Megazyme

www.megazyme.com

ACETIC ACID
(Rapid, Manual, Simple and End-Point AK/PTA Format)

ASSAY PROCEDURE

K-ACETRM 04/20

(*72 Manual Assays per Kit) or
(720 Microplate Assays per Kit)

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

INTRODUCTION:

Given its importance and ubiquitous occurrence in foods, beverages and other materials, acetic acid ranks amongst the most measured analytes. However, unlike some other common analytes, traditional enzymatic methods for this acid present significant limitations. For instance, manual acetyl-coenzyme A synthetase (ACS) based formats are typified by unstable reagents during use, slow reaction times, multiple cuvette additions/absorbance readings and complex calculations. While the advanced acetic acid kit (**K-ACET**) recently developed by Megazyme overcomes the key stability issue relating to the reconstitution of ACS for manual use, the overall format of the assay is still relatively inconvenient, requiring multiple cuvette additions and time-consuming data recording/processing.
Thus Megazyme developed the current kit (**K-ACETRM**) based on acetate kinase (AK) and phosphotransacetylase (PTA) specifically to overcome these limitations experienced by the analyst employing manual assay procedures. Unlike ACS based kits, this product is rapid (approx. 4 min), does not present reagent stability issues, is simple to perform, has extended linearity and gives a change in absorbance stoichiometric with the concentration of acetic acid (i.e. no complex equations are required and the raw absorbance data is processed like most other enzymatic kits). In common with other Megazyme acetic acid kits, **K-ACETRM** also benefits from > 2 years stability during use, as the key enzyme components are supplied as ready-to-use ammonium sulphate suspensions. Polyvinylpyrrolidone (PVP) has also been incorporated into the assay system to prevent interference from particular tannins found in wines (especially red wines). As the pH (7.4) of the buffer system employed is significantly lower than in traditional ACS based kits (8.4), interference from spontaneous ester hydrolysis (causing creep reactions) is also minimised.

PRINCIPLE:

Acetate kinase (AK) in the presence of ATP converts acetic acid (acetate) into acetyl-phosphate and adenosine-5'-diphosphate (ADP) (1).

$$\text{(1)}\quad \text{Acetic acid} + \text{ATP} \xrightarrow{\text{(AK)}} \text{acetyl-phosphate} + \text{ADP}$$

This reaction is significantly accelerated by the rapid conversion of the acetyl-phosphate product into acetyl-CoA and inorganic phosphate, by the action of phosphotransacetylase (PTA) in the presence of coenzyme A (CoA) (2).

(2) Acetyl-phosphate + CoA $\xrightarrow{\text{(PTA)}}$ acetyl-CoA + P_i

The ADP formed in (1) is reconverted into ATP and pyruvate, by phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK) (3).

(3) ADP + PEP $\xrightarrow{\text{(PK)}}$ pyruvate + ATP

In the presence of the enzyme D-lactate dehydrogenase (D-LDH), pyruvate is reduced to D-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the production of NAD^+ (4).

(4) Pyruvate + NADH + H^+ $\xrightarrow{\text{(D-LDH)}}$ D-lactic acid + NAD^+

The amount of NAD^+ formed in the above reaction pathway is stoichiometric with the amount of acetic acid. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for acetic acid.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.063 mg of acetic acid/L of sample solution at the maximum sample volume of 2.00 mL (or to 1.27 mg/L with a sample volume of 0.1 mL). The detection limit is 0.254 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 0.3 to 25 µg of acetic acid 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 2.0 mL, this corresponds to an acetic acid concentration of approx. 0.063 to 0.127 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:

If the conversion of acetic acid has been completed within the time specified in the assay (approx. 4 min), it can generally be concluded that no interference has occurred. However, this can be further checked by adding acetic acid (10 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease 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 acetic acid 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 72 assays in manual format (or 720 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (24 mL, pH 7.4) and sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	NADH, ATP, PEP and PVP. Lyophilised powder. Stable for > 2 years below -10°C.
Bottle 3: (x2)	CoA, lyophilised powder. Stable for > 2 years below -10°C.
Bottle 4:	D-Lactate dehydrogenase, phosphotransacetylase and pyruvate kinase suspension (1.5 mL). Stable for > 2 years at 4°C.
Bottle 5:	Acetate kinase suspension (1.5 mL). Stable for > 2 years at 4°C.
Bottle 6:	Acetic acid standard solution (5 mL, 0.10 mg/mL). 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 15 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. Dissolve the contents of one of bottle 3 in 0.8 mL of distilled water and store below -10°C between use, and keep cool during use if possible. Once dissolved the reagent is stable for > 2 years below -10°C. **Do not** dissolve the contents of the second bottle until required.

