FOOD COMPOSITION AND ADDITIVES

Determination of Ethanol in Kombucha, Juices, and Alcohol-Free Beer by Enzytec™ *Liquid* Ethanol: Single-Laboratory Validation, First Action 2017.07

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Enzytec™ *Liquid* Ethanol is an enzymatic test for the determination of ethanol in kombucha, juices, and alcohol-free beer. The kit contains two components in a ready-to-use format. Quantification is based on the catalytic activity of alcohol dehydrogenase, which oxidizes ethanol to acetaldehyde and converts NAD^+ to NADH. Measurement is performed in 3 mL cuvettes at 340 nm within 20 min. Samples with alcohol contents around 0.5% alcohol by volume need to be diluted 1:20 or 1:50 with water before measurement. Acetaldehyde interferes at concentrations higher than 3000 mg/L, whereas sulfite interferes at concentrations higher than 300 mg/L. The linear measurement range is from 0.03 up to 0.5 g/L ethanol, whereas LOD and LOQ are 1.9 and 3.3 mg/L ethanol, respectively. Kombucha with concentrations between 2.85 and 5.82 g/L showed relative repeatability standard deviation around 1%, whereas juices were below 2%. Results from a reproducibility experiment revealed that at a concentration of 0.1 g/L, the RSD_R was at 2.5%, whereas at higher concentrations between 0.2 and 0.3 g/L, coefficients around 1% were obtained. Trueness was checked by using Cerilliant aqueous ethanol solutions and beer with concentration of 0.4 and 4 g/L (BCR-651 and BCR-652). Spiking of kombucha and juice samples resulted in recoveries between 95% and 104%. Acceptable stability was found for the whole test kit under accelerated conditions at 37°C for 2 weeks. The kit is also not susceptible to short freezing–thawing cycles and harsh transport conditions.

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This method was approved by the Expert Review Panel for Kombucha in Ethanol as First Action.
The Expert Review Panel for Ethanol in Kombucha invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Kombucha is a traditional remedy prepared at home by fermenting sweetened black tea with a symbiotic culture of yeasts and bacteria. The fermented tea is produced by the action of a floating microbial mat/colony consisting of aerobic bacteria and yeasts. The colony's appearance often resembles a surface mold or a mushroom but is actually a floating cellulose mat produced during microbial growth. The kombucha culture has been shown to be a symbiosis of *Acetobacter* and a variety of yeasts. The kombucha recipe may vary; however, it is commonly made by infusing black tea leaves into freshly boiled water, sweetened with 50–150 g/L (5–15%) sucrose, for about 10 min. After removing the tea leaves, the tea is allowed to cool to room temperature and the microbial mat/colony from a previous batch is added to the sweetened tea with about 100 mL of kombucha from a previous fermentation. It is then covered with a clean cotton cloth and incubated at room temperature for about 7–10 days. Carbon conversion of sucrose begins by the yeasts breaking down the sugar into glucose and fructose. Glucose is primarily used up by the yeasts to yield ethanol and carbon dioxide. The ethanol is then oxidized by the bacteria to acetaldehyde and then to acetic acid. As a result, the alcohol content of kombucha is thought to never exceed 10 g/L, and the acetic acid concentrations may rise to levels as high as 10 g/L. The final product is a slightly carbonated beverage composed of fructose, ethanol, organic acids, vitamins, minerals, and tea components, resembling the taste of cider (1).

Ethanol is ubiquitous in its natural occurrence due to the activity of yeasts in sugar-containing food. Its quantitation is not only important in the manufacture of wines, beers, and spirits, but also for low-alcohol and nonalcoholic beverages, juices, and a range of other foodstuffs, including jam, honey, vinegar, and dairy products. Because of its ubiquitous occurrence, determination of ethanol in laboratories requires special attention to all kinds of cross contamination.

Within the Compendium of International Analysis of Methods of the Organisation International de la Vigne et du Vin, there are some fully validated type I methods for quantification of ethanol in wine (2). Besides these worldwide accepted methods, there is no fully validated method to quantify ethanol in kombucha by a simple and robust procedure.

In 2016, AOAC initiated a call for methods based on the *Standard Method Performance Requirements* (SMPR®) 2016.001 for determination of ethanol content in kombucha.

This study investigated the test kit Enzytec™ *Liquid* Ethanol (E 8340; R-Biopharm AG, Darmstadt, Germany) to be applicable for the quantification of ethanol not only in kombucha, but also in fruit juice, vegetable juice, and alcohol-free beer.

The underlying enzymatic reaction of this kit requires one enzyme and one coenzyme only. Ethanol is oxidized by the catalytic activity of alcohol dehydrogenase (ADH) in the presence of nicotinamide-adenine dinucleotide (NAD^+) to acetaldehyde. Because the equilibrium of this reaction lies in favor of ethanol and NAD^+, special experimental conditions were set up to ensure a quantitative reaction to acetaldehyde. In consequence, the test kit only contains two ready-to-use components, which is the basis for a robust and precise simple quantification of ethanol.

AOAC Official Method 2017.07
Ethanol in Kombucha, Juices, and Alcohol-Free Beer ENZYTEC™ *Liquid* Ethanol
First Action 2017

The Enzytec *Liquid* Ethanol quantifies ethanol in diluted or undiluted samples between 30 and 300 mg/L ethanol with a high precision (CV ≤ 2%). The linear range of the system is between 3.3 and 500 mg/L. The LOD is at 1.9 mg/L. All chemically related primary, secondary, and tertiary alcohols show side-chain activity with the exception of methanol. Acetaldehyde interferes at concentrations higher than 3000 mg/L, whereas sulfite interferes at concentrations higher than 300 mg/L.

Caution: see Material Safety Data Sheet

A. Principle

The enzymatic reaction requires one enzyme and one coenzyme only. As can be seen from (I), ethanol is oxidized by the catalytic activity of ADH in the presence of NAD^+ to acetaldehyde and $NADH/H^+$. NADH formed is stoichiometric with amount of ethanol originally present. NADH produced is measured at 340 nm with a spectrophotometer.

$$\text{(I)}\ \text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{(ADH)}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

Because the equilibrium of this reaction lies in favor of ethanol and NAD^+, special experimental conditions were set up to ensure a quantitative reaction to acetaldehyde. In consequence, the test kit only contains two ready-to-use components, which is the basis for a robust and precise simple quantification of ethanol.

B. Apparatus

Apparatus specified has been tested. Equivalent apparatus may be used.

(**a**) *Reaction tube (2 mL); reclosable.*
(**b**) *Disposable plastic cuvettes (1 cm light path).*
(**c**) *Micro-pipettors (20–200 µL and 100–1000 µL).*
(**d**) *Multipette (to dispense 2 mL aliquots of reagent 1).*
(**e**) *Spectrophotometer set at 340 nm.*
(**f**) *Vortex mixer.*
(**g**) *Ultrasound for degassing.*
(**h**) *Centrifuge (min. 3000 g).*

C. Reagents

Items (**a**) to (**c**) are available as a test kit (ENZYTEC *Liquid* Ethanol, E 8340; R-Biopharm Darmstadt, Germany). All reagents are stable as indicated on the label at 2–8°C (36–46°F).

