Ethanol

UV-method

for the determination of ethanol in foodstuffs and other materials

Simplified procedure for the determination of ethanol in alcoholic beverages: see pt. 13

Cat. No. 10 176 290 035

Test-Combination for 33 determinations

interchim
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Enzymatic BioAnalysis / Food Analysis

For use in foodstuff hygiene only.

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

Ethanol is oxidized to acetaldehyde by nicotinamide-adenine dinucleotide (NAD) in the presence of the enzyme alcohol dehydrogenase (ADH) (1).

(1) Ethanol + NAD^+ $\overset{ADH}{\rightleftharpoons}$ acetaldehyde + NADH + H^+

The equilibrium of this reaction lies on the side of ethanol and NAD. It can be completely displaced to the right side at alkaline conditions and by trapping of the acetaldehyde formed. Acetaldehyde is quantitatively oxidized to acetic acid in the presence of aldehyde dehydrogenase (Al-DH) (2).

(2) Acetaldehyde + NAD^+ + H_2O $\xrightarrow{Al\text{-}DH}$ acetic acid + NADH + H^+

NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 100 ml of solution, consisting of:
   potassium diphosphate buffer, pH approx. 9.0
2. Bottle 2 with approx. 30 tablets, each tablet contains:
   NAD, approx. 4 mg; aldehyde dehydrogenase, approx. 0.8 U
3. Bottle 3 with approx. 1.6 ml suspension, consisting of:
   ADH, approx. 7000 U
4. Bottel 4 with ethanol assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one** tablet of bottle 2 with **3 ml** solution of bottle 1 in a beaker or in a centrifuge tube for each assay (blank or samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2*.
3. Use contents of bottle 3 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).
Bring solution 1 to 20°C before use.
The contents of bottle 2 are stable at 2-8°C (see pack label).
Reaction mixture 2 is stable for one day at 2-8°C.
Bring reaction mixture 2 to 20°C before use.
The contents of bottle 3 are stable at 2-8°C (see pack label).

Procedure

Wavelength[1]:	340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]:	1.00 cm light path
Temperature:	20°C
Final volume:	3.150 ml
Read against air	(without a cuvette in the light path), against water or against blank[3]
Sample solution:	0.3-12 µg ethanol/assay[4] (in 0.100-0.500 ml sample volume)

Pipette into cuvettes	Blank	Sample
reaction mixture 2* redist. water sample solution**	3.000 ml 0.100 ml -	3.000 ml - 0.100 ml
Mix***, after approx. 3 min read absorbances of the solutions (A_1). Start reaction by addition of:		
suspension 3	0.050 ml	0.050 ml
Mix***, after completion of the reaction (approx. 5-10 min) read absorbances of the solutions immediately one after another (A_2).		

It is absolutely necessary to stopper the cuvettes, e.g., with Parafilm, during measurement (see "Instructions for performance of assay") in order to prevent the assay mixture form adsorption of ethanol from air.

* For simplification of the assay performance it is also possible to pipette directly 3.000 ml of solution 1 into the cuvette. Afterwards add 1 tablet from bottle 2 and dissolve it (for solubilization crush the tablet with a glass rod or a plastiv spatula). Continue as described in the scheme. The volume error of approx. 1% (the increase of volume caused by one tablet/3.150 ml final volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution; pipette sample solution always under the surface of the reaction mixture 2.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

Determine the absorbance differences (A_2-A_1) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and “Sensitivity and detection limit”, pt.4).

Calculation

According to the general equation for calculating the concentration in reactions in which the amount of NADH formed is stoichiometric to half the amount of substrate:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2 \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient of NADH at:
340 nm = 6.3 [l × $mmol^{-1}$ × cm^{-1}]
Hg 365 nm = 3.4 [l × $mmol^{-1}$ × cm^{-1}]
Hg 334 nm = 6.18 [l × $mmol^{-1}$ × cm^{-1}]

