

## UNITAB™ REAGENT

# L-MALIC ACID

### Enzymatic UV -Method

Product # LMA-60 (30 Tests)

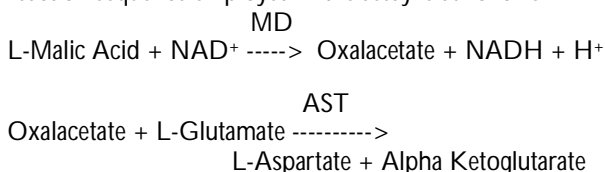
LMA-150 (75 Tests) LMA-500 (250 Tests)

#### INTENDED USE

Unitech Scientific L-Malic Acid Reagent Tablets are intended for the determination of L-malic acid in wine, juice and other liquid samples.

#### METHODOLOGY & CHEMICAL PRINCIPLES

This L-malic acid method is based on the method of Mollering.<sup>1</sup> L-Malic Acid Reagent Tablets are optimized to conform to IFU-Analysis Nr. 21-1964.<sup>2</sup> The enzymatic reaction sequence employed in the assay is as follows:



The primary dehydrogenase reaction is coupled with an aminotransfer reaction. Malate dehydrogenase (MD) catalyzes the oxidation of L-malic acid to oxalacetate with the concomitant reduction of nicotinamide adenine dinucleotide (NAD). The increase in absorbance at 340 nm due to NADH formation is directly proportional to the concentration of L-malic acid in the sample. Removal of oxaloacetate from the reaction system shifts the equilibrium to favor oxidation of malic acid.

#### REAGENTS

Active ingredients are:	Quantity/Kit
	30T 75T 250T
<u>L-Malic Acid Rgt. Tablets</u>	as Formulated 12 30 100
L-Glutamic Acid	90 mM
NAD	5.3 mM
<u>Malic Acid Buffer</u>	60 150 500mL
Diethanolamine Buffer	0.5M
Magnesium	1 mM
<u>Trigger Enzyme</u>	1.3 3.5 11mL
L-Malate Dehydrogenase	2.4 kU/L
Aspartate Transaminase	2 kU/L
Ammonium Sulfate	2.5 M
<u>L-Malic Acid Standard</u>	0.2 g/L 1 1 5mL

#### STORAGE & REAGENT PREPARATION

Components are stable until the labeled expiration date when stored in their original container at 2-8°C; store tablets tightly sealed with the desiccant pack provided. Trigger Enzyme, Buffer and Standard are ready to use and require no reconstitution.

**Working Reagent (WRgt):** Calculate the volume of WRgt required for all samples and standards in the assay. Dissolve each Reagent Tablet in 5 mL Malic Acid Buffer using clean glassware. Mix by gentle inversion. (For high sensitivity assays, refer to NOTES.)

Reconstituted reagent is stable for 4 hours at 2-8°C.

Discard any turbid reagent or reagent having an absorbance greater than 0.7 at 340 nm read against deionized water (DI) blank.

#### PROCEDURE

System requirements: Wavelength 340 nm, absorbance range 0-2A, pathlength 1.0 cm. For alternative samples volumes (SV), wavelengths, etc. refer to NOTES.

Pipet into Cuvettes	Reagent Blank Cuvette	Sample or Standard Cuvettes
Sample		50 µL
DI water	50 µL	
Working Reagent	2 mL	2 mL
Mix cuvettes and wait 3 minutes. Zero spectrophotometer with Reagent Blank. Read $A_{\text{INITIAL}}$ (Initial absorbance).		
Trigger Enzyme	35 uL (1 drop)	35 uL (1 drop)
Mix and wait 10-20 min. Read $A_{\text{FINAL}}$ (Final absorbance).		

1. Label one cuvette for each sample, standard and blank.
2. Prepare sufficient WRgt. Allow WRgt to reach room temperature prior to assay.
3. Pipet standard, samples, and water into cuvettes as shown in the Table above using calibrated pipets.
4. Dispense WRgt into each cuvette, mix and wait 3 minutes. Zero spectrophotometer with the Reagent Blank cuvette. Read the initial absorbance ( $A_{\text{INITIAL}}$ ) values.
5. Gently mix the Trigger Enzyme by inversion and dispense, as shown above. Mix each cuvette, wait 10 -20 minutes and read the final absorbance ( $A_{\text{FINAL}}$ ).

#### CALCULATIONS

1. Calculate the Delta A =  $A_{\text{FINAL}} - A_{\text{INITIAL}}$  for each cuvette.
2. If the Delta A for the Reagent Blank is significant, subtract it from each sample and standard Delta A.:  

$$\text{Net A} = \text{Delta A}_{\text{SAMPLE}} - \text{Delta A}_{\text{BLANK}}$$
3. Samples with Delta A values less than 0.05 should be reassayed with a larger sample volume or less dilute sample.
4. Select one of the following calculation methods:
  - a.) Extinction Coefficient (Use standard to verify recovery.)  

$$\text{L-Malic Acid (g/L)} = \frac{\text{Net A} \times \text{MW} \times \text{TV} \times \text{df}}{(\epsilon)(P)(1000 \text{ mg/g})(\text{SV})}$$

Where: MW = 134.09 g/mole  
 TV = 2.085 mL total reaction volume  
 SV = 0.05 mL sample volume  
 (see Procedure Step 1)  
 $\epsilon$  (absorptivity of NAD) =  
     6.22 @ 334-340nm  
     = 3.4 @ 365nm  
 P = 1 cm light path  
 df = dilution factor