(**a**) *The test kit consists of reagent 1 (2 x) and reagent 2 (2 x).*

(**b**) *Reagent 1*.—50 mL (containing buffer; ready to use).

(**c**) *Reagent 2*.—12.5 mL (containing NAD^+ and ADH; ready to use).

D. Standard Reference Material

Traceability was established by using certified reference materials (AQ01-015, AQ02-015, and AQ03-015) from ACQ Science (Rottenburg-Hailfingen, Germany) with ethanol concentrations from 0.1, 0.2, and 0.3 g/L.

E. Standard and Spike Solution

For spiking experiments, EMSURE® Ethanol (absolute for analysis; Merck, 1.00983.1000) was used. The material is free of water. Because ethanol is volatile, it was decided to use a 20% alcohol by volume (ABV) ethanolic solution for spiking samples. For preparation, a 100 mL volumetric flask was filled with about 50 mL distilled water. Twenty milliliters of absolute ethanol was pipetted into the flask, mixed, and filled with water up to 100 mL.

F. General Preparation

(**a**) Both components of the test kits are ready to use but should be warmed up to room temperature before use.

(**b**) Make sure that the laboratory environment, especially the air, is not contaminated with ethanol, e.g., caused by use of disinfection or cleaning reagents. Otherwise, all results will be biased due to the high sensitivity of the test kit toward ethanol.

(**c**) This test should only be carried out by trained laboratory employees.

(**d**) The instructions for use must be strictly followed.

(**e**) No quality guarantee is accepted after expiration of the kit (see expiration label).

(**f**) Do not interchange individual reagents between kits of different lot numbers.

(**g**) Follow recommendations of the manufacturer of the spectrophotometer for a proper warm-up and maintenance.

G. Sample Preparation

(**a**) *General recommendation.*—(*1*) Ethanol is very volatile; therefore, when diluting sample solutions, always pipette beneath the surface of the diluent; when filtering a sample solution, the filtrate shall not drop but rinse down the wall of the vial; close vial tightly before centrifugation.

(*2*) Use clear, slightly colored, and pH-neutral liquid samples directly, or after dilution into the relevant measurement range of 30–300 mg/L ethanol; slightly acidic or alkaline sample may be used directly after dilution; check strong acidic sample solution for recovery by spiking even after dilution in case of any doubt.

(*3*) Degas samples containing carbon dioxide by a short burst of ultrasound at 0°C (ultrasonic device filled with ice cubes and distilled water).

(**b**) *Sample preparation.*—(*1*) Clear kombucha, alcohol-free beer, and juices: dilute with water.

(*2*) Turbid kombucha, alcohol-free beer, and juices: centrifuge before dilution and dilute supernatant with water.

(*3*) Samples with 0.076–0.76% ABV (0.6 up to 6 g/L ethanol) should be diluted 1 + 19 with water, e.g., 100 µL sample is pipetted into 1900 µL distilled water.

(*4*) Samples with 0.38–3.8% ABV (3 up to 30 g/L ethanol) should be diluted 1 + 99, e.g., 100 µL sample is pipetted into 9.90 mL distilled water.

(*5*) Other dilutions as, e.g., 1:50 or 1:10, are possible if the ethanol concentration of the diluted samples lies within the measurement range (0.03 up to 0.3 g/L).

(*6*) Dilution of ethanol-containing samples with water is very susceptible to pipetted volumes used for dilution. Therefore, pipette at minimum 100 µL ethanol-containing sample into the respected volume of water; lower volumes, e.g., 20 µL, will result in higher CVs.

(*7*) Use diluted sample solutions within 3 days for ethanol measurement (storage temperature 4°C).

H. Determination

(**a**) Place a cuvette for one blank (RB) and each sample/control into a rack and pipette 2000 µL reagent 1 (R1) into each cuvette.

(**b**) Add 100 µL each sample or control into a designated cuvette and 100 µL distilled water into the designated cuvette (blank).

(**c**) Mix with a plastic spatula or another appropriate device.

(**d**) Incubate for 3 min at 20–25°C.

(**e**) Read and document absorbance A1 in a spectrophotometer set at 340 nm for each cuvette.

(**f**) Add 500 µL reagent 2 (R2) in each cuvette and mix well.

(**g**) Incubate for 15 min at 20–25°C.

(**h**) Read and document absorbance A2 in a spectrophotometer set at 340 nm for each cuvette.

I. SLV Parameters

The manufacturer's in-house validation scheme followed the AOAC Appendix K recommendations as described in the SMPR® 2016.001 for ethanol quantification in kombucha and long-lasting practical experiences of the method developer for ready-to-use enzymatic test kits.

(**a**) *Linearity*.—Linearity check over a range of 0.0025 up to 0.7 g/L ethanol (in water) in three different runs with one replicate in each run. Additionally, calculation of a residual plot to characterize range of linearity.

(**b**) *LOD and LOQ*.—According to DIN 32645:2008-11 (based on DIN ISO 11843-2:2008-06) with concentrations ranging from 2.5 up to 45 mg/L ethanol (in water) analyzed in three independent runs ($n = 1$).

(**c**) *Selectivity*.—Experiment using methanol, 1-propanol, 2-propanol, n-butanol, isobutanol, 1-pentanol, 2-pentanol, 3-pentanol, and 1-hexanol at a concentration of 0.2 g/L in comparison with 0.2 g/L ethanol. All solutions were prepared in distilled water.

(**d**) *Precision profile*.—Data were calculated from the linearity data set (aqueous ethanol calibrators). The calculated RSD derived from replicates from three independent runs.

(**e**) *Repeatability*.—Four nonspiked kombucha samples (degassed, centrifuged, diluted 1:50) were used over a period of 3 days by two persons on two occasions per day with two technical replicates per extract and day. Additionally, four nonspiked kombucha samples (degassed, centrifuged, diluted 1:50) were used in $n = 6$ at each occasion over a period of 2 days on three occasions in total with one technical replicate per extract. Four different nonspiked fruit juices were degassed, centrifuged, and tested in $n = 6$ at each occasion over a period of 2 days on three occasions in total with one technical replicate per extract. Three different nonspiked vegetable juices were degassed, centrifuged, and tested in $n = 6$ at each occasion over a period of 2 days on three occasions in total with one technical replicate per extract.

(**f**) *Inter-lot precision*.—Aqueous ethanol solutions with 0.03 (solution A), 0.15 (solution B), and 0.30 g/L (solution C) were analyzed with six replicates on 1 day by one person in three different test kit lots.