It follows for ethanol:

$$c = \frac{3.150 \times 46.07}{\varepsilon \times 1.00 \times 0.100 \times 2 \times 1000} \times \Delta A = \frac{0.7256}{\varepsilon} \times \Delta A \text{ [g ethanol/l sample solution]}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$Content_{ethanol} = \frac{c_{ethanol}\text{ [g/l sample solution]}}{weight_{sample}\text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm **or** 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 For example, when using a double-beam photometer.
4 See instructions for performance of assay
5 Amtliche Sammlung von Untersuchungsverfahren nach §35 LMBG; Untersuchung von Lebensmitteln: Ermittlung des Ethanolgehaltes in Alkohol und alkoholhaltigen Erzeugnissen aller Art (ausser Wein und Bier), L 37.00-1 (Nov. 1982). See also Reference 3.9, page 783. For corrections for the effect of temperature see e.g. Official Journal of the European Communities L 272 (3 October 1990), Legislation, Commission Regulation (EEC) No 2676/90 of 17 September 1990 determining Community methods for the analysis of wines.

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When analyzing ethanol containing samples (beverages, pharmaceuticals, cosmetics), the alcoholic strength by volume at 20 °C is calculated by means of the density of ethanol (at 20 °C) d = 0.78924[5]:

$$\text{Percentage}_{\text{Ethanol}} = \frac{c_{\text{Ethanol}}\ [\text{g/l sample solution}]}{10 \times 0.78924\ [\text{g/ml}]}\ [\%;\ \text{v/v}]$$

1. Instructions for performance of assay

The amount of ethanol present in the assay has to be between 0.5 µg and 12 µg (measurement at 365 nm) or 0.3 µg and 6 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield an ethanol concentration between 0.02 and 0.12 g/l or 0.01 and 0.06 g/l, respectively.

Because of the high sensitivity of the method it has to be taken care that ethanol-free water is used and it is worked in an ethanol-free atmosphere.

Dilution table

Estimated amount of ethanol per liter measurement at 340 or 334 nm	Estimated amount of ethanol per liter measurement at 365 nm	Dilution with water (in parts by volume)	Dilution factor F
< 0.06 g	< 0.12 g	-	1
0.06-0.6 g	0.12-1.2 g	1 up to 10	10
0.6-6.0 g	1.2-12 g	1 up to 100	100
6.0-60 g	12-120 g	1 up to 1000	1000
> 60 g	> 120 g	1 up to 10000	10000

Because of the volatility of ethanol, the dilution of samples should be carried out as follows:
Fill the volumetric flask approx. to the half with water (20°C) and pipette the sample (20°C) with an enzyme test pipette or a piston type pipette under the surface of the water. Fill up to the mark with water (20°C) and mix.

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 0.500 ml. The volume of solution 1 or reaction mixture 2, respectively, remains the same (3.000 ml). The volume of water pipetted into the blank assay must then be increased so as to obtain the same final volume in the assays for sample and blank. The new sample volume (v) and the new final volume (V) must be taken into account in the calculation.

2. Technical information

2.1. Ethanol is very volatile. Therefore it is necessary to be very careful when handling ethanol containing samples, diluting samples and pipetting sample solutions into the assay system.

When filtering solutions the filtrate should not drop into the container but rinse down the wall.

When dispensing ethanol-containing solutions, always pipette these solutions under the surface of water (when diluting) or of buffer (when performing the assay).

2.2 When pipetting highly diluted sample solutions into the assay system, rinse measuring glass pipette (enzyme test pipette) at least 6 times. The tip of the piston type pipette should be rinsed 3 times.

2.3. Do not use the same piston type pipette for diluting the sample and pipetting the sample solution into the assay system.

2.4. Always work in an alcohol-free atmosphere with ethanol-free water.

3. Specificity (Ref. 1)

The influence of aldehydes and ketones is eliminated by the order of reagent addition during the assay. Methanol is not converted because of the unfavourable K_m-values of the enzymes used.

n-Propanol and n-butanol are quantitatively converted under assay conditions, higher primary alcohols lead to sample-dependent creep reactions. Secondary, tertiary and aromatic alcohols do not react. Even higher concentrations of glycerol do not disturb the assay.

In the analysis of commercial ethanol results of approx. 100% have to be expected. (A recovery of less than 100% does not mean an inclompete conversion in the enzymatic reaction, it rather indicates the loss of ethanol during the handling of the sample and preparing the sample solution, as well as when pipetting the diluted ethanol solution into the assay system.)

