Megazyme

L-MALIC ACID (L-MALATE)

ASSAY PROCEDURE

KLMAL100 02/04

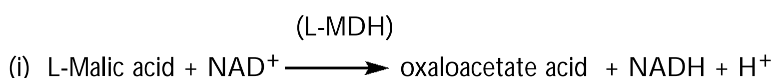
(100 Determinations per Kit)

INTRODUCTION:

As a component of the citric acid cycle, L-malic acid (L-malate) is found in all living organisms. Its quantitative determination is especially important in the manufacture of wine, beer, bread,and fruit and vegetable products,as well as in cosmetics and pharmaceuticals. It is one of the most important fruit acids, and has the highest concentration of all acids in wine. L-Malic acid finds many applications as a food preservative (E296) and flavour enhancing compound, such as in the manufacture of low calorie drinks.

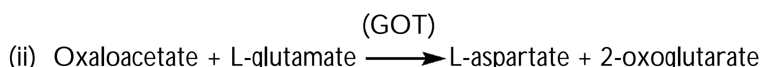
PRINCIPLE:

The detection of L-malic acid requires two enzyme reactions;in the first reaction catalysed by L-malate dehydrogenase (L-MDH), L-malic acid is oxdised to oxaloacetate by nicotinamide-adenine dinucleotide (NAD^+):



$$\text{(i) L-Malic acid} + NAD^+ \xrightarrow{\text{(L-MDH)}} \text{oxaloacetate acid} + NADH + H^+$$

However, since the equilibrium of reaction (i) lies firmly in the favour of L-malic acid and NAD^+, a further reaction is required to "trap" the NADH product,and this is achieved by the conversion of oxaloacetate to L-aspartate and 2-oxoglutarate, in the presence of a large excess of L-glutamate , by glutamate-oxaloacetate transaminase (GOT):



$$\text{(ii) Oxaloacetate} + \text{L-glutamate} \xrightarrow{\text{(GOT)}} \text{L-aspartate} + \text{2-oxoglutarate}$$

The amount of NADH formed in the above coupled reaction is stoichiometric with the amount of L-malic acid in the sample solution. It is the NADH which is measured by monitoring the increase in absorbance at 340 nm.

Methods based on this principle are recommended by IFU, AIJN, MEBAK and OIV, and approved by AOAC International.The method is contained in the food laws of many countries and in European regulations.

ACCURACY:

Relative standard deviations of less than 2 % are achieved. The detection limit is $_{340}$ = 0.010,which corresponds to approximately 0.5 mg of L-malic acid per litre of the solution being assayed. The sensitivity is $_{340}$ = 0.005.

SPECIFICITY:

The assay is specific for L-malic acid. D-malic acid, L-lactic acid, L-aspartic acid and fumaric acid do not react.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (X 2) Glycylglycine buffer, (5 mL, 1 M, pH 10.0), containing 1 M L-glutamate. Store at 4°C.

Bottle 2: NAD^+ (360 mg); freeze dried powder. Store at 4°C.

Bottle 3: Glutamate-oxaloacetate transaminase (GOT) suspension (1.1 mL, 0.30 U/μL). Store at 4°C.

Bottle 4: L-Malate dehydrogenase suspension (1.1 mL, 15 U/μL). Store at 4°C.

Bottle 5: L-Malic acid standard solution (10 mL, 0.15 mg/mL) Store at 4°C.

SAFETY:

The reagents for the determination of L-malic acid are not hazardous. Apply the safety rules normally in use in a chemical laboratory.

PREPARATION OF REAGENTS:

1. Add 45 mL of distilled water to bottle 1. When diluted, this buffer is stable for ~ 6 months at 4°C. Do not dilute the contents of the second bottle until required. Undiluted, the buffer is stable for > 2 years at 4°C.

2. Dissolve contents of bottle 2 in 22 mL distilled water. Divide into appropriately sized aliquots and store at -20°C between use and on ice during use. Stable for 12 months at -20°C.

3. Use contents of bottles 3, 4 and 5 undiluted. Stable for > 2 years at 4°C.

NOTE: The L-malic acid standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used also when the presence of inhibitory compounds in the sample is suspected. Usually, the concentration of L-malic acid can be determined directly from the extinction coefficient of NADH (see page 7).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Cuvettes.- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman (10 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette® - with 5.0 mL Combitip® (to dispense 1.0 mL aliquots of glycylglycine buffer, 0.2 mL aliquots of NAD^+, 0.1 - 1.0 mL of sample solution and 0.8 - 1.0 mL of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA YellowLab Test Tube Shaker TTS).
8. Thermostatted hot-block heater set at 25°C (optional).
9. Stop clock.
10. Whatman No.1 (9 cm) filter papers.

SAMPLE PREPARATION:

The amount of L-malic acid present in the cuvette (i.e. in the 0.1 ml of sample being analysed) should range between 0.5 and 35 µg,and therefore the sample solution should be diluted, if necessary to a concentration of between 0.005 and 0.30 g/L, as illustrated below.

