D-Malic acid, UV method

Alternative Procedures

Introduction

This kit has been developed to work in cuvettes with a standard pathlength of 1 cm, as described in the respective "Product Brochure". However, it can be adapted for use in 96-well microplates or in auto-analysers (micro-volume formats) with minimal assay optimisation. 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 the standard cuvette pathlength of 1 cm. Thus, to perform the calculation of the amount of analyte in the samples under analysis follow one of the three strategies described in the section below.

Strategies for analyte calculation

Auto-analysers use reaction volumes of approximately 0.315 ml and pathlengths from 4 to 8 mm, which is similar to a standard 96-well microplate in which the same reaction volume would have a pathlength of 6 or 7 mm (similar assay volumes). Therefore, in both formats (96-well microplate and auto-analysers systems), the calculation of the analyte must be done by one of the three possible methods described below:

1. Using the pathlength conversion factor

This is the easiest method to perform the calculation of the analyte. However, it requires a microplate reader with pathlength conversion capacity, i.e., the apparatus can detect the pathlength of each well and convert the individual readings to a 1 cm pathlength (cuvette format). In the case of auto-analysers, the absorbance readings should be directly converted to a 1 cm pathlength. This will allow the calculation of the analyte content as described in the "Product Brochure", provided with the kit and available at the NZYTech website.

2. Using one standard curve

In this method, it is necessary to perform a standard curve of the analyte on each microplate that contains the test samples, or in the auto-analyser, and calculate the result from the standard curve of analyte concentration vs. absorbance. The standard curve can be performed by using the control solution provided in the kit.

3. Using two standard curves

The most complicated method is to perform standard curves of the analyte in both the cuvette format (i.e. with a 1 cm of radiation pathlength) and the 96-well microplate or auto-analyser formats, and use these results to obtain a mean conversion factor between the cuvette procedure values and the alternative procedure values. The standard curves can be performed by using the control solution provided in the kit.

V1901

D-Malic acid, UV method

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

Alternative Procedures

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AK0002_IFU_sup_EN_V2301

Instructions for use

AK0002_IFU_EN_V2302

D-Malic acid, UV method

Catalogue number	Presentation
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

$$\text{D-Malate} + NAD^+ \xrightarrow{\text{D-MDH}} \text{Pyruvate} + CO_2 + NADH + H^+$$

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), $MgCl_2$ (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 -30 °C to -15 °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.

Protocol (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 TO 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 $\Delta A_{\text{D-malic acid}}$. The concentration of D-malic acid (g/L), based on the ε of NADH at 340 nm (6300 $L \times mol^{-1} \times cm^{-1}$), is calculated as follows:

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

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 Procedure”, 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:

$$\Delta_{\text{D-Malic acid}} = (A2-A1)_{\text{sample}} - (A2-A1)_{\text{reagent blank}} - (A2-A1)_{\text{sample blank}}$$

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 Commision 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.).

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