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Research Article

FOOD CHEMICAL CONTAMINANTS

Determination of Ethanol Content in Water Kefir Using Headspace Gas Chromatography With Mass Spectrometry Detection: Matrix Extension and Methanol Characterization

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Abstract

Background: Water kefir is a fermented beverage using water, sugar, and cultured microorganism grains as the primary ingredients. Ethanol may be present at varying levels within the final product due to the fermentation process, so it is vital to have a validated method to meet regulatory, quality, and safety requirements.
Objective: This study describes using water kefir as a matrix for the evaluation of the previously validated method employing headspace gas chromatography mass spectrometry (HS-GCMS) detection for ethanol in kombucha. The study objective is to demonstrate the method originally using kombucha is also fit for the analysis of water kefir. This method will also evaluate the determination of methanol within the water kefir samples.
Method: The matrix extension study was performed as per the AOAC INTERNATIONAL guidance documents outlined in Appendix K: Guidelines for Dietary Supplements and Botanicals using HS-GCMS for ethanol determination. Ethanol determination in each water kefir sample is quantified against an external standard calibration curve. The same instrumentation is used for methanol characterization.
Results: RSD_r and HorRat values obtained for from the study demonstrated acceptable precision with RSD_r values of 1.03 to 6.68% and HorRat values determined to be between 0.23 and 1.52 for ethanol determination within kefir samples. Similarly, acceptable values of RSD_r ranging from 1.45 to 3.39% and HorRat ranging from 0.25 to 0.49 were observed with methanol determination. For methanol determination, the limit of detection (LOD) and limit of quantification (LOQ) determined for the method in this study to be 16 and 21 ppm, respectively. The methanol spike recovery study gave overall recoveries ranging from 89 to 91%, demonstrating acceptable method accuracy.
Conclusions: The results of this study demonstrate the previously validated HS-GCMS method for ethanol determination in kombucha can also be used to quantify ethanol in water kefir samples. The method is also suitable for the determination of methanol within water kefir samples.
Highlights: A straightforward method has been adapted to include the the quantification of ethanol and methanol in fermented beverages such as Water Kefir samples.

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Water kefir is a tart and refreshing fermented beverage made up of water, sugar, and kefir grains. The final beverage can be flavored with fruit, juice, or herbs to make a variety of flavor profiles (1, 2). Water kefir is gaining in popularity as the rise in fermented beverages is increasing due to the potential health benefits associated with intestinal health. Traditionally, kefir grains have been used for the fermentation of milk products to produce a fermented dairy beverage (3). This style of beverage has been consumed in parts of Asia for thousands of years and was once commonly fermented naturally in different animal offal, such as hide (1, 4). The kefir grains itself are opaque "beads" that are white in color and consist of a mixture of yeasts and bacteria surrounded by a polysaccharide and protein matrix to give its bead-like texture (4, 5). Kefir grains contain a very diverse and complex mixture of yeasts and bacteria consisting mainly of lactic acid bacteria (LAB), acetic acid bacteria, and yeasts consisting primarily of *Saccharomyces, Candida, Kluyveromyces, Lactobacillus, Lactococcus*, *Leuconostoc*, and *Acetobacter* (5, 6).

During the primary fermentation process, sugar, water, and kefir grains are combined into a fermentation vessel and allowed to ferment aerobically for 24–48 h (7). After the primary fermentation process, the kefir grains are removed and other flavorings such as fruit juice, teas and herbs are added. This process is also known as secondary fermentation and is typically done anaerobically to produce an effervescent type of beverage from the carbon dioxide production. Ethanol, a byproduct of the fermentation process, can be produced as a result, but usually at low levels. As water kefir is marketed as a nonalcoholic beverage and sold in retail grocery stores, it must have an alcohol by volume (ABV) level below <0.5% in the United States and <1.1% ABV in Canada to meet the regulatory requirements of each country.

As with other fermented beverages, it is important to ensure that ethanol levels within the product meet regulatory and quality standards. Recent lawsuits, media reports, and scientific studies describing inaccurate labeling and ABV levels of ethanol >0.5% in kombucha products highlights the potential issues and consequences of the failure to properly monitor and assess ethanol production within fermented beverages (8–12). Due to strict regulatory labeling requirements and the potential public health risk to susceptible populations such as the elderly, young children, and pregnant women, it is imperative to ensure ethanol levels are accurate and true as per the printed label.