4. Sensitivity and detection limit (Ref. 1.3)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 0.500 ml, an assay volume V = 3.550 ml and measurement at 340 nm of an ethanol concentration of approx. 0.1 mg/l sample solution (if v = 0.100 ml and V = 3.150 ml, this corresponds to 0.6 mg/l sample solution).

The detection limit of 0.5 mg/l is derived from the absorbance difference of 0.020 (as measured at 340 nm), a maximum sample volume v = 0.500 ml and an assay volume V = 3.550 ml.

5. Linearity

Linearity of the determination exists from approx. 0.3 µg ethanol/assay (0.5 mg ethanol/l sample solution; sample volume v = 0.500 ml; assay volume V = 3.550 ml) to 12 µg ethanol/assay (0.12 g ethanol/l sample solution; sample volume v = 0.100 ml; assay volume V = 3.150 ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml, an assay volume of V = 3.150 ml and measurement at 340 nm, this corresponds to an ethanol concentration of approx. 0.5-1 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of approx. 0.005-0.01 g/100 g can be expected).

The following data have been published in the literature:

x = 2.5 µg/assay	CV = 1.2 %	n = 16	in series	
x = 5 µg/assay	CV = 0.56 %	n = 16	in series	
x = 10 µg/assay	CV = 0.79 %	n = 16	in series	
x = 5 µg/assay	CV = 1.64 %	n = 30	from day to day	

(Ref. 1.2)

Lager beer	x = 3.585 g/l	r = 0.202 g/l	$s_{(r)}$	= ± 0.071 g/l
		R = 0.368 g/l	$s_{(R)}$	= ± 0.130 g/l
Malt beer	x = 4.755 g/l	r = 0.243 g/l	$s_{(r)}$	= ± 0.086 g/l
		R = 0.591 g/l	$s_{(R)}$	= ± 0.209 g/l

(Ref. 2.13)

For further data see references

7. Interference/sources of error

The presence of ethanol in the redist. water used for the assays or in air results in increased blanks or in creep reactions, respectively. Therefore it is necessary to cover the cuvette during the assay.

8. Recognizing interference during the assay procedure

8.1 If the conversion of ethanol has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding ethanol (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used for an identical final volume.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of ethanol are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaption guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table (in order to avoid a loss of ethanol, it is highly recommended to pipette the sample always under the surface of the diluent), and of a volume up to 0.500 ml;
Filter **turbid solutions** (a loss of small amounts of ethanol is possible);
Degas **samples containing carbon dioxide** (e.g. by filtration, or in order to avoid a loss of ethanol, by the addition of solid KOH or NaOH to bind CO_2 as bicarbonate);
Adjust **acid samples** to pH 8-9 by adding sodium or potassium hydroxide solution;
Adjust **acid and weekly colored samples** to pH 8-9 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
Measure **"colored" samples** (if necessary adjusted to pH 8-9) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyamide or with polyvinylpolypyrrolidone (PVPP), e.g. 2 g/100 ml;
Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;
Deproteinize **samples containing protein** with perchloric acid; alternatively clarify with Carrez reagents;
Extract **samples containing fat** with hot water in a flask with a condenser (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, rinse the condenser with redist. water, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water.

Carrez clarification:

Pipette the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4 \times 7\ H_2O$/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

11. Application examples

Determination of ethanol in fruit juices (Ref. 3.1, 2.6, 2.9, 2.12)

a) Use clear light-colored juices after neutralization or dilution, depending on the ethanol content, for the assay (see dilution table).

b) Decolorize intensely colored juices by addition of 2% polyamide or polyvinylpolypyrrolidone (PVPP) (e.g. 5 ml juice + 100 mg polyamide or PVPP), stir for 2 min (vessel must be stoppered) and filter. Use the mostly clear solution after neutralization for the assay. Decolorization can often be omitted after dilution of the sample.

c) Filter turbid juices and clarify with Carrez-solutions, if necessary: Pipette 10 ml of juice into a 25 ml volumetric flask, add 1.25 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), $K_4[Fe(CN_6)] \times 3\ H_2O$/100 ml), 1.25 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4 \times 7\ H_2O$/100 ml) and 2.50 ml NaOH (0.1 M), mix after each addition, fill up to 25 ml with water, filter (dilution factor F = 2.5). Use the clear sample solution, which may be weakly opalescent, for the assay directly or diluted, if necessary.

