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Method Title: *Determination of Ethanol in Kombucha Tea by Solid Phase Microextraction and GC*

Method Author(s): Katherine K. Stenerson, Olga I. Shimelis

Method Submitter: Katherine Stenerson, SIAL

Primary AOAC Reviewer: Blake Ebersole, Verdure Sciences

Secondary AOAC Reviewer: Rasu Jayabalan, National Institute of Technology Food Microbiology and Bioprocess Lab, India

1 **Determination of Alcohol Content in Kombucha Tea by Headspace Solid Phase**
2 **Microextraction and Gas Chromatography-Mass Spectrometry**

3 Katherine K. Stenerson*, Olga I. Shimelis

4 MilliporeSigma, 595 Harrison Road, Bellefonte PA, U.S.A.

5 Katherine.stenerson@sial.com, 1-814-359-5781

6
7 **Abstract**

8 The fermentation process used to produce Kombucha tea naturally produces small amounts of
9 ethanol (alcohol). In order for Kombucha to be sold as a non-alcoholic beverage in the United
10 States, this content must be < 0.50 percent alcohol by volume (%ABV). Thus, an accurate
11 testing method is required which will allow producers to sell their product in accordance with
12 this requirement. In this study, a headspace solid phase microextraction (HS-SPME) GC-MS
13 method was developed for the determination of alcohol in Kombucha tea. Experimental
14 parameters such as fiber selection, pre-extraction incubation time, temperature and the use of
15 salt and buffer dilution were examined. The optimized method had an LOD and LOQ of 0.01 and
16 0.03 %ABV, respectively. Over a concentration range of 0.10 to 2.00 %ABV, method accuracy
17 ranged from 98 - 100%, with intraday precision of 3%. The method was further validated using
18 certified reference materials, and exhibited average accuracies of 98 - 100%, and
19 reproducibilities of $\leq 5\%$ RSD for analyses on two different instruments by two different
20 analysts. The HS-SPME method was then applied to the analysis of several different varieties of
21 commercially produced Kombucha teas.

23 **Introduction**

24 Kombucha is a fermented tea beverage. It is produced by the addition of a mixture of yeast and
25 acetic acid bacteria, sometimes referred to as “tea fungus”, to a solution of sugar and tea. After
26 fermentation, the result is an effervescent solution containing phenolics, water soluble
27 vitamins, organic acids, as well as some alcohol (ethanol). The health benefits often associated
28 with Kombucha stem from the antioxidant activity of many of these compounds.^{1,2}

29 In order for Kombucha to be sold as a nonalcoholic beverage in the United States, the alcohol
30 content must be $<0.5\%$ by volume. According to the United States Alcohol and Tobacco Tax and
31 Trade Bureau (TTB), if the alcohol content of a commercially produced Kombucha beverage is at
32 or above 0.5% at any time during the production process, the producer is subject to TTB
33 regulations. In addition, if the Kombucha beverage contains $<0.5\%$ alcohol when bottled, but
34 the level rises to or above 0.5% thereafter, it is considered an alcoholic beverage and must be
35 labeled and sold as such. TTB has gone so far as to state that if it obtains a sample of
36 Kombucha beverage from the marketplace and determines the alcohol content to be $\geq 0.5\%$,
37 the producer will be expected to take corrective steps to reduce the content and/or qualify as a
38 producer of alcoholic beverages.³

39 There are AOAC methods for the determination of alcohol by specific gravity in beer and
40 wine.^{4,5} These methods require that the alcohol first be isolated from the sample by
41 distillation, followed by measurement with a pycnometer. The distillation can be a cumbersome
42 process, and the distillate could contain other compounds which distill with the alcohol and
43 affect the accuracy of the final determination.⁶ Gas chromatography is a much more selective

technique, and has been used for the determination of alcohol in a wide variety of matrices.^{6,7,8,9} There is an AOAC method for the determination of alcohol in beer by gas chromatography which describes a very low volume injection (0.2 μ L) of the sample directly into the instrument.¹⁰ However, for samples such as Kombucha tea which contain much lower levels of alcohol, a larger injection volume may be required to achieve the required sensitivity. If these samples contain sugar and other nonvolatile components, the necessity of using a larger injection volume would result in rapid contamination of the GC inlet and column. An alternate approach to direct injection is analysis of alcohol by headspace. Headspace can be used to isolate the volatile alcohol from other constituents in the sample, thus protecting the GC system; and it has been used in the testing of alcohol content in various matrices not amenable to direct injection.^{11,12,13} In the case of commercially produced Kombuchas, some contain sweeteners and flavorings which could be problematic for direct injection GC analysis, thus headspace analysis offers several advantages in the testing of alcohol content: (1) enhanced sensitivity due to the concentration effect of headspace sampling, (2) elimination of potential interferences which could be introduced through a liquid injection, (3) enhanced GC method ruggedness through injection of volatile components only. To date, the only chromatographic method which has been published for the analysis of alcohol specifically in Kombucha uses dynamic headspace GC (purge & trap).¹⁴ Dynamic headspace requires the use of a purge and trap concentrator attached to the GC. Static headspace could also be used, however it typically requires a headspace analyzer to concentrate and transfer the sample to the GC for analysis. Headspace solid phase microextraction (HS-SPME) is an alternative approach to analysis of alcohol content in beverages such as Kombucha. This technique can be

done manually or automated, does not require the use of a concentrator or headspace analyzer external to the GC, and is typically faster and less expensive to perform than other more traditional approaches.