(**g**) *Laboratory-internal reproducibility*.—Measurement of certified reference material (aqueous ethanol solutions from ACQ Science GmbH with ethanol concentrations of 0.1, 0.2, and 0.3 g/L); each material was used directly for measurement and analyzed in four different lots by four different persons with two replicates per measurement. From these values s_r, RSD_r, s_R, and RSD_R were calculated.

(**h**) *Trueness*.—Measurement of certified reference materials (aqueous ethanol solutions from Cerilliant with ethanol concentrations from 0.8 up to 4 g/L and BCR beer with concentrations of 0.05 and 0.5% ABV).

(**i**) *Recovery*.—Four different kombucha samples used for repeatability characterization were spiked to their endogenous ethanol concentrations (between 2.7 and 5.7 g/L); samples were degassed, centrifuged, and diluted in $n = 6$ at each occasion by two persons over a period of 2 days on three occasions in total with one technical replicate per extract per person. For all juices, samples were individually spiked to reach the upper measurement range of 0.3 g/L. Samples were degassed, centrifuged, and tested in $n = 6$ at each occasion over a period of 2 days on three occasions in total with one technical replicate per extract.

(**j**) *Interferences*.—Sulfite and acetaldehyde were tested in presence of 0.15 g/L ethanol ($n = 1$) with concentrations of 0.06 up to 30 and 1.5 up to 30 g/L, respectively.

(**k**) *Robustness*.—Incubation temperature was varied between 18 and 37°C. Incubation time before measuring A2 at 340 nm was varied between 5 and 20 min. An alcohol-free beer sample and a kombucha sample with ethanol concentrations of 3.5 and 2.0 g/L were checked for dilutability.

(**l**) *Stability*.—*See* supplemental material.

J. Calculations

(**a**) Calculate ΔA for every sample or control:

$$\Delta A = (A_2 - df \times A_1)_{sample\ or\ control} - (A_2 - df \times A_1)_{RB}$$

where df is a dilution factor calculated as follows: df = (sample volume + R1) / (sample volume + R1 + R2) = 0.808

(**b**) Calculate concentrations for every sample or control:

$$c = (V \times MW \times \Delta A) / (\varepsilon \times d \times v \times 1000)$$

where V = final volume; MW = molecular weight of ethanol; ε = absorption coefficient of NADH at 340 nm; d = light path within

cuvette; and v = test portion volume explain the ethanol-specific formula:

$$\text{Ethanol, g/L} = (2.6\ \text{mL} \times 46.07\ \text{g} \times \text{mole}^{-1} \times \Delta A)/(6.3\ \text{L} \times \text{mmole}^{-1} \times \text{cm}^{-1} \times 1\ \text{cm} \times 0.1\ \text{mL} \times 1000)$$

or

$$\text{Ethanol, g/L} = 0.190 \times \Delta A$$

If a sample was diluted before measurement, this result has to be multiplied with the dilution factor F and converted to % ABV using the formula:

$$\%\ \text{ABV} = \text{Ethanol [g/L]} / 7.894$$

Results and Discussion

Linearity

The range of concentrations tested for linearity behavior of the enzymatic system is from 0.0025 up to 0.7 g/L ethanol. This set of dilutions was tested three times independently, and results are depicted in Figure 1. It is already visible by eye that at concentrations higher than 0.5 g/L ethanol, the linearity of the system is no longer given.

A correlation factor r^2 is not given in Figure 1 because this factor alone does not result in any relevant information about sufficient linearity. Instead, residuals of ΔOD values were calculated and presented in Figure 2. This plot clearly revealed that the linearity ends between 0.4 and 0.5 g/L ethanol. The lower point of quantification will be characterized in the *LOD and LOQ* section.

LOD and LOQ

The enzymatic system Enzytec *Liquid* Ethanol is very sensitive. Therefore, it is not remarkable that nearly all matrices checked to be ethanol-free contained low but detectable or even quantifiable amounts of ethanol. As a consequence, only calibration data can be used to estimate LOD and LOQ. This procedure is laid down in DIN 32645:2008-11 (based on DIN

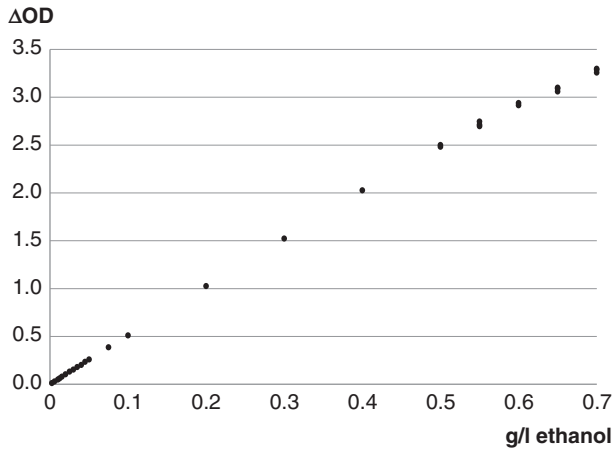

Figure 1. Linearity check over a range of 0.0025 up to 0.7 g/L ethanol in three different runs with one replicate in each run. ΔOD values after subtraction of blank values are shown.

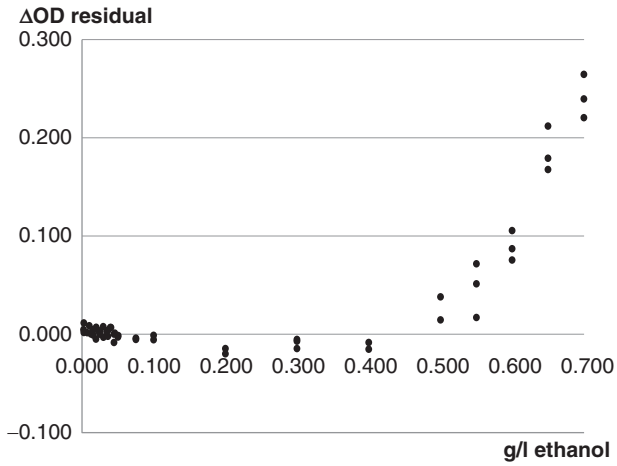

Figure 2. Residual plot of data presented in Figure 1. Range of concentration is from 0.0025 up to 0.7 g/L. For each concentration, three independent measurements were available.

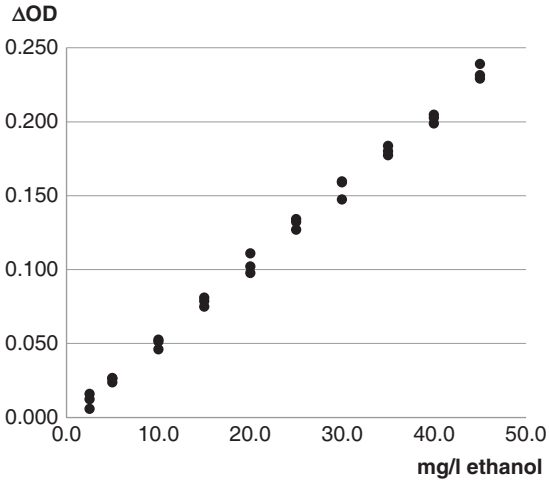

Figure 3. Analysis of calibrators with concentrations near to zero concentration. A range from 2.5 up to 45 mg/L was covered and analyzed in three independent runs (*n* = 1). Calibration function is described by ΔOD = 0.0052 × mg/L ethanol − 0.0004.

