AK0019_IFU_EN_V2302

L-Malic acid, colorimetric method

Catalogue number	Presentation
AK00191	5 x 10 tests

Application

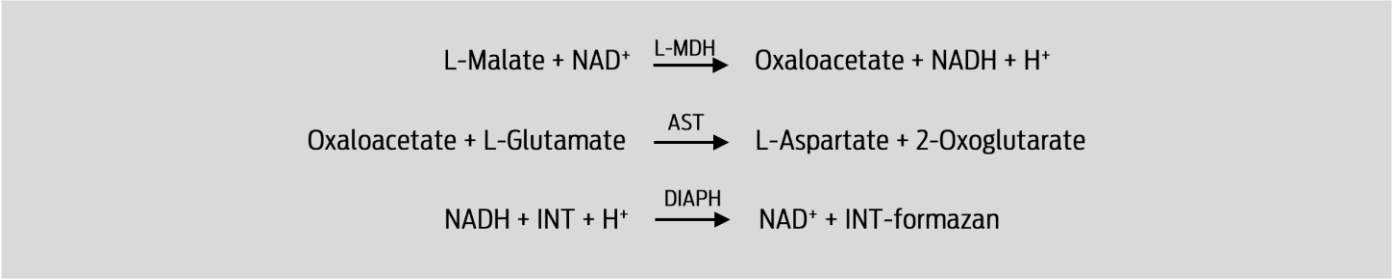
This rapid and simple colorimetric method is used for the determination of L-malic acid (L-malate) in a wide range of matrices. Although specially developed for quantification of L-malic acid in wine industry, this kit is also adequate to L-malic acid measurement in other foodstuffs such as fruit juice, beer, bread, fruit and vegetable products, as well as in cosmetics, pharmaceuticals and biological samples.

Simple, robust, and accurate, this assay can be performed using an inexpensive visible spectrophotometer.

Introduction

L-Malic acid is a relevant component of the citric acid cycle that is found in animals, plants and microorganisms. It is one of the most important fruit acids found in nature and it is the acid present in highest concentrations in wine. Microbial decomposition of L-malic acid leads to the formation of L-lactate; this can be a desirable reaction in the wine industry, where the level of L-malic acid is monitored, along with L-lactic acid, during malolactic fermentation.

Principle



The amount of INT-formazan formed through the action of L-malate dehydrogenase (L-MDH), Aspartate aminotransferase (AST) and Diaphorase measured at 505 nm, is stoichiometric with the amount of L-malic acid present in sample volume.

Specificity

The method is specific for L-malic acid. D-Malic acid, as well as D- and L-lactic acid, L-aspartic acid and fumaric acid do not react. L-Malic acid esters also do not react.

Sensitivity and detection limit

The smallest differentiating absorbance for the assay is 0.01 AU. This corresponds to 10.3 mg/L of sample solution with a sample volume of 20 µL. The detection limit is 20.7 mg/L, which is derived from an absorbance difference of 0.020 and a sample volume of 20 µL.

Linearity and precision

The assay is linear over the range of 0.16 to 16 µg of L-malic acid per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.010 to 0.020 may occur. With a sample volume of 0.020 mL, this corresponds to a L-malic acid concentration of approx. 0.008 to 0.8 g/L of sample solution.

Interferences

Phenolics interfere with the assay by reacting with INT and causing a "creep" reaction. This effect is particularly significant in red wines. When undiluted red wine is to be analyzed, phenolics must be previously removed with PVPP, as described in section *Removal of Phenolics*. A much slower "creep" reaction can occur in undiluted white wines, which is taken in account in the recommended method.

Although PVPP eliminates most of the phenolics from wine, the remaining vestiges can still cause a slight "creep" reaction. Since this reaction is very slow, the potential overestimation of L-malic acid (<0.01 g/L for white wine and < 0.03g/L for red wine) can be ignored and the *Assay Format I* can be used. Still, this overestimation can be taken in account by performing the *Assay Format II*.

Reducing substances such as sulfite and ascorbic acid can interfere with the assay but only at levels not common in wines. Potential minor "creep" reactions can be taken in account by performing *Assay Format II*. Samples containing very high levels of ascorbic acid cannot be tested with this kit.

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.

Kit composition

Solution 1 (2x). Glycylglycine buffer (30 mL, 0.25 M, pH 10.0) plus L-glutamate (0.25 M), Triton X100 (1.25% V/V) and sodium azide (0.02% w/v) as a preservative. Store at 2 °C to 8 °C.