In this work, we have developed an HS-SPME method for the determination of alcohol in Kombucha. GC-MS was used to allow for accurate and confirmative determination. The HS-SPME method developed is quick, simple, accurate, highly sensitive and easy to automate. A very small sample volume was required and refrigeration was not necessary during the sample sequence.

Experimental

Apparatus

Analyses were done on 6890 /5973N and 7890/ 5977C GC/MS systems (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation in both systems was performed on a 30 m x 0.25 mm ID, 0.50 μ m d_f SUPELCOWAX 10 capillary column (MilliporeSigma, Bellefonte PA, USA) with helium carrier gas. The SPME fiber used was fused silica, 100 μ m polydimethylsiloxane (PDMS), 24 gauge, installed in an autosampler-style holder (MilliporeSigma, Bellefonte PA, USA). SPME and injection were performed using an MPS autosampler with cooled agitator (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Deionized water was obtained from a Barnstead Nanopure Diamond water system (ThermoFisher Scientific, Dubuque, IA, USA). Quantitative data analysis was done using Mass Hunter Software (Agilent Technologies, Santa Clara, CA) and method validation parameters were calculated using Microsoft Excel.

87

88 *Reagents*

89 200 proof ethanol (99.5 % purity), was obtained from MilliporeSigma (St. Louis, MO, U.S.A.) and
90 anhydrous ethanol-d6 (99%), was obtained from Cambridge Isotope Laboratories (Andover,
91 MA, U.S.A.) and MilliporeSigma (St. Louis, MO, U.S.A). Sodium chloride (99.5% purity), sodium
92 phosphate-monobasic (99%), and sodium phosphate-dibasic (99%) were obtained from
93 MilliporeSigma (St. Louis, MO, U.S.A). A .05 M phosphate buffer solution containing 25% w/v
94 sodium chloride was prepared by dissolving 7 g of sodium phosphate dibasic and 25 g sodium
95 chloride to a final volume of 1 L in water. The resulting solution was then adjusted to pH 7 with
96 addition of sodium phosphate monobasic. Alcohol calibration standards were prepared at
97 concentrations of 0.10, 0.40, 0.80, 1.00, 1.50 and 2.00 percent alcohol by volume (%ABV) by
98 direct dilution of aliquots of 200 proof ethanol into 25 mL of water. The resulting solutions
99 were stored under refrigeration in 22 mL vials with minimal headspace prior to use. Internal
100 standard solution was prepared at a concentration of 0.08 %ABV by direct dilution of neat
101 ethanol-d6 into the salt/buffer solution. This solution was then used in the dilution of samples
102 prior to SPME. Internal standard/buffer solution was prepared daily, and chilled prior to use.

103 *Samples*

104 All Kombucha samples used were purchased at local grocery stores, and kept under
105 refrigeration until testing. A ginger flavored Kombucha found to have very low alcohol content
106 was used for the preparation of spikes in the method validation process. Certified reference

materials of alcohol in water at 80, 200, and 400 mg/dL were obtained from Cerilliant (Roundrock, TX U.S.A.).

IRMM certified reference materials of low alcohol beer, nominal 0.50% ABV (BCR-651), were obtained from MilliporeSigma. Two separate samples of this reference material were obtained approximately eight months apart.

Calibration standards for HS-SPME were prepared by dilution of 400 μ L of the alcohol calibration solutions in water with 3.6 mL of the salt/buffer solution containing internal standard at .08% ABV. This resulted in a 10X dilution of each standard solution, and a final internal standard concentration of .072 %ABV in each calibration sample.

HS-SPME Procedure

Samples for HS-SPME were prepared by dilution of 400 μ L of sample with 3.6 mL of salt/buffer solution containing .08 %ABV internal standard (see reagent section) in a 10 mL headspace vial. This results in a 10X dilution of the sample and a final internal standard concentration of .072 %ABV. Prior to extraction, samples were incubated at 40°C for 7 min in a heated agitator with agitation speed at 250 rpm. Extraction was performed by exposing a 100 μ m PDMS SPME fiber to the sample headspace for 2 min at 40°C at the same agitation speed. After extraction, the fiber was desorbed in the GC inlet for 3 min at 250°C. A post-bake of the SPME fiber was done after each extraction for 5 min at 260 °C.

Chromatographic Conditions

The GC oven temperature was programmed at 40°C for 5 min, ramped at 8 °C/min to 70°C, ramped at 20°C/min to 250°C and held for 5 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The transfer line to the MSD was set at 250°C. The GC inlet used for SPME desorption was equipped with a 0.75 mm ID liner, and set to a 10:1 split. These same conditions were used on both GC-MS systems.