Previously, a method titled *The Determination of Ethanol Content in Kombucha Products Using Headspace Gas Chromatography With Mass Spectrometry Detection* was validated as per AOAC INTERNATIONAL guidelines (13). Herein, we report the results of a matrix extension study validating the use of this method to determine ethanol content within water kefir samples. Additionally, this method was further validated to detect the presence of methanol in water kefir beverage samples. Methanol can be naturally found in a variety of food and beverage products and can pose a potential health concern at higher levels. The validation of this method's ability for methanol determination further enhances the utility of this method in the evaluation of the quality and safety of fermented beverages such as water kefir.

This validation method follows AOAC guidelines (14). As part of this study, several water kefir market samples were analyzed, and their ethanol and methanol contents were determined and reported.

Experimental

Principle

This is a method using gas chromatography coupled with a headspace autosampler and mass spectrometry detection suitable for the determination of ethanol and methanol in water kefir commercial beverage samples.

Apparatus and Equipment

(a) *GC system*.—Agilent 5975C series GC-MSD (Agilent, ON, Canada) equipped with a CTC Analytics CombiPal autosampler (CTC Analytics AG, Zwigen, Switzerland).
(b) *GC column*.—Agilent J&W DB-624UI (30 m × 0.25 mm, 1.4 μm film).
(c) *Analytical balance*.—Mettler Toledo AE 206 analytical range (±0.1 mg; VWR International, AB, Canada).
(d) *Centrifuge*.—Eppendorf 5804 tabletop centrifuge (VWR International, AB, Canada).
(e) *Vortex mixer*.—Thermolyne Maxi Mix 1 (Thermo Scientific, NC, USA).
(f) *Conical tubes*.—Polypropylene, 50 mL.
(g) *Volumetric flasks*.—10, 25, and 100 mL.
(h) *Beakers*.—2 L.
(i) *Graduated cylinders*.—10, 100, and 1000 mL.
(j) *Volumetric pipets*.—Eppendorf Series 100, 200, 1000, and 5000 μL.
(k) *GC headspace vials*.—20 mL with PTFE with caps.

Note: An equivalent apparatus could be substituted. All glassware used was Class A and must be calibrated.

Test Materials

Water kefir samples were purchased from local grocery stores in the greater Vancouver, British Columbia surrounding areas. A variety of water kefir flavors were randomly selected for purchase for the analysis, which include flavoring types such as lemon, pineapple, blood orange, grape, hibiscus teas, and spirulina. The test sample bottles were all stored in a portable cooler upon purchase with ice packs while transported to the laboratory. Upon arrival at the laboratory, 40 mL of each water kefir sample was aliquoted into 50 mL centrifuge tubes and placed into a freezer until time of analysis. Selected market samples (eight in total) from three local manufacturers were purchased and analyzed for the validation study.

Reagents

ACS grade propan-1-ol (>99.5%) was from Sigma Aldrich (ON, Canada), and 200 proof ACS grade ethanol (>99.8%) was purchased from Greenfield Specialty Alcohols, Inc (ON, Canada). The water used in the sample preparation was obtained from a Barnstead Smart2Pure nanopore system (Thermo Scientific, MA, USA). HPLC grade methanol (>99.8%) used in the study was purchased from VWR International (AB, Canada).

Preparation of Calibration Solutions

(a) *Preparation of propan-1-ol intermediate standard solution*.—Using a volumetric pipet, transfer 5 mL of propan-1-ol to a 100 mL volumetric flask. Add water to the mark and invert 10 times to mix.

(b) *Preparation of propan-1-ol standard working check solution.*—Using a volumetric pipet, transfer 100 μL of the of propan-1-ol intermediate standard solution to a 10 mL volumetric flask. Add water into the volumetric flask to the mark and invert 10 times to mix. Transfer the entire contents into a 20 mL GC headspace vial, cap with a lined PTFE septa cap, and analyze as per the headspace GC-MS conditions.

