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# Enzymatic quantification of ethanol in kombucha using Enzytec™ *Liquid* Ethanol

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## Introduction

In 2016, the Stakeholder Panel on Strategic Food Analytical Methods (SPSFAM) adopted standard method performance requirements (SMPR®) for the quantification of ethanol in kombucha, as laid down in SMPR 2016.001. We would like to present a robust and simple enzymatic test for quantification of ethanol in kombucha and alcohol-free beer that is fit-for-purpose according to this SMPR.

**Picture 1:** Enzytec™ Liquid Ethanol, Art. No. E8340



## Materials and methods

The enzymatic reaction requires one enzyme and one co-enzyme only. As can be seen from (I) ethanol is oxidized by the catalytic activity of alcohol dehydrogenase (ADH) in the presence of nicotinamide-adenine dinucleotide (NAD+) to acetaldehyde Since the equilibrium of this reaction lies in favor of ethanol and $NAD^+$, special experimental

$$\text{Ethanol} + \text{NAD}^+ \xleftrightarrow{\quad \text{(ADH)} \quad} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

conditions were setup 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. Depending on the ethanol content of the samples, and due to the high sensitivity of the system, a simple dilution step might be necessary before measurement. 2 ml of buffer (reagent 1) is mixed with 100 µl sample solution, incubated for 3 min and measured at 340 nm (A1). 500 µl enzyme/NAD-solution (reagent 2) is added and incubated for 15 min. A2 is measured at 340 nm and ethanol concentration is calculated from the difference between A2 and A1.

## Results and discussion of validation

**Table 1:** Calculation of sr, RSD(r), s(R), and RSD(R) for three certified reference materials 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 % |

The linear range is between 3 and 500 mg/l ethanol. Highest precision (CV < 2 %) was shown between 30 and 300 mg/l ethanol. LoD and LoQ were determined according to DIN ISO 11843-2:2008-06 and revealed 1.85 mg/l and 3.26 mg/l, respectively. Other aliphatic alcohols showed side-chain activity but will not disturb the ethanol measurement, since under natural conditions these higher alcohols are present at concentration factor 1000 lower than ethanol. Acetaldehyde interferes at concentrations higher than 3000 mg/l while sulphite interferes at concentrations higher than 300 mg/l. As expected for an uncomplicated enzymatic system, precision is also high (table 1). Analysis of certified reference materials proved that the enzymatic system shows no bias (table 2).

Analysis of spiked kombucha samples from the market are presented in table 3 and revealed as already shown in table 1 excellent precision if four different kombucha samples were analysed repeatedly by two analysts over a period of three days.

Overall, the system is suitable for quick and easy analysis of ethanol in beverages and juices, even by technicians with a low level of experience.



**Table 2:** Measurement of certified reference material (aqueous ethanol solutions from Cerilliant and BCR beer); each material was diluted before measurement and analyzed in duplicate

| Sample | Dilution | Certified value g/l ethanol | Measured g/l ethanol | Recovery % |
|---|---|---|---|---|
| Cerilliant 800 mg/L | 1:20 | 0.8 | 0.81 | 101 |
| Cerilliant 1000 mg/L | 1:20 | 1.0 | 0.98 | 98 |
| Cerilliant 1500 mg/L | 1:20 | 1.5 | 1.47 | 98 |
| Cerilliant 2000 mg/L | 1:20 | 2.0 | 1.97 | 99 |
| Cerilliant 4000 mg/L | 1:20 | 4.0 | 3.90 | 98 |
| BCR-652 0.051% | 1:20 | 0.4 | 0.38 | 96 |
| BCR-651 0.505% | 1:20 | 4.0 | 4.04 | 101 |

**Table 3:** Recovery of ethanol from spiked kombucha samples; samples showed endogenous ethanol concentrations between 2.85 g/l and 5.82 g/l; results are given in g/l ethanol; ethanol background values were subtracted

| | Kombucha 1 | | Kombucha 2 | | Kombucha 3 | | Kombucha 4 | |
|---|---|---|---|---|---|---|---|---|
| Blank | 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** | 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 | |
| CV (%) | 1.15 | | 0.98 | | 1.54 | | 1.56 | |