ISO 11843-2:2008-06) and required calibration data near to zero concentration. Therefore, calibrators between 2.5 and 45 mg/L ethanol were prepared and analyzed in three independent runs (Figure 3).

From this figure, the linear calibration function was calculated to ΔOD = 0.0052 × mg/L ethanol − 0.0004. For the following calculation, the slope of this line (b = 0.0052) is used (Figure 3). According to DIN 32645:2008-11, a quick estimate of LOD and LOQ is possible. In principle, the residual standard deviation $s_{y,x}$ of the measurement signal is "converted" to a standard deviation s_{x0} of the concentration by dividing $s_{y,x}$ with the slope b of the graph, which is given above. This standard deviation of the procedure s_{x0} is multiplied with several factors to estimate LOD and LOQ. In this case, LOD is at 1.85 mg/L (0.0002% ABV), whereas LOQ is at 3.26 mg/L (0.0004% ABV). The estimates from two additional lots revealed LODs at 0.89 and 1.43 mg/L, whereas LOQs were estimated at 1.60 and 2.54 mg/L (raw data and calculation not shown). These three lots were also used to characterize stability of the system. An accelerated stability study was performed over a period of

12 days at 37°C. At the end of this period, LOD and LOQ were estimated again and revealed LOD values of 0.53, 1.33, and 1.25 mg/L, whereas LOQ values were calculated to 0.96, 2.36, and 2.22 mg/L.

Selectivity

Relevant chemically related alcohols that were reported to exist in beverages and juices were tested in the enzymatic system at a concentration of 0.2 g/L (Table 1). Methanol is not reacting in the enzymatic system. In contrast, 1-propanol, 2-propanol, n-butanol, 1-pentanol, and 1-hexanol showed significant or even comparable results to ethanol. This was expected because any alcohol dehydrogenase exerts these kinds of side-chain activities. Theoretically, these alcohols could lead to creep reactions. But the published data on these alcohols reveal that in the best case, a factor of 1000 exists between the concentration of ethanol and the chemically related side-chain active alcohols, so the side-chain activity should not lead to measurable errors.

Precision Profiles

The characterization of precision over the whole measurement range is depicted in Figure 4. These data were calculated from the linearity data set (aqueous ethanol calibrators). The calculated RSD was derived from three independent replicates from three independent runs.

Repeatability

For characterization of repeatability, diluted kombucha samples with endogenous ethanol concentrations between 2.73 and 5.65 g/L were analyzed by two persons over a period of 3 days with two testing series per day; RSDs were somewhat higher (Table 2). But because different persons were involved on different days, the results are still excellent. For this experiment, only one dilution was prepared from each sample to exclude effects due to variation of dilution. If dilution is included into the uncertainty budget, the results are still acceptable (Table 3). At this time, only one analyst performed the experiments, because it was shown in Table 2 that the person has a negligible influence on variation of results. It will be presented in the *Robustness* section that it is absolutely necessary to have a sample intake of 100 µL kombucha for dilution. Otherwise, RSDs will rise up to 4% if, e.g., only 20 µL kombucha is used for dilution before measurement in the enzymatic system (results not shown).

When undiluted fruit juice samples (apple, cranberry, multivitamin, orange) with endogenous ethanol concentrations between 0.034 and 0.329 g/L were analyzed by one person over a period of 2 days with three tests in total, RSDs were below 2%, with the exception of cranberry juice, in which, due to the low ethanol concentrations, the RSD was around 3% (Table 4).

When undiluted vegetable juice samples (tomato, tomato-vegetable, carrot) with endogenous ethanol concentrations between 0.017 and 0.103 g/L were analyzed by one person over a period of 2 days with three tests in total, RSDs were below 1%, with the exception of tomato-vegetable juice, in which, due to the very low ethanol concentrations, the RSD was around 2.3% (Table 5).

Table 1. Selectivity experiment using methanol, 1-propanol, 2-propanol, n-butanol, isobutanol, 1-pentanol, 2-pentanol, 3-pentanol, and 1-hexanol at a concentration of 0.2 g/L in comparison with 0.2 g/L ethanol. All solutions were prepared in distilled water

Substance	Target value, g/L	Concn, g/L	Rec., %
Ethanol	0.200	0.208	103.8
Methanol	0.200	0.000	0.2
1-Propanol	0.200	0.186	93.1
2-Propanol	0.200	0.070	35.2
n-Butanol	0.200	0.146	73.0
Isobutanol	0.200	0.003	1.7
1-Pentanol	0.200	0.047	23.7
2-Pentanol	0.200	0.003	1.4
3-Pentanol	0.200	0.000	0.2
1-Hexanol	0.200	0.049	24.4

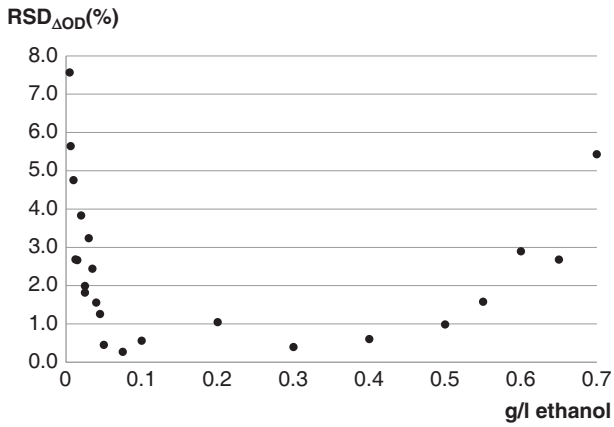

Figure 4. Precision profile of data used to characterize linearity (*see Linearity* section; Figure 1).

Inter-Lot Precision

Because the enzymatic test kit is a proprietary ready-to-use test kit, characterization of inter-lot variation is necessary to show that all lots produced under routine conditions in the production facility at R-Biopharm are comparable with respect to variation. Results are shown in Table 6 and show that all three lots were comparable over the measurement range.