MS Conditions

The 5973N MSD was tuned using a BFB auto-tuning macro and the 5977C was tuned using a high sensitivity autotune macro. Data from both systems was collected in scan mode, covering a range of m/z 25-300. For ethanol, m/z = 45 was used for quantitation and m/z =31, 46 as qualifier ions. For ethanol-d6, m/z = 49 was used for quantitation and m/z =33, 51 as qualifier ions.

Peak Identification and Quantitation

Ethanol (alcohol) was identified in spikes and samples by retention time, the presence of quantitation and qualifier ions, and spectral identification. Retention time of the peak did not vary by more than 4 seconds throughout the course of the testing. Alcohol concentrations as %ABV were determined using peak area, and were calculated relative to the peak area of the internal standard. As described previously, all standards and samples were diluted ten times when prepared for SPME, thus the dilution factor was constant for both. Any samples falling above the highest calibration standard of 2.00 %ABV were diluted further and re-analyzed. An additional dilution factor was then applied in calculation of the final %ABV.

Results and Discussion

Optimization of the HS-SPME Procedure

SPME is a very sensitive technique and is normally applied to testing of analytes at very low levels. The targeted analytical range for this method per standard method performance requirements was 0.10 to 2.00 %ABV, and this is considered very concentrated for SPME. Thus the goal was to develop an SPME method that could be used with high accuracy and reproducibility over this entire range. In order to prevent overloading the fiber and detector, sample dilution and the use of a 10:1 split during desorption of the SPME fiber were necessary. Initially, a carboxen/PDMS fiber was chosen for method optimization. However, linear response could not be obtained over the entire analytical range. Figure 1 shows a comparison of absolute response by GC-FID using the carboxen/PDMS fiber and a 100 μ m PDMS fiber over a range of 0.10 to 1.00 %ABV. Linearity within this range was slightly better using the PDMS fiber, as evidenced by the higher correlation coefficient (r^2) value. Also, the response curve obtained using the carboxen/PDMS fiber started to show some leveling off between 0.80 and 1.00 %ABV, and this was expected to become more pronounced at higher concentrations. Since sufficient sensitivity was obtained using the PDMS fiber, it was chosen for further work.

Obtaining sufficient sensitivity for detection was not a problem by HS-SPME, thus the main focus of further method optimization was reproducibility. After fiber choice; temperature, time, sample additives and sample dilution were parameters evaluated during method development. All were optimized to minimize variability in alcohol response from both water and Kombucha tea samples.

167 For both pre-extraction incubation and extraction of the samples, it was expected that a
168 temperature of $<50^{\circ}\text{C}$, and times in the range of 2-10 minutes would be sufficient to provide
169 adequate response and acceptable reproducibility. Using set equilibration and incubation times
170 of 5 minutes, temperatures of 30°C and 40°C were compared for the analysis of triplicate
171 samples of water spiked at 0.10 %ABV. Reproducibility was compared as the variability in three
172 replicate measurements of absolute response (in area counts) at the different temperatures. At
173 a 30°C extraction temperature, the relative standard deviation (RSD) was 9%, while at 40°C it
174 was 0.09%. Thus 40°C was chosen as the incubation and extraction temperature for the
175 method. Since there many different flavors of Kombucha tea available, the sample incubation
176 and extraction times had to be long enough to provide for reproducible alcohol response in the
177 presence of other volatile compounds. Using the PDMS fiber, the response of alcohol naturally
178 present in a commercially obtained sample of cherry flavored Kombucha tea was studied using
179 different pre-extraction incubation times. The Kombucha was diluted 1:1 with water, spiked
180 with ethanol-d6 at 0.50 %ABV, and analyzed at 40°C using a set extraction time of 2 minutes
181 and incubation times from 2-15 minutes. Figure 2 illustrates absolute response of the alcohol
182 vs. incubation time, and indicates an optimum time of 7 minutes, as response started to drop
183 after this point. Although not shown here, the same behavior was exhibited by the ethanol-d6
184 internal standard added to the test samples. The pre-extraction incubation time was then set at
185 7 minutes. Extraction time was studied from 1 to 10 minutes, however no clear trend in alcohol
186 response was observed, indicating that there was no advantage to using a time greater than 5
187 minutes. Thus, an extraction time of 2 minutes was chosen. This short extraction time in

combination with the 7 minute pre-extraction incubation time provided sufficient sensitivity, while keeping the cycle time of the method short.

In addition to organic acids, phenolics and flavonoid compounds result in Kombucha as a result of the fermentation process, and many commercially available varieties include additional compounds such as seeds and fruit flavors. It was reasoned that dilution of the samples into a pH 7 buffer solution would minimize any potential matrix effects, thus normalizing performance of the method, and improving reproducibility over a wide variety of samples types. The addition of salt was also considered, as it enhances extraction, especially for polar compounds.¹⁵ Increased alcohol response could be beneficial if a very high sample dilution is necessary; thus it was decided to add salt during the dilution process. The effect of salt and buffer dilution on different Kombucha tea samples was evaluated by monitoring response of ethanol-d6 from four different varieties. Samples were spiked at 0.05 %ABV with ethanol-d6, and diluted 1:1 in both plain water and a .05 M phosphate buffer solution at pH 7 containing 25% sodium chloride. For comparison, a spiked water sample was also included. As indicated in Table 1, dilution with the salt/buffer solution decreased variability in response of the ethanol-d6 across the different samples. As predicated, the salt increased response; and this made a larger sample dilution of 10:1 with salt/buffer solution possible. This increased dilution provided adequate minimization of matrix effects, and allowed for good linearity and reproducibility across the entire analytical range. It was also found that adding the internal standard as part of the buffer solution allowed it to be added easily and reproducibly to each sample.

