Megazyme

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D-MALIC ACID (D-MALATE)

ASSAY PROCEDURE

K-DMAL 08/18

(*100 Manual Assays per Kit) or
(1100 Auto-Analyser Assays per Kit) or
(1000 Microplate Assays per Kit)

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

INTRODUCTION:

In Nature, essentially all malic acid is produced in the L-racemic form (the exception being a few micro-organisms which produce D-malic acid). Freshly pressed fruit juices contain D-malic acid at the detection limit (~ 10 mg/L). Malic acid is produced commercially in the D-/L-racemic mixture by the catalytic hydration of maleic anhydride, and can replace citric acid in food products as an acidulant and flavour enhancer. It finds application in flavoured fruit drinks, juices and wines. The detection of D-malic acid in juices or wine indicates that it has been added. The legal situation concerning the addition of D-/L-malic acid to juice or wine varies between countries.

PRINCIPLE:

D-Malic acid (D-malate), in the presence of D-malate dehydrogenase (D-MDH), is quantitatively oxidised to oxaloacetate by nicotinamide-adenine dinucleotide (NAD^+). In turn, D-MDH converts the oxaloacetate to pyruvate and carbon dioxide (1).

$$\text{(1)}\quad \text{D-Malate} + NAD^+ \xrightarrow{\text{(D-MDH)}} \text{pyruvate} + CO_2 + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of D-malic acid. It is the NADH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

D-MDH rapidly oxidises D-malic acid. A slight side activity on L-tartaric acid is also observed; on incubating D-MDH with D-malic acid and L-tartaric acid at the same concentration (i.e. 40 µg per assay of each), the reaction with D-malic acid is unaffected and complete within 5 min, with only a small absorbance contribution from conversion of the L-tartaric acid (i.e. < 3% of that obtained from the D-malic acid). Where there is a high surplus of L-tartaric acid, proceed as described for the determination of D-malic acid in red and white wine [sample preparation example (a), page 10].

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.13 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.26 mg/L, which is derived from an absorbance difference of 0.010 with the maximum sample volume of 2.00 mL. The assay is linear over the range of 0.5 to 40 µg of D-malic 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 a D-malic acid concentration of approx. 0.13 to 0.26 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 D-malic acid has been completed within the time specified in the assay (approx. 6 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-malic acid (approx. 20 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 D-malic acid to the sample in the initial extraction steps.

Tannins in red wine may give rise to a slight inhibition of the assay; this should be checked if there is any doubt. If there is a non-enzymic, slow increase in absorbance, incorporate a sample blank that contains all components, except the D-MDH, and should be measured immediately after the reaction that contains the D-MDH. Use the obtained values in the calculation as follows:

$$\Delta A_{\text{D-malic acid}} = (A_2\text{-}A_1)_{\text{sample}} - (A_2\text{-}A_1)_{\text{reagent blank}} - (A_2\text{-}A_1)_{\text{sample blank}}$$

The accuracy of the absorbance readings can be assessed by including an internal standard as control. This should be performed using the supplied D-malic acid standard solution.

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 100 assays in manual format (or 1100 assays in auto-analyser format or 1000 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 8.0) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+.
Stable for > 5 years below -10°C.

Bottle 3: D-Malate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C or between -10°C to -15°C for > 4 years.

Bottle 4: D-Malic acid standard solution (5 mL, 0.2 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 one of bottle 2 in 11 mL of distilled water. **Stable for > 1 year 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 the bottle to mix contents before use.**
   Stable for > 2 years at 4°C or between -10°C to -15°C for > 4 years.

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

NOTE: The D-malic 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 D-malic acid 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 and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of NAD^+ solution and of buffer (bottle 1)].
   - 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)
Temperature: ~ 25°C
Final volume: 2.42 mL
Sample solution: 0.5-40 µg of D-malic acid per cuvette (in 0.1-2.0 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)	2.00 mL	1.00 mL
sample	-	1.00 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NAD^+)	0.20 mL	0.20 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 3 (D-MDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or 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®.

** for apple juice, the reaction is complete after 5-6 min, 10 min is required for orange and grapefruit juice and approx. 12 min for red and white wine.

CALCULATION:

If the absorbance (A_2) of the sample increases at a greater rate than that of the blank, extrapolate both values back to the time of addition of suspension 3 (D-MDH).

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 $\Delta A_{\text{D-malic acid}}$.

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

The concentration of D-malic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of D-malic 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 D-malic acid:

$$c = \frac{2.42 \times 134.09}{6300 \times 1.0 \times 1.0} \times \Delta A_{\text{D-malic acid}} \quad [g/L]$$

$$= 0.0515 \times \Delta A_{\text{D-malic acid}} \quad [g/L]$$

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 D-malic acid

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

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 D-malic 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 D-malic acid **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^+)	45.6 mL 5.5 mL 5.5 mL (after adding 11 mL of H_2O to bottle 2)
Total volume	56.6 mL

Preparation of R2:

Component	Volume
distilled water suspension 3 (D-MDH)	6.53 mL 0.55 mL
Total volume	7.08 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.38 g/L of D-malic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-malic 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 D-malic 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: 0.242 mL
Linearity: 0.1-4 µg of D-malic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.200 mL	0.100 mL	0.100 mL
sample solution	-	0.100 mL	-
standard solution	-	-	0.100 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* and read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions by addition of:			
suspension 3 (D-MDH)	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. 6 min). If the reaction has not stopped after 6 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 suspension 3.