NOTE: To ensure recovery of sufficient volume, do not invert bottle 3 during dissolution, and always store in an upright position.

4 & 5. Use the contents of bottles 4 and 5 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottles to mix contents before use.**
Stable for > 2 years at 4°C.

6. Use the contents of bottles 6 as supplied.
Stable for > 2 years; store sealed at 4°C.

NOTE: The acetic acid 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 acetic acid is determined directly from the extinction coefficient of NADH (see page 6).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.3 mL aliquots of buffer and 0.2 mL aliquots of NADH/ATP/PEP/PVP 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) and GF/A glass fibre filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.66 mL
Sample solution: 0.3-25.0 µg of acetic acid 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	-	0.10 mL
solution 1 (buffer)	0.30 mL	0.30 mL
solution 2 (NADH/ATP/PEP/PVP buffer)	0.20 mL	0.20 mL
solution 3 (CoA)	0.02 mL	0.02 mL
solution 4 (D-LDH/PTA/PK)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
suspension 5 (AK)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 4 min).		

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

CALCULATION:

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

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

The concentration of acetic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of acetic acid [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 acetic acid:

$$c = \frac{2.66 \times 60.05}{6300 \times 1.0 \times 0.10} \times \Delta A_{\text{acetic acid}} \quad [g/L]$$

$$= 0.2535 \times \Delta A_{\text{acetic acid}} \quad [g/L]$$

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 acetic acid

$$= \frac{c_{\text{acetic acid}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

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

B. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for acetic acid 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 acetic acid **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: 0.266 mL
Linearity: 0.1-2.5 µg of acetic acid 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	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.030 mL	0.030 mL	0.030 mL
solution 2 (NADH/ATP/PEP/PVP)	0.020 mL	0.020 mL	0.020 mL
solution 3 (CoA)	0.002 mL	0.002 mL	0.002 mL
solution 4 (D-LDH/PTA/PK)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 5 (AK)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 4 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).

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 acetic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.3 and 25 µg. The sample solution must therefore be diluted sufficiently to yield an acetic acid concentration between 0.0003 and 0.25 g/L.

Dilution Table

Estimated concentration of acetic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.25	No dilution required	1
0.25-2.5	1 + 9	10
2.5-25	1 + 99	100
> 25	1 + 999	1000

If the value of $\Delta A_{acetic\ acid}$ 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 2.0 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.1 mL and using the new sample volume in the equation.

2. Sample handling.

Acetic acid is volatile, so care should be taken when drying or otherwise heating samples containing this analyte in the acid form. Problems associated with the volatility of acetic acid can be minimised by converting it to the salt form (e.g. sodium acetate or potassium acetate). This is achieved by adjusting the pH of the sample to approx. 7.5 using 1 M NaOH or KOH before drying or heating at elevated temperatures.

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. 7.4 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 to approx. 7.4 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 AK, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(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 acetic acid in wine.

For white wine, use 0.10 mL in the assay. Volumes up to 2.0 mL can be used for samples containing low acid content.

For red wine containing approx. 0.2 g of acetic acid/L, use 0.10 mL of sample without decolourising in the assay. For red wine containing less than 0.1 g of acetic acid/L, decolourise by adding 0.2 g of PVPP per 10 mL of sample and stir for 5 min. Filter an aliquot through Whatman No. 1 filter paper and adjust the pH to approx. 7.4. Adjust the volume to twice that of the original volume of sample taken. Use up to 2.0 mL of sample in the assay, and allow for the dilution and sample volume in the calculations.

When using large sample volumes, the high alcohol concentration of wine samples may retard the activity of the enzymes used in the determination of acetate. In such cases, increase the assay incubation time to 20 min, and take subsequent measurements of absorbance values to confirm the reaction has finished. *Typically, a dilution of 1:5 and a sample volume of 0.1 mL are satisfactory.*

(b) Determination of acetic acid in fruit juices.

