UV assay for the determination of ethanol in foodstuffs and other sample materials
Test combination for 50 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was validated using selected samples of the following matrices: kombucha, juices and alcohol-free beer.

Detailed results and information regarding associated validation data are found in the Validation Report.

This test has been approved as AOAC *Official Method of Analysis*℠ 2024.02 First Action. A publication is available in Journal of AOAC INTERNATIONAL, 106(2), 341–347 (2023).

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

1. Test principle

In the presence of the enzyme alcohol-dehydrogenase (A-DH), ethanol is oxidized to acetaldehyde by nicotinamide-adenine-dinucleotide (NAD):

Ethanol + NAD^+ ——A-DH⟶ acetaldehyde + NADH + H^+

NAD is reduced to NADH. The amount of NADH formed is measured at 340 nm and stoichiometrically corresponds to the amount of ethanol present.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer
- Reagent 2: 2 x 12.5 mL with buffer, NAD, A-DH

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Bring sample solutions to approximately room temperature before measurement.
- Use liquid, **clear** and almost neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Centrifuge turbid solutions.
- Decolorize **strongly** colored samples that are measured undiluted (e.g., juices and wines) using polyvinylpolypyrrolidone (PVPP) if necessary. For example, add 0.1 g of PVPP to 10 mL of sample, stir for 1 minute, and then filter until a **clear** supernatant is obtained. Check the recovery rate by adding a spiking solution.
- Degas samples containing carbon dioxide by a short burst of ultrasound at 0 °C (ultrasonic device filled with ice cubes and distilled water).
- For **strongly** acidic samples, the recovery rate should be verified after dilution with distilled water by the addition of a spiking solution.
- **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 the vial tightly before centrifugation.

3.2. Application examples

- Dilute clear kombucha, non-alcoholic beer, and juices with distilled water.
- Centrifuge turbid kombucha, non-alcoholic beer, and juices before diluting, and dilute the supernatant with distilled water.
- Do not use the same pipette for diluting alcoholic samples or control solutions and for dispensing reagents. **Always use alcohol-free water!**
- Samples with 0.076 – 0.76 % ABV (alcohol by volume; 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.
- Samples with 0.38 – 3.8 % ABV (3 up to 30 g/L ethanol) should be diluted 1 + 99 with water, e.g. 100 µL sample is pipetted into 9.90 mL distilled water.
- Other dilutions, e.g. 1:50 or 1:10, are possible if the ethanol concentration of the diluted samples lies within the measurement range (0.03 – 0.3 g/L).
- 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 Coefficients of variation (CVs).
- Use diluted sample solutions within 3 days for ethanol measurement (storage temperature 4 °C).

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	30 – 300 mg/L

	Reagent blank	**Samples / controls**
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read** absorbance $\mathbf{A_1}$, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **15 minutes at 20 – 37 °C (68 – 99 °F)** and **read** absorbance $\mathbf{A_2}$.		

4.1. Important notes for assay procedure

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated and determined at 25 °C (77 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Due to the high sensitivity of the test, **it is essential to work in an alcohol-free environment** or in airtight cuvettes.
- The sample volume should be at least 100 µl.
- **Reagent 1 should always be added first!** The precision of the test generally depends heavily on the pipetting technique.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip several times before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance
- Always wait for the reaction to end or for the absorbance to stabilize (at least during the first test runs or validation). If the absorbance has not stopped after the recommended incubation time, continue measuring at 5-minute intervals, for example, until a constant absorbance value is reached.
- If a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Calculation of ethanol concentration

The extinction difference ΔA must be calculated for each sample:

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

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume} + R1}{\text{test volume}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible. **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df) accordingly**.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of ethanol is then calculated using Lambert-Beer's law:

$$\mathbf{C}_{\text{ethanol}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.190 \times \Delta A} \times F$$

If the sample solution was diluted before measurement, this result has to be multiplied with the **sample pre-dilution factor F**.

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight ethanol [g/mol]	= 46.07
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of alcohol content by volume

The alcohol content by volume (ABV) can be calculated using the following formula, based on the specific gravity of pure ethanol at 20 °C (68 °F):

$$\text{ABV [\%]} = \frac{C_{\text{ethanol}}\ [g/L]}{7.8924\ (\text{at } 20\ °C / 68\ °F)}$$

5.3. Calculation of solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

$$\text{Content}_{\text{ethanol}}\ [g/100\ g] = \frac{C_{\text{ethanol}}\ [g/L\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/L sample solution}} \times 100$$

5.4. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. For this purpose, we recommend the use of alcohol standard (Art. No. AQ03-015, with 300 mg/L ethanol).

The recovery of this alcohol standard and other *aqueous* control solutions should be 100 ± 5 %.

6. Performance data

6.1. Specificity & side activities

Alcohol-dehydrogenase oxidizes primary alcohols. The recovery of ethanol is around 100 %, whereas other primary alcohols (n-propanol and n-butanol) show lower recoveries.

Secondary and tertiary alcohols can lead to a creep reaction.

6.2. Interferences

Acetaldehyde does not interfere at concentrations below 3000 mg/L. Sulfite does not interfere at concentrations below 300 mg/L.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 500 mg/L ethanol. If the recommended measuring range of 30 to 300 mg/L is exceeded, the samples should be diluted with distilled water to a concentration within the measuring range. The dilution factor has to be considered in the calculation.

The limit of detection (LoD) and the limit of quantification (LoQ) were determined according to the method DIN 32645:2008-11 in buffered aqueous solution for a sample volume of v = 100 µL. This results in an LoD of 1.9 mg/L and an LoQ of 3.3 mg/L.

7. Supporting documents

On request, we offer the following documents:

- Validation reports for Enzytec™ *Liquid* Ethanol are available as AOAC publications via the following links:
  I. Determination of Ethanol in Kombucha, Juices, and Alcohol-Free Beer by Enzytec™ *Liquid* Ethanol: Single-Laboratory Validation, First Action 2017.07 https://doi.org/10.5740/jaoacint.17-0466
  II. Determination of Ethanol in Food by Enzymatic Method Enzytec™ *Liquid* Ethanol, Collaborative Study: Final Action 2017.07 https://doi.org/10.1093/jaoacint/qsac145
- Enzytec™ *Liquid* Ethanol Excel template for results
- Enzytec™ *Liquid* Ethanol Technical Information
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

On request, we offer the following services:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support & technical service

10. Disclaimer

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