Measuring Ethanol in Kombucha Tea by Headspace Solid Phase Microextraction (HS-SPME); a Walk Through Method Development and Validation

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Introduction

Recently, AOAC adopted a method for first action status using headspace solid phase microextraction (HS-SPME) for analysis of ethanol in Kombucha tea.

Described here are some of the parameters that were optimized in development of an HS-SPME method to meet standard method performance requirements (SMPR) as designated by the working group within AOAC.

Method

Per the SMPR, the method had to meet the following requirements:

- Analytical range of 0.1 – 2.0% alcohol by volume (ABV)
- LOQ ≤ 0.05% ABV
- Accuracy over analytical range: 97-102%
- Method repeatability: ≤4% RSD
- Method reproducibility: ≤6% RSD

In developing the final method (**Tables 1 & 2**), the following parameters were optimized:

- **Fiber chemistry** – PDMS chosen over Carboxen/PDMS due to greater capacity - **Figure 1.**
- **Equilibration/Extraction temperature & time** – optimized for best reproducibility, highest sensitivity and lowest method cycle time.
- **Internal standard** – Ethanol-d6 chosen for best accuracy. Addition to samples was done with the diluent for best reproducibility. This I.S. then required use of GC-MS for analysis.
- **Sample diluent** – salt addition and buffer increased response and reproducibility (**Table 3**). Buffer at pH 7 also normalized matrix between sample types.
- **Calibration** - high sample dilution allowed for calibration from water (**Figure 2).**

Table 1. Optimized HS-SPME Method

Sample/matrix: 400 µL tea sample + 3.6 mL salt/buffer soln. (.05M Na2HPO4 at pH 7 w/25% NaCl) containing I.S. in 10 mL headspace vial

Internal standard: ethanol-d6; at .08% ABV in .05M salt/buffer solution (chilled prior to use with samples)

SPME fiber: 100 µm PDMS

Incubation: 7 min, 40 °C

Extraction: 2 min, headspace, 40 °C

Desorption: 3 min, 250 °C, split 10:1

Fiber post-bake: 5 min, 260 °C

Table 2. GC-MS conditions

Column: Supelcowax-10, 30 m x 0.25 mm I.D., 0.50µm

Oven: 40°C (5 min), 8°C/min to 70°C, 20°C/min to 250°C (5 min)

MSD interface: 250°C

Scan range: m/z 25-300

Carrier: helium, 1 mL/min constant flow

Injection: SPME fiber, split 10:1

Liner: 0.75 mm I.D. SPME

Table 3. Effect of salt and buffer on response and reproducibility

	ethanol-d6 response (area counts)	
dilution medium:	salt/buffer	water
Matrix		
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%**

Figure 1. Comparison of ethanol uptake from water; Carboxen vs. 100 µm PDMS fiber

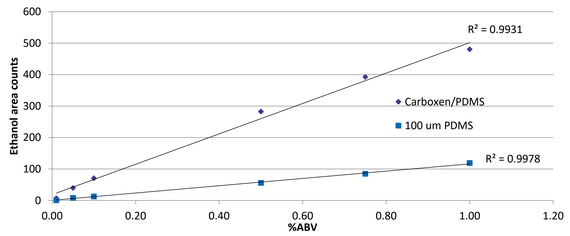
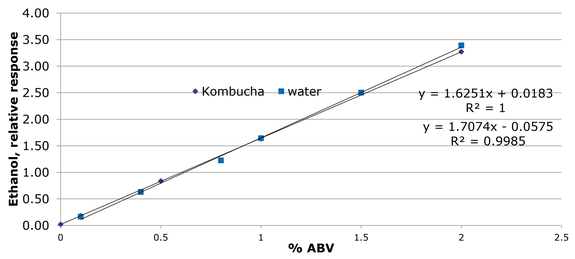


Figure 2. Ethanol calibration; Kombucha vs. water: both diluted 10:1 into salt/buffer diluent.



- From 0.1 – 2% ABV, accuracy was 98-100%, and repeatability 1-3% RSD. (**Table 4**)
- LOD/LOQ was determined at a spike concentration of 0.1% ABV:
  - LOD = .01% ABV
  - LOQ = .03% ABV
  - Accuracy at the LOD and LOQ were 83% and 95%, respectively, for *n*=5 spiked replicates.
- Method accuracy was 98-100% using certified reference materials of ethanol in water and low alcohol beer. Repeatability was 2-5% RSD (**Table 5**).
- Method repeatability determined by triplicate analyses of kombucha samples done on two separate days was from 1-3% RSD. The ethanol levels in these samples ranged from 0.39 to 1.87% ABV (**Table 6**).

Validation Results

Table 4. Method accuracy and repeatability in spiked Kombucha samples from 0.1 to 2.0% ABV

alcohol spiking level (%ABV)	amt. of alcohol measured in unspiked Kombucha (%ABV)	avg. %ABV measured	avg. % ABV less unspiked	repeatability %RSD (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

**n*=7 replicates (used for LOD, LOQ calcs.)

Table 5. Method accuracy and repeatability using certified reference materials analyzed on multiple days using different instruments and analysts

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

Table 6. Method repeatability from different Kombucha tea varieties analyzed in 2 sets of 3 replicates over 2 days.

Sample #	Producer #	Flavor #	"Best by"date	Testing dates	Avg. %ABV	% RSD
1	1	1	10/20/2017	11/29, 12/1/2017	1.87	3
2	1	2	12/25/2017	11/29, 12/1/2017	1.36	3
3	1	3	12/1/2017	11/29, 12/1/2017	0.81	1
4	2	1	1/20/2018	11/29, 12/1/2017	0.66	2
5	3	1	2/28/2018	11/29, 12/1/2017	1.66	1
6	3	2	6/12/2018	11/29, 12/1/2017	1.54	3
7	4	1	12/19/2017	11/30, 12/2/2017	0.39	3
8	5	1	2/6/2018	11/30, 12/2/2017	1.06	2
9*	6	1	NA	11/30, 12/2/2017	0.49	2

*homemade Kombucha provided by a colleague

Summary

In relation to the SMPR:

- **Analytical range of 0.1 – 2.0% ABV:** demonstrated with ≤3% RSD in Kombucha matrix spiked over designated range.
- **LOQ ≤ 0.05% ABV:** demonstrated with LOQ of .03% ABV (verified with spikes at this level)
- **Accuracy over analytical range 97-102%:** demonstrated with range of 98-100% from 0.1 – 2.0 %ABV
- **Method repeatability of ≤4% RSD**: demonstrated with repeatability of ≤3% for multiple replicates analyzed over 2 days.
- **Method reproducibility of ≤6% RSD:** demonstrated using certified reference materials with

% RSDs of ≤5% for multiple analyses done on 2 instruments by 2 different analysts.