(c) *Preparation of intermediate ethanol standard stock solution.*—Use an analytical balance to weigh and record the exact mass of an empty 100 mL volumetric flask. Remove the volumetric flask from the balance and add 5 mL of 200 proof ethanol with a volumetric pipet to the flask. Place the volumetric flask back onto the analytical balance and record the mass of both the ethanol and the flask together. Calculate the mass of the ethanol by calculating the difference between the two recorded masses. Add water to the volumetric flask up to the mark and invert 10 times to mix. Calculate the concentration using the equation in the *Calculations* section below.

(d) *Preparation of intermediate methanol standard stock solution.*—Using an analytical balance, record the exact mass of the 25 mL volumetric flask. Transfer 0.5 mL of methanol solution into the 25 mL volumetric flask using a volumetric pipet. Record the mass of the volumetric flask and methanol together. Determine the mass of methanol by calculating the difference between the two recorded masses. Add water to the flask up to the mark and invert 10 times to mix. Determine the concentration of this stock solution using the equation in the *Calculations* section below.

(e) *Preparation of ethanol standard working check solution.*—Using a volumetric pipet, add 1 mL of the intermediate ethanol standard stock solution to a 10 mL volumetric flask. Add water to the volumetric flask to the mark and invert 10 times to mix. Transfer the entire contents into a 20 mL GC headspace vial, cap with a lined PTFE septa cap, and analyze as per the headspace GC-MS conditions.

(f) *Preparation of methanol standard working check solution.*—Transfer 50 μL of the intermediate methanol standard stock solution into a 10 mL volumetric flask. Add water to the flask up to the mark and invert 10 times to mix. Transfer the entire contents of the flask into a 20 mL glass headspace GC vial and cap. Analyze the vial following the described headspace GC-MS conditions.

(g) *Preparation of ethanol external calibration curve working standard solutions.*—Transfer 100 μL of the propan-1-ol intermediate standard solution into five separate 10 mL volumetric flasks using a volumetric pipet. In each separate 10 mL volumetric flask, prepare the following ethanol standard solutions by diluting an appropriate amount of ethanol standard stock solution with water: 0.2, 2.0, 2.9, 3.8, and 7.8 mg/mL. Invert each flask 10 times to mix, and transfer the entire contents of each flask into separate labeled 20 mL GC headspace vials. Cap each vial with a lined PTFE septa cap, and analyze as per the described headspace GC-MS conditions.

(h) *Preparation of methanol external calibration curve working standard solutions.*—Transfer 100 μL of the propan-1-ol intermediate standard solution into seven individual 10 mL volumetric flask using a volumetric pipet. In each 10 mL volumetric flask, prepare the following methanol standard solutions by diluting the appropriate amount of intermediate methanol standard stock solution with water: 0.015, 0.02, 0.03, 0.05, 0.08, 0.12, and 0.15 mg/mL. Invert each flask 10 times to mix, and transfer the entire contents of each flask into separate labeled 20 mL glass headspace GC vials. Cap each vial with a lined PTFE septa cap, and analyze as per the described headspace GC-MS conditions.

Preparation of Test Solutions

Remove water kefir samples from the freezer and defrost until they are liquid at room temperature. Centrifuge the contents for 5 min at 5000 rpm. Using volumetric pipets, transfer 5 mL of the supernatant and 100 μL of the propan-1-ol intermediate standard solution into to a 10 mL volumetric flask. Add water to the volumetric flask to the mark and invert 10 times to mix. Transfer the entire contents into a 20 mL GC headspace vial, cap with a lined PTFE septa cap, and analyze as per the headspace GC-MS conditions.

Headspace GCMS Operating Conditions

(a) Headspace injector conditions
  (1) *Incubation temperature.*—70°C.
  (2) *Syringe temperature.*—70°C.
  (3) *Incubation time.*—300 s.
  (4) *Agitator speed.*—500 rpm.
  (5) *Injection volume.*—500 μL.
  (6) *Split ratio.*—10:1.

(b) GC operating conditions
  (1) *Injector temperature.*—220°C.
  (2) *Carrier gas.*—Helium.
  (3) *Initial oven temperature.*—35°C.
  (4) *Oven gradient program.*—Initial 35°C. Hold for 4 min, increase 45°C/min to 215°C, and then hold for 2 min.
  (5) *Flow rate.*—1.4 mL/min. (constant flow).
  (6) *Total run time.*—10 min.

(c) MS conditions
  (1) *Source temperature.*—230°C.
  (2) *Quad temperature.*—150°C.
  (3) *Acquisition mode.*—Scan.
  (4) *Scan settings.*—
    (a) *Low mass.*—20.0.
    (b) *High mass.*—100.0.

