with different lot #'s, expiring on 3/25/16 or 3/26/16. Lab #3 received duplicate samples that were split and blinded to expected concentration. Data points combined in the same cell are splits of the same sample. Blank cells = not tested.

Discussion

The practice of making kombucha has historically been on the home or small production scale. Prior to a few years ago, few large-scale production operations of kombucha existed in the United States. Kombucha has been widely consumed in Asia, where its traditional name in Asia translates to 'tea fungus' or 'tea mushroom'. Kombucha is defined as a beverage that results from the fermentation of tea (*Camellia sinensis* L..) leaves, sugar and other natural ingredients, that are combined with a live culture known as SCOBY, short for "symbiotic community (or culture) of bacteria and yeast". Scientifically speaking, SCOBY is traditionally an undefined, uncontrolled mixture of micro-organisms that consume sugar, ethanol and organic acids while producing carbon dioxide, ethanol, organic acids and other byproducts.

Kombucha products that have not arrested fermentation can present technical challenges to the adoption of standardized manufacturing processes and food safety controls. Kombucha may be stabilized by pasteurization and filtration, which may be effective controls for microbial pathogens and other toxins such as mycotoxins, in addition to regulated substances like alcohol.

This study to evaluate alcohol in kombucha products was performed in combination with single lab validation of a GC-FID method. It was initiated in response to regulatory compliance concerns raised by the kombucha industry regarding ethanol levels in excess of the 0.49% legal limit, along with reported imprecise test results. Although published validated methods specifically pertaining to kombucha have not been published, a number of methods have been widely used for the analysis of ethanol in wine, beer and vinegars. These fermentation products contain similar types of interferences as kombucha. According to guidelines for best practices set by standards-setting agencies for health products like FDA, USP and AOAC International, the correct way to prove a test method is fit for its intended purpose is to perform validation using a single method on a suitably specific composition or matrix. In absence of proper method validation, it is possible that interferences may alter reported results, impacting method accuracy.

This study, in addition to the single lab validation, suggests the MP-ETME method is both precise and accurate for the quantification of ethanol in kombucha products. The data supports that the validated GC-FID method used by Lab #1 is suitable for measuring ethanol in kombucha, based on testing of several different types of kombucha and reference materials. Recovery of certified concentrations from reference materials data shows that this method meets applicable accuracy requirements. Lab #2, also running GC-FID appeared to meet typical accuracy requirements for recovery for certified materials. This study supports the addition of kombucha to the list of product types or matrices by which ethanol can be reliably measured using GC-FID.

It is difficult to speculate on the nature of the measurement inconsistencies in results reported by Lab #3 without further investigation. In an informal survey of analytical chemists with expertise in food chemistry, GC-FID, and not GC-MS, was listed as a first choice to measure low levels of ethanol content in regulated consumer products like kombucha. GC-MS is often used for trace levels of contaminants, and has potential limitations like any analytical method.

This preliminary investigation did not seek to answer all possible questions relating to reliable analysis and handling of kombucha samples, but to offer some initial data to inform next steps in the study plan. Testing on additional commercial samples, after different sample handling and storage parameters, should be replicated independently and with greater rigor.

One question that remains unanswered is the extent to which alcohol increases over the shelf life of kombucha, particularly for products in which fermentation has not been arrested. For example, allowing the product to warm to room temperature for short periods of time is thought to trigger fermentation and increase ethanol content. To determine to a preliminary extent whether shelf life and short durations of storage in room temperature impact ethanol results, a commercial kombucha (exp. 1/20/2016) was tested by Lab #1 near the end of its shelf life, both before and after one month of additional refrigeration. Another kombucha sample from a different lot (exp. 1/17/2016) was tested by Lab #1 before and after one month refrigeration, plus 48 hours in room temperature. Both GC-FID and GC-MS at Lab #1 were used to test these samples. Results on the same manufacturing lots were generally consistent between methods and dates tested. A potential trend for increased concentrations was observed, although the difference in sample means between the time points was less than 10%. This data indicates that changes in storage and shelf life conditions like those that could be observed during product transportation and distribution may not be likely to change ethanol concentrations substantially, although a more rigorous analysis needs to be done.

Another question to be answered is whether alternative and/or cheaper methods can serve as an adequate screening analyses or field tests for kombucha products containing alcohol content. In the preliminary study, three samples of kombucha were tested by four different methods (GC-FID, GC-MS, NIR Alcolizer, and a traditional distillation/densitometric method). Results from the NIR and distillation methods were comparable to those from GC-based methods at Labs #1 and #2.

Lastly, changes in analytical parameters such as vial type and handling procedure which can be common causes of analyte loss through volatility could make a difference in results. This study sought to investigate in a preliminary fashion whether the addition of a sample transfer step affected results. Results from certified reference material (CRM) samples split into smaller vials containing some headspace suggested a possible trend toward lower results compared to the unopened CRM ampoules. However, no statistical differences between unopened ampoules and resampled vials were observed, and all CRM samples analyzed as one population were within the 90-110% percent recovery range.

Next steps should include submission of the Covance method MP-ETME for review as an AOAC Official Method of Analysis (OMA). Then, multi-lab validation and proficiency studies using the same OMA and Certified Reference Materials should be conducted. Additional work should be done to investigate ways to ensure products found on the shelves can meet the legal limit for alcohol content, using validated test methods integral to their control.

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