Dilution Table

Estimated concentration of L-malic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.30	No dilution required	1
0.3 – 3.0	1 + 9	10
3.0 – 30	1 + 99	100
> 30	1 + 999	1000

If the absorbance difference measured A ($A_2 - A_1$) for the sample is less than 0.1 absorbance units larger than that of the blank, the sample volume may be increased to 1.0 mL (making sure the sum of the sample and distilled water components in the reaction is 1.0 mL and using the new sample volume "v" in the equation). If A is still too low (e.g. less than 0.1), weigh out more sample, or dilute it less strongly.

Liquid samples: Clear/slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

Acidic samples: If an acidic sample is to be used undiluted (such as red wine or coloured fruit juice), increase the pH of the solution to between 8.0 and 10.0 using 2 M NaOH, and incubate at room temperature for 30 min.

Carbon dioxide: Degass samples containing carbon dioxide either by stirring, e.g. with a glass rod for 5 min, or by filtration.

Coloured samples: Perform a sample blank, i.e. sample with no L-malate dehydrogenase enzyme, in the case of coloured samples (see procedure).

Strongly coloured samples: If used undiluted, decolorise strongly-coloured samples by adding 1 g polyvinylpolypyrrolidone (PVPP) per 100 mL of solution. Stir for 1 min and filter.

Solid samples: Homogenise solid samples, such as fruit products, extract the L-malic acid in distilled water and filter.

Examples:

(a) Determination of L-malic acid in wine.

The free L-malic acid concentration [F] of white and red wine can generally be determined without any sample treatment (except dilution according to the dilution table).

(b) Determination of L-malic acid and its esterified derivatives in wine.

The concentration of both free and esterified L-malic acid [F + E] in white and red wine can be determined as follows: add 6 mL of 2 M NaOH to 20 mL of wine and heat under reflux for 30 min with stirring. After cooling, carefully adjust the pH of the solution to pH 10 with 1 M H_2SO_4 and adjust the volume to 50 mL with distilled water. Then analyse the sample according to the general procedure. The concentration obtained is the sum of the free and esterified L-malic acid [F + E], and thus the esterified L-malic acid concentration alone [E] can be calculated as follows:

$$[E] = ([F + E] - [F]) \quad g/L$$

(c) Determination of L-malic acid in fruit juice, concentrates and related beverages.

The L-malic acid concentration of clear, neutral solutions can generally be determined without any sample treatment (except dilution according to the dilution table).

Turbid liquids generally only require filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an appropriate L-malic acid concentration. However, if coloured solutions require analysis undiluted, they may need decolorising as follows: stir 10 mL of liquid sample for 1 min with 0.1 g of PVPP and then

filter. Use the clear/slightly coloured filtrate directly in the assay.

(d) Determination of L-malic acid in beer.

Remove carbon dioxide by either stirring 5-10 ml of beer with a glass rod for 5 min or by filtration. Increase the pH of the solution to 10 with 2 M NaOH and dilute according to the dilution table.

(e) Determination of L-malic acid in solid foodstuffs.

Homogenise ~ 10 g of solid foodstuffs (weighed accurately) using a mortar and pestel or an electric blender. Extract 2 g of representative material in 40 ml of distilled water for 30 min,with heating at 60°C where necessary. Quantitatively transfer the extract to a 50 mL volumetric flask and adjust to volume with distilled water. Filter the turbid solution and dilute if necessary (according to the dilution table) before analysis.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.22 mL
Read against air (without a cuvette in the light path) or against water.
Sample solution:0.5-35 µg L-malic acid per cuvette (in 0.10-1.5 mL sample volume)

Pipette into cuvettes	Blank	Sample
Bottle 1 (Glycylglycine buffer, L-glutamate)	1.00 ml	1.00 ml
Bottle 2 (NAD^{+})	0.20 ml	0.20 ml
Bottle 3 (GOT suspension)	0.01 ml	0.01 ml
Sample	-	0.10 ml
Distilled water	1.00 ml	0.90 ml
Mix* the reagents in the cuvette and read the absorbance (A_1; when stable) after ~ 3 min. Start the reaction by the addition of:		
Bottle 4 (L-MDH suspension)	0.01 ml	0.01 ml
Mix the reagents in the cuvette and after completion of the reaction (5 – 6 min) read the absorbance, A_2, of the blank and sample.		

* for example with a plastic spatula, by slow/repeated aspiration with a 1 mL pipette, or by gentle swirling after sealing the cuvette with Parafilm.®

CALCULATIONS:

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

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW \times A}{\times d \times v \times 1000} \quad [g/L]$$

where:

V = final volume [mL]
v = sample volume [mL]
MW = molecular weight of L-malic acid [g/mol]
d = light path [cm]
= extinction coefficient of NADH at 340 nm
= 6.3 [$l \times mmol^{-1} \times cm^{-1}$]

It follows for L-malic acid:

$$c = \frac{2.22 \times 134.09 \times A_{L\text{-malic acid}}}{6.3 \times 1 \times 0.1 \times 1000} \quad [g/L]$$

$$= 0.473 \times A_{L\text{-malic acid}} \ [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the appropriate 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 L-malic acid

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

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Megazyme International Ireland Ltd.,
Bray Business Park, Bray,
Co. Wicklow,
IRELAND

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

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