Determination

(a) *Retention time determination for propan-1-ol, ethanol and methanol peaks, and system suitability tests.*—Analyze the ethanol standard working check solution, the methanol standard working check solution, and the propan-1-ol standard working check solution on the GC-MS as per the operating conditions and confirm the identities of the ethanol, methanol and propan-1-ol peaks through their mass spectrometry spectrums. Record the retention times of the ethanol, methanol, and propan-1-ol peaks and review the chromatograms to confirm the absence of any contaminants. Make eight replicate injections of the 2.0 mg/mL of the ethanol working standard calibration solution and analyze on the GC-MS as per the operating conditions above. Use a new cap after each injection. Determine the ratio of the peak areas for the propan-1-ol and ethanol peaks for each injection and calculate the RSD. The system is considered suitable if the RSD values of the peak area ratios for all injections is $\leq$4.0%.

(b) *External calibration: Ethanol determination.*—Analyze all ethanol external calibration curve working standard solutions using the described HSGC-MS operating conditions.

Determine each peak area, and calculate the ratio of the peak response of ethanol to the peak response of propan-1-ol. Record the peak ratios and plot the concentration of ethanol to the ratio of the ethanol peak area to propan-1-ol peak area for each standard. Use linear regression to calculate the slope, y-intercept, and coefficient of determination (r^2). The calculated r^2 value is determined to be satisfactory if the value is ≥99.5%.

(c) *Eternal calibration curve: Methanol determination.*—Analyze all methanol external calibration curve working standard solutions using the described GC-MS operating conditions. Determine each peak area, and calculate the ratio of the peak response of methanol to the peak response of 1-propan 1-ol. Record the peak ratios and plot the concentration of methanol to the ratio of the ethanol peak area to propan-1-ol peak area for each standard. Use linear regression to calculate the slope, y-intercept, and coefficient of determination (r^2). The calculated r^2 value is determined to be satisfactory if the value is ≥99.5%.

Test Sample Analysis

Prepare and analyze all test samples following the stated GC-MS operating conditions. Determine the ratios of both the peak area of ethanol to the peak area of propan-1-ol and the peak area of methanol to the peak area of propan-1-ol in each of the test solutions. Calculate the concentration of both the ethanol and methanol using the formulas in the calculations below.

Calculations

The concentration of ethanol (mg/mL) in the intermediate ethanol standard stock solutions is calculated using the following calculation:

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

C_{stock} = the concentration of the ethanol in the intermediate ethanol stock solution in mg/mL; m_{total} = the mass in mg of the volumetric flask with 5 mL of ethanol added; m_{flask} = the mass in mg of the volumetric flask itself.

The ethanol concentration in the test solution vial (mg/mL) was calculated using the following calculation:

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

C_{vial} = the concentration of ethanol in the test solution vial in mg/mL; P_0 = the response ratio of the peak area of the ethanol peak to the peak area of the propan-1-ol peak determined for the vial; b = the y-intercept; a = the slope of the calibration curve determined from the analysis of the calibration standards.

The concentration of ethanol (mg/mL) in the kefir sample was calculated using the following calculation:

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

C_{sample} = the concentration of ethanol in the test sample in mg/mL; C_{vial} = the concentration of ethanol in the test solution vial in mg/mL.

The concentration of ethanol in the kefir sample (mg/mL) is then converted to % ABV using the following calculation:

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

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

Methanol Calculations

The intermediate methanol standard stock solution concentration of methanol was calculated using the following calculation:

$$M_{stock} = \frac{(B_{total} - B_{flask})}{25}$$

M_{stock} = the concentration of the methanol stock solution in mg/mL; B_{total} = the mass in mg of the 25 mL volumetric flask with 0.5 mL of methanol added; B_{flask} = the mass of the 25 mL volumetric flask itself.

The methanol concentration in the test solution vial in mg/mL was calculated using the following calculation:

$$M_{vial} = \left(\frac{A_0 - d}{c}\right)$$

M_{vial} = the concentration of methanol in the test solution vial in mg/mL; A_0 = the response ratio of the peak area of the methanol peak to the peak area of the propan-1-ol peak determined for the vial; d = the y-intercept; c = the slope of the calibration curve determined from the analysis of the calibration standards.