Laboratory-Internal Reproducibility (Intermediate Precision)

Table 7 contains data from R-Biopharm's QC department, where real-time stability tests are performed. For QC tests, certified reference materials from ACQ are used because they have optimal volumes of 1.5 mL, which makes it possible to use a new vial for each testing. As can be seen from Table 7, four different lots were tested by four technicians on different days in duplicate. Using the AOAC excel work sheet for collaborative tests, results from Table 7 were taken to calculate s_r, RSD_r, s_R, and RSD_R (Table 8). Reproducibility

Table 2. Repeatability—Kombucha (nonspiked). Sample was degassed, centrifuged, diluted (1:50), and used over a period of 3 days by two persons on two occasions per day. Two technical replicates per extract and day. Result in g/L ethanol

	Kombucha 1		Kombucha 2		Kombucha 3		Kombucha 4	
	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2
Day 1, test 1	5.69	5.68	3.15	3.17	3.70	3.69	2.75	**2.72**
	5.71	5.68	3.15	3.13	3.70	3.67	2.75	2.70
Day 1, test 2	5.67	5.66	3.14	3.14	3.69	3.73	2.74	2.74
	5.66	5.66	3.14	3.12	3.69	3.67	2.73	2.70
Day 2, test 1	5.69	5.67	3.13	3.16	3.71	3.67	2.74	2.73
	5.66	5.65	3.13	3.15	3.66	3.65	2.71	2.72
Day 2, test 2	5.67	5.69	3.13	3.14	3.66	3.68	2.71	2.71
	5.68	5.63	3.16	3.13	3.70	3.67	2.73	2.72
Day 3, test 1	5.68	5.60	3.15	3.10	3.69	3.90	2.79	2.69
	5.65	5.61	3.14	3.11	3.71	3.74	2.82	2.74
Day 3, test 2	5.64	5.59	3.13	3.00	3.68	3.62	2.72	2.68
	5.63	5.57	3.11	3.09	3.61	3.62	2.71	2.71
Mean, g/L	5.65		3.13		3.69		2.73	
SD, g/L	0.035		0.033		0.054		0.030	
RSD, %	0.62		1.05		1.47		1.10	

relative standard deviations for concentrations between 0.1 and 0.3 g/L were between 0.91 and 2.53%, which is quite excellent.

Table 3. Repeatability including dilution—Kombucha (nonspiked). Sample was degassed, centrifuged, and diluted (1:50) in *n* = 6 at each occasion over a period of 2 days on three occasions in total. One technical replicate per extract. Result in g/L ethanol; 100 µL kombucha was pipetted into 4.90 mL water

	Kombucha 1	Kombucha 2	Kombucha 3	Kombucha 4
Day 1, test 1	5.80	3.28	3.72	2.83
	5.80	3.30	3.75	2.87
	5.77	3.34	3.76	2.86
	5.75	3.35	3.77	2.85
	5.78	3.36	3.78	2.86
	5.76	3.29	3.80	2.85
Day 1, test 2	5.91	3.34	3.74	2.87
	5.83	3.31	3.82	2.81
	5.98	3.34	3.78	2.84
	5.91	3.38	3.68	2.92
	5.84	3.31	3.77	2.88
	5.84	3.33	3.69	2.87
Day 2, test 1	5.86	3.26	3.68	2.84
	5.87	3.31	3.75	2.86
	5.68	3.33	3.79	2.83
	5.82	3.26	3.75	2.84
	5.84	3.29	3.76	2.84
	5.76	3.29	3.72	2.84
Mean, g/L	5.82	3.32	3.75	2.85
SD, g/L	0.070	0.034	0.041	0.024
RSD, %	1.20	1.02	1.11	0.84

Trueness

Several certified reference materials (CRM) are available, although most of them are aqueous ethanol solutions. Only two beers are available. In consequence, most of the data presented in this chapter are results for aqueous ethanol solutions. In the opinion of the method developer, this is not a general problem because all samples with ethanol concentrations around the threshold of 0.5% ABV (3.945 g/L) need to be diluted by a factor of 20, which minimizes the influence of matrix effects. The *Recovery* section will deal with possible matrix effects. Table 9 shows the results for CRMs from Cerilliant and two BCR beers

Table 4. Repeatability including dilution—Four different fruit juices (nonspiked) were degassed, centrifuged, and tested in *n* = 6 at each occasion over a period of 2 days on three occasions in total. One technical replicate per extract

	Apple	Cranberry	Multivitamin[a]	Orange
Mean, g/L	0.175	0.034	0.080	0.329
SD, g/L	0.003	0.001	0.001	0.004
RSD, %	1.66	3.11	1.83	1.08

[a] Apple, orange, pineapple, acerola, passion fruit, mango, lemon, banana, nectarine.

Table 5. Repeatability including dilution—Three different vegetable juices (nonspiked) were degassed, centrifuged, and tested in *n* = 6 at each occasion over a period of 2 days on three occasions in total. One technical replicate per extract. Result in g/L ethanol

	Tomato	Tomato-vegetable	Carrot
Mean, g/L	0.099	0.017	0.103
SD, g/L	0.001	0.000	0.001
RSD, %	0.98	2.28	0.87

Table 6. Inter-lot precision using aqueous ethanol solutions with 0.03 (solution A), 0.15 (solution B), and 0.30 g/L (solution C). Six replicates were analyzed on one day by one person in three lots

	Lot 1 g/L	Lot 2 g/L	Lot 3 g/L
	Solution A		
Mean	0.029	0.030	0.029
SD	0.0001	0.0005	0.0002
RSD, %	0.49	1.68	0.85
	Solution B		
Mean	0.145	0.146	0.145
SD	0.0008	0.0027	0.0009
RSD, %	0.53	1.83	0.59
	Solution C		
Mean	0.290	0.288	0.288
SD	0.0011	0.0009	0.0012
RSD, %	0.38	0.33	0.43

(BCR-651 and BCR-652). Compared with the certified value, all results were excellent and only differed in one case by 4% (beer with 0.051% ABV, corresponding to 0.4 g/L ethanol)

Recovery

All kombucha samples from the market showed ethanol concentrations between 2.85 and 5.82 g/L (*see* top of Table 10). It was decided to spike these samples as high as their endogenous ethanol concentration. The four different kombucha samples were analyzed by two persons on three occasions over a period of 2 days as a 6-fold replication. Every sample of these six replicates was diluted and not stored for the next occasion. The concentration of the spiked samples was calculated by subtraction of the endogenous ethanol concentration from the analytical result. From this, the recovery was calculated. Results of this recovery experiment are depicted in Table 10 and revealed very high precision (RSDs smaller than 1.6%). This high precision was only achievable if 100 µL kombucha was used for dilution. If only 20 µL was used, RSD was less than 4% (results not

Table 8. Calculation of s_r, RSD_r, s_R, and RSD_R for three certified reference materials (raw data in Table 7) from QC data

		Reference 1	Reference 2	Reference 3
		0.1 g/L	0.2 g/L	0.3 g/L
Total number	p	13	13	13
Total number of replicates	Sum(n(L))	26	26	26
Overall mean of all data (grand mean)	XBARBAR	0.101 g/L	0.202 g/L	0.299 g/L
Repeatability standard deviation	s(r)	0.0006 g/L	0.0014 g/L	0.0021 g/L
Reproducibility standard deviation	s(R)	0.0025 g/L	0.0018 g/L	0.0049 g/L
Repeatability relative standard deviation	RSD(r)	0.58%	0.71%	0.72%
Reproducibility relative standard deviation	RSD(R)	2.53%	0.91%	1.65%