With fruit juices containing a high level of acetic acid (approx. 0.3 g/L), dilute an aliquot of the sample with an equal volume of water and use 0.1 mL for assay. If a large volume of sample is required, adjust the pH of the solution to approx. 7.4 before analysis. Coloured juices should be decolourised as described in "General considerations (e)" on page 10. Use 0.10 to 2.00 mL of sample for the assay (adjust to pH 7.4 if larger volumes are required). *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(c) Determination of acetic acid in vinegar.

Dilute the sample according to the dilution table for the assay. *Typically, a dilution of 1:500 and sample volume of 0.1 mL are satisfactory.*

(d) Determination of acetic acid in sour dressings and sauces.

Separate the solids from the liquid component. Add 1 g of sample to 40 mL of water and adjust the volume to 100 mL. Store the solution at 4°C for 20 min to obtain separation of fat. Filter an aliquot of the aqueous layer, discarding the first few mL. Dilute an aliquot of the filtrate according to the dilution table (on Page 9), if necessary. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(e) Determination of acetic acid in beer.

Degass the beer by filtration or by stirring for 5 min with a glass rod. Analyse the sample without dilution. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

(f) Determination of acetic acid in hard cheese.

Accurately weigh approx. 2 g of ground cheese into a 100 mL volumetric flask and add 60 mL of distilled water. Incubate the flask at approx. 60°C for 20 min, with intermittent shaking. Cool the flask to 20-25°C and fill to the mark with distilled water. Store the flask at 4°C for 30-60 min and then filter an aliquot of the solution through Whatman GF/A glass fibre filter paper. Use the clear filtrate in the assay. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

(g) Determination of acetic acid in mayonnaise or yogurt.

Accurately weigh approx. 5 g of sample into a 100 mL volumetric flask and add approx. 50 mL of distilled water. Heat in a water bath at 50-60°C for 20 min with intermittent shaking. Cool the flask to approx. 20°C and adjust to the mark with distilled water. Place the flask in a refrigerator for 30 min. Filter the solution through Whatman GF/A glass fibre filter paper and use the clear or slightly turbid solution for the assay. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(h) Determination of acetic acid in biological samples.

If necessary, heat biological samples at approx. 80°C for 20 min to denature any enzymes present that may interfere with the assay (alternatively, deproteinise using Carrez reagents as described on page 9). After filtering, use the clear supernatant, with dilution if necessary, for the assay. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

REFERENCES:

1. Beutler, H. O. (1988). Determination with Acetyl-CoA Synthetase. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 639-645, VCH Publishers (UK) Ltd., Cambridge, UK.

2. AOAC Official Methods of Analysis (2002). 17th ed., Chapter 32, pp. 47-48.

3. Green, A. (1971). "*Biochemistry of fruits and their products*." (Hulme, C. H., ed.), Vol 2, Chapter 11, Academic Press, London and New York.

4. Chemistry of Winemaking (1964). Advances in Chemistry Series, 137, A. Dinsmoor Webb. American Chemical Society, pp. 136-137.

5. Rankine, B. (2002). *Making good wine*. Pan Macmillan Australia Pty. Ltd., Sydney, Australia.

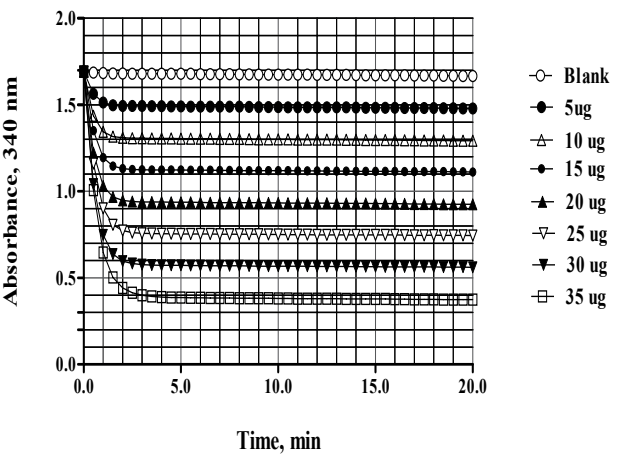

Figure 1. Decrease in absorbance at 340 nm on incubation of 0-35 µg of acetic acid with acetate kinase in the acetic acid AK/PTA format.

NOTES:

Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com