Mixture 2 (5 x). NAD^+ (30 mg) plus INT (1.1 mg) and FAD (75 µg). Store at 2 °C to 8 °C. (Long-term storage at -30 °C to -15 °C)

Dissolve content of 1 bottle in 12 mL of *Solution 1*. Stable for at least 48 h.

Suspension 3. Aspartate aminotransferase (AST/GOT), L-Malate dehydrogenase (LMDH) and Diaphorase in 3.2 M ammonium sulphate (2.2 mL). Store at 2 °C to 8 °C. Swirl bottle before use.

Solution 4. L-Malic acid standard solution (5 mL, 0.4 mg/mL). Store at 2 °C to 8 °C.

This standard solution can be used when there is some doubt about the method accuracy.

Powder 5: PVPP (polyvinylpolypyrrolidone; 10 g) Store at 2 °C to 8 °C.

Protocol (endpoint analysis)

Assay format I (simple format)

Wavelength: 505 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~25 °C

Final volume: 3.04 mL

Sample solution: 0.16-16 µg of L-malic acid per cuvette (in 20 µl sample volume)

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

PIPETTE INTO CUVETTES (mL)	BLANK	SAMPLE
Distilled water	1.80	1.78
Sample	-	0.02
Solution 1+2	1.20	1.20
Mix, measure the absorbances of the above solutions (A1) after approx. 2 min and start the reactions by addition of		
Suspension 3 (GOT+LMDH+DIAPH)	0.04	0.04
Mix, measure the absorbances of the above solutions (A2) at the end of the reaction (approx. 5 min)		

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

To confirm that the reaction is concluded, take extra measurements (A2) at 1 min intervals beyond 5 min measure. The reading should remain the same over 1 min interval (Figure 1).

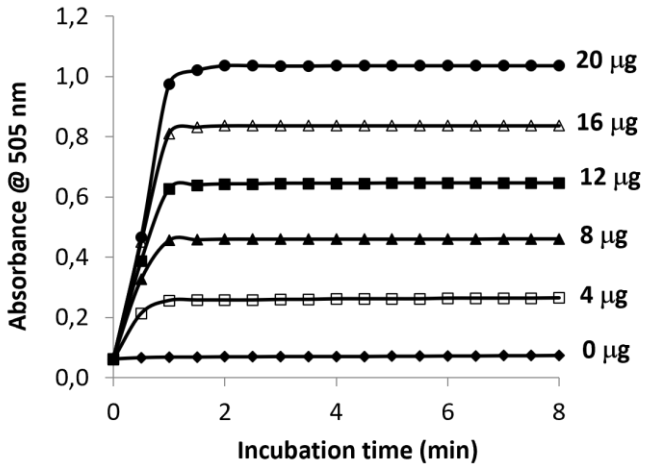


Figure 1. Increase in absorbance at 505 nm on incubation of 0-20 µg of L-malic acid, performed with NZYTech colorimetric kit (Assay format I) at 25 °C using 1 cm pathlength cuvettes.

Assay format II ("creep" corrected format)

Wavelength: 505 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~25 °C

Final volume: 3.04 mL

Sample solution: 0.16-16 µg of L-malic acid per cuvette (in 20 µl sample volume)

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

Pipette into cuvettes (mL)	**Blank**	**Sample**
Distilled water	1.80	1.78
Sample	-	0.02
Solution 1+2	1.2	1.20
Mix, measure the absorbances of the above solutions (A0) after approx. 1 min. Measure the absorbances again, after exactly 5 min (A1) and start the reactions by addition of		
Suspension 3 (GOT+LMDH+Diaph)	0.04	0.04
Mix, measure the absorbances of the above solutions (A2) at the end of the reaction (approx. 5 min)		

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

To confirm that the reaction is concluded, take extra measurements (A2) at 1 min intervals beyond 5 min measure. The reading should remain the same over 1 min interval (Figure 2).