Determination of ethanol in low-alcohol and non-alcoholic beer (Ref. 2.1, 2.10, 2.11, 2.13)

Add solid potassium hydroxide or solid sodium hydroxide to approx. 100 ml sample in a beaker while stirring carefully until a pH value of approx. pH 8-9 is obtained. Use solution, diluted according to the dilution table, if necessary, for the assay.

Determination of ethanol in vinegar

Filter, if necessary and neutralize vinegar. Neutralization can be omitted after dilution of the sample.

Determination of ethanol in alcoholic beverages

a) *Wine* (Ref. 2.2, 2.8) Dilute wine with redist. water to the appropriate concentration (see dilution table). Decolorization and neutralization are not necessary.

b) *Beer*: To remove carbonic acid, stir approx. 5-10 ml of beer in a beaker for approx. 30s using a glass rod or filter. Dilute the sample 1:1000 (1 + 999) with water and use the diluted sample solution for the assay.

c) *Liqueur*: Pipette liquid liqueurs for dilution into an appropriate volumetric flask and fill up to the mark with water.

Weigh approx. 1 g of viscous liqueurs (e.g. egg liqueur) accurately into a 100 ml volumetric flask, fill up to the mark with redist. water, mix, keep it in a refrigerator for separation of fat, and filter.
Dilute the clear solution 1:100 (1 + 99) with water and use it for the assay. Calculate result in g/100 g.

d) *Brandy*: Take care as mentioned when taking samples of alcoholic beverages and dilute to a certain concentration (e.g. 1 + 9999 , e.g. in two steps). Convert the measured values (g ethanol/l solution) into volume percentage (v/v) with the aid of conversion tables, or the specific gravity of ethanol[5].

Determination of ethanol in chocolates, sweets and other alcohol-containing chocolate products

Chocolates with liquid filling compound (brandy balls, brandy cherries):
Open, e.g., one brandy ball carefully, pipette 0.500 ml of the liquid filling into a 50 ml volumetric flask filled with approx. 25 ml water, taking care that the tip of the pipette dips into the water. Fill up to the mark with water, stopper and mix. Dilute the solution with water in a ratio of 1:20 (1 + 19). Use 0.100 ml of the diluted solution for the assay (dilution factor F = 2000).

Chocolate products with highly viscous filling:
Accurately weigh the filling of one or several sweets or chocolates into a 50 ml volumetric flask filled with approx. 5 ml water (when the sample is weighed by means of a pipette, the tip of the pipette must **not** touch the water surface). Fill up to the mark with water, mix, filter, if necessary, and dilute until the alcohol content of the sample is less than 0.12 g/l.

Determination of ethanol in jam (Ref. 2.7)

Homogenize sample thoroughly (mixer, etc.) and accurately weigh approx. 10-20 g into a beaker. Add some water, mix and neutralize the mixture with KOH, if necessary. Transfer the mixture quantitatively into a 100 ml volumetric flask and fill up to the mark with redist. water.

Decolorize solution with 2% polyamide or PVPP, if necessary (see pt. 10) and filter. Use the filtrate for the assay undiluted.

Determination of ethanol in honey

Accurately weigh approx. 20 g honey into a 100 ml volumetric flask and dissolve with some water under slight agitation at approx. 50°C (ascending tube!), cool to 20°C and fill up to the mark with redist. water. Use the solution for the assay, clarify with Carrez-solutions (see pt. 10), if necessary (dilution factor F = 2.5). Use the clear solution for the assay after filtration.

Determination of ethanol in dairy products (e.g. curds, kefir)

Accurately weigh approx. 10 g of the homogenized sample into a 100 ml volumetric flask, add approx. 50 ml water and keep the flask (ascending tube!) at 50°C for 15 min under slight agitation. For protein precipitation add 5 ml Carrez-I-solution, 5 ml Carrez-II-solution and 10 ml NaOH (0.1 M) (see pt. 10), mix after each addition. Allow to cool to 20°C and fill up to the mark with water. Mix and filter. Use the clear, possibly slightly turbid solution for the assay.

Determination of ethanol in protein-containing samples

Deproteinize protein-containing sample solutions with perchloric acid (1 M) in a ratio of 1:3 (1+2) and centrifuge. Neutralize an aliquot of the supernatant with KOH (2 M)

12. Further applications

The method may also be used in the examination of cosmetics (Ref. 3.3), pharmaceuticals (Ref. 3.4), and in research when analyzing biological samples.

