

D-Malic acid, UV method

Catalogue number: AK00021, 100 tests (manual) / 1000 tests (microplate)

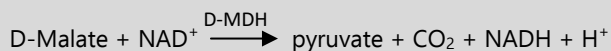
Application

This rapid and simple stereo-specific enzymatic method is used for the determination of D-malic acid (D-malate) in foodstuffs such as wine, fruit and vegetable products, as well as in pharmaceuticals and biological samples.

Introduction

D-Malic acid practically does not occur in nature; it is a metabolite produced only by some microorganisms. Analysis of freshly pressed fruit juices shows the presence of D-malic acid at the detection limit. Hence, the D-Malic legal limit for fruit and fruit juices is recommended as 10 mg/L. D-Malic acid is a component of the (racemic) D-/L-malic acid as prepared chemically. Since natural products are practically free of D-malic acid, the detection of D-malic acid could indicate that D-/L-malic acid has been added *e.g.* to wine or to fruit juice. This may be allowed or forbidden and thus the legal situation in the different countries has to be taken into consideration.

Principle



The amount of NADH formed through the action of D-malate dehydrogenase (decarboxylating; D-MDH), measured at 340 nm, is stoichiometric with the amount of D-malic acid in sample volume.

Specificity

D-MDH rapidly converts D-malic acid; a side activity of D-MDH (decarb.) leads to the reaction with L-tartaric acid, although at much lower rate. A small amount of L-tartaric acid causes a slight creep reaction which may be eliminated by extrapolation.

Sensitivity and detection limit

The sensitivity of the determination is based on 0.005 AU and a sample volume of 2.00 mL. This corresponds to a D-malic acid concentration of 0.13 mg/L sample solution when measured at 340 nm. The detection limit of 0.26 mg/L is derived from the absorbance difference of 0.010 (340 nm) and a maximum sample volume of 2.00 mL.

Linearity and precision

Linearity of the assay exists from 0.5 to 40 µg D-malic acid per assay ($v = 2.00$ mL). In a double determination using one sample solution, a difference of 0.005 to 0.010 AU may occur. With a sample volume of 2.00 mL, this corresponds to a D-malic acid concentration of 0.13 to 0.26 mg/L. The coefficient of variation is approx. 1%.

Kit composition

Solution 1. Glycylglycine buffer (25 mL, 1 M, pH 8.0) plus KCl (0.5 M), MgCl₂ (0.5 M) and sodium azide (0.02 % w/v) as a preservative. Store at 2 °C to 8°C.

Solution 2 (×2). NAD⁺ (250 mg). Store at 2 °C to 8°C (Long term storage: -30 °C to -15 °C)

Dissolve (each bottle) in 11 mL of distilled water, divide into appropriately sized aliquots and store in PP tubes at 2 °C to 8°C between use and on ice during use.

Suspension 3. D-Malate dehydrogenase (EC 1.1.1.83) suspension (2.2 mL, 374 U/mL). Store at 2 °C to 8°C. Swirl bottle before use.

Solution 4. D-Malic acid standard solution (5 mL, 0.20 mg/mL). This standard solution can be used when there is some doubt about the method accuracy. Store at 2 °C to 8°C.

Safety

Reagents that are used in the determination of D-malic acid are not hazardous materials (see Hazardous Substances Regulations). However, the concentrated buffer contains sodium azide as a preservative. The general safety measures that apply to all chemical substances should be followed.

Procedure (endpoint analysis)

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 (mL)	Blank	Sample
Distilled water (at ~25 °C)	2.00	1.00
Sample	-	1.00
Solution 1 (glycylglycine buffer)	0.20	0.20
Solution 2 (NAD ⁺)	0.20	0.20
Mix, measure the absorbances of the above solutions (A1) after approx. 3 min and start the reaction by addition of		
Suspension 3 (D-MDH)	0.02	0.02
Mix, measure the absorbances of the above solutions (A2) at the end of the reaction (approx. 6 min)*		

Mixtures can be obtained with a plastic spatula or by gentle inversion after sealing with a cuvette cap or Parafilm®.

* for orange and grapefruit juice are required approx. 10 min, and for red and white wine approx. 12 min.

Calculation

Determine the absorbance difference for both blank and sample (A2-A1). Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-malic acid}}$. The concentration of D-malic acid (g/L), based on the ϵ of NADH at 340 nm ($6300 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$), is calculated as follows:

$$C (D\text{-malic acid}) = 0.05151 \times \Delta A_{D\text{-malic acid}} \quad [\text{g/L}]$$

If the sample has been diluted or a different sample volume was used during the reaction, the result must be multiplied by the corresponding dilution/concentration factor.