The concentration of methanol (ppm, wt/v) in the kefir sample was calculated using the following calculation:

$$M_{sample} = \frac{M_{vial} * 10}{5} \times 1000$$

M_{sample} = the concentration of methanol in the test sample in ppm (wt/v); M_{vial} = the concentration of methanol in the test solution vial in mg/mL.

Single-Laboratory Validation (SLV) Parameters

This method was previously validated as per AOAC guidelines for ethanol determination using kombucha as a matrix (13). This matrix extension study was performed to validate the method to include water kefir samples. Both kombucha and water kefir are fermented products and can be considered similar matrixes. The use of HS-GCMS as an analysis method also mitigates many of the issues associated with matrix interferences. Given these conditions, the procedures that were used to evaluate several of the performance characteristics for kombucha in the previous SLV study were considered applicable for the evaluation of these same performance characteristics for kefir water in this study. As such, several of the performance characteristics for ethanol determination had been assessed and established in the previous single-laboratory validation study. A further extension study was performed to validate this method for the determination of methanol concentration within water kefir samples.

(a) *Selectivity.*—The retention times for the peaks corresponding to ethanol, methanol, and propan-1-ol were confirmed through analysis of their respective working check solutions. Identity of the peaks were confirmed through

comparison of the obtained mass spectrometry spectrums against the NIST database. Chromatograms of water kefir samples were reviewed to ensure there was adequate separation between the analyte peaks and any other peaks observed in the samples.

(b) *Linearity*.—The linear response of ethanol over the desired analytical range had already been assessed in the previous kombucha validation study. For the purposes of this study, five-point calibration curves covering the expected analytical range for the samples being analyzed was used. The linear response of methanol was determined through the preparation of nine separate seven-point calibration curves prepared using the procedure described above. The r^2 values were determined for each calibration curve, and each curve was visually inspected to confirm linearity over the range. An r^2 value >0.995 was considered acceptable.

(c) *Method detection level and limit of quantification (LOQ)*.—The limits of detection and quantification for ethanol were determined previously in the kombucha validation study. For methanol, the limit of detection (LOD) was determined through the analysis of seven replicates of water samples containing no methanol. As per the International Union of Pure and Applied Chemistry (IUPAC) recommendations described in the AOAC guidance document, the LOD was defined as the mean response plus three times the standard deviation obtained from analysis of these samples (14). The LOQ for methanol was defined as 10 times the standard deviation that was used to determine the LOD.

(d) *Accuracy*.—The accuracy of the ethanol determination for this method had been assessed previously in the kombucha study. For methanol, the accuracy of the method was assessed using a spike recovery study. The spike recovery study was performed at three levels: 27, 71, and 105 ppm. Water was used as the blank sample matrix. The appropriate amount of methanol was spiked into the blank matrix and then analyzed in quadruplicate as per the described method. The mean recovery was calculated for each spike level and evaluated against AOAC recommended guidelines for acceptable recoveries (14).

(e) *Precision*.—Precision for ethanol determination was assessed through the analysis of the obtained market samples. Eight water kefir samples in quadruplicate were prepared and analyzed on each of three separate days, and ABVs of each sample were determined. For methanol, there was a paucity of samples with quantifiable levels of methanol available on the market. As such, data to evaluate precision were obtained from the spiked samples used in the accuracy study. The within-day, between-day, and total standard deviations of the obtained results were calculated. HorRat values and relative standard deviation (RSD_r, %) were determined and used to evaluate the precision of the method. As per SLV guidelines published by AOAC, a HorRat value of <2.0 was considered acceptable.

Results and Discussion

Method Validation Results

(a) *Selectivity*.—Ethanol, methanol, and propan-1-ol peaks on the chromatogram were confirmed using their mass spectrums, and all showed adequate separation (*see* Figures 1–3 for the chromatograms showing the analysis of standards and water kefir samples). In each respective figure, the chromatograms show peaks for ethanol, propan-1-ol, and methanol and all are clearly separated from all other peaks seen in the sample.

