UNITECH SCIENTIFIC CALIFORNIA, U.S.A.

FLEX-REAGENT™

L-MALIC ACID

Product #: LMA-F60 (30 Tests), LMA-F150 (75 Tests) LMA-F500 (250 Tests)

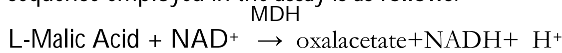
Enzymatic UV-Method

INTENDED USE

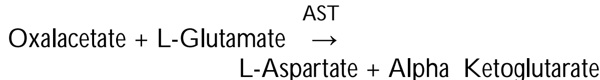
Unitech Scientific L-Malic Acid FLEX-Reagents™ are intended for the determinations of L-malic acid in wine, juice and other liquid samples.

METHODOLOGY & CHEMICAL PRINCIPLES

The assay methodology of this reagent is based on the method of Mollering.[1] L-Malic Acid FLEX-Reagents are optimized to conform to IFU-Analysis Nr. 21-1964.[2] The enzymatic reaction sequence employed in the assay is as follows:



$$\text{L-Malic Acid} + NAD^+ \xrightarrow{MDH} \text{oxalacetate} + NADH + H^+$$



$$\text{Oxalacetate} + \text{L-Glutamate} \xrightarrow{AST} \text{L-Aspartate} + \text{Alpha Ketoglutarate}$$

The primary dehydrogenase reaction is coupled with an amino transfer reaction. Malate Dehydrogenase (MDH) catalyzes the oxidation of L-malic acid to oxalacetate with the concomitant reduction of nicotinamide adenine dinucleotide (NAD). The increase in absorbance at 340nm 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

L-Malic Acid FLEX-Reagent active ingredients are:

	Concentration as Formulated
1. Mali-Lactic Buffer	
Glycylglycine	1.5 M
L-Glutamic Acid	100 mM
Stabilizers, pH 10	
2. Mali-Lactic NAD	50 mM
3. GOT Suspension	400 U/mL
4. MDH Suspension	6 KU/mL
5. L-Malic Acid Standard	(refer to label concentration)

A 5-Level L-Malic Acid Standards Kit is available from Unitech Scientific.

REAGENT PREPARATION & STORAGE

1. Mali-Lactic Buffer, NAD Solution, GOT and MDH enzyme suspensions and standards are ready to use; gently mix suspensions by inversion prior to use.
2. Reagent components are stable until labeled expiration date when stored in their original container at 2-8°C.

PROCEDURE

Working Reagent (WRgt): Working Reagent: Prepare sufficient WRgt for all samples and standards in the assay, using clean glassware, according to the examples in the following tables.

MANUAL TESTING	1 Test	8 Tests	13 Tests	24Tests
Mali-Lactic_Buffer (Bottle1)	1.0 mL	5 mL	8 mL	15 mL
NAD Solution (Bottle2)	0.3 mL	1.5 mL	2.4 mL	4.5 mL
GOT Suspension (Bottle3)	0.015mL	0.075mL	0.12mL	0.225mL
Deionized Water	2.0 mL	10 mL	16 mL	30 mL
WRgt (Approx.Total)	**3mL**	**16.5mL**	**26.4mL**	**49mL**

CHEMWELL (AUTOMATED)	30 Tests	45 Tests	60Tests	100 Tests
Mali-Lactic_Buffer (Bottle1)	3 mL	4 mL	5 mL	8 mL
NAD Solution (Bottle2)	0.9 mL	1.2 mL	1.5 mL	2.4mL
GOT Suspension (Bottle3)	0.045mL	0.060mL	0.075mL	0.120mL
Deionized Water	4.5mL	6 mL	7.5mL	12.0
WRgt (Approx.Total)	8 mL	11mL	14mL	22mL

(# of Tests accounts for Reagent Bottle dead volume)

Working reagent is stable for 8 hrs when refrigerated; discard any turbid working reagent or if the 340 nm absorbance is greater than 0.5 (1cm path) when read against distilled water.

1. Pipet water into the Reagent Blank cuvette and pipet standards, controls, samples into cuvettes as shown.

Pipette into Cuvettes	Reagent Blank Cuvette	Reaction Cuvettes
Sample		50µL
DI water	50µL	
Working Reagent	2 mL	2 mL
Mix cuvettes and incubate 3 minutes Zero spectrophotometer with Reagent Blank Read $A_{INITIAL}$ (Initial ABS)		
MDH Suspension	10 uL	10 uL
Mix and incubate 10-20 min. Read A_{FINAL} (Final ABS)		

System parameters: Wavelength 340 nm, Absorbance Range 0-2A, pathlength 1.0 cm. Refer to NOTES for alternatives.

