

L-Malic acid

UV-method

for the determination of L-malic in foodstuffs and other materials

Cat. No. 10 139 068 035

Test-Combination for 30 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

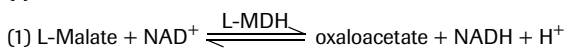
For *in vitro* use only

Store at 2-8°C

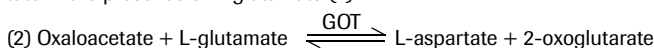
For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

L-Malic acid (L-malate) is oxidized to oxaloacetate by nicotinamide-adenine dinucleotide (NAD) in the presence of L-malate dehydrogenase (L-MDH) (1).



The equilibrium of this reaction lies on the side of L-malate. Removal of oxaloacetate from the reaction system causes displacement of the equilibrium in favour of oxaloacetate. In the reaction catalyzed by the enzyme glutamate-oxaloacetate transaminase (GOT), oxaloacetate is converted to L-aspartate in the presence of L-glutamate (2).



The amount of NADH formed is stoichiometric to the amount of L-malate. The increase in NADH is measured by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 30 ml solution, consisting of: glycyglycine buffer, pH approx. 10.0; L-glutamic acid, 440 mg
2. Bottle 2 with approx. 210 mg NAD lyophilizate
3. Bottle 3 with approx. 0.4 ml glutamate-oxaloacetate transaminase suspension, approx. 160 U
4. Bottle 4 with approx. 0.4 ml L-malate dehydrogenase solution, approx. 2400 U
5. Bottle 5 with L-malic acid assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Use contents of bottles 1, 3 and 4 undiluted.
2. Dissolve contents of bottle 2 with 6 ml redist. water.

Stability of reagents

The contents of bottles 1, 2, 3 and 4 are stable at 2-8°C (see pack label).

Bring solution 1 to 20-25°C before use.

Solution 2 is stable for 3 weeks at 2-8°C and for 2 months at -15 to -25°C.

Procedure

Wavelength¹: 340 nm, Hg 365 nm or Hg 334 nm

Glass cuvette²: 1.00 cm light path

Temperature: 20-25°C

Final volume: 2.220 ml

Read against air (without a cuvette in the light path) against water or against blank³

Sample solution: 0.5-35 µg L-malic acid/assay⁴ (in 0.100-1.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1	1.000 ml	1.000 ml
solution 2	0.200 ml	0.200 ml
suspension 3	0.010 ml	0.010 ml
sample solution*	-	0.100 ml
redist. water	1.000 ml	0.900 ml
Mix**, read absorbances of the solutions (A ₁) after approx. 3 min (see also instructions pt. 10). Start reaction by addition of:		
solution 4	0.010 ml	0.010 ml
Mix**, after completion of the reaction (approx. 5-10 min) read absorbances of the solutions immediately one after another (A ₂) (see pt. 7).		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

Determine the absorbance differences (A₂-A₁) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADH at:

$$340 \text{ nm} = 6.3 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 365 \text{ nm} = 3.4 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 334 \text{ nm} = 6.18 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

It follows for L-malic acid:

$$c = \frac{2.220 \times 134.09}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{2.977}{\epsilon} \times \Delta A \text{ [g L-malic acid/l sample solution]}$$

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

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

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

1. Instructions for performance of assay

The amount of L-malic acid present in the assay has to be between 1 µg and 35 µg (measurement at 365 nm) or 0.5 µg and 20 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a L-malic acid concentration between 0.08 and 0.35 g/l or 0.04 and 0.2 g/l, respectively.

Dilution table

Estimated amount of L-malic acid per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.2 g	< 0.35 g	-	1
0.2-2.0 g	0.35-3.5 g	1 + 9	10
2.0-20 g	3.5-35 g	1 + 99	100
> 20 g	> 35 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 1.000 ml. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.

2 If desired, disposable cuvettes may be used instead of glass cuvettes.

3 For example, when using a double-beam photometer

4 See instructions for performance of assay

2. Technical information

- 2.1 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as L-malic acid (molar mass 134.09 g/mol) or as L-malate (molar mass 132.07 g/mol). (In enzymatic determinations, the L-malate ion is measured.)
- 2.2 In evaluating the analytical results, it should be taken into account that in the acidimetric determination of "total acid calculated as L-malic acid" protons are measured and in enzymatic determinations the L-malate ions are measured. It is thus not possible to compare such results directly.

3. Specificity (Ref. 1)

The method is specific for L-malic acid. The D-isomer does not react. Also L-lactic acid, D-Lactic acid, L-aspartic acid and fumaric acid are not converted. L-Malic acid esters do not react (Ref. 3.3).

In the analysis of commercial L-malic acid, results of approx. 99 % have to be expected.

4. Sensitivity and detection limit (Ref. 1.2)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume $v = 1.000$ ml and measurement at 340 of a L-malic concentration of 0.25 mg/l sample solution (if $v = 0.100$ ml, this corresponds to 2.5mg/l sample solution).

The detection limit of 0.5 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume $v = 1.000$ ml.

5. Linearity

Linearity of the determination exists from 0.5 µg L-malic acid/assay (0.5 mg L-malic acid/l sample solution; sample volume $v = 1.000$ ml) to 35 µg L-malic acid/assay (0.35 g L-malic acid/l sample solution; sample volume $v = 0.100$ ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of $v = 0.100$ ml and measurement at 340 nm, this corresponds to a L-malic acid concentration of approx. 2-5 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.02-0.05 g/100 g can be expected.)

