D-Malic acid

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

UV-method

for the determination of D-malic acid in foodstuffs and other materials

Cat. No. 1 215 558

Test-Combination for 3 × approx. 10 determinations

For use in *in vitro* only

Store at 2-8°C

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

Principle (Ref. 1)

D-Malic acid (D-malate) is oxidized to oxaloacetate by nicotinamide-adenine dinucleotide (NAD) in the presence of D-malate dehydrogenase (D-MDH). Oxaloacetate is immediately split by the same enzyme to pyruvate and carbon dioxide.

$$\text{D-Malate} + NAD^+ \xrightarrow{\text{D-MDH, decarb.}} \text{pyruvate} + CO_2 + NADH + H^+$$

The amount of NADH formed is stoichiometric to the amount of D-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: hepes[1] buffer, pH approx. 9.0
2. Bottle 2 with approx. 210 mg NAD, lyophilizate
3. Three bottles 3 with D-MDH, decarb., lyophilizate, each approx. 8 U
4. D-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 content of bottle 1 undiluted.
2. Dissolve contents of bottle 2 with 4 ml redist. water.
3. Dissolve contents of one bottle 3 with 0.6 ml redist. water.

Stability of reagents

The contents of bottle 1 are stable at 2-8°C (see pack label).
Bring solution 1 to 20-25°C before use.
The contents of bottle 2 are stable at 2-8°C (see pack label).
Solution 2 is stable for 3 weeks at 2-8°C, or for 2 months at −15 to −25°C.
The contents of the bottles 3 are stable at 2-8°C (see pack label).
Solution 3 is stable for 5 days at 2-8°C.

Procedure

Wavelength[2]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[3]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 2.950 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 1-50 µg D-malic acid/assay[4] (in 0.100-1.800 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1	1.000 ml	1.000 ml
solution 2	0.100 ml	0.100 ml
sample solution*	-	0.100 ml
redist. water	1.800 ml	1.700 ml
Mix**, and read absorbances of the solutions (A_1) after approx. 6 min. Start reaction by addition of:		
solution 3	0.050 ml	0.050 ml
Mix**, wait for completion of the reaction (approx. 20 min) and read absorbances of blank and sample (A_2).		

* 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)

1 hepes = 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

2 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.

3 If desired disposable cuvettes may be used instead of glass cuvettes.

4 See instructions for performance of assay

Determine the absorbance differences (A_2-A_1) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

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

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:

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

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 nm = 6.3 [l × mmol^{-1} × cm^{-1}]
Hg 365 nm = 3.4 [l × mmol^{-1} × cm^{-1}]
Hg 334 nm = 6.18 [l × mmol^{-1} × cm^{-1}]

It follows for D-malic acid:

$$c = \frac{2.950 \times 134.09}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{3.956}{\varepsilon} \times \Delta A \text{ [g D-malic acid/sample solution]}$$

If the sample has been diluted during 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 calculated from the amount weighed:

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

1. Instructions for performance of assay

The amount of D-malic acid present in the assay has to be between 2 µg and 50 µg (measurement at 365 nm) or 1 µg and 30 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a D-malic acid concentration between 0.1 and 0.5 g/l or 0.06 and 0.3 g/l, respectively.

Dilution table

Estimated amount of D-malic acid per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.3 g	< 0.5 g	-	1
0.3-3.0 g	0.5-5.0 g	1 + 9	10
3.0-30 g	5.0-50 g	1 + 99	100
> 30 g	> 50 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 1.800 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.

2. Technical information

2.1 Note, that in the case of trace level analysis of D-malic acid all reagents used for sample preparation must be absolutely free of D-malic acid.

2.2 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as D-malic acid (molar mass 134.09 g/mol) or as D-malate (molar mass 132.07g/mol). (In enzymatic determinations, the D-malate ion is measured.)

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3. Specificity (Ref. 1)

D-Malic acid reacts fast. A side activity of the enzyme reacts with L-tartaric acid at a lower speed. At the same concentrations of L-tartaric acid and D-malic acid a slight "creep reaction" appears which may be eliminated by extrapolation. At a high surplus of L-tartaric acid, proceed as described for the determination of D-malic acid in wine and grape juice.

Note:

Commercial D-malic acid may contain approx. 1-4 % L-malic acid (Ref. 1).

4. Sensitivity and detection limit (Ref. 1)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 1.800 ml and measurement at 340 of a D-malic concentration of 0.2 mg/l sample solution (if v = 0.100 ml, this corresponds to 3 mg/l sample solution).

The detection limit of 0.35 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 1.800 ml.

5. Linearity

Linearity of the determination exists from approx. 1 µg D-malic acid/assay (0.35 mg D-malic acid/l sample solution; sample volume v = 1.800 ml) to 50 µg D-malic acid/assay (0.5 g D-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 D-malic acid concentration of approx. 3-6 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 0.03-0.06 g/100 g can be expected.)