(b) *Linearity*.—The linearity for ethanol over the desired range had already been established in the previous kombucha SLV study. The linearity for this analyte was further confirmed through the observation of linear curves and the calculated r^2 values >0.999 for all standard curves used for ethanol quantification in this study. For methanol, all calibration curves prepared appeared linear upon visible inspection. The calculated r^2 values of all methanol calibration curves prepared was >0.999, which demonstrated the linear response of the analyte over the evaluated range.

(c) *Repeatability and intermediate precision*.—The intermediate precision for ethanol was determined using quantitative data from the three days of analysis of samples with four replicates to calculate the between-day, within-day, and standard deviations for the method. Intermediate precision values ranged from 1.03 to 6.68% RSD_r. Mean ABV values calculated from these samples ranged from 0.14 to 1.70%. ABV and HorRat values were determined to be 0.23–1.52. A summary of these data is shown in Table 1.

For methanol determination, RSD_r values for all three levels ranged from 1.45 to 3.39% and HorRat values ranged from 0.25 to 0.49. All HorRat values were determined to be <2.0, demonstrating acceptable precision is achieved for both analytes with this method.

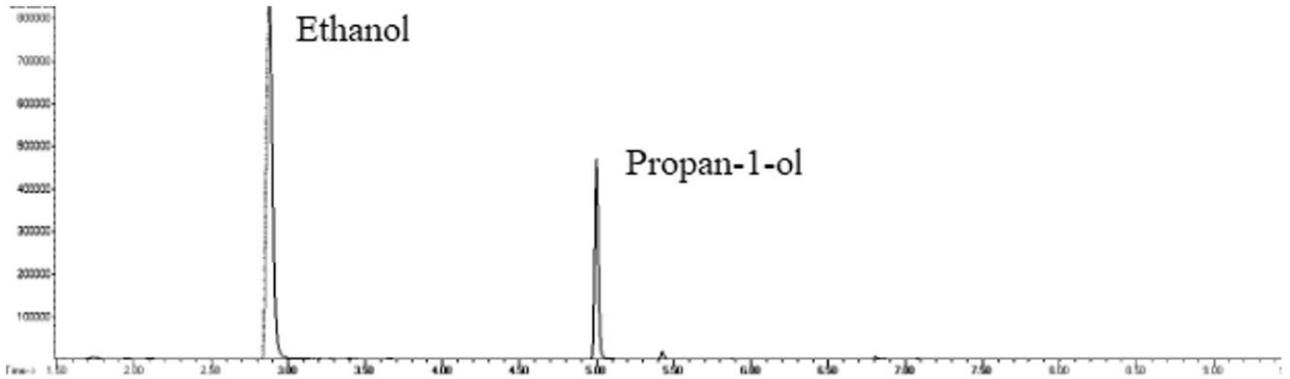

Figure 1. Chromatogram obtained from analysis of a water kefir sample 2785-2.

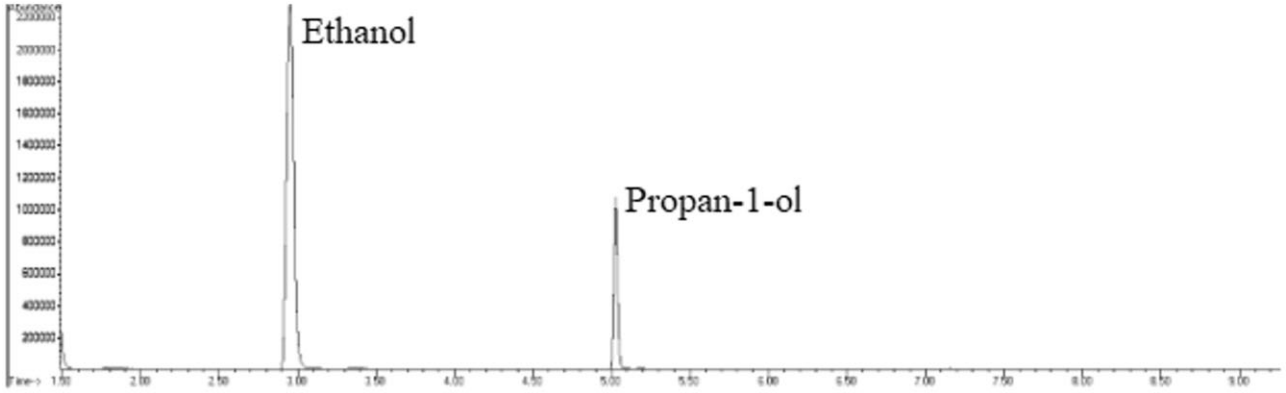

Figure 2. Chromatogram obtained from analysis of an ethanol standard solution.