The 10X final dilution of samples prior to SPME reduced matrix effects enough to allow for calibration standards to be prepared in water; with excellent linearity obtained from 0.10 to 2.00 %ABV. Figure 3 shows a comparison of calibration curves generated from HS-SPME analysis of alcohol standards prepared in water and Kombucha tea. Alcohol response was the same from both sample matrices, indicating that the 10X dilution with salt/buffer solution normalized response. Thus, the calibration standards used for quantitation of Kombucha spikes and samples were prepared in water.

Method Validation

Accuracy of the HS-SPME GC/MS method was evaluated using ginger flavored Kombucha tea spiked at 0.10, 0.50, 1.00 and 2.00 %ABV, and analyzed in sets of a minimum of five replicates. Average amounts measured in %ABV were determined along with percent repeatability, reported as %RSD; and these results are summarized in table 2. The method accuracy study was conducted over two days, using two separate bottles of ginger Kombucha for spiking. The Kombucha used for spiking on each of these days was analyzed without spiking, and the %ABV measured was subtracted from the average amounts measured in the spikes prior to calculating accuracy. Water blanks were run at the beginning, and end of each sample batch to monitor for alcohol contamination and/or carryover. For the Kombucha spikes, good linearity was obtained, with a linear correlation coefficient of 0.999 from 0.10 to 2.00 %ABV. The average amount of alcohol measured in each set of spikes vs. the amount spiked is reported as percent accuracy. Repeatability at each spiking level was acceptable, with RSD values <4%. Alcohol was not detected in any of the water blanks run throughout the sequence, indicating no carryover

or cross-contamination during the HS-SPME procedure. The limit of detection (LOD) and limit of quantitation (LOQ) for the method were calculated from the set of Kombucha tea samples spiked at 0.10 %ABV ($n=8$). LOD was calculated as 3 times the standard deviation and LOQ as 10 times the standard deviation of the average amount measured. These values are reported in table 3. The calculated LOD and LOQ were verified with the analysis of Kombucha samples spiked at .010 and .030% ABV. For $n=5$ spikes at each concentration, accuracy at the LOQ was 95% with a repeatability value of 6% RSD. Accuracy at the LOD was 83% with repeatability of 21% RSD. These low accuracy and poor repeatability values at the LOD can be attributed to the poor response of ethanol at this level; as the signal to noise ratio was approximately 2X lower than that obtained at the LOQ, and 4.5X times lower than the 0.1% spiking level. If it was necessary to quantitate at .01 %ABV, simple modifications to the method, such as using a splitless SPME injection, could be used to increase the ethanol response.

Analysis of Certified Reference Materials

The method was further validated using certified reference materials of low alcohol beer and pre-prepared solutions of alcohol in water, and the results are summarized in table 4. Each was analyzed multiple times over different days, which allowed for the determination of reproducibility. Two separate samples of beer were analyzed by two analysts on two different instruments. The ethanol in water solutions were also analyzed on two different systems, with the 200 and 400 mg/dL samples done by two different analysts. The concentrations of alcohol in the water materials were converted from mg/dL to %ABV using the density of ethanol at 21°C. Average accuracies and reproducibilities obtained for all these certified reference

materials fell within the 97-102% and <6% RSD values described in the method performance requirements. The daily accuracies ranged from 96-101% for the beer samples and 94-103% for the alcohol in water solutions.

Analysis of Kombucha tea samples

A total of thirteen different Kombucha tea samples from six different producers were tested using the optimized HS-SPME GC/MS procedure. All samples, except where indicated, were analyzed in duplicate, and the average alcohol levels measured as %ABV are reported in table 5. Measurement reproducibility was determined as the difference in the two measurements (%Diff), and was calculated for each sample set as the absolute difference in the two measurements divided by the average. The “best by” dates indicated on each bottle, along with the testing date, are indicated in the table. Sample 2, analyzed one month after the “best by” date, had a %ABV level above 2.00%, and had to be diluted an additional 10X in order to be quantitated within the calibration range of the standards. It was noted that the Kombuchas with alcohol levels >0.50 % were all labeled as “raw” on the sample containers, and contained noticeable amounts of sediment. Those with levels < 0.50% were not indicated as raw, and were relatively clear. Two samples, nos. 3 and 8, had measurement reproducibility greater than 10%. The alcohol levels measured in duplicates of sample 8 were 0.20 and 0.23 %ABV, thus the absolute difference in measurements is very small. Sample 3 was a very viscous matrix which contained seeds, and was centrifuged prior to taking an aliquot for testing. Sample 13 also contained seeds and was viscous, and a different approach was used when measuring out the aliquot used for SPME. The 400 µL was measured using a pipette with a wide tip, to prevent

clogging. Thus the seeds in the sample contributed to the final volume used for the alcohol determination. This yielded a more reproducible measurement, with an RSD value of 3% for 3 replicate measurements.