At 340 nm,  
 L-Malic Acid (g/L) =  $\frac{\text{Net A} \times 134.09 \times 2.085}{6.22 \times 1 \times 1000 \times 0.05}$   
 = 0.899 X Net A

Adjust calculations if alternate SV and TV are used or if diluted samples are reassayed. Note that calibration accuracy of pipets affects results with extinction coefficient calculations.

b.) A single point standard, e.g. 0.2 g/L L-Malic Acid  
 L-Malic Acid, g/L = Conc. Standard x df x  $\frac{\text{Net A}_{\text{SAMPLE}}}{\text{Net A}_{\text{STANDARD}}}$   
 = 0.2 x df x  $\frac{\text{Net A}_{\text{SAMPLE}}}{\text{Net A}_{\text{STANDARD}}}$

c.) A Multi-point Standard (e.g. Unitech 5-Point Standards). Sample concentrations are calculated from the best-fit standard curve.

## NOTES

1. Wavelength: NADH absorbance maximum is 340 nm. Therefore, 334-340 nm determinations provide the best analytical discrimination. While less sensitive, 365 nm analysis provides approximately 1.5-fold broader measuring range (e.g. 0.05 – 0.8 g/L @ 334-340 nm vs. 0.08 – 1.2 @ 365 nm, assuming sample volume of 100  $\mu\text{L}/2$  mL).

2. SV and Extended Measuring Range: Assay sensitivity increases with a higher SV. For extended measuring range assays, reduce SV. For example:

$$\begin{array}{ll} 50 \mu\text{L sample}/2 \text{ mL WRgt} & 0.05 - 0.8 \text{ g/L} \\ 25 \mu\text{L sample}/2 \text{ mL WRgt} & 0.1 - 1.6 \text{ g/L} \end{array}$$

Adjust calculations using appropriate SV and TV values. Select standard(s) within the linear assay range.

3. Sample Dilution: Results are linear to 0.8 g/L. Dilute samples with  $A_{\text{FINAL}}$  values near instrumentation absorbance limits (typically 3.0 O.D.) with deionized water and reassy.

<u>Estimated L-Malic Acid</u>	<u>Dilution</u>
0.8 to 8 g/L	1:10
< 0.8 g/L	neat (or 1:3)*

Multiply the result by the dilution factor. For example, when diluting 1 part sample with 9 parts DI water, the dilution factor is "10".

Note that if phenolic interference from dark red wines is suspected, and you do not want to decolorize wine prior to assay, predilute samples 1:3 prior to assay.

4. Creep reactions occasionally occur as a result of endogenous sample enzymes or pigments interfering with the enzymatic reactions. If necessary, prepare a sample blank, i.e. prepare two tubes:

- Sample Blank [Rgt + Sample]
- Reaction [Rgt + Sample + Trigger]

Calculate corrected delta A =  
 $\text{delta A}_{\text{REACTION}} - \text{delta A}_{\text{BLANK}}$

Use this corrected delta A to calculate results.

## SAMPLE PREPARATION

### Free Malic Acid Determination

Follow the procedure as given above. If the initial absorbance drifts due to the presence of fermentation enzymes, adjust the sample to approximately pH 10 and incubate for 30 minutes prior to measurement.

### Total L-Malic Acid Determination

Both free L-malic acid and its esters of polyphenols or anthocyanins may be measured in wine following alkaline hydrolysis. Heat 20 mL of wine and 6 mL sodium hydroxide (2 M) for 30 minutes while stirring under a reflux condenser. (Ammonium ions interfere with this hydrolysis.) After cooling, neutralize with 1M sulfuric acid; quantitatively transfer to volumetric flask, Q.S. with distilled water to 50 mL, and assay L-malic acid.

### Clarification

Turbid samples should be filtered. Fermentation samples may be clarified by centrifugation (if necessary) and placed into a water bath at 80°C to inactivate fermentation enzymes.

### Decolorization

If an unacceptably high sample blank absorbance is obtained, mix 10 mL juice and approximately 0.1 g polyamide powder or polyvinylpyrrolidone (PVPP), stir for 1 minute and filter. Red wine typically needs decolorization only when SV larger than 100  $\mu\text{L}/2$  mL reagent are used.

## SIGNIFICANCE OF MEASUREMENTS

Both free and esterified L-malic acid are commonly found in fruits and vegetables. Its abundance drops from 8 to perhaps 1 g/L in grape must as the ripening process proceeds. Up to 30% of the malic acid may be consumed by yeast fermentation. A secondary fermentation is typical in wine; L-malic acid is converted to L-lactic acid and carbon dioxide by lactic bacteria. Malo-lactic fermentation can be prevented by filtration and increased sulfite.<sup>3</sup>

## QUALITY CONTROL

Low and high level controls should be included in each set of assays. For quality control, calculate the "% Expected" for controls with established L-malic acid values. Factors that may affect the performance of this test include proper instrument function, temperature control, glassware cleanliness, and pipetting accuracy.

## REFERENCES

- Mollering, H. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd ed., vol. 3, pp. 1589-1593, Verlag Chemie, Weinheim/Academic Press, Inc., New York.
- Mollering, H. (1985) In: Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., vol. VII, pp. 39-47, Verlag Chemie, Weinheim, Deerfield Beach/Florida.
- Peynaud, E. (1984) Eng. Trans, John Wiley & Sons, p.41.

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