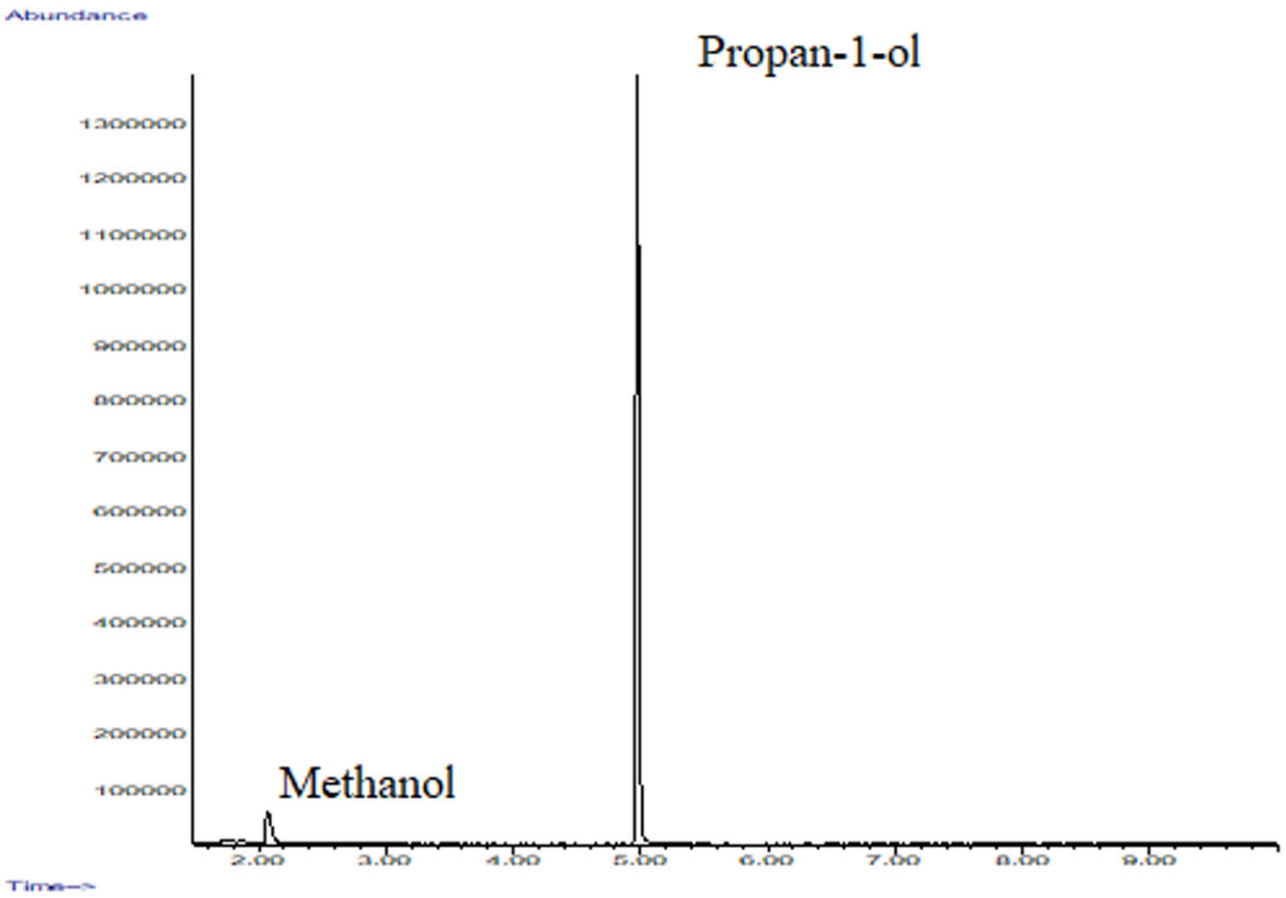

Figure 3. Chromatogram obtained from methanol spike recovery study.

(d) *Accuracy*.—Accuracy for this study was previously determined in the validation study of ethanol determination in kombucha. For methanol, the three spike levels gave mean recoveries of 91, 90, and 89%. These levels are within AOAC guidelines for typical acceptable recovery values for these analyte concentrations, demonstrating the method possesses acceptable accuracy for methanol determination (14).

