UV assay for the determination of L-malic acid in foodstuffs and other sample materials
Test combination for 50 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was evaluated using selected samples of the following matrices: wine, beer, lemonades, fruit and vegetable products.

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

1. Test principle

L-Malic acid (L-malate) is oxidized by nicotinamide adenine dinucleotide (NAD) in the presence of the enzyme L-malate dehydrogenase (L-MDH) to form oxaloacetate and NADH:

L-Malate + NAD^+ $\xrightarrow{\text{L-MDH}}$ oxaloacetate + NADH + H^+

Oxaloacetate + L-glutamate $\xrightarrow{\text{GOT}}$ L-aspartate + 2-oxoglutarate

NAD is reduced to NADH during this process. The amount of NADH formed is proportional to the concentration of L-Malic acid in the sample and is measured at a wavelength of 340 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer, L-glutamate, GOT
- Reagent 2: 2 x 12.5 mL with buffer, NAD, L-MDH

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Samples solutions should be brought to room temperature before measurement.
- Neutralize **strongly** acidic samples to a pH of approx. 7 – 8 by adding 1 M KOH or NaOH or adding HCl to alkaline samples.
- For turbid test samples: Filter by using fluted paper filter or syringe filter or centrifuge the test solution in a reaction tube (recommended 3000 rpm for at least 5 minutes) until a clear filtrate or supernatant is obtained.
- Degas samples containing carbon dioxide (e.g., beer) by stirring in a beaker.
- If necessary, decolorize **strongly** colored samples with polyvinylpolypyrrolidone (PVPP, e.g., 1 g/100 mL sample). Stir or shake for 1 minute and filter or centrifuge at 3000 rpm for at least 5 minutes until a clear supernatant is obtained.
- Grind and homogenize solid or semi-solid samples, weigh in suitable sample quantity and extract with water. Filter, centrifuge, or use Carrez clarification if necessary.
- Clarify samples containing proteins or fat with Carrez clarification: Weigh or pipette the appropriate sample quantity into a 100 mL volumetric flask and add approx. 60 mL distilled water. Then add 5 mL Carrez I solution (3.60 g potassium hexacyanoferrate(II)-trihydrate $K_4[Fe(CN)_6]$ x 3 H_2O/100 mL), 5 mL Carrez II solution (7.20 g zinc sulfate $ZnSO_4$ x 7 H_2O/100 mL) and 10 mL 0.1 M NaOH. Mix well after each addition. Fill the measuring flask with distilled water up to the mark, mix and filter (discard first milliliters).
- For samples with a high fat content, weigh e.g. 5 g into a 100 mL volumetric flask, fill halfway with water, and heat in a water bath at 50 – 60 °C (122 – 140 °F) for 20 minutes. After cooling, fill the flask to the mark and place it in the refrigerator for about 20 minutes to separate the fat. Then use a pleated filter to obtain a clear or slightly cloudy sample.
- In case of higher sample volumes (up to 1000µL), check the pH value of the test solution and neutralize in case of any doubt.

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	15 – 500 mg/L (for 100 µL)

	Reagent blank	**Samples / controls**
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read absorbance A_1**, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **15 minutes at 20 – 37 °C (68 – 99 °F)** and **read absorbance A_2**.		

4.1 Important notes for assay procedure

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated at 25 °C (77 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance.
- Always wait for the reaction to end or for the absorbance to stabilize (at least during the first test runs or validation). If the absorbance has not stopped after the recommended incubation time, continue measuring at 5-minute intervals, for example, until a constant absorbance value is reached.

- If a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Concentration of L-malic acid

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_2 - df \times A_1)_{\text{sample or control}} - (A_2 - df \times A_1)_{RB}$$

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume + R1}}{\text{test volume}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df)** accordingly.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of L-malic acid is calculated using Lambert-Beer's law:

$$\mathbf{C_{L\text{-malic acid}}\ [g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.5534 \times \Delta A} \times F$$

If the sample solution was diluted before measurement, this result has to be multiplied with the sample **pre-dilution factor F.**

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight L-malic acid [g/mol]	= 134.09
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

$$\text{Content}_{\text{L-malic acid}}\ [g/100\ g] = \frac{C_{\text{L-malic acid}}\ [g/L\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/L sample solution}} \times 100$$

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. Therefore, we recommend Enzytec™ *Liquid* Multi-Acid Standard *low* (Art. No. E8460; 0.250 g/L L-malic acid).

The recovery of this multi-standard *low* and other aqueous control solutions should be 100 ± 5 %.

6. Performance data

6.1. Specificity

The test is specific for L-malic acid.

6.2. Interferences

Potential interferences were investigated by testing structurally related organic acids at defined concentrations.

Acetic acid, ascorbic acid, butyric acid, isobutyric acid, D-lactic acid, L-lactic acid and propionic acid showed no influence on the determination of L-malic acid, even at concentrations up to 50 g/L.

Citric acid, maleic acid, succinic acid, tartaric acid, D-malic acid and 2-hydroxybutyric acid did not interfere with the assay at concentrations below 5 g/L.

Due to limited solubility, L-aspartic acid and fumaric acid could be tested only up to 4.0 g/L and 4.9 g/L, respectively, and showed no influence at these concentrations.

Significant interference was observed for meso-tartaric acid and oxaloacetate. Reliable quantification of L-malic acid is ensured only when oxaloacetate concentrations are below 0.5 g/L and meso-tartaric acid concentrations are below 0.005 g/L.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 500 mg/L L-malic acid (sample volume of 100 µL) with a recommended measuring range of 15 – 500 mg/L.

The limit of detection (LoD) was determined according to the DIN 32645:2008-11 method in a buffered aqueous solution. For a sample volume of 100 µL, the calculated LoD is 8.0 mg/L.

The limit of quantification (LoQ) was determined by precision profile. The calculated LoQ is 15.0 mg/L for a sample volume of 100 µL.

The smallest absorbance difference reliably distinguishable by the method is ΔA = 0.005. By increasing the sample volume, the sensitivity of the assay can be improved accordingly.

6.4. Automation with Pictus 500

6.4.1. Limit of quantification (LoQ)

P500 application	LoQ
High Range	125 mg/L
Basic Range	15 mg/L
Sensitive Range	3.5 mg/L

6.4.2. Measuring ranges

P500 application	Measuring range
High Range	to 2.5 g/L
Basic Range	to 500 mg/L
Sensitive Range	to 50 mg/L

6.4.3. Precision and accuracy

Data from the measurement of an aqueous solution are shown here.

High Range

Target concentration, mg/L	200	500
Mean value, mg/L	203.6	505.7
SD, mg/L	4.02	4.44
RSD, %	1.98	0.88
Recovery, %	101.8	101.1

Basic Range

Target concentration, mg/L	200	500
Mean value, mg/L	204.7	501.3
SD, mg/L	0.92	1.29
RSD, %	0.45	0.26
Recovery, %	102.4	100.3

Sensitive Range

Target concentration, mg/L	20	50
Mean value, mg/L	20.42	49.94
SD, mg/L	0.09	0.35
RSD, %	0.43	0.69
Recovery, %	102.1	99.9

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* L-Malic acid Validation Report
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* L-Malic Excel template for results
- Enzytec™ *Liquid* L-Malic Technical information
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

Upon request, we offer the following services, among others:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support & technical service

10. Disclaimer

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