The following data have been published in the literature:

x =	31.3 µM	sample solution	$\Delta E_{339\ nm} = 0.199$	CV = 1.01 %	n = 10
x =	61.7 µM	sample solution	$\Delta E_{339\ nm} = 0.387$	CV = 0.83 %	n = 10
x =	125.4 µM	sample solution	$\Delta E_{339\ nm} = 0.791$	CV = 0.79 %	n = 10

(Ref. 1)

Wine:
r ~ 5%, corresponding $r = 0.05 \times x_i$
R ~ 10%, corresponding $R = 0.10 \times x_i$
x_i = content of D-malic acid in g/l (Ref. 2.1)

$r = 0.05 \times x_i$
$R = 0.1 \times x_i$
x_i = content of D-malic acid in g/l (Ref. 2.2)

7. Interference/sources of error

Tannins contained in the sample could effect a slight inhibition of the assay: the presence of 50 µg pyrogallol delays the conversion of D-malic acid for approx. 5 min.

A "creep reaction" appears with plant dyes. Use a sample blank, if necessary: the test procedure contains all components except the starting-enzyme; the absorbances of blank, sample and sample blank should be measured immediately one after another and are used for the calculation of the absorbance differences:

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

A "creep reaction" of 0.5 mA/min also appears with 2-oxoglutarate at a concentration of 100 µg/assay.

An inhibition is also recognized with lead ions, 0.1 µg/assay: after 20 min under the reaction conditions approx. 85% of D-malic acid is to be found.

8. Recognizing interference during the assay procedure

8.1 If the conversion of D-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 D-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 D-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 and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 1.800 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to approx. pH 8-9 by adding sodium or potassium hydroxide solution;
Adjust **acid and weakly colored samples** to approx. pH 8-9 by adding sodium or potassium hydroxide solution and incubate for approx. 30 min;
Measure **"colored" samples** (if necessary adjusted to pH 8-9) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with activated charcoal e.g. 6 g/100 ml or with poly-vinylpolypyrrolidone (PVPP; e.g. 8-20g/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 D-malic acid in fruit juices (Ref. 3.1)

a) Colorless or faintly colored juices (citrus fruits, pear, pineapple, apricot, apple):
Adjust 25 ml of the fruit juice to pH 7-8 with KOH (2 M) and make up to 50 ml with redist. water. Add 3 g of activated charcoal or 4 g PVPP, mix, stir for 2 min and filter. Use 1.000 to 1.800 ml of the filtrate for the determination.

b) Intensely colored juices (cherry, black and red currant):
Adjust 25 ml of the fruit juice to pH 7-8 with KOH (2 M) and make up to 50 ml with redist. water. Add 3 g of activated charcoal or 4 g PVPP, mix, stir for 2 min and filter. Use 0.100 to 0.200 ml of the filtrate for the determination.

c) White grape juice:
Add 250 mg solid calcium hydroxide and 10 ml ethanol (approx. 98%) to 50 ml fruit juice and stir for 2 min. Adjust the pH to 7-8 with HCl (2 M). Transfer the liquid quantitatively into a 100 ml volumetric flask, make up to the mark with water, mix and filter. Use 0.500 to 1.500 ml of the filtrate for the determination.

d) Red grape juice:
Add 250 mg solid calcium hydroxide and 10 ml ethanol (approx. 98 %) to 50 ml fruit juice and stir for 2 min. Adjust the pH to 7-8 with HCl (2 M). Transfer the liquid quantitatively into a 100 ml volumetric flask, make up to the mark with redist. water, mix and filter. Add 3 g of activated charcoal, mix, stir for 2 min and filter again. Use 0.100 to 0.200 ml of the filtrate for the determination.

Determination of D-malic acid in wine (acc. to Beutler, Ref. 1.)

Mix 25 ml of wine, 125 mg calcium hydroxide and 5 ml ethanol (approx. 98%) for 2 min; adjust pH 7-8 with potassium hydroxide solution; transfer quantitatively into a 50 ml volumetric flask and fill up to the mark with redist. water. Filter and use clear, colorless filtrate with a volume of v = 1.000 - 1.800 ml for the assay.
Strongly colored filtrates or filtrates, which show "creep reactions", must be decolorized and treated as follows:

Mix 10 ml of filtrate and 2 g of wet polyvinylpolypyrrolidone (PVPP), stir for 2 min and filter using a fluted filter paper. Use the filtrate with a volume of v = 1.000-1.800 ml for the assay.
If the reaction does not stop (e.g. in the analysis of wine) after 20 min, read the absorbance in 2 min time intervals until a constant absorbance increase for each 2 min interval is reached.
If the absorbances A_2 increase constantly, extrapolate the absorbances to the time of addition of solution 3 (D-MDH, decarb.).