Two Kombucha tea samples were spiked with a known amount of alcohol and used as matrix spikes to determine the accuracy of measurement from matrix. These are reported in table 6 with corresponding measurement accuracy, determined as amount spiked vs. amount measured. Measurement accuracy from sample 1 was acceptable, while from sample 12 was lower than expected at 93%. This could be due to the uncertainty of the ethanol level measured in the unspiked sample, which was near the LOQ.

Since Kombucha tea represents a complex matrix, the 400 μ L sample size taken for dilution prior to SPME was evaluated to determine if it was a representative enough for an accurate alcohol measurement. Several Kombucha tea samples included in table 5 were retested using larger sample aliquots diluted into higher volumes of salt/buffer solution. Samples 1, 5, 8, and 10 were diluted 10X using 2.5 mL sizes diluted to 25 mL in salt/buffer solution. Sample 6 was diluted 10X using 100 mL diluted to 1 L in salt/buffer solution. Aliquots of 4 mL of each diluted sample were then used for HS-SPME analysis. The results obtained using these larger sample sizes for dilution, compared to those using the 400 μ L to 4 mL dilution, are compared in table 7. As indicated, results using the larger sample sizes were similar to those using the 400 μ L aliquot. With the exception of sample 8, the results were within 10% of each other.

293 *Method Repeatability*

294 Repeatability of the method was studied using nine Kombucha tea samples analyzed in
295 triplicate, and then analyzed a second time 48 hours later on the same instrument with the
296 same operator. With the exception of one sample, all were different varieties than those tested
297 previously (table 5). Sample 10 was homemade Kombucha, donated by a colleague. Each round
298 of testing was conducted as a single analytical batch, with a calibration curve, followed by a
299 certified reference material of ethanol in water (run in duplicate) and blank. Mid-point
300 calibration standards were run every 10th sample as calibration checks, along with water blanks.
301 The results of this testing is summarized in table 8. The samples ranged in %ABV from 0.49 to
302 1.87. Repeatability was determined for each Kombucha sample as the percent relative
303 standard deviation (%RSD_R) of the 2 sets of measurements (six total for each) taken on
304 separate days. Values were < 4% for all varieties, per SMPR requirements. It should be noted
305 that as seen with previously tested Kombucha tea samples in table 5, most had %ABV values >
306 0.5%. Consequently, these were indicated on the label as “raw” Kombucha. While it is
307 assumed that the homemade Kombucha was also raw, ironically it had a %ABV value < 0.5%.

308 **Conclusions**

309 An HS-SPME method was developed which can be used to accurately and precisely measure
310 alcohol content in Kombucha tea samples. The optimized method allowed for accurate
311 determination in the range of 0.1 to 2.0 %ABV as is currently designated by AOAC standard
312 method performance requirements for Kombucha; however the limit of quantitation indicates
313 that accurate measurement is possible down as far as 0.03 %ABV. Method repeatability, as
314 demonstrated with analysis of 9 different Kombucha varieties, was demonstrated as <4 %RSD_R

for replicate measurements made over two days. The dilution approach used in the procedure minimizes matrix effects, thus making it possible to use this HS-SPME method for other low alcohol matrices such as non-alcoholic beer and wine. Applying the HS-SPME method to 20 different varieties of commercially available Kombucha tea samples from nine producers, it was found that those designated as “raw” had alcohol levels above 0.5% ABV. This indicates that either the current methodology used for alcohol measurement of these products is not accurate, and/or fermentation is continuing after bottling, despite refrigeration, and resulting in elevation of alcohol level.