(e) *Limits of detection and quantitation*.—The previous SLV had reported the LOD and LOQ for ethanol as being 0.0002% ABV and 0.002% ABV, respectively. For methanol, the LOD and LOQ were determined for this method in this study to be 16 and 21 ppm, respectively.

Conclusions

New flavor profiles and the diversity of fruit combinations have made fermented beverages such as water kefir more desirable to a broader consumer base to include young children, adults, and the elderly. With the rise in consumption, it is imperative to ensure that alcohol levels within such products are accurately conveyed to consumers and meet regulatory

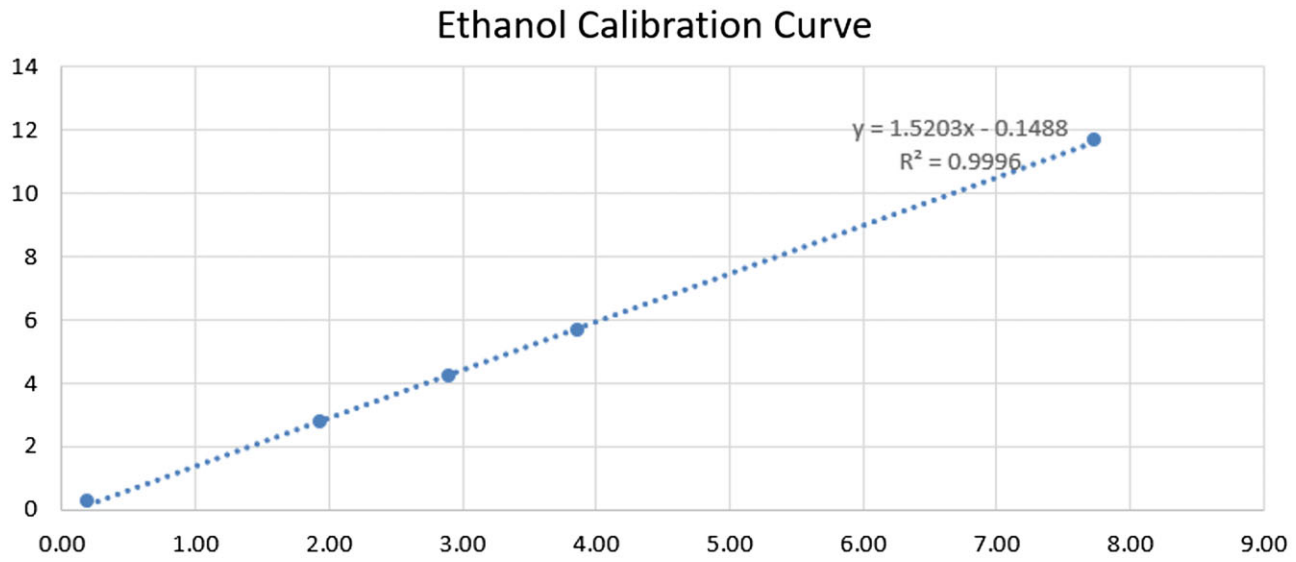

Figure 4. Representative standard curve prepared from the kefir water SLV. The standard curve displays a linear relationship and the calculated r^2 is 99.9%.

Table 1. Statistical summary on the analysis of ethanol in water kefir beverages us HS-GC-MS

Water kefir sample	Mean ethanol concentration (ABV, %)	RSD, %	HorRat value
1	0.54	6.68	1.52
2	0.48	1.84	0.41
3	0.54	1.72	0.39
4	1.70	2.14	0.58
5	0.49	1.03	0.23
6	0.58	1.23	0.28
7	0.22	3.76	0.75
8	0.14	4.62	0.86

requirements. This study provides validation data that demonstrate a previously established method for ethanol determination in kombucha can also be reliably used for the analysis of water kefir samples. The study also demonstrates that this method can quantify methanol within this matrix. Demonstrating the fitness of this method to analyze another fermented beverage matrix and another analyte of interest provides the fermented beverage industry with an invaluable tool to assess their products and to ensure product quality, safety and regulatory requirements are met.

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Conflict of Interest

All authors declare no conflict of interest.

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