Table 7. Measurement of certified reference material (aqueous ethanol solutions from ACQ Science GmbH). Each material was used directly for measurement and analyzed in four different lots by four different persons with two replicates per measurement

			ACQ reference 1 Certified: 0.1 g/L		ACQ reference 2 Certified: 0.2 g/L		ACQ reference 3 Certified: 0.3 g/L	
			Rep.[a] 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2
Lot	Date	Person	g/L ethanol		g/L ethanol		g/L ethanol	
1	2017-02-01	A	0.103	0.103	0.205	0.203	0.305	0.304
1	2017-04-19	B	0.097	0.096	0.204	0.203	0.304	0.305
2	2017-02-01	A	0.102	0.102	0.203	0.203	0.303	0.302
2	2017-04-19	B	0.096	0.096	0.203	0.203	0.302	0.303
3	2017-02-01	A	0.101	0.102	0.202	0.202	0.303	0.302
3	2017-04-19	B	0.097	0.097	0.202	0.202	0.302	0.302
4	2017-03-07	C	0.102	0.101	0.201	0.199	0.300	0.299
4	2017-03-14	D	0.102	0.104	0.203	0.202	0.291	0.297
4	2017-03-14	D	0.102	0.103	0.202	0.201	0.294	0.298
4	2017-03-14	D	0.101	0.102	0.201	0.206	0.290	0.295
4	2017-03-14	D	0.102	0.102	0.206	0.203	0.292	0.298
4	2017-03-14	D	0.102	0.102	0.201	0.199	0.293	0.293
4	2017-03-14	D	0.102	0.102	0.201	0.199	0.293	0.294

[a] Rep. = Replicate.

Table 9. Measurement of certified reference material (aqueous ethanol solutions from Cerilliant and BCR beer). Each material was diluted before measurement and analyzed

Sample	Dilution	Certified value g/L ethanol	Measured g/L ethanol	Rec. %
Cerilliant 800 mg/L	1:10	0.8	0.80	100
Cerilliant 800 mg/L	1:20	0.8	0.81	101
Cerilliant 1000 mg/L	1:10	1.0	1.01	101
Cerilliant 1000 mg/L	1:20	1.0	0.98	98
Cerilliant 1500 mg/L	1:10	1.5	1.50	100
Cerilliant 1500 mg/L	1:20	1.5	1.47	98
Cerilliant 2000 mg/L	1:10	2.0	1.99	99
Cerilliant 2000 mg/L	1:20	2.0	1.97	99
Cerilliant 4000 mg/L	1:10	4.0	3.93	98
Cerilliant 4000 mg/L	1:20	4.0	3.90	98
BCR-652 0.051%	1:10	0.4	0.40	100
BCR-652 0.051%	1:20	0.4	0.38	96
BCR-651 0.505%	1:10	4.0	4.01	100
BCR-651 0.505%	1:20	4.0	4.04	101

shown). Table 11 contains recovery data converted to percentage of recovery. Mean recovery varied from 96 up to 102%, whereas the range of individual recoveries was between 95 and 104%.

Fruit juice samples from the market showed ethanol concentrations between 0.034 and 0.175 g/L (*see* top of Table 12). It was decided to spike these samples at about 0.3 g/l in total because the linear range of the calibration is limited, and the spiked samples should be treated like unspiked samples (undiluted). The three different juice samples were analyzed by one person on three occasions over a period of 2 days as a 6-fold replication. The concentration of the spiked samples was calculated by subtraction of the endogenous ethanol concentration from the analytical result. From this, the recovery was calculated. Results of this recovery experiment are depicted in Table 12 and revealed very high precision (RSDs smaller than 2%). Table 12 also contains recovery data converted to percentage of recovery. Mean recovery varied from 95 up to 97%, whereas the range of individual recoveries was between 90 and 101%.

Vegetable juice samples (tomato, tomato-vegetable, and carrot) from the market revealed ethanol concentrations between 0.017 and 0.103 g/L (*see* top of Table 13). It was decided to spike these samples at about 0.3 g/L in total because the linear range of the calibration is limited, and the spiked samples should be treated like unspiked samples (undiluted).

Table 10. Recovery—Kombucha. Four different kombucha samples with endogenous ethanol concentrations between 2.85 and 5.82 g/L were spiked with ethanol between 2.70 and 5.70 g/L (*see* top of table). Samples were degassed, centrifuged, and diluted in *n* = 6 at each occasion by two persons over a period of 2 days on three occasions in total. One technical replicate per extract per person. Result in g/L ethanol. The concentration of the spiked samples is calculated by subtraction of the endogenous ethanol concentration from the analytical result; 100 µL kombucha was pipetted into 4.90 mL water

	Kombucha 1		Kombucha 2		Kombucha 3		Kombucha 4	
Blank[a]	5.82		3.32		3.75		2.85	
Spike	5.70		3.20		3.70		2.70	
	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2
Day 1, test 1	5.56	5.49	3.26	3.24	3.78	3.60	2.69	2.72
	5.47	5.47	3.26	3.28	3.58	3.62	2.68	2.73
	5.52	5.51	3.27	3.27	3.68	3.63	2.70	2.78
	5.62	5.52	3.30	3.28	3.67	3.66	2.74	2.77
	5.54	5.50	3.23	3.29	3.62	3.63	2.66	2.76
	5.51	5.57	3.34	3.24	3.64	3.66	2.69	2.74
Day 1, test 2	5.58	5.40	3.26	3.21	3.64	3.61	2.66	2.70
	5.49	5.47	3.25	3.21	3.59	3.67	2.70	2.67
	5.30	5.47	3.27	3.23	3.55	3.60	2.68	2.71
	5.53	5.47	3.27	3.20	3.66	3.57	2.70	2.68
	5.51	5.47	3.24	1.53[b]	3.58	3.70	2.63	2.68
	5.43	5.48	3.24	3.23	3.60	3.57	2.69	2.70
Day 2, test 1	5.43	5.40	3.28	3.23	3.58	3.54	2.64	2.60
	5.44	5.41	3.24	3.21	3.58	3.54	2.64	2.65
	5.44	5.42	3.27	3.24	3.64	3.55	2.67	2.65
	5.50	5.41	3.32	3.21	3.66	3.54	2.66	2.68
	5.40	5.41	3.27	3.21	3.56	3.53	2.64	2.62
	5.53	5.42	3.24	3.23	3.56	3.54	2.67	2.64
Mean, g/L	5.47		3.25		3.61		2.68	
SD, g/L	0.063		0.032		0.056		0.042	
RSD, %	1.15		0.98		1.54		1.56	

[a] *See* Table 3.

[b] Outlying value.