2. Refer to the Notes if using a SV greater than 0.1 mL or using alternative assay parameters.
3. Dispense WRgt, mix and incubate 3 minutes. Zero spectrophotometer with the Reagent Blank cuvette. Read the initial absorbance ($A_{INITIAL}$) values.
4. Gently mix the MDH Suspension and dispense as shown above. Mix each cuvette, incubate and read the final absorbance (A_{FINAL}).

CALCULATIONS

1. Calculate the absorbance difference for the sample: (Delta A) = A_{FINAL} - $A_{INITIAL}$. If the Reagent Blank (Step 1) "Delta A" is significant, subtract the Blank Delta A from that of each sample and standard. Net A = Delta A – Delta A_{Blank}.

2. Compute L-Malic Acid Levels by one of the following:

a. Extinction Coefficient (Use the 0.2 G/L Standard as a control to verify recovery.)

$$\text{Malic Acid (G/L)} = \frac{\text{Net A} \times \text{MW} \times \text{T.V.}}{(\varepsilon)(P)(1000\text{mG/G})(SV)} = \frac{\text{Net A} \times 134.09 \times 2.06}{6.22 \times 1 \times 1000 \times 0.05} = \text{Net A} \times 0.888$$

Where:

Net A = Delta A – Delta A_{Blank}

Delta A = A_{FINAL} - $A_{INITIAL}$

MW =134.09G/mole for malic acid

*TV = 2.06 mL total reaction volume

*SV = 0.05 mL sample volume (See Notes)

ε = absorptivity of NAD =6.22 @334-340nm; 3.4 @ 365nm

P = 1 cm light path

* Recalculate if alternate WRgt & SV are used. See Notes. Inaccuracy in SV delivery will affect results with this calculation method. Use calibrated micropipettes.

b. A single point standard, for example 0.2 G/L.

$$\text{Malic Acid G/L} = \text{Conc. Standard} \times \frac{\text{Net } A_{SAMPLE}}{\text{Net } A_{STANDARD}} = 0.2 \times \frac{\text{Net } A_{SAMPLE}}{\text{Net } A_{STANDARD}}$$

c. A multi-point standard curve run with each assay. Sample concentrations are calculated from the best-fit standard curve.

If samples have been diluted during preparation, multiply the results by the dilution factor.

SAMPLES

Significance of Measurements

Both free and esterified L-Malic acid is commonly found in fruit 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 concentration[3]

Clarification and Decolorization

Test solutions should be clear liquids. Centrifuge or filter cloudy or turbid samples.

Due to enzyme content of some samples, absorbance may be affected by secondary (i.e. "creep") reactions in some samples. Fermentation samples may be placed into a water bath at 80°C to inactivate fermentation enzymes following clarification. Red wine typically does not need decolorization; consider decolorizing if unusually high Sample Blank absorbance is obtained. Mix 10mL juice and approximately 0.1g polyamide powder or polyvinyl-polypyrrolidone (PVPP), stir for 1 minute and filter.

Free Malic Acid Determination

Follow the procedure as given above. If the initial Abs 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 anthocyans may be measured in wine following alkaline hydrolysis. Heat 20mL of wine and 6mL sodium hydroxide (2M) for 30 minutes while stirring under a reflux condenser. (Ammonium ions interfer with this hydrolysis.) After cooling, neutralize with 1M sulfuric acid; quantitatively transfer to volumetric flask and Q.S. with distilled water to 50mL. Proceed with L-malic acid determination.

QUALITY CONTROL

A low and high level control should be included in each set of assays. Commercially available control material with established L-malic acid values may be used for quality control. Factors that may affect the performance of this test include proper instrument function, temperature control, cleanliness of glassware and accuracy of pipetting.

Notes:

Selection of SV and Wavelenth: The NADH absorbance maximum is 340nm; 334-340nm analysis provides the best analytical discrimination. While less sensitive, analysis at 365nm provides a broader measuring range, e.g. 0-0.6 g/L @ 334-340nm compared to 0–0.9 @ 365nm. An alternate assay range and SV may be appropriate.

Preparation of Working Reagent: For SV $\geq$ 100 µL:

1) reduce water such that the total "DI water + SV" is between 2 and 2.1 mL and 2) decolorize red wines.

L-Malic Acid, G/L			D.I Water
334-340nm	365nm	SV	per mL Buffer
0.03 – 0.6	0.06 – 0.9	50µL	2.0 mL
0.03 -- 0.3	0.05 – 0.6	150µL	1.85 mL
0.01 -- 0.1	0.02 -- 0.2	500 µL	1.5 mL
0.002 -- 0.02	0.004 – 0.04	2000µL	none

Select standards within the assay range.

REFERENCES

1. 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.

2. 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.

3. Peynaud, E. p.41, Eng.Trans, John Wiley & Sons, 1984.

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