The following data have been published in the literature:

$x = 2.97$ g/l wine $CV = 1.8$ % $n = 12$ (Ref. 1.1, 1.2)

Fruit juice: $r = 0.014 + 0.030 \times x$ $R = 0.032 + 0.070 \times x$
 $x =$ content of L-malic acid in g/l (Ref. 2.6)

Wine: $r = 0.03 + 0.034 \times x_1$ $R = 0.05 + 0.071 \times x_1$
 $x_1 =$ content of L-malic acid in g/l (Ref. 2.10, 2.11)

7. Interference/sources of error

Traces of glutamate dehydrogenase (GIDH) in GOT result in reagent-dependent creep reactions. An extrapolation of the measuring value with A_2 is not necessary if the absorbances of blank and sample are read immediately one after another.

8. Recognizing interference during the assay procedure

- 8.1 If the conversion of L-malic acid has been completed according to the time given under "Procedure" it can be concluded in general that no interference has occurred.
- 8.2 On completion of the reaction, the determination can be restarted by adding L-malic acid (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.
- 8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

- 8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

- 8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of L-malic acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:

Use **clear, colorless or slightly colored and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 1.000 ml;

Filter **turbid solutions**;

Degas **samples containing carbon dioxide** (e.g. by filtration);

Adjust **acid samples** which are used undiluted for the assay, to pH 8-10 by adding sodium or potassium hydroxide solution and incubate for approx. 30 min; Measure **"colored" samples** (if necessary adjusted to pH 8-10) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam, especially, if there is a creep reaction before the addition of solution 4 (L-MDH);

Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpyrrolidone (PVPP; e.g. 1 g/100 ml);

Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary.

11. Application examples

Determination of L-malic acid in wine

Free L-malic acid can be determined in white or red wine directly or after a dilution acc. to the dilution table normally without prior decolorization.

Determination of free and esterified L-malic acid in wine (Ref. 3.3)

To determine the total L-malic acid content (the sum of free and esterified L-malic acid), white wine or red wine, respectively, should be treated as follows:

Heat 20 ml of wine and 6 ml sodium hydroxide (2 M) for 30 min under a reflux condenser while stirring (do not use ammonia for alkaline hydrolysis since an excessively high concentration of ammonium ions inhibits the reaction!), allow to cool to 20-25°C, and neutralize with sulfuric acid (1 M) (indicator paper).

Transfer quantitatively into a 50 ml volumetric flask and fill up to the mark with water. Use the sample for the assay according to the standard procedure (this results in total L-malic acid content = the sum of free and esterified L-malic acid).

Determination of L-malic acid in fruit juice, concentrates and in beverages

Use clear, liquid, almost neutral samples directly or after dilution with water (concentration of L-malic acid approx. 0.04-0.35 g/l) for the assay.

Filter turbid juices and dilute to obtain an L-malic acid concentration of approx. 0.04 to 0.35 g/l. The diluted solution can be used for the assay even if it is slightly colored.

Only intensely colored juices require previous decolorization when they are used undiluted for the assay. In such cases, proceed as follows:

Mix 10 ml of juice and approx. 0.1 g of polyamide powder or polyvinylpyrrolidone (PVPP), stir for 1 min, and filter. Use the clear, slightly colored solution for the assay.

When using colored juices or red wine as sample material which is used undiluted for the assay, occasionally it may occur that the absorbance A_1 is not constant because of the change in pH value (sample is acidic; assay mixture shows pH 10). In this case it is recommended to adjust the sample to pH 10 before assaying and to incubate for approx. 30 min.

Determination of L-malic acid in beer

To remove the carbonic acid, stir about 5-10 ml of beer for approx. 1 min using a glass rod or filter; dilute the largely CO₂-free sample according to the dilution table. Alternatively, alkalize the beer sample by the addition of solid sodium or potassium hydroxide.

Determination of L-malic acid in solid foodstuffs

Homogenize solid or semi-solid samples (e.g. fruit and vegetable products) with a mortar, meat grinder, or homogenizer. Weigh out a well mixed sample and extract with water - heated to 60°C, if necessary. Transfer quantitatively

into a volumetric flask and fill up to the mark with water. Filter and use the clear solution for the assay. Dilute solution depending on the L-malic acid content, if necessary (see dilution table)

12. Further applications

The method may also be used in research when analyzing biological samples.

For details of sampling, treatment and stability of the sample see Gutmann, I. & Wahlefeld, A. W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, pp. 1586-1587, Verlag Chemie, Weinheim/Academic Press, Inc., New York and London, as well as Ref. 1.2, pp. 43-44

The method may also be used in the examination of cosmetics and pharmaceuticals, e.g. infusion solutions.

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L-Malic acid assay control solution (Bottle 5)

Concentration: see bottle label

L-Malic acid assay control solution is a stabilized aqueous solution of L-malic acid. It serves as assay control solution for the enzymatic determination of L-malic acid in foodstuffs and other materials.

Application:

1. *Addition of L-malic assay control solution to the assay mixture:*

Instead of sample solution the assay control solution is used for the assay.

2. *Restart of the reaction, quantitatively:*

After completion of the reaction with sample solution and measuring of A_2 , add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 10 min). Calculate the concentration from the difference ($A_3 - A_2$) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the data stated on the bottle label.

Also available:

Test-Combination D-Malic acid, Cat. No. 11 215 558 035

3. Internal standard:

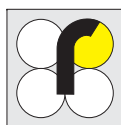
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 1	1.000 ml	1.000 ml	1.000 ml	1.000 ml
solution 2	0.200 ml	0.200 ml	0.200 ml	0.200 ml
suspension 3	0.010 ml	0.010 ml	0.010 ml	0.010 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	1.000 ml	0.900 ml	0.900 ml	0.900 ml

Mix, and read absorbances of the solutions (A_1) after approx. 5 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100 [\%]$$



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