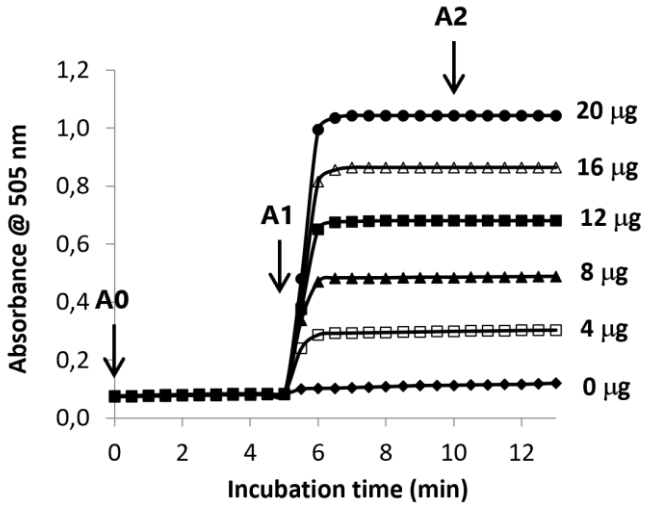


Figure 2. Increase in absorbance at 505 nm on incubation of 0-20 µg of L-malic acid, performed with NZYTech colorimetric kit (Assay format II) at 25 ºC using 1 cm pathlength cuvettes. The analysed sample was red wine treated with PVPP and filtration and fortified with different quantities of L-malic acid (0-20 µg).

Calculation

Assay format I: Determine the absorbance difference (A2-A1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{L-malic acid}}$.

Assay format II: Determine the absorbance difference [(A2-A1)-(A1-A0)] for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{L-malic acid}}$.

The concentration of L-malic acid (g/L) is calculated as follows (this equation derived from the standard curve in Figure 3):

$$C\ (\text{L-Malic acid}) = 1.033 \times \Delta A_{\text{L-Malic acid}} \quad [g/L]$$

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.

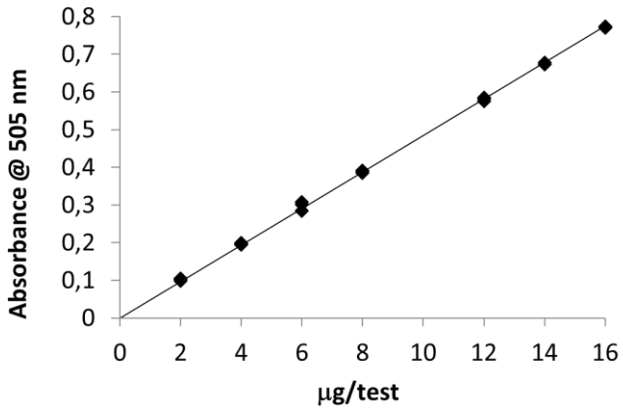


Figure 3. Standard curve relating L-malic acid concentration (µg/test) to absorbance at 505 nm (25 ºC)

General information on sample preparation

Removal of Phenolics

This procedure is usually required only for red wines. Add 0.2 g of PVPP (Powder 5) to 10 mL of wine. Shake vigorously for 5 minutes and then filter through Whatman Nº 1 filter paper. A no darker than light pink filtrate should be obtained. However, strong coloured wines may need to be treated with an additional 0.2 g of PVPP per 10 mL of sample. Recover filtrate and analyse as described above.

Sample dilution

The amount of L-malic acid present in the cuvette should range between 1.6 and 16 µg. Thus, if a sample volume of 0.02 mL is used the sample solution must be diluted to yield a L-malic acid concentration between 0.008 and 0.8 g/L.

Examples of sample preparation

Determination of L-malic acid in grape juice and white wine

L-malic acid of these matrices can be quantified without any treatment. Usually, no dilution (wine at the end of malolatic fermentation) or 1:10 dilution (grape juice) and a sample volume of 0.02 mL are satisfactory.

Determination of L-malic acid in red wine

Remove phenolics as discribed above to obtain a clear, light pink coloured solution adequate to analysis. Usually, no dilution is required for red wine at late stage of malolactic fermentation and a sample volume of 0.02 mL is satisfactory.

Determination of L-malic acid in grapes

Add 200 mL of distilled water to a measuring cylinder, then add grapes to increase volume to at least 400 ml. If needed, add distilled water in a volume equivalent to the volume above 400 mL. With this procedure, a 1:2 dilution of the volume of the grapes will be obtained. Homogenize water+grapes with a kitchen blender for at least 3 minutes. Filter an aliquot of this solution using Whatman Nº 1 filter paper. Reject the first few mL and collect the next approx. 5-10 mL. Analyze the filtrate. Usually, an additional 1:10 dilution and a sample volume of 0.02 mL are satisfactory.

References

Mollering, H. (1985). L-Malate. In: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 3rd ed., Vol.VII, pp. 39-47, VCH Publishers (UK), Cambridge, UK.

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