Megazyme

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ACETIC ACID

ASSAY PROCEDURE

K-ACET 04/18

(*53 Assays per Kit)

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

INTRODUCTION:

Acetic acid (acetate) occurs in a wide range of foods and beverages, and in many other materials such as paper, pharmaceuticals and industrial products. In the wine industry, it is one of the most important quality parameters and is measured throughout the entire vinification process. This advanced kit (**K-ACET**) benefits from greatly increased shelf life as the acetyl-coenzyme A synthetase (ACS) is supplied as a highly stable ready-to-use ammonium sulphate suspension, rather than as a lyophilised powder. Polyvinylpyrrolidone (PVP) has also been incorporated into the assay to prevent interference from particular tannins found especially in red wines.

This kit is recommended for manual analysis, while two additional kits have been optimised for auto-analysis applications; **K-ACETAF** has extended calibration linearity up to 30 µg/mL (in the final assay), and also benefits from a stabilisation system that significantly reduces the rate of deterioration (increase in absorbance) of prepared reagent, a problem exhibited by some other products; **K-ACETAK** is a new assay format based on acetate kinase. As an indicator reaction is not involved in this case, a change in absorbance stoichiometric with the amount of acetic acid is achieved, resulting in excellent linear calibration ($R^2 > 0.999$). Booklets for both auto-analyser format kits are available at www.megazyme.com.

PRINCIPLE:

Acetyl-coenzyme A synthetase (ACS) in the presence of adenosine-5'-triphosphate (ATP) and coenzyme A (CoA) converts acetic acid (acetate) into acetyl-CoA (1), with the formation of adenosine-5'-monophosphate (AMP) and pyrophosphate. Citrate synthase (CS), in the presence of acetyl-CoA, converts oxaloacetate into citrate (2). The oxaloacetate required in reaction (2) is formed from L-malate and nicotinamide-adenine dinucleotide (NAD^+) in the presence of L-malate dehydrogenase (L-MDH) (3). In this reaction, NAD^+ is reduced to NADH.

$$(1)\quad \text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{(ACS)}} \text{acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

$$(2)\quad \text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\text{(CS)}} \text{citrate} + \text{CoA}$$

$$(3)\quad \text{L-Malate} + NAD^+ \underset{}{\overset{\text{(L-MDH)}}{\rightleftharpoons}} \text{oxaloacetate} + \text{NADH} + H^+$$

The determination is based on the formation of NADH which is measured by the increase in absorbance at 340 nm. As the preceding reaction is an equilibrium reaction, the amount of acetic acid present must be calculated by way of the equation on page 6.

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 0.07 mg/L of sample solution at the maximum sample volume of 2.0 mL (or to 1.4 mg/L with a sample volume of 0.1 mL). The detection limit is 0.14 mg/L, which is derived from an absorbance difference of 0.010 with the maximum sample volume of 2.0 mL.

The assay is linear over the range of 0.3 to 20 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 2.00 mL, this corresponds to an acetic acid concentration of approx. 0.07 to 0.14 mg/L of sample solution. Reproducibility data for a range of samples has been published by Beutler[1] with c.v. values ranging from 0.6 to 2.8% for various samples. 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:

Acetic acid esters (e.g. ethyl acetate) are frequently found in the presence of acetic acid. Ethyl acetate is slowly hydrolysed under assay conditions and is responsible for "creep" reactions. The effect of acetic acid esters may be eliminated by extrapolating the value of A_2 back to the time of ACS addition. This is most easily performed by taking additional absorbance readings at 16 and 20 min and using the MegaCalc™ Excel based calculation tool (available free from where this kit appears on the Megazyme website). This corrected A_2 value will provide the acetic acid concentration. Total acetate concentration (including esters) can be determined by allowing the reaction to reach the end-point (until the absorbance value stabilises).

If the conversion of acetic acid has been completed within the time specified in the assay (approx. 10-12 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding acetic acid (approx. 10 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 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 53 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (30 mL, pH 8.4) plus L-malic acid and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+ plus ATP, PVP and CoA.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: L-Malate dehydrogenase plus citrate synthase suspension, 1.1 mL.
Stable for > 2 years at 4°C.

Bottle 4: Acetyl-coenzyme A synthetase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 5: Acetic acid standard solution (5 mL, 0.10 mg/mL).
Stable for > 2 years 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 5.5 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes 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.