Acknowledgments

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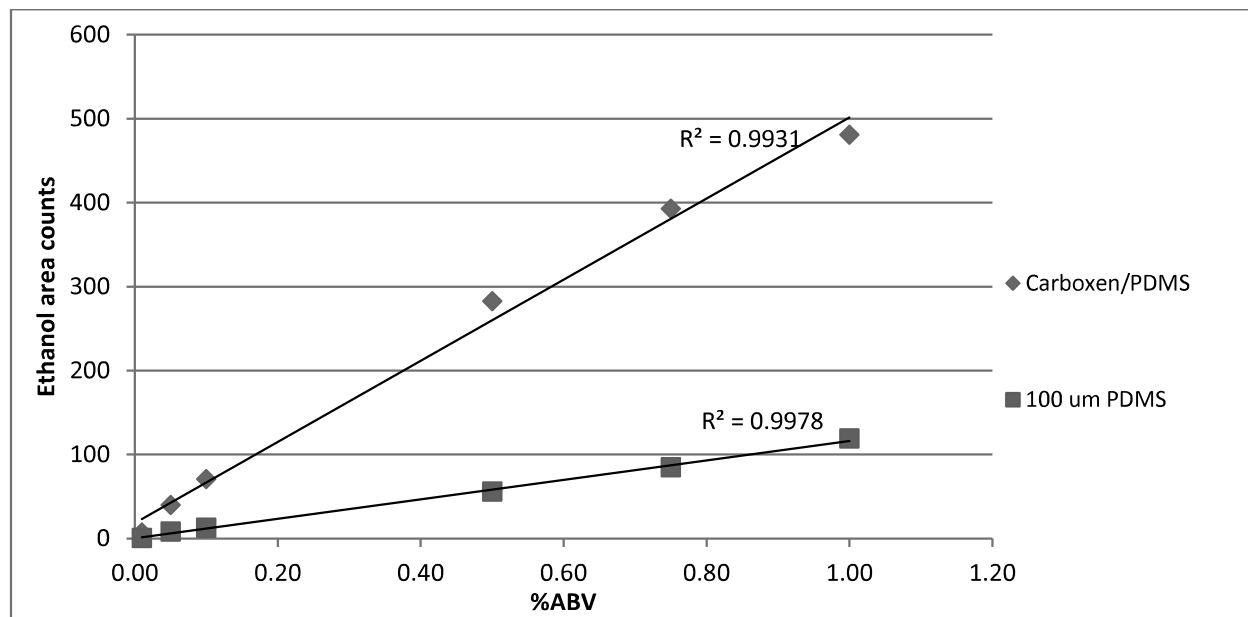
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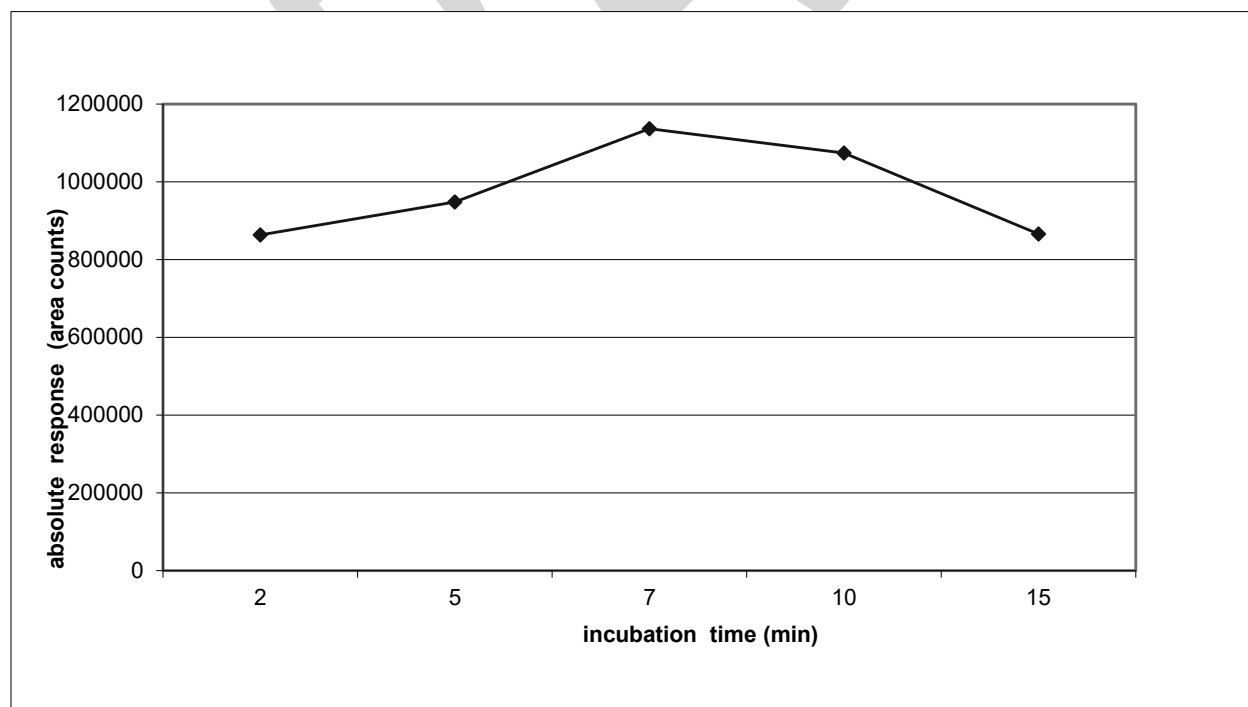
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354 Figure 1. Comparison of alcohol response (GC-FID) from water obtained by HS-SPME; 100 μ m
355 PDMS and Carboxen/PDMS fibers.



356

357 Figure 2. Alcohol response (GC-MS) from cherry flavored Kombucha tea (diluted 1:1 with water)
358 using the 100 μ m PDMS SPME fiber with differing pre-extraction incubation times.



359

360

Figure 3. Comparison of standards prepared in water and Kombucha tea (ginger flavored).

