Enzymatic assay for Ethanol in foodstuff and other sample materials
AOAC® *Official Method℠* 2017.07 for kombucha, juices, and alcohol-free beer
2 x 50 ml R1 + 2 x 12.5 ml R2 (50 assays)

For *in vitro* use only

Store between +2 and +8°C

Principle

Enzymatic test with Alcohol-Dehydrogenase (ADH). NADH is produced and is measured at 340 nm:

Ethanol + NAD^+ ⟶ ADH ⟶ Acetaldehyde + NADH + H^+

Reagents

The reagents are ready-to-use.
Reagent 1: two vials ≥ 50 ml (buffer)
Reagent 2: two vials ≥ 12.5 ml (NAD, ADH)

The reagents are stable up to the end of the indicated month of expiry if stored at 2 - 8 °C, even after repeated opening (if not contaminated during handling). Do not freeze the reagents. Let the reagents reach the laboratory temperature before use (20 - 25 °C).

The general safety rules for working in chemical laboratories should be applied. Do not swallow! Avoid contact with skin and mucous membranes.

This kit may contain hazardous substances. For hazard notes on the contained substances, please refer to the appropriate material safety data sheets (MSDS) for this product, available online at www.r-biopharm.com. After use, the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

Sample preparation

- Use liquid and clear samples directly, or after dilution into the relevant measuring range (see test performance)
- Filter or centrifuge turbid solutions
- Degas samples containing carbon dioxide
- Clarify samples containing proteins with Carrez clarification
- Crush and homogenize solid or semi-solid samples and extract with water (e.g. 30 min at 60 - 70°C). Filter or centrifuge, or apply Carrez clarification if necessary.
- For fat containing samples, extract with hot water, cool down to separate the fat (fridge or ice), remove the fatty layer and filter the aqueous part

Assay procedure

Wavelength:	340 nm
Optical path:	1 cm
Temperature:	20 – 25 °C / 37 °C
Measurement:	Against air or against water
Sample solution:	3 – 500 mg/l

	Reagent Blank (RB)	**Samples**
Reagent 1	2000 µl	2000 µl
Sample / Standard	-	100 µl
Dist. water	100 µl	-
Mix, incubate for 1 min. at 37 °C or 3 min. at 20 - 25 °C. Read absorbance A1 , then add:		
Reagent 2	500 µl	500 µl
Mix, wait until the end of the reaction (incubation for approx. 10 min. at 37°C or approx. 15 min. at 20 - 25 °C). Read absorbance A2.		

Reagent blank must be performed once for every run, and subtracted from every sample during calculation of results.

In order to avoid evaporation, the sample must be pipetted into the reagent 1 dispensed before.

Calculation of results

$\Delta A = (A_2 - df \times A_1)_{sample} - (A_2 - df \times A_1)_{RB}$
With df = dilution factor of optical densities through reagent volumes:
df = (sample volume + R1) / (sample volume + R1 + R2) = 0.808

c = (V x MW x Δ A) / (ε x d x v x 1000) [g/l of Ethanol]
c = (2.600 x 46.07 x Δ A) / (ε x 1 x 0.1 x 1000)
It results for the determination at 340 nm (ε = 6.3 l x $mmol^{-1}$ x cm^{-1}):

$c_{Ethanol}$ [g/l] = **0.190** x ΔA

Alcohol-by-volume [vol. %] = $C_{Ethanol}$ [g/l] / 7.894

Notes

The assay is very sensitive. Ethanol from air (e.g. disinfection or cleaning agents) causes a creep reactions and false results. It is necessary to run the assay in Ethanol free air, or to work with closed (air dense) cuvettes.

Because of the very volatile characteristics of Ethanol, it is necessary to follow appropriated procedures:

- When diluting sample solution, pipetting of the sample must be always under the surface of the dilution solution.
- Use at minimum 100 µl of sample for dilution.
- When filtering sample solution, the filtrate has not to drop but rinse down the wall of the container.
- Use diluted samples within one day.
- Precision is highly dependent on pipetting technique

Test performance

Specificity

ADH oxidizes primary alcohols. The recovery of Ethanol is around 100%, whereas other primary alcohols (n-Propanol and n-butanol) show a lower recovery. Secondary and tertiary alcohols can lead to a creep reaction.

Linearity and measuring range

The test is linear up to 500 mg/l Ethanol. The recommended measuring range lies between 20 and 300 mg/l, in order to keep ΔA ≅ 1.5 (A). When values exceed this range, samples should be diluted into the range 50 – 300 mg/l with dist. water. The dilution factor has to be considered in the calculation.

Sensitivity

The Limit of Detection (LoD) and Limit of Quantification (LoQ) where determined according to the method DIN 32645:2008-11:

- LoD = 1.9 mg/l
- LoQ = 3.3 mg/l

Precision and recovery

For kombucha, juices and alcohol-free beer:
- relative standard deviation for repeatability [RSD(r)] < 2%
- relative standard deviation for reproducibility [RSD(R)] < 3%
- recovery = 95-105%

Disclaimer

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