For details of sampling, treatment and stability of the sample see Bernt, E. & Gutmann, I. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed. vol. 3, p. 1500, Verlag Chemie, Weinheim/Academic Press. Inc. New York and London.

Determination of ethanol in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a water-bath at 80°C for 15 min (cover the tube because of the volatility of ethanol) to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization may be carried out with perchloric acid or with Carrez-solutions. See the above-mentioned examples. Homogenize gelatinous agar media with water and treat further as described.

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13. Simplified procedure for the determination of ethanol in alcoholic beverages and other samples containing at least 6 g ethanol/l

Preparation

Dissolve 10 tablets from bottle 2 in 30 ml solution from bottle 1, add 0.5 ml suspension from bottle 3, and mix.

(Note: Prepare reagent solution using alcohol-free redist. water in an alcohol-free atmosphere. Store in a tightly stoppered container.)

Stability

The reagent solution is stable for 8 hours at 20°C

Sample preparation

Dilute beer, wine, brandy, and other samples that contain ethanol, according to the dilution table.

The amount of ethanol present in the assay should range between 0.6 µg and 12 µg. The sample solution must therefore be diluted sufficiently to yield an ethanol concentration between 0.006 and 0.12 g/l

Due to the high sensitivity of the method care must be taken to use ethanol-free water and to work in an ethanol-free atmosphere.

Dilution table

Estimated amount of ethanol per liter	Dilution with water (in parts by volume)	Dilution factor F
6 - 120 g	1 up to 1000	1000
>120 g	1 up to 10000	10000

Because of the volatility of ethanol, the dilution of samples should be carried out as follows:

Fill half of the volumetric flask with redist. water and pipette the sample under the surface of water, using an enzyme test pipette or a piston type pipette. Fill up to the mark with redist. water and mix.

Procedure

Pipette into tubes	Blank	Sample
reagent solution	3.000 ml	3.000 ml
Read absorbances of the solutions (A_1). Start reaction by the addition of:		
diluted sample*)	-**)	0.100 ml
Mix. After completion of the reaction (approx. 5-10 min) read absorbances of the solutions immediately one after the other (A_2).		

*) Note: Glass pipettes must be pre-rinsed with the (diluted) sample solution at least 6 times, and the plastic tips of piston type pipettes 3 times. It is also recommended to pipette the sample solution under the surface of the reagent solution in the cuvette.
It is essential to stopper the cuvettes during measurement (see "Instructions for performance of assay") e.g. with Parafilm (trademark of American Can Company, Greenwich, Ct., USA) in order to prevent the assay mixture form adsorption of ethanol form the air.

**) Pipetting of 0.100 ml redist. water is not necessary.

Calculation

Determine the absorbance differences (A_2-A_1) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$$

$$c = \frac{0.714}{\varepsilon} \times \Delta A \times F \text{ [g ethanol/l sample]}$$

F = diluition factor
ε = extinction coefficient of NADH at:
340 nm = 6.30 [l × mmol^{-1} × cm^{-1}]
Hg 365 nm = 3.40 [l × mmol^{-1} × cm^{-1}]
Hg 334 nm = 6.18 [l × mmol^{-1} × cm^{-1}]

$$\text{Percentage}_{Ethanol} = \frac{c_{Ethanol} \text{ [g/l sample solution]}}{10 \times 0.78924 \text{ [g/ml]}} \quad \text{[\%; v/v]}$$

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Ethanol assay control solution (Bottle 4)

Concentration: see bottle label

Ethanol assay control solution is a stabilized aqueous solution of ethanol. It serves as an assay control solution for the enzymatic determination of ethanol in foodstuffs and other materials.

Application:

1. Addition of ethanol assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay.

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring of A_2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 5 -10 min). Calculate the concentration from the difference of (A_3-A_2) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the data stated on the bottle label.

3. Internal standard:
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
reaction mixture 2	3.000 ml	3.000 ml	3.000 ml	3.000 ml
redist. water	0.100 ml	-	-	-
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
Mix, and read absorbances of the solutions (A_1) after approx. 3 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

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