Table 11. Recovery—Kombucha. Results are expressed as percentage recovery calculated from values in Table 10. The concentration of the spiked samples is calculated by subtraction of the endogenous ethanol concentration from the analytical result; from this, the recovery was calculated

	Kombucha 1		Kombucha 2		Kombucha 3		Kombucha 4	
	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2	Person 1	Person 2
Day 1, test 1	98	96	102	101	102	97	100	101
	96	96	102	102	97	98	99	101
	97	97	102	102	99	98	100	103
	99	97	103	103	99	99	101	103
	97	96	101	103	98	98	98	102
	97	98	104	101	98	99	100	102
Day 1, test 2	98	95	102	100	98	98	99	100
	96	96	102	100	97	99	100	99
	93	96	102	101	96	97	99	100
	97	96	102	100	99	96	100	99
	97	96	101	—[a]	97	100	97	99
	95	96	101	101	97	96	100	100
Day 2, test 1	95	95	102	101	97	96	98	96
	95	95	101	100	97	96	98	98
	95	95	102	101	98	96	99	98
	97	95	104	100	99	96	99	99
	95	95	102	100	96	96	98	97
	97	95	101	101	96	96	99	98
Mean, rec. %	96.0		101.6		97.5		99.4	
SD, rec. %	1.11		0.99		1.50		1.55	

[a] Outlying value; *see* Table 10.

The concentration of the spiked samples was calculated by subtraction of the endogenous ethanol concentration from the analytical result. From this, the recovery was calculated. Results of this recovery experiment are depicted in Table 13 and revealed very high precision (RSDs smaller than 1.6%). Table 13 also contains recovery data converted to percentage of recovery. Mean recovery varied from 96 up to 97%, whereas the range of individual recoveries was between 93 and 99%.

Interferences

The aliphatic alcohols 1-propanol, 2-propanol, and n-butanol that showed the highest side-chain activities (*see Selectivity* section) were again tested at different levels between 0.015 and 0.15 g/L in presence of 0.15 g/L ethanol.

As can be seen in Figure 5, the three tested higher alcohols exert significant positive interferences to the ethanol quantification. As expected from selectivity experiments, the graph is quite linear. Nevertheless, the relationship between ethanol concentrations and concentration of these interfering alcohols will always be greater than factor 1000 under practical conditions. In the present case, this would be, e.g., a 1-propanol concentration of 0.00015 g/L. This will never influence the ethanol determination significantly. Results for other possible interferants are shown in Table 14.

Sugars and short-chain organic acids exert no interfering effect (results not shown). Up to 300 mg/L, sulfite does not interfere with the ethanol measurement. Higher values are not practical because even in wine the thresholds are between 200 and 300 mg/L. Kombucha, alcohol-free beer, and juices are normally not conserved by sulfite. Acetaldehyde does not interfere up to 3000 mg/L. Because acetaldehyde is only a fermentation byproduct and normally present at very low concentrations in final products, it is assumed that this interference has no influence on ethanol quantification under practical conditions.

Robustness

Enzymatic systems are sometimes susceptible to variations in incubation times and incubation temperatures. Therefore, it was checked if the assay still produces true results if the temperature is lowered to 18°C or increased up to 37°C. Incubation time before measuring A2 at 340 nm was varied between 5 and 20 min. There is no influence when varying the incubation temperature or incubation time (results not shown).

Due to the small measurement range between 30 and 300 mg/L, it will often happen in praxis that a sample shows ethanol concentration higher than 300 mg/L. Therefore, an alcohol-free beer sample and a kombucha sample with ethanol concentrations of 3.5 and 2.0 g/L were checked for dilutability. As can be clearly seen in Figure 6, both samples are dilutable over the whole range.

Discussion

The test kit Enzytec *Liquid* Ethanol investigated in this validation study was proven to be applicable for the quantification of ethanol in kombucha, fruit juice, vegetable juice, and alcohol-free beer. It consists of two components, in which one

Table 12. Recovery—Fruit juice. Three different juice samples with endogenous ethanol concentrations between 0.034 and 0.175 g/L were spiked with ethanol between 0.125 and 0.270 g/L (*see* top of table). Samples were degassed, centrifuged, and tested in *n* = 6 at each occasion over a period of 2 days on three occasions in total. One technical replicate per extract. The concentration of the spiked samples is calculated by subtraction of the endogenous ethanol concentration from the analytical result and is given in g/L ethanol; from this, the recovery was calculated and is presented in parentheses

	Apple	Cranberry	Multivitamin
Blank[a]	0.175	0.034	0.08
Spike	0.125	0.270	0.220
Day 1, test 1	0.121 (97%)	0.253 (94%)	0.209 (95%)
	0.122 (97%)	0.250 (93%)	0.262 (—)[b]
	0.121 (97%)	0.257 (95%)	0.215 (98%)
	0.126 (101%)	0.257 (95%)	0.210 (95%)
	0.125 (100%)	0.258 (96%)	0.211 (96%)
	0.122 (98%)	0.266 (98%)	0.209 (95%)
Day 1, test 2	0.119 (95%)	0.258 (95%)	0.210 (95%)
	0.121 (96%)	0.255 (94%)	0.207 (94%)
	0.120 (96%)	0.253 (94%)	0.215 (98%)
	0.122 (98%)	0.258 (95%)	0.206 (93%)
	0.120 (96%)	0.257 (95%)	0.210 (95%)
	0.120 (96%)	0.260 (96%)	0.209 (95%)
Day 2, test 1	0.125 (100%)	0.244 (90%)	0.245 (—)[b]
	0.121 (97%)	0.255 (95%)	0.200 (91%)
	0.116 (93%)	0.252 (93%)	0.211 (96%)
	0.124 (100%)	0.258 (96%)	0.211 (96%)
	0.123 (98%)	0.257 (95%)	0.210 (95%)
	0.120 (96%)	0.259 (96%)	0.213 (97%)
Mean, g/L	0.122 (97%)	0.256 (95%)	0.210 (95%)
SD, g/L	0.002 (1.9%)	0.005 (1.7%)	0.004 (1.6%)
RSD, %	1.96	1.78	1.68

[a] See Table 4.

[b] Outlier.

