Megazyme

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ACETALDEHYDE

ASSAY PROCEDURE

K-ACHYD 01/20

(*50 Manual Assays per Kit) or
(500 Auto-Analyser Assays per Kit) or
(500 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

Acetaldehyde is generated in many metabolic processes and it therefore occurs in all living organisms.[1] Wherever fermentation processes play a role in the production of food and beverages, the concentration of acetaldehyde rises considerably. Acetaldehyde has been found in wine at concentrations up to 100 mg/L and beer at up to 20 mg/L. Acetaldehyde in human blood originates from ethanol that has been ingested in foods and beverages. Extreme ethanol consumption can lead to acetaldehyde poisoning following oxidation of the ethanol in the liver by alcohol dehydrogenase.

PRINCIPLE:

Acetaldehyde is quantitatively oxidised to acetic acid in the presence of aldehyde dehydrogenase (Al-DH) and nicotinamide-adenine dinucleotide (NAD^+) (1).

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

The amount of NADH formed in this reaction is stoichiometric with the amount of acetaldehyde. It is the NADH that is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Al-DH acts on aldehydes other than acetaldehyde but at a reduced rate. Under the assay conditions described, acetaldehyde and propionaldehyde are quantitatively converted, as is glycoaldehyde if the reaction time is extended to ~ 20 min. The presence of benzaldehyde and glyceraldehyde may lead to sample dependent creep reactions, that can be accounted for by extrapolation back to the time of addition of Al-DH.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.044 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.176 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.5 to 20 µg of acetaldehyde per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to an acetaldehyde concentration of approx. 0.044 to 0.088 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

Compounds in animal extracts do not interfere with the assay. However, polyphenolics from plant materials may reduce the speed of the reaction.

Alcohols interfere with the assay only when present at very high concentrations. The assay is not affected by reducing substances such as ascorbic acid and sulphur dioxide.

If the conversion of acetaldehyde has been completed within the time specified in the assay (approx. 3-4 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding acetaldehyde (approx. 5 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding acetaldehyde to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays in manual format (or 500 assays in auto-analyser format or 500 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (12 mL, pH 9.0) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: NAD^+.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: Aldehyde dehydrogenase solution (2.75 mL).
Stable for > 2 years below -10°C.

Bottle 4: Acetaldehyde control powder. Acetaldehyde ammonia trimer (~ 2 g).
Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl bottle 3 to mix contents before use.**
Stable for > 2 years below -10°C.

4. Dissolve approx. 80 mg of acetaldehyde ammonia trimer (which corresponds to approx. 50 mg of acetaldehyde) in 1 L of distilled water. Store in a well-sealed Duran® bottle and use on the day of preparation (see safety note on page 2 & sample handling note on page 9).

NOTE: The acetaldehyde standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of acetaldehyde is determined directly from the extinction coefficient of NADH (see page 6).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL and 100 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of Buffer 1 and NAD^+ solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Note: It is essential to stopper the cuvettes
Temperature: ~ 25°C
Final volume: 2.55 mL
Sample solution: 0.5-20.0 µg of acetaldehyde per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample solution	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NAD^+)	0.20 mL	0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:		
solution 3 (Al-DH)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 3-4 min). If the reaction has not stopped after 3-4 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of solution 3 (using MegaCalc™).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{acetaldehyde}$.

The value of $\Delta A_{acetaldehyde}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of acetaldehyde can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{acetaldehyde} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of acetaldehyde [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for acetaldehyde:

$$c = \frac{2.55 \times 44.05}{6300 \times 1.0 \times 0.10} \times \Delta A_{acetaldehyde} \quad [g/L]$$

$$= 0.1783 \times \Delta A_{acetaldehyde} \quad [g/L]$$

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

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of acetaldehyde

$$= \frac{c_{acetaldehyde}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for acetaldehyde can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of acetaldehyde **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water solution 1 (buffer) solution 2 (NAD^+)	48.68 mL 5.50 mL 5.50 mL (after adding 12 mL of H_2O to bottle 2)
Total volume	59.68 mL

Preparation of R2:

Component	Volume
distilled water solution 3 (Al-DH)	6.00 mL 1.35 mL
Total volume	7.35 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 4 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.183 g/L of acetaldehyde using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for acetaldehyde can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of acetaldehyde **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.255 mL
Linearity: 0.1-2.0 µg of acetaldehyde per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NAD^+)	0.020 mL	0.020 mL	0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
solution 3 (Al-DH)	0.005 mL	0.005 mL	0.005 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 4 min). If the reaction has not stopped after 4 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min**.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of solution 3.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of acetaldehyde present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.5 and 20 µg. The sample solution must therefore be diluted sufficiently to yield an acetaldehyde concentration between 0.005 and 0.20 g/L.