Determination of D-malic acid in wine and grape juice (acc. to Hunger et al., Ref. 3.2)
Weigh 2.5 (solid) potassium chloride into a 50 ml-volumetric flask, add 25 ml wine resp. grape juice, and dissolve potassium chloride either by rigourously shaking of the volumetric flask or by means of a magnetic stirrer. Add 0.5 ml glacial acetic acid, mix, make up to the mark with portions of ethanol (96 %; v/v). Mix and store volumetric flask in a refrigerator overnight.

Filter solution, adjust 20 ml filtrate to pH 9 with KOH (first with 2 M KOH to pH 8, and then with 0.2 M KOH to pH 9), transfer into a 25 ml volumetric flask, and make up to the mark with redist. water. For decolorization add 0.5 g activated charcoal, mix and filter through a 0.2 µm-filter. Use the filtrate for the determination. (The addition of activated charcoal is also recomended for the analysis of white wine.)

Note:
Samples which do not contain L-tartaric acid are prepared as follows: adjust 20 ml sample to pH 9 with KOH, transfer into a 25 ml volumetric flask, and make up to the mark with redist. water. For decolorization add 0.5 g activated charcoal, mix and filter through a 0.2 µm-filter. Use the filtrate for the determination.

Determination of D-malic acid in apples
Mince and homogenize the apples. Weigh approx. 40 g of sample material into a 100 ml beaker and add approx. 20 ml of boiling redist. water. Boil for 10 min and allow to cool to 20-25°C. Adjust to pH 7-8 with potassium hydroxide solution (1 M). Transfer the content of the beaker quantitatively into a 100 ml volumetric flask and fill up to the mark with redist. water. Mix the solution and filter using a fluted filter paper. Add 0.6 g activated charcoal to 10 ml filtrate, stir for 2 min and filter using a fluted filter paper. Use a volume v = 0.500 ml of the clear filtrate for the assay.

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

References

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2.1 Recueil des méthodes internationales d'analyse des vins et des moûts, Complément n° 1 à l'édition officielle de juin 1990, OFFICE INTERNATIONAL DE LA VIGNE ET DU VIN, Annexe A, S. 1-3
2.2 Amtsblatt der Europäischen Gemeinschaften L 272 (3. Oktober 1990) Rechtsvorschriften: Verordnung (EWG) Nr. 2676/90 der Kommission vom 17. September 1990 zur Festlegung gemeinsamer Analysenmethoden für den Weinsektor (S. 106-108) Official Journal of the European Communities L 272 (3 October 1990), Commission Regulation (EEC) No 2676/90 of 17 September 1990 determining Community methods for the analysis of wines (pp. 106-108) ; L 99 (14. April 1999) Commission Regulation (EU) No 761/1999 of 12 April 1999 for the change of the Commision Regulation (EEC) No 2676/90 determining Community methods for the analysis of wines
2.3 Europäische Norm/European Standard EN 12138 (Dez. 1997) Frucht- und Gemüsesäfte: Enzymatische Bestimmung des Gehaltes an D-Äpfelsäure - Spektralphotometrische Bestimmung von NAD (Fruit and vegetable juices - Enzymatic determination of D-malic acid content - NAD spectrometric method)
2.4 Deutsche Norm DIN EN 12138 (1997) Frucht- und Gemüsesäfte, Teil 13: Enzymatische Bestimmung des Gehaltes an D-Äpfelsäure; Spektralphotometrische Bestimmung von NAD
2.5 International Federation of Fruit Juice Producers (IFU, Methods of Analysis, no. 64-1995); contained in "Code of Practice for Evaluation of Fruit and Vegetable Juices" (1996) edited by Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community (A.I.J.N.)
3.1 Beutler, H.-O. & Ara, V. (1992) Enzymatische Bestimmung von D-Äpfelsäure in Fruchtsäften, Flüssiges Obst **59,** 552-554; Enzymatic Determination of D-Malic Acid in Fruit Juices, Fruit Processing **2,** 140-141
3.2 Hunger, Ch., Schuch, R. & Hörtner, H., Bundesanstalt für Lebensmitteluntersuchung und -forschung, Wien (1995) Enzymatic Determination of D-Malic Acid in Wine and Fruit Juices, in Current Status and Future Trends in Analytical Food Chemistry, Proceedings of the Eight European Conference on Food Chemistry (EURO FOOD CHEM VIII), Vol. 3, 715-718

D-Malic acid assay control solution

Concentration: see bottle label

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

Application:

1. Addition of D-malic acid 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 of the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 20 min). Calculate the concentration from the difference of (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.

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	0.100 ml	0.100 ml	0.100 ml	0.100 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	1.800 ml	1.700 ml	1.700 ml	1.700 ml
Mix, and read absorbances of the solutions (A_1) after approx. 6 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:

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

Also available:

Test-Combination L-Malic acid, Cat. No. 0 139 068

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