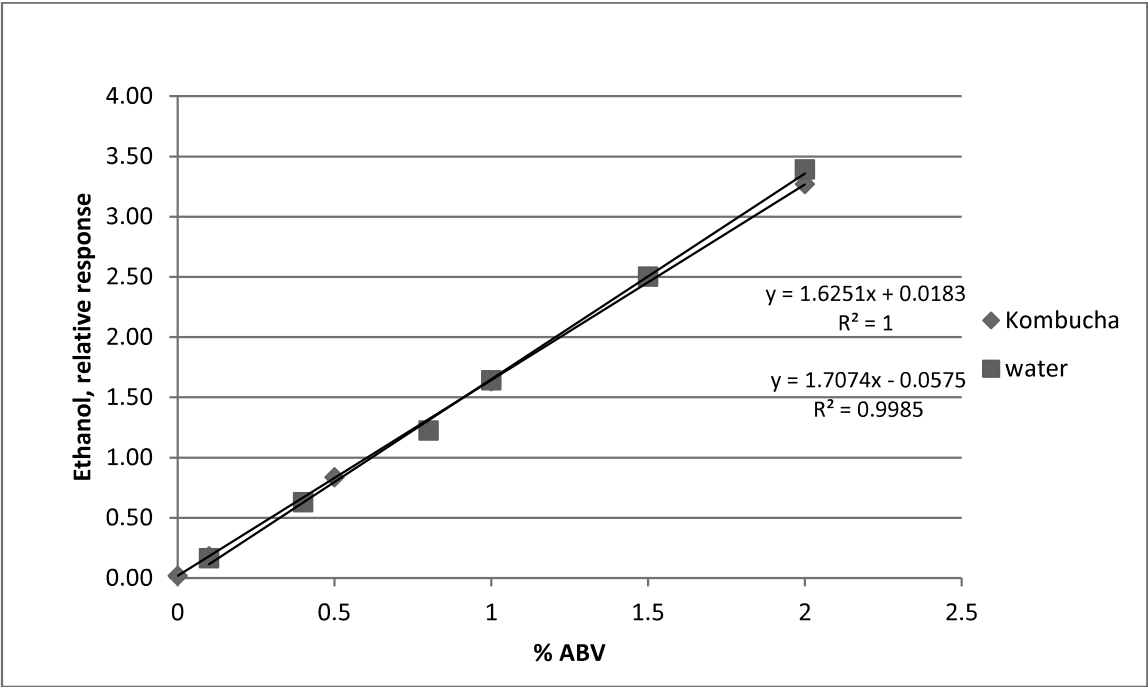


Table 1. Reproducibility of internal standard (ethanol-d6) response; the effect of diluting Kombucha samples 1:1 with water vs. salt/buffer solution.

dilution medium:	ethanol-d6 response (area counts)	
	salt/buffer	water
water	24958	19623
raspberry lemon Kombucha	24879	17289
mango Kombucha	26986	22006
cherry Kombucha	23684	17677
cranberry Kombucha	25785	14527
average response	25258	18224
%RSD	5%	15%

370 Table 2. HS-SPME Method accuracy and repeatability from 0.10 % to 2.00 %ABV; spiked
371 Kombucha tea.

alcohol spiking level (%ABV ^a)	amt. of alcohol measured in unspiked Kombucha (%ABV)	avg. %ABV measured	avg. % ABV less unspiked	repeatability %RSD ^b (n=5)	% accuracy
0.10	.011	0.11	.098	3*	98
0.50	.011	0.51	0.50	2	100
1.00	.011	1.00	0.99	1	99
2.00	.026	1.99	1.96	1	98

372 ^a alcohol by volume

373 ^brelative standard deviation

374 *n=8

375
376 Table 3. Method LOD and LOQ; determined using Kombucha tea spiked at 0.10 %ABV.

	spiking level (%ABV ^a)	avg. amount measured (%ABV)	s ^b (n=8)	LOD ^c (%ABV)	LOQ ^d (%ABV)
ethanol	0.10	0.11	0.003	0.01	0.03

377 ^a alcohol by volume

378 ^bstandard deviation

379 ^c limit of detection

380 ^d limit of quantitation

381 ^e relative standard deviation

382

383 Table 4. Method accuracy and reproducibility, determined using certified reference materials.

sample	certified conc. (%ABV ^a)	# of replicates	# instruments/ analysts	range ^b (%ABV)	avg. amt. measured (%ABV)	avg. % accuracy	reproducibility %RSD ^c
low alcohol beer	0.51	10	2/2	0.48-0.51	0.50	98	2
alcohol in water, 80 mg/dL	0.10	4	2/1	.095-0.11	.099	98	5
alcohol in water, 200 mg/dL	0.25	3	2/2	0.24-0.26	0.25	99	3
alcohol in water, 400 mg/dL	0.51	7	2/2	0.49-0.52	0.51	100	3

384 ^a alcohol by volume

385 ^brange of replicate measurements

386 ^c relative standard deviation

387

388 Table 5. %ABV levels measured by HS-SPME in commercially obtained Kombucha tea samples;
 389 thirteen different flavors from 6 different producers.

sample	producer	flavor	"best by" date on bottle	date tested	%ABV ^a	%diff ^b
1	1	1	5/2/2016	4/1/2016	1.10	8
2	1	2	3/13/2016	4/5/2016	2.21	2
3	1	3	4/8/2016	4/1/2016	0.99	13
4	1	4	4/17/2016	4/5/2016	1.04	0.3
5	1	5	5/16/2016	4/7/2016	1.18	7
6	1	6	5/3/2016	4/18/2016	1.28	4
7	2	1	8/10/2016	4/1/2016	0.16	1
8	2	2	5/4/2016	4/7/2016	0.22	15
9	3	1	6/24/2016	4/1/2016	0.86	4
10	4	1	9/8/2016	4/7/2016	1.51	1
11	5	1	7/3/2016	4/7/2016	1.52	8
12	6	1	8/8/2016	4/7/2016	0.05	5
13	1	7	12/23/2016	12/21/2016	0.82*	3*