Dilution Table

Estimated concentration of acetaldehyde (g/L)	Dilution with water	Dilution factor (F)
< 0.20	No dilution required	1
0.20-2.00	1 + 9	10
2.00-20.0	1 + 99	100
20.0-200	1 + 999	1000
> 200	1 + 9999	10000

If the value of $\Delta A_{acetaldehyde}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample handling.

(a) Acetaldehyde is extremely volatile (boiling point ~ 20°C), so all samples and assay controls must be stored in tightly closed containers.

(b) Solutions containing acetaldehyde should always be dispensed into the buffer or aqueous solutions to minimise loss through evaporation.

(c) Acetaldehyde is readily oxidised by atmospheric oxygen. Consequently, it is essential to analyse samples as soon as possible after preparation. Control solutions should be used on the day of preparation.

(d) The preferred assay control material is acetaldehyde ammonia trimer as it is less volatile than acetaldehyde and is not as readily oxidised or polymerised.

3. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

4. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be analysed undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 9.0 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH of the solution to approx. 9.0 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no Al-DH, may need to be performed in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of activated carbon. Stir for 2 min and then filter.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for

15-30 min and then filter. Discard the first few mL of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of acetaldehyde in white wine.

Adjust the pH of white wine to approx. 9.0 with 2 M NaOH and use immediately for assay (0.10-0.50 mL). If the wine contains sulphite, then "total aldehyde" is measured, i.e. the sum of free and sulphite-bound acetaldehyde. *Typically, no dilution will be required and a sample volume of 0.2 mL is satisfactory.*

(b) Determination of acetaldehyde in red wine.

If necessary, decolourise red wine before using in the assay. This is achieved as follows: in a sealed Duran® bottle, mix 25 mL of red wine with 0.5 g of polyvinylpolypyrrolidone (PVPP), stir for approx. 5 min and then immediately filter an aliquot of the slurry through Whatman No. 1 filter paper. Collect into a Duran® bottle and seal immediately after collection. Use the clear, essentially colourless filtrate for assay. *Typically, no dilution will be required and a sample volume of 0.2 mL is satisfactory.*

(c) Determination of acetaldehyde in beer and champagne.

De-gas samples containing carbon dioxide by increasing the pH to approx. 9.0 with 2 M NaOH and gentle stirring. *Typically, no dilution will be required and a sample volume of 0.2 mL is satisfactory.*

(d) Determination of acetaldehyde in fruit juice, concentrates and related beverages.

The acetaldehyde concentration of clear, neutral solutions can generally be determined without any sample treatment (except dilution according to the dilution table and pH adjustment to approx. 9.0). Turbid liquids generally only require filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an appropriate acetaldehyde concentration. However, if coloured solutions require analysis undiluted, they may need decolourising as follows: in a sealed Duran® bottle, mix 25 mL of red wine with 0.5 g of polyvinylpolypyrrolidone (PVPP), stir for approx. 5 min and then immediately filter an aliquot of the slurry through Whatman No. 1 filter paper. Collect into a Duran® bottle and seal immediately after collection. *Typically, no dilution will be required and a sample volume of 0.2 mL is satisfactory.*

(e) Determination of acetaldehyde in vegetable products.

Homogenise vegetable products in a kitchen blender. Accurately weigh the homogenised sample into a 100 mL volumetric flask, add 60 mL of distilled water and shake the closed flask vigorously to extract the acetaldehyde. Fill the volumetric flask to the mark and clarify an aliquot by filtration through Whatman GF/A glass fibre filter paper. Collect into a Duran® bottle and seal immediately after collection. Further clarify with Carrez reagents if required. Dilute if necessary, and use a sample volume of 0.10-0.50 mL. *Typically, no dilution will be required and a sample volume of 0.2 mL is satisfactory.*

(f) Determination of acetaldehyde in liqueurs such as brandy.

No pre-treatment of brandies is required. *Typically, a dilution of 1:2 and a sample volume of 0.2 mL are satisfactory.*

REFERENCE:

Beutler, H. O. (1988). Acetaldehyde. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 606-613, VCH Publishers (UK) Ltd., Cambridge, UK.

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