CALCULATION (Microplate Assay Procedure):

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

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 D-malic acid present in the cuvette (i.e. in the 1.0 mL of sample being analysed) should range between 0.5 and 40 µg. The sample solution must therefore be diluted sufficiently to yield a D-malic acid concentration between 0.005 and 0.04 g/L.

Dilution Table

Estimated concentration of D-malic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.04	No dilution required	1
0.04-0.4	1 + 9	10
0.4-4.0	1 + 99	100

If the value of $\Delta A_{\text{D-malic acid}}$ 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 to 2.00 mL, making sure that the sum of the sample and the distilled water components in the reaction is 2.00 mL and using the new sample volume in the equation.

2. 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.

3. 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 used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 8.0 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 to approx. 8.0 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: a sample blank, i.e. sample with no D-MDH, 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 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 by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-malic acid in red and white wine.
Add 125 mg of calcium hydroxide to 25 mL of wine and mix for 2 min. Adjust the pH to 7-8 with 1 M KOH, quantitatively transfer the solution to a 50 mL volumetric flask and fill to the mark with distilled water. Transfer the solution to a 100 mL beaker, add 0.5 g of PVPP and stir the suspension for 5 min on a magnetic stirrer. Filter an aliquot of the solution through Whatman No. 1 (9 cm) filter paper and use 1.0-2.0 mL of the clear, colourless filtrate in the assay. Determine the absorbance difference (A_2-A_1) for both blank and

sample after approx. 12 min, with subsequent measurements at 3 min intervals to determine the creep rate (if necessary).

(b) Determination of D-malic acid in apple juice.

Adjust 25 mL of the apple juice to pH of approx. 7-8 with 2 M KOH, transfer the solution to a 50 mL volumetric flask and fill to the mark with distilled water. Transfer the solution to a 100 mL beaker, add 0.5 g of PVPP and stir the suspension for 5 min on a magnetic stirrer. Filter an aliquot of the solution through Whatman No. 1 (9 cm) filter paper and use 1.0-2.0 mL of the clear, colourless filtrate in the assay. Determine the absorbance difference (A_2-A_1) for both blank and sample after approx. 5-6 min.

(c) Determination of D-malic acid in juice from citrus fruits (e.g. grapefruit and orange).

Adjust 25 mL of the fruit juice to pH of approx. 7-8 with 2 M KOH, transfer the solution to a 50 mL volumetric flask and fill to the mark with distilled water. Transfer the solution to a 100 mL beaker, add 0.5 g of PVPP and stir the suspension for 5 min on a magnetic stirrer. Filter an aliquot of the solution through Whatman No. 1 (9 cm) filter paper and use 1.0-2.0 mL of the clear, colourless filtrate in the assay. Determine the absorbance difference (A_2-A_1) for both blank and sample after approx. 10 min, with subsequent measurements at 2 min intervals to determine the creep rate (if necessary).

(d) Determination of D-malic acid in intensely coloured juices (e.g. cherry, black and red currant).

Adjust 25 mL of the fruit juice to pH of approx. 7-8 with 2 M KOH and transfer the solution to a 100 mL volumetric flask and fill to the mark with distilled water. Transfer the solution to a 200 mL beaker, add 1 g of PVPP and stir the suspension for 5 min on a magnetic stirrer. Filter an aliquot of the solution through Whatman No. 1 (9 cm) filter paper and use 1.0-2.0 mL of the clear, colourless filtrate in the assay. Determine the absorbance difference (A_2-A_1) for both blank and sample after approx. 12-14 min, with subsequent measurements at 2 min intervals to determine the creep rate (if necessary).

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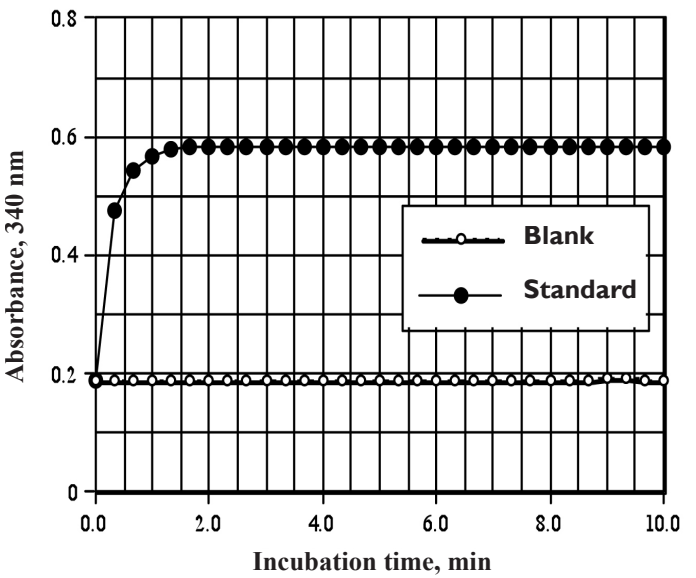

Figure 1. Increase in absorbance at 340 nm on incubation of 20 µg of D-malic acid with hexokinase and glucose 6-phosphate dehydrogenase in the presence of NAD^+.

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