Table 13. Recovery—Vegetable juice. Three different juice samples with endogenous ethanol concentrations between 0.017 and 0.103 g/L were spiked with ethanol between 0.200 and 0.280 g/L (*see* top of table). Samples were degassed, centrifuged, and tested in *n* = 6 at each occasion over a period of 2 days on three occasions in total. One technical replicate per extract. The concentration of the spiked samples is calculated by subtraction of the endogenous ethanol concentration from the analytical result and is given in g/L ethanol; from this, the recovery was calculated and is presented in parentheses

	Tomato	Tomato-vegetable	Carrot
Blank[a]	0.099	0.017	0.103
Spike	0.200	0.280	0.200
Day 1, test 1	0.190 (95%)	0.269 (96%)	0.194 (97%)
	0.192 (96%)	0.267 (95%)	0.199 (99%)
	0.190 (95%)	0.268 (96%)	0.195 (97%)
	0.195 (97%)	0.267 (95%)	0.197 (99%)
	0.192 (96%)	0.266 (95%)	0.196 (98%)
	0.192 (96%)	0.268 (96%)	0.197 (98%)
Day 1, test 2	0.196 (98%)	0.268 (96%)	0.191 (96%)
	0.193 (96%)	0.266 (95%)	0.192 (96%)
	0.193 (96%)	0.265 (95%)	0.186 (93%)
	0.196 (98%)	0.266 (95%)	0.196 (98%)
	0.194 (97%)	0.265 (95%)	0.195 (97%)
	0.194 (97%)	0.267 (95%)	0.196 (98%)
Day 2, test 1	0.191 (95%)	0.266 (95%)	0.192 (96%)
	0.193 (96%)	0.270 (97%)	0.194 (97%)
	0.193 (97%)	0.272 (97%)	0.196 (98%)
	0.191 (96%)	0.268 (96%)	0.196 (98%)
	0.193 (97%)	0.271 (97%)	0.198 (99%)
	0.194 (97%)	0.270 (96%)	0.192 (96%)
Mean, g/L	0.193 (96%)	0.268 (96%)	0.195 (97%)
SD, g/L	0.002 (0.9%)	0.002 (0.7%)	0.003 (1.5%)
RSD, %	0.90	0.77	1.57

[a] *See* Table 5.

component contains a buffer and the second component alcohol dehydrogenase and NAD. For sample preparation, kombucha, juices, and beer were centrifuged and diluted if necessary. Measurement is monitored at 340 nm and is finished within 20 min. Because only a few steps are necessary to finish sample preparation and measurement, the assay is especially useful for technicians with base-level laboratory experience. The in-house validation included a linearity study, estimation of LOD and LOQ, selectivity and inferences, different types of precision, characterization of trueness, recovery, a lot-to-lot comparability, stability testing, and ruggedness testing. Experiments showed excellent linearity and high precision that is mainly driven by precision of pipettes and not test kit components. The assay fulfills all requirements listed in the AOAC SMPR 2016.001. Due to the low LOQ of 3.3 mg/L ethanol (0.0004% ABV), kombucha and alcohol-free beer need to be diluted before measurement, typically around 1:20 or 1:50. Therefore, possible matrix interferences due to, e.g., color or pH are often

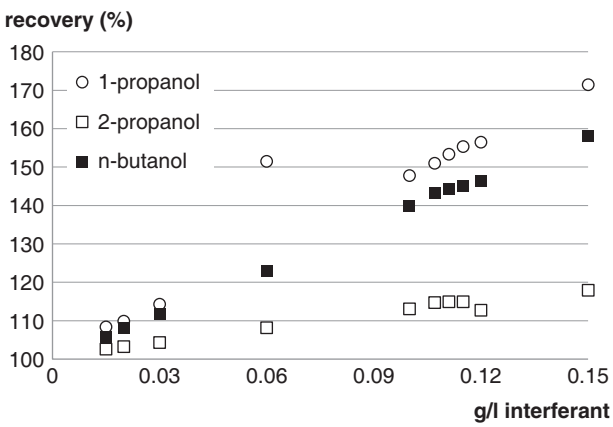

Figure 5. Interference of other alcohols. Addition of different amounts of 1-propanol, 2-propanol, and n-butanol (0.015 up to 0.15 g/L) to 0.15 g/L ethanol (*n* = 1).

Table 14. Interferants sulfite and acetaldehyde at different concentrations in presence of 0.15 g/L ethanol (*n* = 1)

Probe	Interferant, g/L	Measured in g/L	Rec., %
Ethanol 0.15 g/L	—[a]	0.152	101
Ethanol 0.15 g/L	—	0.150	100
Ethanol 0.15 g/L + sulfite	30[b]	0.667[b]	445[b]
	3[b]	0.227[b]	151[b]
	1.5[b]	0.187[b]	125[b]
	0.6[b]	0.166[b]	111[b]
	0.3	0.157	105
	0.15	0.155	103
	0.06	0.150	100
Ethanol 0.15 g/L + acetaldehyde	30[b]	0.115[b]	77[b]
	3	0.144	96
	1.5	0.149	99

[a] — = Not applicable.

[b] Significant interferences.

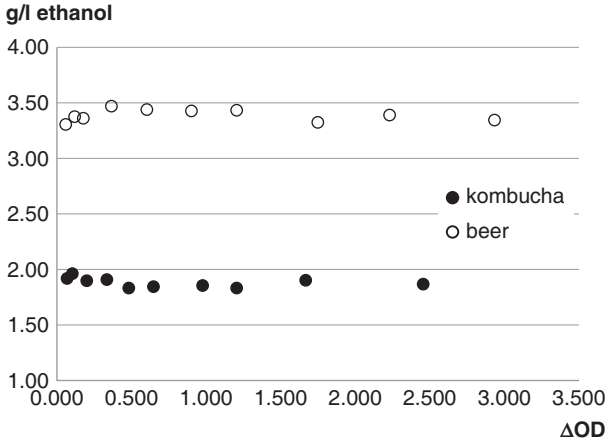

Figure 6. Dilutability of kombucha and alcohol-free beer over the whole measurement range. Samples were diluted 1:4 up to 1:150 (kombucha) and 1:6 up to 1:300 (beer) with water.

not relevant. In the case of juices, ethanol concentrations were low, and no dilution was necessary. Nevertheless, precision and recovery were not affected. The enzyme contained in the test kit will also convert other primary aliphatic alcohol as, e.g., 1-propanol, n-butanol, and 1-pentanol, but not methanol. These primary alcohols are only contained at very low levels after alcoholic fermentation. Typically, concentrations of higher alcohols are 1000 times lower than ethanol. Therefore, this side-chain activity is not relevant under practical conditions for measurement of ethanol in kombucha, juices, and alcohol-free beer. Acetaldehyde interferes at concentrations higher than 3000 mg/L, whereas sulfite interferes at concentrations higher than 300 mg/L. Under practical conditions, this is also not relevant because kombucha, juices, and beer are not treated with sulfite, and acetaldehyde is never present at 3 g/L after a normal fermentation process. Trueness was analyzed using aqueous ethanol standard reference solutions and a certified reference material (alcohol-free beer). A thorough robustness testing included the analysis of incubation temperature (18, 25, and 37°C) and incubation time (5, 10, 15, and 20 min). No parameter was found to influence the result in a way that could be critical under practical conditions. Kombucha and beer samples are dilutable. Care should be taken when alcohol-containing sample are diluted, because the volume of sample used for dilution is critical. It is strongly recommended to use 100 µL at minimum for dilution. At lower volumes (e.g., 20 µL), results will turn out to be less precise. The test kit is stable for 2 weeks at 37°C and until now a real time stability of 12 months is covered.

Conclusions

In summary, the data of the in-house validation study proved that the performance claims for kombucha are fulfilled and are in accordance with AOAC SMPR 2016.001.

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