Alternative procedures (micro-volumes)

Although this kit has been developed to work in cuvettes, it can be easily adapted for use in 96-well microplates or in auto-analysers. Basically, the assay volumes for the cuvette format have to be reduced approximately 10-fold for use in microplate format or in auto-analyser format. However, when using these micro-volume formats, you must be aware that the radiation pathlength is usually smaller than 1 cm, which is the standard cuvettes pathlength. Thus, to perform the calculation of the amount of analyte in the samples follow one of the three possible strategies described in the "Alternative Procedures Brochure", available on the NZYTech website.

Interferences

An internal standard should be included during sample analysis if the presence of interfering substances is suspected. A quantitative recovery of this standard should be expected. Identification of losses in sample handling and extraction may be identified by performing recovery experiments, i.e., by adding L-malic acid to the sample in the initial extraction steps.

If present, tannins in red wine may give rise to a slight inhibition of the assay. If there is a non-enzymatic, slow increase in absorbance, incorporate a sample blank containing all components except the D-MDH. This reaction should be measured immediately after the reaction that contains the D-MDH. Use the obtained values in the calculation as follows:

$$\Delta A_{D\text{-malic acid}} = (A2-A1)_{\text{sample}} - (A2-A1)_{\text{reagent blank}} - (A2-A1)_{\text{sample blank}}$$

General information on sample preparation

The amount of D-malic acid present in the cuvette should range between 0.5 and 40 μg . Thus, if a sample volume of 0.10 mL is used the sample solution must be diluted to yield a D-malic acid concentration between 5 and 400 mg/L. However, the sample volume can vary from 0.10 to 2.00 mL, by replacing water (analyte range from 0.25 to 400 mg/L).

To implement this assay use clear, colorless or slightly colored and practically neutral liquid samples directly, or after dilution; 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; adjust acid and weakly colored samples to pH 8-10 and incubate for approx. 30 min; measure "colored" samples (if necessary adjusted to pH 8-10) against a sample blank; treat "strongly colored" samples that are used undiluted or with a higher sample volume with PVP; crush or homogenize solid or semi-solid samples, extract with water or dissolve.

Examples of sample preparation

Determination of D-malic acid in red and white wine

To 25 mL of red or white wine add 125 mg of calcium hydroxide and mix for 2 min. Adjust pH to 7-8 with 1 M KOH. Quantitatively transfer the solution to a 50 mL volumetric flask and fill with distilled water. In a 100 mL beaker, mix the solution with 3 g of activated charcoal by stirring the suspension for 2 min. An aliquot of the solution should be filtered in a Whatman GF/A glass fibre filter paper and use 1.0-2.0 mL of the clear, colorless filtrate in the assay. If necessary, determine the absorbance difference (A2-A1) for both blank and sample after approx. 12 min, with subsequent measurements at 3 min intervals to determine the creep rate.

Determination of D-malic acid in juice from citrus fruits (e.g. grapefruit and orange) and in intensely coloured juices (e.g. cherry, black and red currant)

Take 25 mL of the fruit juice and adjust pH to approximately 7-8 with 2 M KOH. Quantitatively transfer the solution to a 50 mL volumetric flask and fill with distilled water. Transfer the solution to a 100 mL beaker, add 3 g of activated charcoal and stir the suspension for 2 min on a magnetic stirrer. An aliquot of the solution should be filtered in a Whatman GF/A glass fibre filter paper and use 1.0-2.0 mL of the clear, colorless filtrate in the assay. If necessary, determine the absorbance difference (A2-A1) for both blank and sample after approx. 10 min, with subsequent measurements at 2 min intervals to determine the creep rate.

References

Rader, F. & Knichel, W. (1988). D-(+)-Malate. In: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 3rd ed., Vol VII, pp. 53-59, VCH Publishers (UK) Ltd., Cambridge, UK.

Elkins, E.R. & Freund, W. (1994). Detection of adulteration in apple juice by L-malic/total malic acid ratio: Collaborative Study. J.AOAC Int., 77, 411-415.

Official Journal of the European Communities L 272 (3 Oct. 1990), Legislation: 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 Commission Regulation (EEC) No 2676/90 determining Community methods for the analysis of wines.

Recommendations

This method is recommended/approved by the:

- European Commission regulation (EEC) (analysis of wine);
- European and German standards (EN, DIN);
- Recommended by the International Wine Office (OIV), the International Federation of Fruit Juice Producers (IFU), and by the

Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Community (A.I.J.N.).

V2201

Certificate of Analysis

Test	Criteria	Result
Test Performance	Reaction completed within time stated	Meets specification
	Target value for recommended standard material +/- 10%	Meets specification
Blank reaction absorbance	+/- 10% of the blank value	Meets specification

Approved by:



Patrícia Ponte
Senior Manager, Quality Systems

For research use only

Please enquire info@nzytech.com to obtain any additional information about this kit, including additional specific applications.



Estrada do Paço do Lumiar, Campus do Lumiar - Edifício E, R/C, 1649-038 Lisboa, Portugal Tel.:+351.213643514 Fax: +351.217151168

www.nzytech.com