3 & 4. Use the contents of bottles 3 and 4 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.

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years 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 0.2 mL aliquots of NAD^+/ATP/PVP/CoA solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water and 0.5 mL aliquots of buffer).
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.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.84 mL
Sample solution: 0.3-20.0 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)	0.50 mL	0.50 mL
solution 2 (NAD^+/ATP/PVP/CoA)	0.20 mL	0.20 mL
Mix*, read the absorbances of the solutions (A_0) after approx. 3 min and start the reactions by addition of:		
suspension 3 (L-MDH/CS)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
suspension 4 (ACS)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 12 min). If the reaction has not stopped after 12 min, continue to read the absorbances at 4 min intervals until 20 min, and then use the Mega-Calc™ Excel based tool to take into account the "creep" rate.		

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

CALCULATION:

If the absorbance (A_2) of the sample slowly increases after the initial rapid reaction, this linear "creep" rate should be taken into account by taking additional absorbance readings at 16 and 20 min. This data can be input into the Mega-Calc™ calculator, yielding a corrected A_2 value.

If not using Mega-Calc™ determine the absorbance differences (A_1-A_0) and (A_2-A_0) for both blank and sample. With the equilibrium of the preceding indicator reaction (3), there **is not** a linear proportionality between the measured absorbance difference and the acetic acid

concentration, and thus $\Delta A_{\text{acetic acid}}$ must be calculated using the equation below:

$$\Delta A_{\text{acetic acid}} = \left[(A_2\text{-}A_0)_{\text{sample}} - \frac{(A_1\text{-}A_0)^2_{\text{sample}}}{(A_2\text{-}A_0)_{\text{sample}}} \right] - \left[(A_2\text{-}A_0)_{\text{blank}} - \frac{(A_1\text{-}A_0)^2_{\text{blank}}}{(A_2\text{-}A_0)_{\text{blank}}} \right]$$

The value 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:

$$c = \frac{2.84 \times 60.05}{6300 \times 1 \times 0.10} \times \Delta A_{\text{acetic acid}} \quad [g/L]$$

$$= 0.2707 \times \Delta A_{\text{acetic acid}} \quad [g/L]$$

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).

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 20 µg. The sample solution must therefore be diluted sufficiently to yield an acetic acid concentration between 0.03 and 0.20 g/L.

Dilution Table

Estimated concentration of acetic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.20	No dilution required	1
0.20-2.0	1 + 9	10
2.0-20	1 + 99	100
> 20	1 + 999	1000

If the value of $\Delta A_{\text{acetic acid}}$ 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. 8.4 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. 8.4 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 ACS, 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. 8.4. 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 incubation times 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. 8.4 before analysis. Coloured juices should be decolourised as described in "General considerations (e)" (page 8). Use 0.10 to 2.00 mL of sample for the assay (adjust to pH 8.4 if larger volumes are required). *Typically, no dilution is required and 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, 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.*

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.

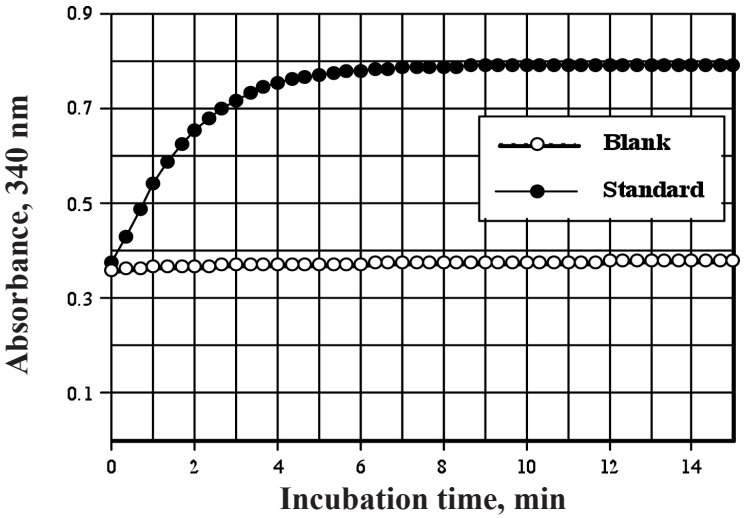

Figure 1. Increase in absorbance at 340 nm on incubation of 15 µg of acetic acid with L-malate dehydrogenase, citrate synthase and acetyl-coenzyme A synthetase in the presence of NAD^+.

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