*avg. 3 replicates

^a alcohol by volume

^b percent difference of duplicate measurements

393 Table 6. Measurement accuracy from Kombucha tea matrix, alcohol spiked at a known amount.

sample	amount spiked (%ABV ^b)	amount in unspiked (%ABV)	avg. amount measured (%ABV)	avg. amount measured less unspiked (%ABV)	% accuracy	%diff ^c
1: MS/MSD ^a	0.40	1.10	1.51	0.41	104	1
12: MS/MSD ^a	0.40	0.05	0.42	0.37	93	0.2

^a matrix spike and matrix spike duplicate

^b alcohol by volume

^c difference of duplicate measurements

398

399 Table 7. Comparison of % ABV levels measured using 400 uL Kombucha sample size for dilution
400 vs. larger sample size.

Sample	sample size used for dilution (mL)	amount measured (%ABV ^a)	amount measured using 400 uL sample size for dilution (%ABV)
1	2.5	1.12	1.10
5	2.5	1.18	1.18
6	100	1.23	1.25
8	2.5	0.17	0.22
10	2.5	1.41	1.51

401 ^aalcohol by volume

402

403 Table 8. Method repeatability using 9 different Kombucha varieties, analyzed in triplicate on
 404 separate days (same instrument, same operator).

Sample	Producer	Flavor	"Best by" date on bottle	date tested	% ABV	avg. %ABV	% RSD _R
1	1	2	10/20/2017	11/29/2017 2:48 PM	1.86		
1				11/29/2017 3:14 PM	1.86		
1				11/29/2017 3:40 PM	1.76		
1				12/1/2017 1:10 PM	1.88		
1				12/1/2017 1:36 PM	1.94		
1				12/1/2017 2:02 PM	1.91		
1						1.87	3%
2	1	7	12/25/2017	11/29/2017 4:06 PM	1.31		
2				11/29/2017 4:32 PM	1.33		
2				11/29/2017 4:58 PM	1.36		
2				12/1/2017 2:28 PM	1.38		
2				12/1/2017 2:54 PM	1.42		
2				12/1/2017 3:20 PM	1.36		
2						1.36	3%
3	1	8	12/1/2017	11/29/2017 5:24 PM	0.80		
3				11/29/2017 5:50 PM	0.81		
3				11/29/2017 6:16 PM	0.81		
3				12/1/2017 3:46 PM	0.81		
3				12/1/2017 4:12 PM	0.82		
3				12/1/2017 4:38 PM	0.83		
3						0.81	1%
4	7	1	1/20/2018	11/29/2017 7:34 PM	0.66		
4				11/29/2017 8:00 PM	0.66		
4				11/29/2017 8:26 PM	0.66		
4				12/1/2017 5:57 PM	0.64		
4				12/1/2017 6:23 PM	0.68		
4				12/1/2017 6:49 PM	0.67		
4						0.66	2%
5	8	1	2/28/2018	11/29/2017 8:52 PM	1.65		
5				11/29/2017 9:18 PM	1.66		
5				11/29/2017 9:44 PM	1.67		
5				12/1/2017 7:15 PM	1.65		
5				12/1/2017 7:41 PM	1.66		
5				12/1/2017 8:07 PM	1.67		
5						1.66	1%
6	8	2	6/12/2018	11/29/2017 10:11 PM	1.51		

6				11/29/2017 10:37 PM	1.55		
6				11/29/2017 11:03 PM	1.49		
6				12/1/2017 8:33 PM	1.51		
6				12/1/2017 8:59 PM	1.58		
6				12/1/2017 9:25 PM	1.60		
6						1.54	3%
7	9	1	12/19/2017	11/30/2017 1:39 AM	0.40		
7				11/30/2017 2:05 AM	0.40		
7				11/30/2017 2:31 AM	0.40		
7				12/2/2017 12:03 AM	0.38		
7				12/2/2017 12:29 AM	0.37		
7				12/2/2017 12:56 AM	0.39		
7						0.39	3%
8	4	2	2/6/2018	11/30/2017 2:57 AM	1.04		
8				11/30/2017 3:23 AM	1.06		
8				11/30/2017 3:49 AM	1.07		
8				12/2/2017 1:23 AM	1.06		
8				12/2/2017 1:49 AM	1.11		
8				12/2/2017 2:16 AM	1.04		
8						1.06	2%
9	10	1	NA (homemade)	11/30/2017 5:07 AM	0.49		
9				11/30/2017 5:33 AM	0.51		
9				11/30/2017 5:59 AM	0.49		
9				12/2/2017 3:35 AM	0.49		
9				12/2/2017 4:02 AM	0.48		
9				12/2/2017 4:29 AM	0.49		
9						0.49	2%

405

406

